Role of *AccMGST1* in oxidative stress resistance in *Apis cerana cerana*

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Abstract

As detoxification enzymes, proteins in the glutathione S-transferase (GST) superfamily are reported to participate in oxidative stress resistance. Nevertheless, microsomal GSTs (MGSTs), a unique subclass of the GST superfamily associated with membranes, are rarely studied in insects. Here, we isolated an *MGST* gene in *Apis cerana cerana* (*AccMGST1*) and verified its role in oxidative stress response. We found higher expression of *AccMGST1* in protective or defensive tissue, that is, the epidermis, which indicated its role in stress resistance. Real-time quantitative PCR (qRT-PCR) analysis indicated that *AccMGST1* was upregulated by oxidative stresses at the transcriptional level. In contrast, *AccMGST1* expression was inhibited when the antioxidant vitamin C (VC) was fed to experimental bees. Through western blotting, we found that the protein level of *AccMGST1* under oxidative stress corresponded to the transcript level. Disc diffusion and mixed-function oxidation (MFO) assays suggested that *AccMGST1* can protect not only cells but also DNA against oxidative damage. Furthermore, we discovered that the expression patterns of known antioxidant genes were changed in *A. cerana cerana* after *AccMGST1* was silenced by RNA interference (RNAi). Thus, we concluded that the gene *AccMGST1* exerts a significant role in the antioxidant mechanism.

Keywords *Apis cerana cerana* · Oxidative stress · *AccMGST1* · Expression · RNA interference

Abbreviations

BLAST	Basic local alignment search tool
CDNB	Xenobiotic substrate 1-chloro-2,4-dinitrobenzene
dsRNAs	Double-stranded RNAs
GST	Glutathione S-transferase
GSH	Glutathione
H ₂ O ₂	Hydrogen peroxide
IPTG	Isopropyl-β-D-thiogalactopyranoside

LTA4	Leukotriene A4
LB	Lysogeny broth
MGST	Microsomal glutathione S-transferase
MFO	Mixed-function oxidation
MAPK	Mitogen-activated protein kinase
MAPEG	Membrane-associated proteins in eicosanoid and glutathione metabolism
NCBI	National Center for Biotechnology Information
O ₂ [−]	Superoxide anion
ORF	Open reading frame
OD	Optical density
POD	Peroxidases
qRT-PCR	Real-time quantitative PCR
RNAi	RNA interference
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SOD	Superoxide dismutase
SDS-PAGE	Sulfate-polyacrylamide gel electrophoresis
UV	Ultraviolet
VC	Vitamin C

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Introduction

For living organisms, environmental stress is inevitable. Specifically, reactive oxygen species (ROS) are induced by environmental stress, such as suboptimal temperatures, heavy metals, direct oxidation damage, ultraviolet (UV) radiation, and pesticides (Lushchak 2011; Kottuparambil et al. 2012; Jordan et al. 2012; Meng et al. 2009). ROS, as a general term, includes superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and other species associated with macromolecular damage, which is associated with diseases such as aging, atherosclerosis, and diabetes (Ray et al. 2012). The altered redox balance resulting from ROS accumulation initiates elevated inflammatory signaling and metabolic dysfunction (Forrester et al. 2018). Overall, ROS contributes to damage to living organisms.

To control the effects of ROS, a variety of nonenzymatic and enzymatic antioxidative agents have evolved. Although the complete antioxidant mechanism remains unclear, some nonenzymatic molecules, such as glutathione (GSH) and thioredoxin, and enzymatic antioxidative biomacromolecules, such as glutathione S-transferases (GSTs), superoxide dismutase (SOD), catalase, and peroxidases (POD), have been reported to resist oxidative stress (Monteiro et al. 2010). Among these, GSH, which is related to H_2O_2 detoxification as a crucial molecule in oxidative stress, may be modified by several enzymes (Rahantaniaina et al. 2018). GSTs, which are one type of detoxification enzymes (phase II), catalyze glutathione conjugation with a large variety of interior and exterior electrophilic compounds to protect cellular macromolecules against some kinds of attack by reactive electrophiles (Townsend and Tew 2003).

The GST superfamily is a multifunctional superfamily with important roles in vital activities. For example, several studies have demonstrated that GSTs exert a regulatory influence on the mitogen-activated protein kinase (MAPK) signaling pathway, especially when the MAPK signaling pathway takes part in cellular survival and death signaling caused by UV irradiation, H_2O_2 treatment, and other stresses (Adler et al. 1999; Yin et al. 2000). Moreover, mtDNA mutations caused by ROS can enhance tumor cell metastasis, while GST expression is up-regulated in a large variety of tumor types, as previously reported (Tang and Tu 1994; Mapp et al. 2002; Segura-Aguilar and Paris 2014; Ishikawa et al. 2008). GSTs are divided into three categories: cytosolic, microsomal, and mitochondrial; only two types of GSTs (cytosolic and microsomal) have been found in insects (Wang et al. 2019). MGSTs differ from cytosolic GSTs in two aspects. First, MGSTs are located in microsomes and are integral membrane proteins (Mannervik et al. 2005). Second, MGSTs are also members of the membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamilies, which are related to inflammatory reactions (Ekström et al. 2003). Moreover, each member of the MAPEG superfamily plays a separate role in eicosanoid

and glutathione metabolism (Jakobsson et al. 2010). For example, MGST2 has been reported to accelerate the conjugation of the xenobiotic substrate 1-chloro-2,4-dinitrobenzene (CDNB), as well as that of leukotriene A4 (LTA4), with glutathione, while MGST1 is linked to cellular defense against external substances or metabolites resulting from oxidative stress (Jakobsson et al. 2010; Scoggan et al. 1997; Morgenstern et al. 1996; Dejong et al. 1988; Mosialou et al. 1995; Ahmad et al. 2013).

