Tannic Acid Induction of a Glutathione *S*-transferase in *Micromelalopha troglodyta* (Lepidoptera: Notodontidae) Larvae[1]

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Abstract *Micromelalopha troglodyta* (Graeser) (Lepidoptera: Notodontidae) is an important pest of poplar trees, *Populus* spp. (family Salicaceae), in China. Herein, we report the first cloning and sequencing of a full-length cDNA of a delta class (Class I) glutathione *S*-transferase (GST), *MtGSTd1*, from the larval midgut of *M. troglodyta*. The open reading frame of the *MtGSTd1* cDNA was 657 bp and encoded 219 amino acid residues in *M. troglodyta*. Furthermore, specific activities of GSTs were induced in fat bodies and midguts of *M. troglodyta* by tannic acid. *MtGSTd1* mRNA also was induced by tannic acid in the fat bodies and midguts. GST activities increased following the elevated expression of GST mRNA in *M. troglodyta*. These findings indicate that the GSTs may have an antioxidant role in the metabolism of plant secondary substances in *M. troglodyta* larvae.

Key Words *Micromelalopha troglodyta*, tannic acid, glutathione *S*-transferases, *MtGSTd1*, mRNA expression

The glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a superfamily of multifunctional detoxification enzymes. The GSTs catalyze the conjugation of the tripeptide glutathione to diverse electrophilic substrates such as insecticides or their primary toxic metabolic products (Nay et al. 1999). Furthermore, GSTs have long been demonstrated to be involved in the protection from oxidative damage and intracellular transport of hormones, endogenous metabolites, and exogenous chemicals (Clark 1989, Listowsky et al. 1988, Rushmore and Pickett 1993). They therefore protect the cell from attack by a range of reactive electrophilic compounds. Indeed, glutathione-dependent conjugation plays a role in detoxification mechanisms in insects and mammals (Clark et al. 1984).

Earlier classification systems for enzymes had GSTs of insects divided into two types (Class I and Class II) and differed from compounds involved in immune reactions (Fournier et al. 1992, Hemingway 2000). Class I GSTs are also referred to as the delta class, while the Class II GSTs are the sigma class (Chelvanayagam et al. 2001), which is in line with the nomenclature of the existing vertebrate GST classes. Recently, an additional insect GST class (Class III, also known as epsilon-class GSTs) was established (Ranson et al. 2001, Sawicki et al. 2003). The Class I and Class III enzymes are reportedly involved in imparting resistance to insecticides

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(Agianian et al. 2003), as well as providing defense mechanisms against plant alleochemicals.

Micromelalopha troglodyta (Graeser) (Lepidoptera: Notodontidae) is an important feeding-leaf pest of poplar trees, *Populus* spp. (family Salicaceae), in many parts of China. Actively feeding larvae may consume tannic acid produced by the host plant trees as a secondary substance to provide a direct defense to the phytophagous insects. Detoxification enzymes in insects are known to be responsible for insect adaptation to such secondary plant substances. GSTs are among these enzymes, and their production in insects has been induced (Lindroth et al. 1990, Yu 1982).

The induction mechanism of GST by tannic acid in *M. troglodyta* has not yet been demonstrated. Our objectives in this study were to (1) clone the cDNA from class I, or delta class, GST genes in *M. troglodyta* larvae and (2) elucidate the tannic acid induction mechanism of GST activity and gene expression in *M. troglodyta* larvae. Additionally, we hoped to define the role of GSTs in the metabolism and excretion of plant secondary substances and thus provide initial insights into the evolution and functions of the GSTs in *M. troglodyta*.

Materials and Methods

Larvae used in these assays were from a colony initially established by collecting *M. troglodyta* from poplar trees in Nanjing (31°56′17.00″N, 118°22′35.98″E), Jiangsu Province, China. Larvae were supplied fresh poplar leaves in a room maintained at 26 ± 1°C and 70–80% relative humidity with a photoperiod of 16:8 (light:dark).

GST induction by tannic acid was studied by first feeding larvae on poplar leaves that had been immersed in tannic acid solutions. The tannic acid (Sigma Chemical, St. Louis, MO) was dissolved in a small aliquot of ethanol and then serially diluted in distilled water to test concentrations of 10, 1, 0.1, 0.01, and 0.001 mg/ml. Freshly collected poplar leaves were immersed in the various solutions and then allowed to air-dry. Once dried, the treated leaves were placed individually into triangle bottles with newly molted third instars and placed on the shelf in the rearing room. Controls consisted of larvae fed on leaves immersed in distilled water. Larvae were allowed to feed on treated leaves for 96 h when they were removed for dissection.

