A novel 1-Cys thioredoxin peroxidase gene in *Apis cerana cerana*: characterization of *AccTpx4* and its role in oxidative stresses

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Abstract Thioredoxin peroxidase (Tpx), also named peroxiredoxin (Prx), is an important peroxidase that can protect organisms against stressful environments. *AccTpx4*, a 1-Cys thioredoxin peroxidase gene from the Chinese honey bee *Apis cerana cerana*, was cloned and characterized. The *AccTpx4* gene encodes a protein that is predicted to contain the conserved PVCTTE motif from 1-Cys peroxiredoxin. Quantitative real-time PCR (Q-PCR) and Western blotting revealed that *AccTpx4* was induced by various oxidative stresses, such as cold, heat, insecticides, H₂O₂, and HgCl₂. The in vivo peroxidase activity assay showed that recombinant AccTpx4 protein could efficiently degrade H₂O₂ in the presence of DL-dithiothreitol (DTT). In addition, disc fusion assays revealed that AccTpx4 could function to protect cells against oxidative stresses. These results indicate that *AccTpx4* plays an important role in oxidative stress responses and may contribute to the conservation of honeybees.

Keywords *Apis cerana cerana* · Thioredoxin peroxidase · Oxidative stress

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Introduction

Oxidative stress arises from a significant increase in the amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS) or a decrease in their detoxification mechanisms. Living organisms are constantly exposed to many natural sources of oxidative stress that are caused by environmental oxidants, toxic heavy metals, ionizing irradiation, heat shock, and inflammation (Wang et al. 2008). To protect against excessive ROS, organisms have developed various antioxidative enzymes; among the family of cysteine-based peroxidases, thioredoxin peroxidase (Tpx, also named peroxiredoxin) is an important thiol-specific antioxidant protein (McGonigle et al. 1998; Pushpamali et al. 2008; Rhee et al. 2001; Corona et al. 2006). Tpxs are antioxidant enzymes that are present ubiquitously in mammals, plants, yeast, and bacteria (Pedrajas et al. 2010; Perez-Sanchez et al. 2011; Srinivasa et al. 2012). The antioxidative activity of peroxiredoxin is the first to be studied and is known as the compound's most general biological function. However, recent research has shown that peroxiredoxins also function in signal transduction (Jarvis et al. 2012; Poole et al. 2011; Wood et al. 2003a) and the regulation of phospholipid metabolism (Chen et al. 2000; Manevich and Fisher 2005; Manevich et al. 2013; Nevalainen 2010). However, the inactivation of peroxiredoxins at high H₂O₂ levels can turn off peroxide defenses and preserve the pool of reduced thioredoxin; thus, peroxiredoxin can be used to repair proteins vital to survival (Karpus and Poole 2012).

Peroxiredoxins are responsible for antioxidant defense in bacteria (AhpC), yeast (thioredoxin peroxidase), and trypanosomatids (tryparedoxin peroxidase). They also appear to be fairly promiscuous with respect to the hydroperoxide substrates, and the specificities for the donor substrate—e.g., glutathione (GSH), thioredoxin, tryparedoxin, and the

analogous CXXC motifs of bacterial AhpF proteins, vary considerably between subfamilies (Hofmann et al. 2002). Peroxiredoxins are a family of cysteine-based peroxidases that are classified into 1-Cys Prx, typical 2-Cys Prx, and atypical 2-Cys Prx based on the number of conserved cysteine residues. The second conserved Cys that is present near the C terminus distinguishes the “2-Cys” from “1-Cys” groups of Prx enzymes. However, Prxs share a common fold and active site as well as a catalytic cycle that uses a conserved Cys residue, called Cys47 (Hall et al. 2009; Poole et al. 2011; Wood et al. 2003b). In the typical catalytic mechanism for Prxs, sulfenic acid is captured by the active site, and H_2O_2 is reduced to H_2O while the active site cysteine is oxidized to sulfenic acid (Karplus and Poole 2012; Poole et al. 2011).