Hebert and Jegerschold found that MGST1 formed a homotrimer in the membrane and that the MGST1 monomer consisted of four membrane-spanning helices (Hebert and Jegerschold 2007). Moreover, previous studies have demonstrated that only 1/3 of the active sites of MGST1 are activated toward glutathione (Ahmad et al. 2015; Ålander et al. 2009). MGST1, as an antioxidant enzyme with a specific structure and character, is thought to play a significant role in some activities. For example, MGST1 exhibits POD and GST activities and can be activated by reactive nitrogen species (RNS) and ROS (Kowalski 2014; Morgenstern et al. 2011). Moreover, MGST1 represses the negative effects of lipid peroxidation intermediates (Siritantikorn et al. 2007). The results of previous research show that, when the level of MGST1 is decreased, complex gene rearrangement occurs, and the antioxidant properties of nondifferentiated PC12 cells are altered (Kowalski 2014). We also considered that, with age, the expression level and activity of MGST1 tend to decline (Maeda et al. 2008; Li et al. 2011). However, its precise effects on oxidative stress in insects are not yet understood.

Apis cerana cerana (*A. cerana cerana*), with an evolutionary history of 70 million years, is a unique native bee species in China. Furthermore, as a major pollinator, it is significant in maintaining the ecological balance and ensuring food and oil safety (Hepburn and Radloff 2011; Gallai et al. 2009). Overall, *A. cerana cerana* plays an increasingly important role in human life. However, because of chemical residues, pesticide abuse, and harsh environmental conditions, the survival of *A. cerana cerana* is currently in crisis (He and Liu 2011; Potts et al. 2010; Diao et al. 2018). In addition, we cannot ignore that *A. cerana cerana* may encounter many stressors in the process of survival. Under these circumstances, studying the environmental stress resistance of *A. cerana cerana* is vital, so that we can not only find new ways to improve their survival rate, but also understand their environmental adaptation mechanisms. Our laboratory is dedicated to studying the molecules and mechanisms that function in resisting biotic and abiotic stresses in *A. cerana cerana*.

In this study, we analyzed the role of *AccMGST1* in the oxidative stress response. We investigated the changes in *AccMGST1* expression when *A. cerana cerana* responded to simulated environmental stresses. Using a prokaryotic expression system, we tested the function of *AccMGST1* protein by disc diffusion and mixed-function oxidation (MFO) assays.

With RNA interference (RNAi), we analyzed the changes in the expression profiles of known antioxidant genes in *A. cerana cerana*. According to our work, *AccMGST1* may play a crucial role in the antioxidant mechanism. Our study establishes a foundation for further study of the overall antioxidant mechanism in the future.

Materials and methods

Insects and treatments

The artificially cultured *A. cerana cerana* in our experiments were obtained from Shandong Agricultural University (Taian, China). The bees/ bees just out of the hive were collected and cultured under artificial conditions (constant temperature at 32 °C, 80% relative humidity, without environmental stresses). We selected 15- to 30-day-old adult worker bees as the experimental insects. As Alaux et al. (2010) described, worker honeybees were collected at the access points of the hive and were maintained under suitable temperature, humidity, and available food. The collected honeybees were divided into 9 groups. Among these groups, groups 1–3 were exposed to suboptimal temperatures (4 °C, 14 °C, 44 °C) for 0, 0.5, 1, 2, and 3 h, respectively. Group 4 was exposed to UV radiation (30 mJ/cm² UV, 254 nm) for 0, 0.5, 1, 2, and 3 h. In addition, group 5 was fed with H₂O₂ (0.5 µL 50 mM H₂O₂/worker) for 0, 0.5, 1, and 2 h, while group 6 was fed with VC (2 mg/L) for 0, 1, 2, and 3 h (1 µL per bee). Furthermore, worker bees in group 7 was injected with 1 µL HgCl₂ (2 mg/L) for 0, 1, 2, 3, and 4 h on the back, and group 8 was injected with 1 µL CdCl₂ (2 mg/L) for 0, 1, 2, 3, and 4 h on the back. The bees in group 9 were exposed to paraquat (1.6 µg/mL) as described for 0, 0.5, 1, 2, and 3 h (Jia et al. 2017). Untreated bees were used as a control. The samples were frozen in liquid nitrogen transiently and stored at –80 °C permanently for further study. In addition, we collected the head, pereion, abdomen, muscle, honey bag, and epidermis of experimental bees by ice dissection and stored them at –80 °C for tissue-specific expression analysis. All the treatments were performed with three repeats.