Fat bodies and midguts were removed from individual larvae by dissection on ice. Peritrophic membranes associated midgut contents were removed, and midguts were gently shaken to free all contents and rinsed in 1.15% ice-cold KCl. The tissues were homogenized in sodium phosphate buffer (pH 6.5, 0.1M) containing ethylene diamine tetraacetic acid (EDTA) (1 mM), phenylmethanesulfonyl fluoride (PMSF) (1 mM, dissolved in absolute alcohol), and DL-dithiothreitol (DTT) (1 mM), using an ice-cold mortar. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4°C, and the supernatant was used to determine the enzyme activity. All experiments were performed in triplicate.

GST activities were measured according to methods of Habig et al. (1976). The 1.2-ml reaction mixture consisted of 1.0 ml sodium phosphate (0.1 M, pH 6.5), 60 μl of 20 mM glutathione, 40 μl of 30 mM 1-chloro-2,4-dinitrobenzene (CDNB), and 100 μl of enzyme extract. The reaction was conducted at 25°C, and the absorbance of

Table 1. Primers used in molecular analyses of glutathione *S*-transferases in *Micromelalopha troglodyta*.

Primer Name	Primer Sequences (5′–3′)*	Use
GSTN	AATCCTCAACACACAGT	cDNA fragment amplification
GSTC	CTGARGTCTGCCAYRGTC	cDNA fragment amplification
GST-3RF	GCCGGTACCTGGTGAAACAAGTATGG	3′ RACE** amplification
GST-5′ outer	ACACCTTTTCCATTGGCGTCTT	5′ RACE amplification
GST-5′ inner	ACGAGGGCTTCTTCTAGTTTCTT	5′ RACE amplification
GST-ORF F	ATGTCGATCGACCTGTACTACCTG	ORF proving amplification
GST-ORF R	AGCTTCTTACAGCTCTGTTTTAGC	ORF proving amplification

* Degeneracy bases in primers, Y = C or T; R = A or C.
** Abbreviations: RACE, rapid amplification of cDNA ends; ORF, open reading frame.

conjugate formation was monitored kinetically at 340 nm for 2 min. Enzyme activity was documented as nmol/min, and the specific activity as nmol/min/mg protein using an extinction coefficient of 9.6 L/mM/cm. Protein quantification was subjected to the Bradford (1976) assay with bovine serum albumin as the standard.

Genomic DNA was isolated from the larval midguts according to the instructions provided in the DNA isolation kit (AxyPrep gDNA Extraction Kit, Axygen Biotechnology Ltd., Hangzhou, China). Total RNA was extracted from 50 mg of midgut tissues using Trizol reagent (Invitrogen™, Thermo Fisher Scientific, Inc., Waltham, MA) according to the manufacturer's instructions. RNA samples were treated with PrimeScript™ RT reagent kit and gDNA eraser (Clontech Laboratories, Inc, Mountain View, CA) to eliminate contamination of genomic DNA prior to reverse transcription and eventual quantification and purity analysis of RNA and DNA. The cDNA synthesis was conducted using PrimeScript RT reagent kit with gDNA eraser, and the synthesized cDNA was kept at −80°C.

A pair of degenerate primers (GSTN and GSTC) were designed according to the conserved sequences of Class I and Class III GST genes from other lepidopteran species (Table 1). The GST gene fragment was obtained using degenerate primers by rapid amplification. The rapid amplification of cDNA ends (RACE) was performed to isolate the full-length cDNA of the GST gene. The specific primers (GST-3RF) were synthesized based on the cDNA fragment obtained and were used as the 3′-RACE primer (Table 1). The 3′ RACE polymerase chain reaction (PCR) amplification of the GST gene was performed using a Smart Race cDNA amplification kit (Clontech Laboratories, Inc.) by following the manufacturer's instructions. The touchdown PCR was used for RACE-PCR, and the sequence

Table 2. Primers used for polymerase chain reaction amplifications in molecular analyses of glutathione *S*-transferases in *Micromelalopha troglodyta*.