Among the types of peroxiredoxins, 1-Cys Prx, a homodimer with a subunit molecular weight of 25 kDa, has been studied the least. Recently, *TgPrx2* of *Toxoplasma gondii* (Deponte and Becker 2005), 1-Cys-Prx of *Bombyx mori* (BmPrx) (Wang et al. 2008), *BiPrx1* of *Bombus ignites* (Hu et al. 2010), and 1-Cys-Prx of *Plamodium vivax* (Pv1-Cys-Prx) and *Plamodium knowlesi* (Pk1-Cys-Prx) (Hakimi et al. 2013) have been characterized. These proteins share high homology and play important roles in oxidation resistance; however, the roles of 1-Cys Prx in bees have not been investigated extensively.

As an important indigenous species of the Chinese honeybee, *Apis cerana cerana* (*A. cerana cerana*) not only provides rich nutrition via bee products but also plays an important role in crop pollination. It has been reported that bees are one of the animals that function as fresh power, promoting the rapid development of the agricultural economy due to their pollination function. Furthermore, Prxs play an important role in the antioxidant system of the honey bee (Corona and Robinson 2006). In this study, we cloned a 1-Cys Prx and tested its antioxidant activity against H_2O_2 . The results suggest that *AccTpx4* may function in the oxidative stress response of *A. cerana cerana*.

Materials and methods

Apis cerana cerana and treatments

The Chinese honeybee (*A. cerana cerana*) was collected in mimetic hives at experimental apiary of Shandong Agricultural University (Taian, China). The adult worker honeybees were caught at the 50th day after emergence. These honeybees were housed 30 per group and kept in an incubator with a constant temperature 34 °C, 60 % relative humidity under a 24-h dark condition. They were fed with basic adult food containing 30 % honey from the source colonies, 70 % powdered sugar, and water (Alaux et al. 2010) for 1 day before treatments. Group 1 was fed only the pollen-and-sucrose

solution during the treatments as control. Groups 2–4 were incubated under 4, 25, and 42 °C conditions. Groups 5–8 were treated as follows: H_2O_2 (0.5 μL of a 2 mM dilution was injected on the thoracic notum of each worker bee), HgCl_2 (3 mg/mL), and pesticides (cyhalothrin, acetamiprid, and phoxime which were diluted to the final concentration 20 mg/L) were added to food for stress. The worker honeybees also collected based on the age and shape named as L4–L6 (the fourth to the sixth day instar larvae), Pw (white-eyed pupae), Pp (pink-eyed pupae), Pb (brown-eyed pupae), Pd (dark-eyed pupa), A1 (1-day post-emergence adult), A15 (15-day post-emergence adult), and A30 (30-day post-emergence adult). At appropriate time after inoculation, the bees were flash-frozen in liquid nitrogen and stored at -80 °C.

Primers and PCR amplification conditions

The sequences of primers and PCR amplification conditions used in the study are listed in Table 1 and Table 2, respectively.

RNA extraction, cDNA synthesis

The total RNA was extracted with Trizol reagent (TransGen Biotech, Beijing) based on the manufacturer's instructions. The RNA samples for different treatments were digested with RNase-free DNase-I and used for complementary DNA (cDNA) synthesis with the reverse transcriptase system (TransGen Biotech, Beijing).

Isolation of the full-length cDNA of *AccTpx4*

The full-length cDNA of *AccTpx4* was amplified using reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends PCR (RACE-PCR) as described previously (Yu et al. 2011).

Bioinformation and phylogenetic analysis

Sequence identity of *AccTpx4* 1-Cys Prxs was analyzed using NCBI bioinformatics tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Multiple alignment analysis, the theoretical isoelectric point, and molecular weight prediction were conducted using DNAMAN software 7.0.2 (Lynnon Biosoft). Tertiary structure was predicted using SWISS-MODEL (<http://swiss-model.expasy.org/>). The phylogenetic tree was constructed by the neighbor-joining method using MEGA software 4.0.