In nature, organisms are inevitably exposed to temperature fluctuations and UV radiation, which may cause the accumulation of ROS, resulting in oxidative stress (Lushchak 2011; Kottuparambil et al. 2012; Jordan et al. 2012; Meng et al. 2009). The suitable temperature for the *A. cerana cerana* population ranges from 32 °C to 35 °C. Hence, we first selected 4 °C, 14 °C, and 44 °C as simulated stress temperatures. *A. cerana cerana*, which pollinates flowers in fields, is inevitably subjected to pesticides and the pollution caused by heavy metals. Paraquat is recognized as an ROS generator (Jia et al. 2017; Jordan et al. 2012; Landis et al. 2004), and HgCl₂ and CdCl₂ have been reported as factors for ROS accumulation (Lushchak 2011; Park and Park 2007; Li et al.

2005). We fed bees with H₂O₂ as an indirect oxidative treatment or VC, which suppresses the production of ROS, as an antioxidant treatment (Kim et al. 2008). The treatment concentration and times were selected based on previous researches (Jia et al. 2017; Zhai et al. 2018) in our laboratory.

RNA extraction, cDNA synthesis, and real-time quantitative PCR

Using the manufacturers' specifications, total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase, and cDNA was compounded with EasyScript First-Strand cDNA Synthesis (TransGen Biotech, Beijing, China). We used 25-µL PCR mixtures (1.0 µL of each primer (10 mM each), 12.5 µL SYBR Premix Ex Taq, 2.0 µL cDNA, and ddH₂O up to 25 µL) and the reaction protocol corresponding to SYBR Premix Ex Taq (95 °C predenaturation, 30 s; 40 cycles of denaturation and annealing at 95 °C for 5 s and 60 °C for 30 s; incubation at 95 °C for 10 s; and finally, a melt curve from 65 °C to 95 °C) to perform real-time quantitative PCR (qRT-PCR) by the CFX96™ Real-Time PCR Detection System. The expression profiles of *AccMGST1* were contrasted with the *β-actin* gene in *A. cerana cerana* [GenBank ID:XM640276] under various conditions. In addition to the *β-actin* gene, we used two reference genes, *Rps 18* [GenBank ID:XM_625101] (Zhao et al. 2014) and *Arp1* [GenBank ID:NM_001185145] (Ni et al. 2013), to detect the tissue specificity of *AccMGST1*. All primers used in the study are shown in Table 1 and Supplementary Table 1. The amplification specificity of the four quantitative qPCR primers is shown in Supplemental Fig. 1. In addition, the efficiency for *β-actin*, *Rps 18*, *Arp1*, and *AccMGST1* were 95.0%, 86.1%, 97.5%, 107.1%, respectively.

Supplemental Fig 1: qPCR amplification specificity. (a) Amplified fragments of the three reference genes and target gene *AccMGST1*. Agarose gel (2%) electrophoresis shows the specific PCR product with the expected size for each gene. DNA ladder with sizes of 500, 250 and 100 bp is shown. (b) Melting curves for the three reference genes and *AccMGST1* showing a single peak. The temperature is displayed on the x axis, and the derivative reporter signal is displayed on the y axis.

Cloning of the full-length coding sequence of the *AccMGST1* gene

The specific primers M1F and M1R (as shown in Table 1) were designed based on the genomic sequence of *AccMGST1*. The reaction volume (25 µL) was mixed following Zhao et al. (2017). The reaction protocol was designed according to the manufacturers' specifications: 94 °C, 10 min; 35 cycles at 94 °C for 40 s, 48 °C for 40 s, and 72 °C for 40 s; 72 °C, 10 min.

Table 1 The designed primer in this study

Primer	Primer sequence (5'-3')	Description
M1	ATGTCTTTAAATACCTCTCTTATAGG	cDNA sequence primer, forward
M2	TTACATGTAAAATAATAAAGTATTAGCAG	cDNA sequence primer, reverse
Me1	GGTACCATGTCTTTAAATACCTCTCTTATAGG	Primer for protein expression, forward
Me2	GGATCCCATGTAAAATAATAAAGTAT	Primer for protein expression, reverse
Mr1	TAATACGACTCACTATAGGGCGAATGTCTTTAAATA	Primer for silence, forward
Mr2	TAATACGACTCACTATAGGGCGACATGTAAAATAAT	Primer for silence, reverse
Mq1	CAAATCCTGAAGATGCTG	Primer for qRT-PCR, forward
Mq2	GCTCTGCGTACACGTTCAATCT	Primer for qRT-PCR, forward

Bioinformatic analysis of *AccMGST1*

DNAMAN software was used to align the *AccMGST1* protein sequence with homologous *MGST1*s found by searching with the Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To test whether transmembrane domains are present in *AccMGST1*, we predicted membrane-spanning regions with TMHMM server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>). Swiss-Model (<http://swiss-model.expasy.org/>) was used to predict the tertiary structures of the *AccMGST1* protein and to compare its structure with rat *MGST1* protein, which has previously been investigated. To determine the genetic relationships of *AccMGST1* with the different subclasses of GSTs in different species, we performed a phylogenetic analysis with the Molecular Evolutionary Genetic Analysis software by the neighbor-joining method. To understand the possible functions of *AccMGST1*, its upstream promoter sequence was obtained from NCBI, and the cis-acting elements in the promoter region were predicted with TFBIND software.

