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Identification and characterisation of a novel 1-Cys thioredoxin peroxidase gene (*AccTpx5*) from *Apis cerana cerana*

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ABSTRACT

Thioredoxin peroxidases (Tpxs), members of the antioxidant protein family, play critical roles in resisting oxidative stress. In this work, a novel 1-Cys thioredoxin peroxidase gene was isolated from *Apis cerana cerana* and was named *AccTpx5*. The open reading frame (ORF) of *AccTpx5* is 663 bp in length and encodes a 220-amino acid protein with a predicted molecular mass and isoelectric point of 24,921 kDa and 5.45, respectively. Promoter sequence analysis of *AccTpx5* revealed the presence of putative transcription factor binding sites related to early development and stress responses. Additionally, real-time quantitative PCR (Q-PCR) analysis indicated that *AccTpx5* was primarily present in some developmental stages, with the highest expression levels in the first-instar larvae. The expression level of *AccTpx5* was up-regulated under various abiotic stresses, including 4 °C, 42 °C, $HgCl_2$, H_2O_2, phoxim and acaricide treatments. Conversely, it was down-regulated by UV and pyriproxyfen treatments. Moreover, H_2O_2 concentration dramatically increased under a variety of stressful conditions. Finally, the purified recombinant AccTpx5 protein protected the supercoiled form of plasmid DNA from damage in the thiol-dependent mixed-function oxidation (MFO) system. These results suggest that *AccTpx5* most likely plays an essential role in antioxidant defence.

1. Introduction

As a pollinator of flowering plants, the Chinese honeybee (*Apis cerana cerana*) is critical in maintaining the balance of regional ecologies and agricultural enterprise (Weinstock et al., 2006). However, recent colony losses raise concerns. Multiple factors have been investigated as potential causes of or factors contributing to the losses, including honeybee pathogens and exogenous stressors associated with the generation of increased reaction oxygen species (ROS) and subsequent oxidative damage (Narendra et al., 2007; Brennan et al., 2008). Therefore, there is a need to understand the antioxidant system of *A. cerana cerana* and its ROS defence mechanisms and address the new challenges in antioxidant stress.

Naturally, organisms living in aerobic environments generate excessive ROS that could damage all of the major classes of biological macromolecules, causing lipid peroxidation, protein oxidation, DNA base modifications and strand breaks (Halliwell and Gutterridge, 1989; Lee et al., 2005). To protect themselves from the damage caused by ROS, insects have evolved a complex network of enzymatic antioxidant systems including enzymatic and non-enzymatic components (Felton and Summers, 1995). The dominant components of the antioxidant system in insects include thioredoxin peroxidases (Tpxs, which are also named peroxiredoxins), catalases (CATs), glutathione-S-transferases (GSTs) and superoxide dismutases (SODs) (Yu et al., 2011; Wang et al., 2012; Yan et al., 2012, 2013).

Tpxs are a ubiquitous family of antioxidant enzymes that play an important role in the detoxification of ROS and balancing the redox environment in the insect system (Radyuk et al., 2001, 2003; Tsuda et al., 2010; Yao et al., 2013a). All Tpxs contain one conserved cysteine residue that catalyses peroxide reduction during the reaction with the hydroperoxide. According to the number and location of the cysteine residues directly involved in catalysis, Tpxs are divided into two categories, namely 1-Cys Tpxs and 2-Cys Tpxs (Wood et al., 2003). In contrast to the 2-Cys Tpxs, the 1-Cys Tpxs lack a second conserved resolving cysteine residue.

To date, 1-Cys Tpxs have been identified in several organisms, and these proteins show unique functional characteristics. The human 1-Cys Tpx protein, hORF06, is a bifunctional enzyme because it has phospholipase A_2 (PLA_2) activity in addition to its peroxidase function (Fisher, 2011). Mutational studies in the mouse indicated that 1-Cys PRDX6 plays important roles in resisting certain exogenous sources of oxidative stress (Muller et al., 2007). In insects, the two 1-Cys Tpxs in

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Drosophila melanogaster possess distinctive temporal patterns of expression, and both function to protect against ROS resulting from airborne oxidants and xenobiotics (Radyuk et al., 2001). It was also shown that a 1-Cys Tpx in *Bombyx mori* was induced by an external temperature stimulus (Wang et al., 2008).

However, the molecular characterisation of the 1-Cys *Tpxs* gene in honeybees, particularly in Chinese honeybees, has not been performed. In this paper, we cloned a novel Tpx gene (*AccTpx5*) encoding a 1-Cys thioredoxin peroxidase from *A. cerana cerana*. We evaluated its expression patterns in different developmental stages and under oxidative stresses including cold, heat, ultraviolet light, $HgCl_2$, phoxim, acaricide and pyriproxyfen. We evaluated the antioxidant activity of the purified recombinant AccTpx5 by expressing the recombinant enzyme in *Escherichia coli*. Based on these results, we speculate that *AccTpx5* might play an important role in alleviating oxidative stress.