Primer Name	Primers	Length of Products (bp)
MtGSTd1 (target)		
Sense	5′-CTCTGGTCGACTTGAGGCTGGAC-3′	313
Antisense	5′-TCTTCGTAGCCAGGTGCGGTAGAC-3′	
Actin (internal standard)		
Sense	5′-CTCTGGTCGACTTGAGGCTGGAC-3′	241
Antisense	5′-CTCTGGTCGACTTGAGGCTGGAC-3′	

was: 94°C for 4 min; then 94°C for 30 s, 70°C for 45 s, and 72°C for 2 min for five cycles. This was followed by 94°C for 1 min, 55°C for 50 s, and 72°C for 1 min for 30 cycles; and 72°C for 10 min for elongation. The GST-5′ outer and GST-5′ inner primers were designed and used in a modified 5′ RACE procedure to obtain the 5′ end (Table 1). One microgram of RNA was used to obtain the 5′ end of the cDNA by using the 5′-Full RACE Kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) according to the manufacturer's instructions. The PCR was done at 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min; followed by 72°C for 10 min. The PCR products were purified using the Generay DNA Recovery kit (Shanghai Generay Biotechnology Co., Ltd., Shanghai, China). The purified cDNA fragments were ligated to pMD19-T vectors (Takara Biotechnology (Dalian) Co., Ltd.) and cloned into DH5α competent cells. At least three clones were sequenced by the GenScript Biological Technology Co. Ltd. (Nanjing, China). For each plasmid insert, both strands of DNA were sequenced at least twice.

A primer pair was designed on the basis of the open reading frame (ORF) that was predicted according to the 5′ and 3′ rapid amplification production. The PCR was conducted at 94°C for 4 min; 33 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min 35 s; followed by 72°C for 10 min. These PCR products were purified, ligated, cloned, and sequenced using the same methods as previously described.

The total RNA was extracted using 50 mg of either midguts or fat bodies according to the instruction of the TRIzol Reagent kit (Invitrogen), dissolved in 30 μl diethylpyrocarbonated-treated water. The integrity and quality of total RNA were tested by running 1% agarose gel electrophoresis and measuring absorbance at 260 and 280 nm using a Thermo Scientific NanoDrop2000. According to the instructions of PrimeScript RT reagent Kit with gDNA Eraser, 2μ total RNA was used to reverse-transcribe into cDNA and then stored at −80°C or used for the determination of GST mRNA expression.

Specific primers (Table 2) were designed based on nucleotide sequences of *MtGSTd1* (accession no. FJ532064) and an actin gene, beta action (accession no.

GU262991), of *M. troglodyta*. Primers were designed using the DNAMAN 5.2 software and synthesized by Shanghai Generay Biotechnology Co., Ltd.

Quantitative real-time PCR (qRT-PCR) was performed on a 7500 Real-Time PCR system (Applied Biosystems, Foster, CA) to compare the expression of *MtGST* mRNA in the midguts and fat bodies of *M. troglodyta* larvae exposed to tannic acid. The *MtGST* mRNA expression was studied by using the Real-Time PCR Kit (Takara Biotechnology (Dalian) Co., Ltd.). The amplification of cDNA by qRT-PCR was performed in a 20-μl mixture, which contained about 1 μl cDNA, 10 μl of SYBR Premix Ex Taq, 0.4 μl of Rox Reference Dye (50×), 0.4 μl of both sense and antisense primers of GST, and 7.8 μl double-distilled water. As an endogenous control, the GST gene primers were replaced by a pair of actin gene primers (0.4 μl for each) in the amplification reaction using the same cDNA as the template. The qRT-PCR reaction conditions were as follows: the reaction mixture was first kept at 95°C for 30 s, then 40 cycles of 95°C for 5 s, 60°C for 34 s were run. To confirm the amplification of specific products, melting-curve cycles were continued under the following conditions: 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. All experiments were performed in triplicate. The relative expression level of *MtGST* mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method (Giulietti et al. 2001).

Data collected from these assays were subjected to analysis of variance using InStat software (GraphPad, San Diego, CA) with significance defined as $P < 0.05$. Tukey's test was used for multiple comparisons.