Overexpression and purification of *AccMGST1* protein

The *AccMGST1* open reading frame (ORF) with added restriction enzyme cutting sites at the ends was amplified by RT-PCR with the primers ME1 and ME2 (as shown in Table 1). The expression vector pET-32a, which was constructed by double digestion and T4 ligase, was integrated into *Escherichia coli* (*E. coli*) Transetta (DE3) competent cells. We selected positive clones and cultured them in lysogeny broth (LB) overnight at 37 °C, part of which was cultured a second time at 37 °C to reach an optical density (OD) 600 of 0.5. Then, 0.2 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to induce the expression of *AccMGST1* recombinant protein at 25 °C for 6 h (refer to Uno et al. (2013)). We identified the recombinant protein by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue staining. On this basis, the recombinant *AccMGST1* protein was purified by nickel column affinity chromatography (Meng et al. 1984).

Furthermore, we detected the concentration of the purified protein using a BCA Protein Assay Kit from Nanjing Jiancheng Bioengineering Institute.

Disc diffusion assay

The overexpressed *AccMGST1* and the empty pET-32 a (+) vector were separately integrated into *E. coli* Transetta cells, and the bacteria were spread on LB-ampicillin agar plates and incubated for 1 h inverted at 37 °C. The empty pET-32a (+) vector in *E. coli* Transetta cells was used as the control group. We placed sterile filter discs in the same location on agar plates and saturated these discs with different concentrations of cumene hydroperoxide. Subsequently, the plates were inverted and cultured at 37 °C for 15 h. Finally, we put the prepared discs into the centers of the plates and measured the killing zones following Burmeister et al. (2007). To prove the function of *AccMGST1* sufficiently, as Zhu et al. (2016) described, we used *AccSOD2* [GenBank:JN637476], a proven antioxidant gene in *A. cerana cerana* (Jia et al. 2014), as a positive control.

Enzyme activity measurements and DNA cleavage assay with the MFO system

Following the instructions and Habig's description (Habig et al. 1974), we measured glutathione S-transferase activity in *AccMGST1* with 1-chloro-2,4-dinitrobenzene (CDNB) at 412 nm by spectrophotometry with a GST kit. Because POD can catalyze the hydrogen peroxide reaction, the POD activity in *AccMGST1* was tested with a POD kit by spectrophotometry at 420 nm according to the manufacturer's instructions. Both GST activity and POD activity were measured with the detected concentrations, and both the GST kit and POD kit are from Nanjing Jiancheng Bioengineering Institute.

The integrated MFO system contains 100 mM HEPES buffer, 10 mM DTT, 3 mM FeCl₃, 300 ng pUC19 plasmid DNA, ddH₂O, and purified protein with an increased concentration gradient (from 0.96 to 7.68 μ g) (Meng et al. 1984). HEPES buffer, DTT, FeCl₃, target protein, and water were mixed to reach 25 μ L and then incubated in a water bath at

37 °C for 30 min. Furthermore, we added 300 ng pUC19 plasmid DNA and incubated the mixture in a water bath at 37 °C for 3 h. Agarose gel electrophoresis (2.0%) was used to detect the extent of pUC19 plasmid DNA cleavage. Three systems without the target protein were used as a control. System 1 lacked only the target protein; system 2 lacked the target protein and FeCl₃; system 3 lacked the target protein, FeCl₃, and DTT.

Protein extraction, antibody preparation, and western blot analysis

We extracted the total protein from the treatment groups using the Tissue Protein Extraction Kit (ComWin Biotech, Beijing, China) according to the instructions. As described by Chen

et al., we used 5 white mice (China Biologic Products, Inc., Shandong) to prepare the anti-AccMGST1 (Chen et al. 2015). The antisera containing anti-AccMGST1 were stored at – 80 °C for further analysis. The anti- α -tubulin (Beyotime Biotechnology, Shanghai, China) antibody was used as a control during western blot analysis, as previously described (Chen et al. 2015).

RNA interference (RNAi) of *AccMGST1*

We amplified a special *AccMGST1* internal fragment containing the T7 polymerase promoter with the designed primers MT1 and MT2 by PCR and obtained purified DNA fragments of *AccMGST1* at high concentrations using the Agarose Gel Extraction kit (Solarbio, Beijing, China). The DNA fragment

Fig. 1 Amino acid sequence analysis of the gene *AccMGST1*. **a** Multiple alignment analysis of AccMGST1 with microsomal GSTs from *Apis mellifera* (AmMGST1), *Apis florea* (AfMGST1), *Bombus terrestris* (BtMGST1), *Homo sapiens* (HmMGST1), *Macaca fascicularis* (MfMGST1), and *Rattus norvegicus* (RnMGST1). The conserved amino acids are marked with black shading, and some amino acid residues at specific positions are labeled with boxes: Arg at 72 and 73, Asn at 77 and 81, His at 75, Asp at 78, and Asn at 80. **b** Phylogenetic analysis of MGST1 and the Sigma, Omega, Zeta, and Delta GST classes. Eight main groups are shown. Acc, *Apis cerana cerana*; Am, *Apis mellifera*; Af, *Apis florea*; Ad, *Apis dorsata*; Bt, *Bombus terrestris*; Mr, *Megachile rotundata*; Nv, *Nasonia vitripennis*; Px, *Plutella xylostella*