Protein expression and purification

The coding region of *AccTpx4* was cloned into the pET-30a (+) expression vector using primers YH1 and YH2. The recombinant plasmid pET-30a (+)-*AccTpx4* was then

Table 1 The primers used in this study

Abbreviation	Primer sequence (5'-3')	Description
TP1	GATTGGCAAGGAGATTCATGGGTTG	cDNA sequence primer, forward
TP2	CAGAAGGCATTGACACTTTATCCACTC	cDNA sequence primer, reverse
3P1	TCTTGCGAGTTATAGATTCCTTGC	3' RACE forward primer, outer
3P2	GATGATCCAGAACAAGCGTT	3' RACE forward primer, inner
5P1	CAACCCATGAATCTCCTTGCCAAT	5' RACE reverse primer, outer
5P2	TCATGATCAGCAATTATCGGGT	5' RACE reverse primer, inner
AAP	GGCCACGCGTCGACTAGTAC(G) ₁₄	Abridged anchor primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged universal amplification primer
B26	GACTCTAGACGACATCGA(T) ₁₈	3' RACE universal primer, outer
B25	GACTCTAGACGACATCGA	3' RACE universal primer, inner
QP1	ATGAGAATTAATTCTATTGTGCCG	Full-length cDNA primer, forward
QP2	TTAATAATTTGTGGTTGTGCGGAC	Full-length cDNA primer, reverse
β -s	TTATATGCCAACACTGTCCTTT	Standard control primer, forward
β -x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
YH1	GGTACCATGAGAATTAATTCTATTGTG	Protein expression primer, forward
YH2	GGATCCATAATTTGTGGTTGTGCGGAC	Protein expression primer, reverse
TY1	GCGTTAACTGTTCTGTGCTTT	Real-time PCR primer, forward
TY2	CAAGGAATCTATAACTCGCAAG	Real-time PCR primer, reverse

transformed into BL21 (DE3) *Escherichia coli* cells, and expression in *E. coli* was induced by 1.0 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 37 °C. Purification of the fusion protein was performed as described previously (Yu et al. 2011).

Western blot

The purified protein was injected subcutaneously into white mice for the generation of antibodies as described by Yan et al. (2014). Total proteins were extracted from whole adult bees using the Tissue Protein Extraction Kit (CoWin Bioscience Co., Beijing, China). These total proteins were quantified using the BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Western blotting was performed according to the procedure by Zhang et al. (2013) using wet transfer method for preparing the replica.

Disc diffusion assay

Disc diffusion assays under oxidative stress were performed according to Zhang et al. (2013). Approximately, 5×10^8 bacterial cells were coated plates on LB-kanamycin agar plates and incubated at 37 °C for 1 h. Next, filter discs (8-mm diameter) soaked in different concentrations of cumene hydroperoxide (0, 10, 20, 50, or 100 mM) or t-butylhydroperoxide (0, 10, 30, 50, or 100 mM) were placed on the surface of the culture medium. The cells were cultivated at 37 °C for 24 h before the inhibition zones around the paper discs were measured.

In vitro peroxidase activity

Reduction of H₂O₂ by purified recombinant AccTpx4 protein was estimated as described previously (Yu et al. 2011).

Table 2 PCR amplification conditions

Primer pair	Amplification conditions
TP1/TP2	10 min at 94 °C; 40 s at 94 °C; 40 s at 54 °C; 40 s at 72 °C for 32 cycles; 10 min at 72 °C
3P1 B26	10 min at 94 °C; 40 s at 94 °C; 40 s at 50 °C; 40 s at 72 °C for 28 cycles; 10 min at 72 °C
3P2 B25	10 min at 94 °C; 40 s at 94 °C; 40 s at 53 °C; 30 s at 72 °C for 35 cycles; 10 min at 72 °C
5P1 AAP	10 min at 94 °C; 40 s at 94 °C; 40 s at 50 °C; 40 s at 72 °C for 28 cycles; 10 min at 72 °C
5P2 AUAP	10 min at 94 °C; 40 s at 94 °C; 40 s at 54 °C; 30 s at 72 °C for 35 cycles; 10 min at 72 °C
QP1/QP2	10 min at 94 °C; 40 s at 94 °C; 40 s at 50 °C; 50 s at 72 °C for 32 cycles; 10 min at 72 °C
YH1/YH2	10 min at 94 °C; 40 s at 94 °C; 40 s at 50 °C; 50 s at 72 °C for 30 cycles; 10 min at 72 °C

Increasing concentrations (0, 20, 40, 60, 80, and 100 $\mu\text{g/mL}$) of the purified recombinant AccTpx4 proteins were added into the reaction system, respectively, and then added H_2O_2 (final concentration of 200 mmol in reaction system) to initiate the reactions and incubated for 10 min at 37 °C. The decrease in absorbance at 475 nm was used to measure the peroxidase activity.