Results

Induction of GST activity. The specific activities of GSTs in the fat bodies of *M. troglodyta* larvae were induced by tannic acid consumed on treated leaves and varied with tannic acid concentration, reaching a maximum activity value after exposure to 0.01, 0.1, 1.0, and 10 mg/ml (Fig. 1A). Similarly, GST activities also increased in the midguts of larvae exposed to tannic acid compared the water control. The maximum level of GST induction was observed in the midgut from larvae exposed to 0.1 mg/ml tannic acid and was 2.18-fold higher than the control (Fig. 1B).

Cloning of a GST gene. The GST cDNA of *M. troglodyta* was cloned using the technology of RT-PCR, 3′ RACE and 5′ RACE. The 854-bp cDNA of GST from *M. troglodyta* (*MtGST*) was sequenced (Fig. 2). The full-length ORF region was 657 bp and encoded in a 219 amino acid peptide. The amino acid sequence of the MtGSTd1 protein was deduced using Bio XM software and shown in alignments (Fig. 2). The comparison of the amino acid sequence of MtGSTd1 with other insect GSTs is shown in Fig. 3. The derived amino acid sequence showed 78%, 76%, and 73% identity to Class III (delta class) GSTs from *Helicoverpa armigera* (Hübner), *Bombyx mori* (L.), and *Papilio xuthus* L., respectively. The phylogenetic tree was built upon the alignment of GST sequences obtained from protein blast in Genbank, and it was constructed using Neighbor-joining, placed the *M. troglodyta* GST in the Class I (delta class) of insect GSTs (Fig. 4).

To characterize the genomic structure, the *MtGSTd1* gene was amplified from *M. troglodyta* genomic DNA using the primers designed based on the sequences of the *MtGSTd1* cDNAs. The PCR products were cloned and sequenced. The genomic

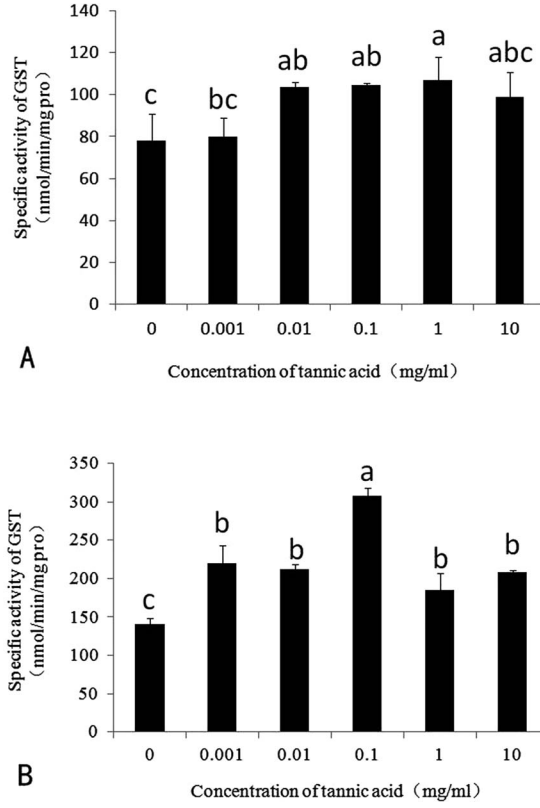


Fig. 1. Mean specific activities of glutathione *S*-transferases in fat bodies (A) and midguts (B) of *Micromelalopha troglodyta* following exposure to various concentrations of tannic acid. Standard errors are indicated by bars above means; means with the same lowercase letter are not significantly different (Tukey's test, $P < 0.05$).

PCR product sequences were identical to the *MtGSTd1* cDNAs. The comparison of the genomic sequence with the sequence of the cDNA revealed that there were four exons and three introns in the *MtGSTd1* gene. The sequences at the exon-intron boundaries conformed to consensus eukaryotic splice sites, including an invariant GT at the intron 5′ boundary and an invariant AG at its 3′ boundary. The genomic DNA sizes from translation start codon to stop codon were 5690 bp for *MtGSTd1*.

Induction of *MtGSTd*1 mRNA expression by tannic acid in *M. troglodyta*. Expression of *MtGSTd1* mRNA in the midgut and fat body of *M. troglodyta* larvae was clearly induced by exposure to different concentrations of tannic acid for 96 h. The expression of *MtGSTd1* mRNA in the fat bodies was induced by the concentration of 0.001, 0.01 and 0.1 mg/ml tannic acid. In addition, the highest inducing multiple of *MtGSTd1* mRNA is 2.78 in the fat bodies from larvae exposed to 0.1 mg/ml tannic acid (Fig. 5A). Similar results were obtained in the midguts treated with tannic acid. The expression of *MtGSTd1* mRNA reached the maximum

Fig. 2. Nucleotides and deduced amino acid sequences of the glutathione *S*-transferase gene from *Micromelalopha troglodyta*.

level in the midguts from larvae exposed to 0.001 mg/ml tannic acid; the expression level was 3.69 times higher than that observed in the controls (Fig. 5B).