Fig. 2 Predicted membrane topology and tertiary structure of *AccMGST1*. **a** Predicted transmembrane regions of *AccMGST1*. The four regions composed of red lines are the predicted transmembrane regions. **b** Predicted secondary structure type and tertiary structure of *AccMGST1*. The tertiary structure of *AccMGST1* is composed of α -helices with three subunits (A, B, C). Each α -helix in A, B, and C subunit is marked with A1 to A4, B1 to B4, and C1 to C4, respectively

Fig. 3 Expression pattern of *AccMGST1* in different tissues. EP, epidermis; AB, abdomen; MU, muscle; HB, honey bag; HE, head; PE, pereion. The error bars represent the mean $\pm$ SEM of three independent repeats. The letters above the error bars represent significant differences ($P < 0.05$) as analyzed by ANOVA and Duncan's tests with SPSS software version 17.0

of the *GFP* [GenBank accession number:U87974] gene was obtained as above. The Ribo MaxTM T7 Large Scale RNA Production System (Promega, Madison, WI, USA) was used to synthesize the double-stranded RNAs (dsRNAs) dsRNA-*AccMGST1* and dsRNA-*GFP* from the DNA fragments. The synthetic dsRNA-*AccMGST1* was injected into the abdomens of the experimental honeybees. Meanwhile, synthetic dsRNA-*GFP* and ddH₂O were injected into the honeybees to provide a reference (Chen et al. 2015). Each of the three groups contained 30 experimental honeybees. We detected the effects of *AccMGST1* silencing and the expression profiles of antioxidant genes in *A. cerana cerana* by qRT-PCR. The experiments were performed with three repeats.

Results

Identification and sequence analysis of *AccMGST1*

The internal fragment sequence of *AccMGST1* was isolated by RT-PCR and found to contain a 450 bp ORF that encodes 149 amino acids. The predicted relative molecular mass of the protein formed by these 149 amino acids is 17.143 kDa, and this protein has an isoelectric point of 9.74. Sequence alignment between *AccMGST1* and other MGST proteins showed that *AccMGST1* contains a relatively conserved domain with 7 nonhydrophobic residues, which is significant for the interaction between *AccMGST1* and GSH (Fig. 1a) (Busenlehner et al. 2007). Through sequence alignment, we also found that *AccMGST1* is highly homologous to *Apis mellifera* MGST1 (*AmMGST1*) (92.62%). The phylogenetic analysis reflected the close relationship between *AccMGST1* and other insect MGST1s. Among the 5 subclasses of the GST superfamily, *AccMGST1* was classified as part of the microsomal class (Fig. 1b). In addition, we predicted the transmembrane regions of *AccMGST1*, and the prediction showed four transmembrane domains (Fig. 2a). Then, we predicted the three-dimensional structure of *AccMGST1*. As shown in Fig. 2b, there are 12 α -helices in *AccMGST1*.

Based on the functional prediction of the promoter of *AccMGST1*, the TATA box was found to be the most important element in the promoter region (Elias-Neto et al. 2010). Furthermore, we detected the binding sites of some transcription factors, such as AP1, CREB, NFY, STAT, and HSF, in the promoter region of *AccMGST1* (Supplemental Fig. 2). These transcription factors are reported to participate in responses to environmental stresses, especially oxidative stress. For example, STAT may be induced by oxidative stress (Carballo et al. 1999). NFY can regulate the expression of the H-ferritin gene, a gene that is associated with oxidative stress (Falbo et al. 2009). In addition, the CREB transcription factor is a regulator of several diseases that are induced by oxidative stress (Zou and Crews 2006; Grösch and Kaina 1999).

Fig. 4 Expression patterns of *AccMGST1* at the transcriptional level when *A. cerana cerana* undergo environmental stresses. Total RNA from experimental bees treated with simulated environmental stresses was extracted at the appointed times. The mRNA levels of *AccMGST1* after treatment with 4 °C (a), 14 °C (b), 44 °C (c), paraquat (d), 1 μ L CdCl₂ (2 mg/L) (e), 1 μ L HgCl₂ (2 mg/L) (f), H₂O₂ (0.5 μ L 50 mM H₂O₂/worker) (g), UV radiation (30 mJ/cm²) (h), and 1 μ L VC (2 mg/L) (i) were detected by qRT-PCR. The error bars represent the mean $\pm$ SEM of three independent repeats. The letters above the error bars represent significant differences ($P < 0.05$) as analyzed by ANOVA and Duncan's tests with SPSS software version 17.0

Supplemental Fig. 2 Nucleotide sequence and predicted cis-acting elements of the *AccMGST1* promoter region. The transcription site and translation start sites are marked with a box and an arrow, respectively. The TATA box and predicted cis-acting elements are also marked with boxes. CREB, cAMP-response element binding protein; HSF: heat transcription factor; NFY: nuclear factor Y; AP1: active protein; STAT: signal transducers and activators of transcription.

