Journal of Insect Physiology 58 (2012) 782–791

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

# The identification and oxidative stress response of a zeta class glutathione S-transferase (GSTZ1) gene from *Apis cerana cerana*

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ARTICLE INFO

*Article history:*
Received 9 November 2011
Received in revised form 11 February 2012
Accepted 13 February 2012
Available online 20 February 2012

*Keywords:*
Glutathione-S-transferase
Antioxidant activity
Oxidative stress
*Apis cerana cerana*

ABSTRACT

Glutathione-S-transferases (GSTs) play an important role in protecting organisms against the toxicity of reactive oxygen species (ROS). However, no information is available for GSTs in the Chinese honey bee (*Apis cerana cerana*). In this study, we isolated and characterized a zeta class GST gene (*AccGSTZ1*) from the Chinese honey bee. This gene is present in a single copy and harbors five exons. The deduced amino acid sequence of AccGSTZ1 shared high sequence identity with homologous proteins and contained the highly conserved features of this gene family. The temporal and spatial expression profiles of *AccGSTZ1* showed that *AccGSTZ1* was highly expressed in fourth instar larvae during development, and the mRNA level of *AccGSTZ1* was higher in the epidermis than that in other tissues. The expression pattern under oxidative stress revealed that *AccGSTZ1* transcription was significantly upregulated by external factors, such as temperature challenges and $H_2O_2$ treatment. The characterization of the purified protein revealed that AccGSTZ1 had low glutathione-conjugating activity, but the recombinant AccGSTZ1 protein displayed high antioxidant activity under oxidative stress. These data suggest that AccGSTZ1 is an oxidative stress-inducible antioxidant enzyme that plays an important role in the protection against oxidative stress and may be of critical importance for the survival of the honey bees.



## 1. Introduction

The Chinese honey bee, *Apis cerana cerana*, is an important species that plays a critical role in the balance of regional ecologies and agricultural economics as a pollinator of flowering plants. However, farming this species is extremely difficult due to the environmental temperature challenge. Yang et al. (2010) recently demonstrated that induced thermal stress was associated with the generation of increased reactive oxygen species (ROS) and the production of oxidative damage. Thus, understanding the antioxidant system of the honey bee and its ROS defense mechanisms has become a primary issue for this industry.

ROS, including superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (·OH), are generated naturally during aerobic metabolism. In normal conditions, there is a balance between the generation of ROS and antioxidant processes, but exogenous stressors, such as prooxidants, heavy metals, pesticides and biotic infections, can break this balance and cause excessive production or accumulation of ROS (Narendra et al., 2007; Brennan et al., 2008). The excess ROS can result in lipid peroxidation which disrupts cell membrane fluidity and can lead to apoptosis (Green and Reed, 1998). The sperm storage of *Apis mellifera* is also affected by ROS (Collins et al., 2004) . Oxidative damage to proteins can range from specific amino acid modifications and peptide scission to loss of enzyme activity (Stadtman, 1986; Stadtman and Levine, 2003). Surplus ROS can also lead to DNA damage in the form of mutations, base deletions, degradation, single-strand scission and rearrangements (Imlay and Linn, 1988). To prevent ROS-mediated damage, complex enzymatic and non-enzymatic defense systems have evolved. The dominant antioxidant enzymes include glutathione-S-transferase (GST), glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) (Rameshthangam and Ramasamy, 2006; Dubovskiy et al., 2008).

Among the antioxidant enzymes, GSTs are the major detoxifying enzymes that are primarily localized to the cytosol of the cells in all living organisms (Lantum et al., 2002), and play pivotal roles in oxidative stress resistance and in detoxifying endogenous and xenobiotic compounds in cells (Huang et al., 2011). Considering the significant functions in which they are involved in, GSTs have been predominantly studied in mammals and humans. Insect GSTs,

*Abbreviations:* ROS, reactive oxygen species; GSTs, glutathione S-transferases; cDNA, complementary DNA; bp, base pair(s); UTR, untranslated region; ORF, open reading frame; mRNA, messenger ribonucleic acid; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; IPTG, isopropyl-1-thio-β-galactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1, 2-dichloro-4-nitrobenzene; PVDF, polyvinylidine difluoride; PBS, phosphate buf fered saline.

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doi:10.1016/j.jinsphys.2012.02.003

which have been grouped into six GST classes, delta, epsilon, omega, sigma, theta and zeta (Tu and Akgül, 2005), are of particular interest because of their role in insecticide resistance (Vontas et al., 2002; Enayati et al., 2005). All of the GSTs directly implicated in insecticide metabolism to date belong to the delta and epsilon classes, which are believed to be insect specific (Li et al., 2008; Yu et al., 2008). Most recently, insect GSTs have been studied for their role in mediating oxidative stress responses (Kim et al., 2011). Kampkotter et al. (2003) showed that overexpressing an *Onchocerca volvulus* omega class GST (OvGST3) in *Caenorhabditis elegans* increased resistance to oxidative stress in the transgenic worm lines, and the authors speculated that OvGST3 plays an important role in the protection of the parasite against reactive oxygen species derived from the host's immune system. Putative binding sites and regulatory/response elements involved in the induction of GST expression in response to oxidative stress have been found in epsilon and delta GST gene promoters from anophelines, supporting the antioxidant physiological role of some GSTs (Ding et al., 2005; Udomsinprasert et al., 2005). Increased GST activity was also found in *Helicoverpa armigera* adults under oxidative stress resulted from Ultraviolet light (Meng et al., 2009).