Discussion

GSTs in insects are considered as being responsible for the detoxification and recovery from oxidative stress (Kohji et al. 2005). They may be involved in detoxificating secondary plant compounds that the insect encounters when feeding on the plant. Thus, these secondary plant compounds may induce of GSTs within the feeding insect (Gao et al. 1997, Lee 1991, Wadleigh et al. 1987, Yu 1996, Zhang et al. 2009).

Tannic acid is characterized as a plant polyphenol and is commonly distributed in many plants. Our results suggest that GST activities in insects are induced by feeding upon or exposure to tannic acid. This is in agreement with previous studies

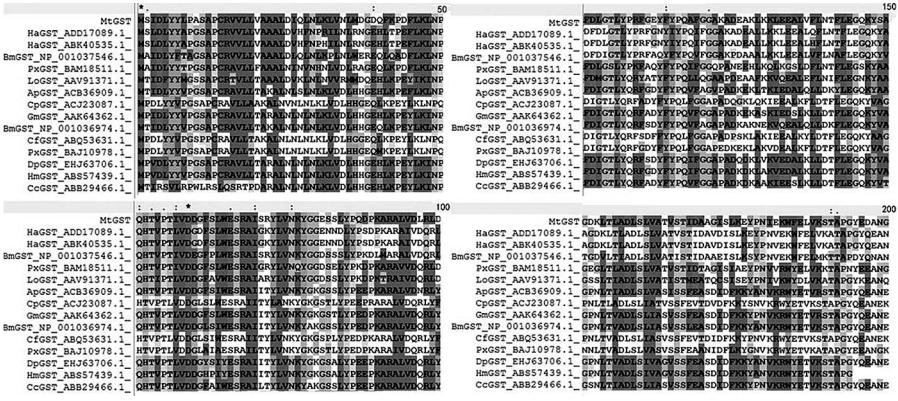
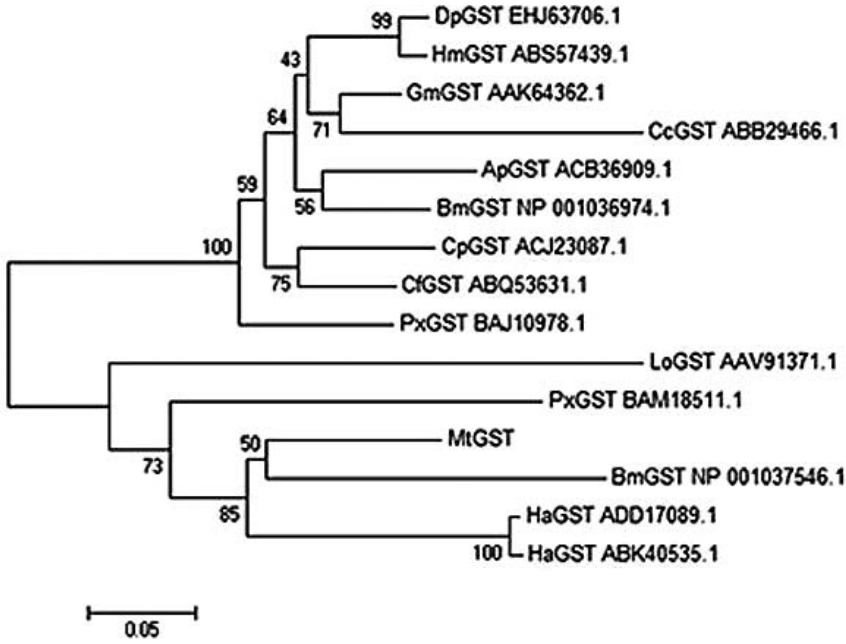