Tissue-specific expression analysis of *AccMGST1*

To understand the expression pattern of *AccMGST1* in different tissues, we detected the mRNA levels of *AccMGST1* in the head, pereion, abdomen, epidermis, muscle, and honey bag (Fig. 3). qRT-PCR analysis showed that *AccMGST1* is expressed in multiple tissues. The highest expression of *AccMGST1* was found in the

Fig. 5 Expression patterns of *AccMGST1* at the protein level by western blot analysis. Total protein from experimental bees treated with UV radiation (30 mJ/cm²) (a), H₂O₂ (0.5 μ L 50 mM H₂O₂/worker) (b), and 1 μ L VC (2 mg/L) (c) was extracted. The *AccMGST1* protein in the total protein was immunoblotted with anti-*AccMGST1*. The protein level of α -tubulin in the total protein was used as a control. The error bars represent the mean $\pm$ SEM of three independent repeats. The letters above the error bars represent significant differences ($P < 0.05$) as analyzed by ANOVA and Duncan's tests with SPSS software version 17.0

Fig. 6 Disc diffusion assays. *E. coli* cells overexpressing *AccMGST1* (a) and *AccSOD2* (b) were plated on agar plates, and filter discs soaked in different concentrations of cumene hydroperoxide were placed in the agar plates. The numbers marked on the filter discs indicate the increasing concentrations of cumene hydroperoxide. The inhibition zones around each filter disc were measured with a ruler. The error bars represent the mean $\pm$ SEM of three independent repeats

epidermis, and relatively high expression of *AccMGST1* was found in the honey bag, while *AccMGST1* was expressed at lower levels in the head, pereion, abdomen, and muscle.

Fig. 7 Expression and purification of recombinant *AccMGST1* with SDS-PAGE analysis and MFO assay. **(a)** SDS-PAGE analysis. M, prestained protein marker; lane 1, noninduced; lane 2, induced; lane 3–lane 5, purified proteins. **(b)** MFO assay. SF, supercoiled form; NF, nicked form; lane 1, pUC19 plasmid DNA; lane 2, pUC19 plasmid DNA + FeCl_3 ; lane 3, pUC19 plasmid DNA + FeCl_3 + DTT; lane 4, 0.96 μg *AccMGST1* + pUC19 plasmid DNA + FeCl_3 + DTT; lane 5, 1.92 μg *AccMGST1* + pUC19 plasmid DNA + FeCl_3 + DTT; lane 6, 2.88 μg *AccMGST1* + pUC19 plasmid DNA + FeCl_3 + DTT; lane 7, 3.84 μg *AccMGST1* + pUC19 plasmid DNA + FeCl_3 + DTT; lane 8, 5.76 μg *AccMGST1* + pUC19 plasmid DNA + FeCl_3 + DTT; lane 9, 7.68 μg *AccMGST1* + pUC19 plasmid DNA + FeCl_3 + DTT

Expression profiles of *AccMGST1* in different treatments

To test whether the expression of *AccMGST1* is affected when *A. cerana cerana* undergoes oxidative stress, we analyzed the expression patterns of *AccMGST1* under various simulated environmental stresses related to ROS accumulation. As Fig. 4 shows, all the treatments in this study could affect *AccMGST1* expression in 15-day-old adult workers. When experimental bees were exposed to 4 °C for 0.5 h, the expression of *AccMGST1* was upregulated, reaching a maximum at 2 h (Fig. 4a). In addition, when experimental bees were exposed to 14 °C for 0.5 h, the expression of *AccMGST1* was upregulated to its highest level (Fig. 4b). Analogously, when experimental bees were exposed to 44 °C, its expression profile was similar to that at 14 °C (Fig. 4c). Then, we investigated the expression of *AccMGST1* in *A. cerana cerana* treated with paraquat, CdCl_2 , and HgCl_2 . The results indicated that paraquat, CdCl_2 , and HgCl_2 could also induce expression of *AccMGST1* (Fig. 4d, f). After CdCl_2 and HgCl_2 treatment, the expression of *AccMGST1* was highest at 2 h, while the maximum was reached after 0.5 h of paraquat treatment. Next, we treated *A. cerana cerana* with UV, H_2O_2 , and VC. UV and H_2O_2 treatment induced the expression of *AccMGST1*, while VC inhibited *AccMGST1* expression (Fig. 4g, i). All these data show the impact of oxidative stress on *AccMGST1* expression at the transcriptional level.

Table 2 The concentration of purified proteins

Sample	Blank	Standard	1	2	3
A_{562}	0.031	0.144	0.092	0.146	0.062
concentration ($\mu\text{g/mL}$)	/	/	303.9	573.0	154.5

Table 3 GST enzymatic activity

Sample	Blank	Standard	Control	Purified protein
A ₄₁₂	0.038	1.182	0.452	0.425
concentration (U/mgprot)	/	/	/	9.88

To further verify the influence of oxidative stress on *AccMGST1*, we detected the protein levels of *AccMGST1* during oxidative treatments by western blotting. Total proteins were extracted after the treatments, and the AccMGST1 protein was detected by anti-AccMGST1. The protein level of *AccMGST1* was compared with that of α -tubulin. As shown in Fig. 5, the expression of the AccMGST1 protein was induced by H₂O₂ treatment at 1 h and UV radiation at 0.5 h. The expression of AccMGST1 protein decreased after VC treatment at 1 h. Furthermore, the transcript levels of the *AccMGST1* gene corresponded to the protein levels basically.