2. Material and methods

2.1. Animals and various treatments

The Chinese honeybees (*A. cerana cerana*) used in this study were reared routinely in the experimental apiary at Shandong Agricultural University, Taian, P.R. China. The bees were staged according to the criteria established by Thompson (1978), Michelette and Soares (1993) and Elias-Neto et al. (2010); the stages included egg, larvae, pupae and adults. The second or third day eggs; the first to fifth instar larvae (L1–L5); pupae, including prepupae (P0), white-eyed (Pw), pink-eyed (Pp), brown-eyed (Pb) and dark-eyed (Pd); newly emerged adults (A1, 1 day post-emergence); nurses (A7, usually 7–10 days post-emergence); and foragers (A15, older than 15 days post-emergence) were collected from the hive, frozen in liquid nitrogen and stored at −70 °C for the analysis of developmental expression patterns. Ten-day-old adult bees were collected randomly from the hive and reared under artificial conditions (34 °C, 60% relative humidity and constant darkness). The bees were randomly divided into 8 groups, and each group contained 30 individuals. Groups 1, 2 and 3 were exposed to 4 °C, 42 °C and UV (254 nm, 30 MJ/cm^2), respectively. Groups 4–8 were treated with H_2O_2 (0.5 μL of 2 mM H_2O_2 was injected to the thoracic notum of bees), $HgCl_2$ (3 mg/mL was added to the basic adult diet), and pesticides (acaricide, phoxim and pyriproxyfen, 2.0 mg/L was added to the basic adult diet), respectively. The control group fed on a normal diet containing water, 70% powdered sugar and 30% honey from the source colonies. The whole bodies of the bees were flash-frozen in liquid nitrogen at the appropriate time and stored at −70 °C.

2.2. RNA extraction, cDNA synthesis, and DNA preparation

Total RNA was isolated from adult bees using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The extracted RNA was incubated with RNase-free DNase I (Promega, Madison, WI, USA). Then, the RNA was used to obtain the first-strand cDNA using the EasyScript First-strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) following the manufacturer's instructions. Genomic DNA was extracted from the adult bees using the EasyPure Genomic DNA Extraction Kit (TransGen Biotech) according to the manufacturer's instructions.

2.3. Primers and PCR amplification conditions

The primers and PCR amplification conditions are listed in Tables 1 and 2, respectively.

2.4. Cloning of the full-length cDNA of AccTpx5

To obtain internal conserved gene fragments, two PCR primers PF and PR were designed and synthesised based on the conserved regions of Tpxs in several insects. Then, rapid amplification of cDNA ends PCR (RACE-PCR) was used to obtain the 5′ and 3′ cDNA fragments using specific primers (5PW/5PN and 3PW/3PN, respectively) as described by Yan et al. (2012). After assembly, specific primers for CSF and CSR were designed to clone the complete encoding sequence of AccTpx5. All of the PCR products were purified, cloned into the *pEASY*-T3 vectors (TransGen Biotech), and then transformed into *E. coli* competent cells for sequencing. The PCR primers and amplification conditions are shown in Tables 1 and 2, respectively.

Table 1
The primers in this study.

Abbreviation	Primer sequence (5′–3′)	Description
PF	CGCTACTATGGTTTTGTTAGGTG	cDNA sequence primer, forward
PR	CGATACAATGCGCACGTAAGA	cDNA sequence primer, reverse
5PW	TGGCAGTCATAGGTATACC	5′RACE reverse primer, outer
5PN	TAGTGCAATAACTTTCACTCCT	5′RACE reverse primer, inner
3PW	TCTTATATCCTGCTACTACT	3′RACE forward primer, outer
3PN	TCACTACAATTAACTGAGAAG	3′RACE forward primer, inner
AAP	GGCCACGCGTCGACTAGTAC$(G)_{14}$	Abridged Anchor Primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged Universal Amplification Primer
B25	GACTCTAGACGACATCGA	3′RACE universal primer, outer
B26	GACTCTAGACGACATCGA$(T)_{18}$	3′RACE universal primer, inner
CSF	CGCTACTATGGTTTTGTTAGGTG	Full-length cDNA sequence primer, forward
CSR	TTACAGCGGTTGCGATACAATG	Full-length cDNA sequence primer, reverse
DLF	GGGGTATTCTATTTTCGCATCCA	Real-time PCR primer, forward
DLR	CCATTTACGATGAGAATCGACTGA	Real-time PCR primer, reverse
β-s	TTATATGCCAACACTGTCCTTT	Standard control primer, forward
β-x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
G1	CGCTACTATGGTTTTGTTAGGTG	Genomic sequence primer, forward
G2	TCAACTTCTAAAGGATCTAAC	Genomic sequence primer, reverse
G3	CTTATATCCTGCTACTACTGG	Genomic sequence primer, forward
G4	TTACAGCGGTTGCGATACAATG	Genomic sequence primer, reverse
YHF	GATATCATGGTTTTGTTAGGTGAA	Protein expression primer, forward
YHR	GGATCCCAGCGGTTGCGATACAAT	Protein expression primer, reverse
QS1	GTACAACTGAGTTAGCTCGAG	IPCR forward primer, outer
QS2	GTCTTACGAATTATCTAGCCAATC	IPCR reverse primer, outer
QS3	CAGTCGATTCTCATCGTAAA	IPCR forward primer, inner
QS4	CACCTAACAAAACCATAGTAGCG	IPCR reverse primer, inner
QYZ1	TCAGCCTGCTATTATAAAACTCAT	Promoter special primer, forward
QYZ2	CACCTAACAAAACCATAGTAGCG	Promoter special primer, reverse