In *A. mellifera*, proteins involved in oxidative stress, such as vitellogenin and juvenile hormone, have been reported by Corona et al. (2007), but few are available in *A. cerana cerana*. Given the important roles of GSTs in protecting tissues against oxidative damage and oxidative stress (Li et al., 2008; Kim et al., 2011), we decided to study the oxidative stress response of GSTs in *A. cerana cerana*. Our studies were undertaken to identify the *A. cerana cerana* GST supergene family in response to oxidative stress through molecular cloning studies and analysis of the responses to oxidative stresses at both the mRNA and protein levels. Our work provides a better understanding of the role that a zeta class GST plays in the defense against oxidative stress in *A. cerana cerana* and contributes to our knowledge of this versatile superfamily.

## 2. Materials and methods

### 2.1. Insects and treatments

The Chinese honey bees (*A. cerana cerana*) used in this study were maintained at the experimental apiary of Shandong Agricultural University (Taian, China). Larval and pupal worker honey bees were generally classified according to age, eye color and shape. The whole bodies of the first to fifth larval instars (L1–L5) and pupae including prepupae (PP), white eyes (Pw), pink eyes (Pp), brown eyes (Pb) and dark eyes (Pd) were obtained from the hive, while the adult worker honey bees (A10-ten day post-emergence and A30-thirty day post-emergence) were collected at the entrance of the hive. The brain, epidermis, muscle and midgut of adults (15 days) were dissected on ice, freshly collected and used to examine tissue specific expression. Two-week-old adult worker honey bees, collected randomly from combs in outdoor beehives, were caged in eight groups of 40 individuals and reared on an artificial diet in an incubator with 60% relative humidity at 34 °C under a 24 h dark regimen.

Groups 1–5 were placed at 4, 15, 25, 34 or 43 °C, respectively. The bees kept at 34 °C served as a control. Worker bees in group 6 were injected with 20 μl (50 μM of $H_2O_2$/worker) of $H_2O_2$ between the first and second abdominal segments using a sterile microscale needle. Bees in group 7 injected with phosphate buffered saline (PBS) (20 μl/worker) were the injection controls. Prior to injection, the worker bees were placed on ice for 5 min. Control bees in group 8 were fed a pollen-and-sucrose solution only. The whole bodies of the bees were flash-frozen in liquid nitrogen at the appropriate time and stored at −80 °C until use.

### 2.2. Primers and PCR amplification conditions

Primers and PCR amplification conditions used are listed in Table 1 and Table 2, respectively.

### 2.3. Isolation of the full-length AccGSTZ1 cDNA

Total RNA was extracted using the Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. To eliminate potential DNA contamination, total RNA was digested with RNase-free DNaseI, and then the first-strand cDNA was synthesized using reverse transcriptase (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. An internal fragment was obtained by reverse transcription-PCR (RT-PCR) using the primers GZ1 and GZ2, which were designed based on the *GSTZ1* gene consensus sequences from *Nasonia vitripennis*, *Drosophila melanogaster*, *Homo sapiens* and *Bombyx mori*. According to the

**Table 1**
Details of the primers used in this study.

| Abbreviation | Primer sequence (5′–3′) | Description |
|---|---|---|
| GZ1 | GCGGAGTTCTTGTTCGTGGAG | cDNA sequence primer, forward |
| GZ2 | CAGGCTGATTATTTGGATGAGCAG | cDNA sequence primer, reverse |
| 5W | CGATGTGGTCTGGTTTCTTCC | 5′ RACE reverse primer, outer |
| 5N | GTACCTGCTCCATTGGATTAATCTC | 5′ RACE reverse primer, inner |
| 3W | GAAAAGAGCACGCGTCAGAG | 3′ RACE forward primer, outer |
| 3N | CGTAAAAAGGAATGGGCACAAC | 3′ RACE forward primer, inner |
| AAP | GGCCACGCGTCGACTAGTAC$(G)_{14}$ | Abridged Anchor Primer |
| AUAP | GGCCACGCGTCGACTAGTAC | Abridged Universal Amplification Primer |
| B26 | GACTCTAGACGACATCGA$(T)_{18}$ | 3′ RACE universal adaptor primer |
| B25 | GACTCTAGACGACATCGA | 3′ RACE universal primer |
| QC1 | GTCTGGCAATCGAATAAAAGG | Full-length cDNA sequence primer, forward |
| QC2 | TAAATATATGACAAATGTTTCTACAAAAT | Full-length cDNA sequence primer, reverse |
| N1 | GCGGAGTTCTTGTTCGTGGAG | Genomic sequence primer, forward |
| N2 | CTCTAGTAATCCAATGTTGTGCCC | Genomic sequence primer, reverse |
| N3 | GCACTTCATATTGACAATCACAC | Genomic sequence primer, forward |
| N4 | CACCTTTTGCCACTATAGAACTAC | Genomic sequence primer, reverse |
| BDL1 | CGAATCCGTCCATCAAAGCC | Semi-quantitative RT-PCR primer, forward |
| BDL2 | GTTGTGCCCATTCCTTTTTACG | Semi-quantitative RT-PCR primer, reverse |
| β-Actin-s | GTTTTCCCATCTATCGTCGG | Standard control primer, forward |
| β-Actin-x | TTTTCTCCATATCATCCCAG | Standard control primer, reverse |
| YH1 | GGTACCATGTCCGTTATGGGAAAGCC | Protein expression primer, forward |
| YH2 | GAGCTCCTTGGTTGCCTCTGGAGGAC | Protein expression primer, reverse |
| TZ1 | CGAATAAAAGGGGAGGGAGGAG | Primer of the probe used in Southern blot, forward |
| TZ2 | CTCCACGAACAAGAACTCCGC | Primer of the probe used in Southern blot, reverse |