Characterization of recombinant AccMGST1 (disc diffusion, enzyme activity, MFO system)

To investigate the function of the AccMGST1 protein in oxidative stress, we obtained recombinant AccMGST1 protein by prokaryotic expression and performed analyses. The result of disc diffusion shows that cumene hydroperoxide inhibits the growth of *E. coli* cells, and varying concentrations of this compound cause different effects. However, the diameter of the inhibition zone in *AccMGST1*-overexpressing cells was smaller than that in the control group (Fig. 6a). Moreover, the results were consistent with the antioxidant levels of *AccSOD2*-expressing cells (Fig. 6b).

Then, we purified the recombinant AccMGST1 protein to study its function by enzymatic analysis (Fig. 7a). First, we detected the concentration of the purified protein. As shown in Table 2, a high concentration of purified protein was used for further study. To identify the enzymatic activities of the purified AccMGST1 protein, the GST enzyme activity and POD enzyme activity mentioned above (Morgenstern et al. 2011) were measured. As shown in Tables 3 and 4, the GST enzyme activity of the purified AccMGST1 protein is 9.88 U/mg prot, while the POD enzyme activity is 32.4 U/mg prot.

Furthermore, we used the MFO system to analyze the protection AccMGST1 can afford DNA against oxidative damage. A previous study reported that, in the presence of DTT

and FeCl₃, hydroxyl radicals that are formed in the MFO system may break supercoiled pUC19 plasmid DNA (Zhai et al. 2018). As shown in Fig. 7b, with increasing concentration of AccMGST1, the amount of intact supercoiled pUC19 plasmid DNA is increased while the quantity of damaged DNA is reduced. In summary, AccMGST1 protects DNA from oxidative damage.

AccMGST1 knockdown and the effects on antioxidant gene expression in *A. cerana cerana*

The above results suggested that AccMGST1 possesses antioxidant capability, and we wanted to further investigate the role of *AccMGST1* in the antioxidant system in *A. cerana cerana*. Thus, we knocked down the *AccMGST1* gene by RNAi and detected the expression patterns of oxidative resistance genes. Through qRT-PCR detection, we found it was only 12 h that after *dsAccMGST1* injection the expression level of the *AccMGST1* gene had declined (Fig. 8). Moreover, greater silencing effects were found at 24 h and 36 h. At 36 h after *dsAccMGST1* injection, antioxidant genes belonging to the GST superfamily in *A. cerana cerana*, including *AccGSTO2*, *AccGSTS4*, *AccGSTD*, and *AccGSTZ*, as well as the *AccTpx3*, *AccTpx4*, *AccTpx5*, *AccTrxr1*, and *AccCAT* genes, were induced (Fig. 9). In short, silencing *AccMGST1* may influence oxidative resistance.

Discussion

MGSTs are members of the GST family of detoxification enzymes, which protect cells against foreign particles and metabolic substances such as pesticides and ROS (Townsend and Tew 2003; Mannervik et al. 2005; Ahmad et al. 2013). Researchers have reported that some classes of GSTs may participate in the MAPK pathway, which is related to the oxidative stress response (Adler et al. 1999; Yin et al. 2000), but MGSTs, which have structures and characteristics

Fig. 8 *AccMGST1* knockdown. The expression patterns of *AccMGST1* after RNA interference at the indicated times were compared with those in two controls: the expression patterns of *AccMGST1* after injection of the experimental bees with sterile water and ds-GFP, a double-stranded RNA from a gene that does not exist in the *A. cerana cerana* genome. The error bars represent the mean $\pm$ SEM of three independent repeats. The letters above the error bars represent significant differences ($P < 0.05$) as analyzed by ANOVA and Duncan's tests with SPSS software version 17.0

Table 4 POD enzymatic activity

Sample	Blank	Purified protein
A ₄₂₀	0.111	0.278
concentration (U/mgprot)	/	32.4

Fig. 9 Effects of *AccMGST1* knockdown on the mRNA levels of some reported antioxidant genes in *A. cerana cerana*. **a** *AccGSTO2* [GenBank ID:JX434029], **b** *AccGSTS4* [GenBank ID:JN008721], **c** *AccGSTZ* [GenBank ID:JN637474], **d** *AccGSTD* [GenBank ID:JF798572], **e** *AccTrx1* [GenBank ID:JX844651], **f** *AccTpx3* [GenBank ID:JX456217], **g** *AccTpx4* [GenBank ID:KJ551847], **h** *AccTpx5*

[GenBank ID:KF745893], and **i** *AccCAT* [GenBank ID:KF765424]. The error bars represent the mean $\pm$ SEM of three independent repeats. The letters above the error bars represent significant differences ($P < 0.05$) as analyzed by ANOVA and Duncan's tests with SPSS software version 17.0

distinct from those of cytoplasmic GSTs, have rarely been studied, especially in insects. To investigate the functions of MGSTs, we isolated a presumptive MGST gene in *A. cerana cerana*, identified it as belonging to the MGSTs subclass and named it *AccMGST1*.