Fig. 3. Multiple sequence alignment of *Micromelalopha troglodyta* glutathione *S*-transferase (GST) with other insect GST genes as performed using clustalX 2.0. Conserved regions are shaded. (*Helicoverpa armigera* ADD17089.1, *Helicoverpa armigera* ABK40535.1, *Bombyx mori* NP-001037546.1, *Papilio xuthus* BAM18511.1, *Lonomia obliqua* Walker AAV91371.1, *Antheraea pernyi* (Guérin-Méneville) ACB36909.1, *Cydia pomonella* (L.) ACG69436.1, *Galleria mellonella* (L.) AAK64362.1, *Bombyx mori* NP-00136974.1, *Choristoneura fumiferana* (Clemens) ABQ53631.1, *Plutella xylostella* (L.) BAJ10978.1, *Danaus plexippus* (L.) EHJ63706.1, *Heliconius melpomene* L. ABS57439.1, *Corcyra cephalonical* Staint ABB29466.1).

of the response of insect herbivores to plant allelochemicals including the induction of GST activity by tannic acid in *H. armigera* feeding on cotton (Chen et al. 2003), induction of GST activity in *Musca domestica* L. by phenolbarbital (Hayaoka et al. 1982), and increased GST activity in *Spodoptera frugiperda* (J.E. Smith) fed mustard leaves, radish leaves, and cowpea leaves (Yu 1982).

We also sequenced and characterized the cDNA clones, including the full-length ORF, from *M. troglodyta*. The GST from *M. troglodyta* (*MtGSTd1*) cDNA was 854 bp long and contained an ORF of 657 nucleotides being capable of encoding a 219–amino acid polypeptide with a calculated molecular weight of 24,272.8 and a theoretical isoelectric point of 5.03 as analyzed by software DNAMAN, an online analysis software (http://us.expasy.org/tools/protparam.html). The ORF had both a start (ATG) and stop codon (TAA), indicating that the sequence contained the complete coding region. A putative polyadenylation signal, AATAAA, was located at nucleotide positions 812–817. Multiple sequence alignments of the deduced protein sequences of *MtGSTd1* cDNA with the available insect GST sequences from GenBank databases were compared and showed that the GST genes of *M. troglodyta* shared some of the same domains with other insect GST genes, implying that these GSTs may be expressed in response to similar stresses. Alignment with the deduced amino acid sequence of *MtGSTd1* cDNA indicated that the *MtGSTd1* sequence was closely related to the Class I (delta class) GST from *H. armigera* (ADD17089.1 and ABK40535.1, 78% protein sequence identity), *B. mori* (NP-



• (*Helicoverpa armigera* ADD17089.1, *Helicoverpa armigera* ABK40535.1, *Bombyx mori* NP-001037546.1, *Papilio xuthus* BAM18511.1, *Lonomia oblique* AAV91371.1, *Antheraea pernyi* ACB36909.1, *Cydia pomonella* ACG69436.1, *Galleria mellonella* AAK64362.1, *Bombyx mori* NP-00136974.1, *Choristoneura fumiferana* ABQ53631.1, *Plutella xylostella* BAJ10978.1, *Danaus plexippus* EHJ63706.1, *Heliconius melpomene* ABS57439.1, *Corcyra cephalonical* ABB29466.1)

Fig. 4. Phylogenetic tree analysis of *Micromelalopha troglodyta* and other insect glutathione *S*-transferase sequences with numbers at nodes representing the branch lengths. The sequences were indicated as follows with accession numbers in parentheses: (*Helicoverpa armigera* ADD17089.1, *Helicoverpa armigera* ABK40535.1, *Bombyx mori* NP-001037546.1, *Papilio xuthus* BAM18511.1, *Lonomia obliqua* AAV91371.1, *Antheraea pernyi* ACB36909.1, *Cydia pomonella* ACG69436.1, *Galleria mellonella* AAK64362.1, *Bombyx mori* NP-00136974.1, *Choristoneura fumiferana* ABQ53631.1, *Plutella xylostella* BAJ10978.1, *Danaus plexippus* EHJ63706.1, *Heliconius melpomene* ABS57439.1, *Corcyra cephalonical* ABB29466.1).

001037546.1, 76% protein sequence identity), and *P. xuthus* (BAM18511.1, 73% protein sequence identity), all belonging to the Class I (delta class) GSTs. Based on the phylogenetic tree generated from the aligned amino acid sequences of insect GSTs, *MtGSTd1* was proposed to belong to the Class I (delta class) GSTs (Fig. 5). The gene was named *MtGSTd1*, in accordance with the nomenclature proposed by Yamamoto et al. (2005).

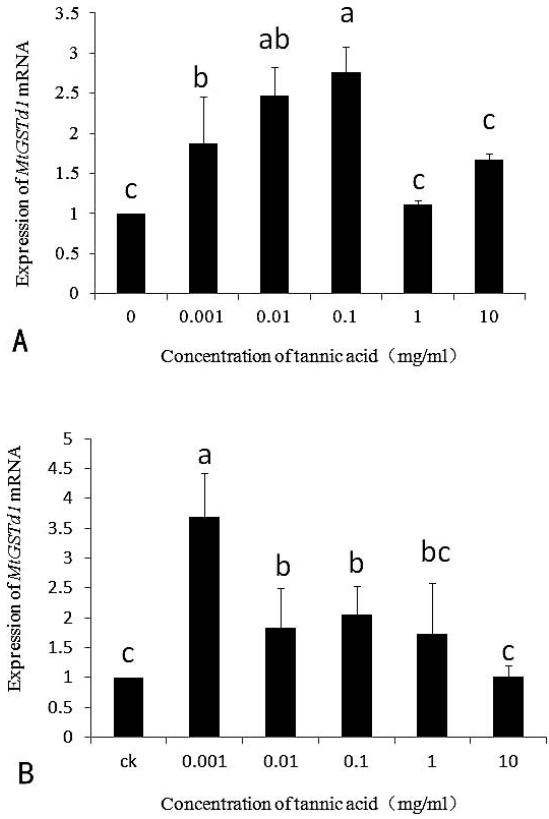


Fig. 5. The expression of *MtGSTd1* mRNA in fat bodies (A) and midguts (B) of *Micromelalopha troglodyta* treated with tannic acid. Standard errors are indicated by bars above means; means with the same lowercase letter are not significantly different (Tukey's test, $P < 0.05$).

GST subunits consist of two domains, each containing two binding sites—a G site and an H site. The highly conserved G site, binding the tripeptide glutathione, is largely composed of amino acid residues found in the N-terminal domain. The H site, or substrate binding site, is more variable in structure and is largely formed from residues at the C terminus (Mannervik 1985). Our results were consistent with these general characteristics of GST in that the *MtGSTd1* had a G site binding the tripeptide glutathione in the N-terminal region and an H site, which was a substrate binding site in the C terminus.

Class I and Class III GST enzymes are involved in insect resistance to insecticides (Agianian et al. 2003). The Class I GSTs are the most numerous, and were thought to approximate the mammalian theta class (Pemble and Taylor 1992, Ranson et al. 1997), but are now defined as the insect-specific delta class (Board et al. 1997). With the development of molecular biology techniques, the GST gene sequences in many insects have been cloned and identified (Ding et al. 2003,

Toung et al. 1993, Yu et al. 2008), including that of the spruce budworm, *Choristoneura fumiferana* (Clemens) (Feng et al. 1999, Zheng et al. 2007). The delta class GST of *Drosophila melanogaster* Meigen also exhibited a major antioxidant role with its considerable conjugating activity of lipid peroxidation products (Agianian et al. 2003).

GST enzymes in insects are inducible. Many exogenous toxic substances, such as barbital, pesticides, and plant secondary compounds, can induce GST activity in insects. This induction process appears to be an adaptation mechanism of organisms to counter chemical stress (Chen 2003). Our studies showed that the GST activities were induced in *M. troglodyta* following exposure to tannic acid. This conclusion is underscored by the expression of *MtGSTd1* mRNA induction by exposure to tannic acid. These results are consistent with other reports, including increased GST activity owing to the elevated expression of GST mRNA in *H. armigera* (Tang et al. 2005) and the induction of gstD1 and gstD21 mRNA in *D. melanogaster* by phenobarbital (Tang and Tu 1995). Increased GST activities in these studies were mainly due to the transcription level.

In conclusion, we analyzed the GST activity and mRNA expression in *M. troglodyta* following exposure to tannic acid. Our analyses might facilitate an understanding of the induction effect of GST by exposure to plant secondary compounds. Additional research efforts should identify the regulating site of *MtGST*d1 and clarify the relationship between *M. troglodyta* GST and insecticides' agent resistance and the relationship between *M. troglodyta* GST and the induction of plant secondary compounds, which might prove instrumental in regulating the expression of GST and delaying the development of resistance to insecticides.

Acknowledgments

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