Different tissues have unique functions in *A. cerana cerana*. For example, the epidermis is a barrier between bees and the environment that guards these organisms against environmental stresses (Marionnet et al. 2003). Interestingly, we found the highest expression of *AccMGST1* in the epidermis. In other words, *AccMGST1* may contribute to withstanding environmental stresses and defending bees in disadvantageous situations. Similarly, *AccGSTO2* and *AccGSTD* expression was high but not highest in the epidermis, while *AccGSTS1* expression was very low (Zhang et al. 2017; Yan et al. 2013a, b). Intriguingly, *AccGSTO2*, *AccGSTD*, and *AccGSTS1* expression are also high in protective tissues such as the midgut, fat body, or tissues that need sufficient oxidative defense, such as the brain (Zhang et al. 2017; Yan et al. 2013a, b). These expression patterns may reflect the tissue-specific and antioxidant function of the GST superfamily. Furthermore, we investigated the expression patterns at the transcription and translation levels of *AccMGST1* under oxidative stress. In this

investigation, all the simulated environmental stresses resulting in oxidative stresses were designed to reflect the lifestyle of *A. cerana cerana*. Remarkably, the expression of *AccMGST1* at the transcriptional level was upregulated by inaptitude temperatures, UV radiation, H₂O₂, heavy metals, and paraquat. Reportedly, under oxidative treatments, some genes in *Drosophila melanogaster* encoding proteins that function in antioxidant defense, such as glutathione S-transferases, show increased expression levels (Jordan et al. 2012; Nair and Choi, 2011; Landis et al. 2004). As reported in *A. cerana cerana*, some antioxidant genes may be induced by different oxidative treatments (Zhang et al. 2017; Yan et al. 2013a, b; Jia et al. 2014). Therefore, when *A. cerana cerana* suffers from oxidative stress, *AccMGST1*, as an antioxidant gene, will be expressed to resist it. Compared the influences caused by directed oxidative treatment with antioxidant treatment, we obtained opposite results that feeding H₂O₂ to *A. cerana cerana* induces the expression of *AccMGST1*, while feeding VC reduces. The expression patterns of *AccGSTO2* and *AccGSTS1* under H₂O₂ treatment were similar to that of *AccMGST1* (Zhang et al. 2017; Yan et al. 2013b). However, the expression patterns of *AccGSTO2* and *AccGSTS1* under VC treatment have not been

reported. Our results proved that when ROS accumulates, *AccMGST1* is expressed to scavenge it. Furthermore, when ROS is consumed, *AccMGST1* will be downregulated to restore ROS to normal levels. Considering these results, we also conclude that *AccMGST1* is very sensitive to alterations in oxidative stress and may function by maintaining the oxidative balance.

Western blot showed that the expression patterns of *AccMGST1* at the translational level are similar to those at the transcriptional level. In most cases, the protein level is coincident with the mRNA level, but the regulation of gene expression after transcription may affect the protein level. The consistent results obtained for *AccMGST1* indicated that *AccMGST1* was increased to translate more protein to defend against oxidative stress, considering the function of GSTs.

Overexpressing *AccMGST1* can protect cell growth by resisting the effects of cumene hydroperoxide. The purified recombinant AccMGST1 protein has enzymatic activity, including GST activity and POD activity. In addition, AccMGST1 can protect DNA against oxidative stress. All of these results prove the antioxidant function of the AccMGST1 protein.

Previous studies have reported that in *Drosophila*, loss of the antioxidative gene Trx-2 enhances phenotypes that depend on oxidative stress (Tsuda et al. 2010). In *A. cerana cerana*, several genes, such as GSTs and Trxs, have been reported to participate in oxidative stress (Meng et al. 1984; Yan et al. 2013b; Yao et al. 2013; Yan et al. 2014). We knocked down *AccMGST1* by RNAi and found that the expression of other antioxidant genes in *A. cerana cerana* was upregulated. The molecules may play diverse roles when participating in antioxidant defense that various results present after the loss of them. For example, silencing *AccSTIP1*, *AccSCO2* downregulated the expression of antioxidant genes (Jia et al. 2017; Zhai et al. 2018). We conclude that, due to *AccMGST1* silencing, ROS accumulation became uncontrolled, resulting in oxidative stress. Then, antioxidant genes in *A. cerana cerana* were expressed to respond to this oxidative stress. In short, *AccMGST1* may play a significant role in oxidative stress response.

Collectively, although the mechanism by which *AccMGST1* perceives that *A. cerana cerana* is subjected to oxidative stress is still not clear, we investigated the possible role of *AccMGST1* in antioxidant defense. When *A. cerana cerana* suffers from oxidative stresses, *AccMGST1* will be induced to generate enough AccMGST1 protein to scavenge excess ROS to defend against oxidative stresses until no oxidative stresses are present. In addition, we concluded that *AccMGST1* is essential for maintaining redox homeostasis in the body of *A. cerana cerana*. Our study also provides a reference for antioxidant research and development.

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