Quantitative real-time PCR

To identify the expression pattern of *AccTpx4*, the *AccTpx4* primers TY1/TY2 and the β -actin gene (XM640276) primers β -s/ β -x were used for Q-PCR with a modified volume containing 1.6 μL of diluted cDNA from different samples, 10.0 μL of Takara SYBR® premix Ex Taq™, 0.4 μL of each primer (10 pmol/mL), and 7.6 μL of ddH₂O from Yao et al. 2013. The Q-PCR amplification condition was as follows: initial denaturation at 95 °C for 30 s; 40 cycles (95 °C for 5 s, 55 °C for 15 s, and 72 °C for 15 s) and a single melt cycle from 65 to 95 °C. The linear relationship, amplification efficiency, and data analysis were conducted using CFX Manager Software version 1.1. The analysis of significant difference was determined by Duncan's multiple range tests using Statistical Analysis System (SAS) software version 9.1.

Results

Cloning and molecular characterization of *AccTpx4*

The full-length cDNA sequence of *AccTpx4* (GenBank accession number: KJ551847), containing a 73-base pair (bp) 5' untranslated region (UTR) and a 196-bp 3' UTR, was cloned by RT-PCR and RACE-PCR. The 663-bp open reading frame (ORF) encoded a protein of 220 amino acids that was predicted to be localized in the cytoplasm and to have a molecular mass of approximately 25.087 kDa and an isoelectric point of 6.30. Analysis of the deduced amino acid sequence indicated that the PVCTTE motif was conserved in 1-Cys peroxiredoxin (Wood et al. 2003b). Figure 1 indicated the peroxidatic cysteine and nearby histidine. Comparisons of AccTpx4 with other 1-Cys peroxiredoxins showed that AccTpx4 had the highest similarity to AmPrx (*Apis mellifera*, NP_001164444.1), BiPrx (*Bombus ignitus*, ACP44068.1), AePrx6 (*Acromyrmex echinator*, EGI64430.1), and GoPrx (*Gryllotalpa orientalis*, AAX18657.1), ranging from 100 to 76.82 %. To investigate the evolutionary relationship of the 1-Cys peroxidases in insects, a phylogenetic tree (Fig. 2) obtained using a neighbor-joining method showed that AccTpx4 was closely related to AmPrx through evolution.

Furthermore, to understand the conserved peroxiredoxin active site and catalytic mechanism of AccTpx4, the tertiary structure of AccTpx4 (Fig. 3) was predicted by the SWISS-

MODEL. The catalytic site is surrounded by a loop-helix structural motif that forms a narrow pocket, contributing to peroxide substrate attack by reaching across the dimer interface to interact with the other subunit of the domain-swapped homodimer. In addition, the sulfur atom of C47, which binds to arginine in the monomer, forms a new hydrogen bond with the nearby histidine, forming a dimer, and this interaction is conserved for 1-Cys Prx (Mizohata et al. 2005; Wood et al. 2003b).

Temporal and spatial expression patterns of *AccTpx4*

Peroxiredoxins are a widespread and highly expressed family of cysteine-based peroxidases that react very rapidly with H_2O_2 , organic peroxides, and peroxynitrite (Nelson et al. 2011). This large protein family, which has diverged from thioredoxin-like redox proteins, is widespread across phylogeny, and one or more members are typically expressed at high levels in many cell types (Wood et al. 2003b). To determine the expression profile of AccTpx4 during different developmental stages, the transcript levels from larvae, pupae, and adults were measured using Q-PCR. Figure 4a showed that *AccTpx4* was expressed at high levels in larvae, pupa, and adult bees. In addition, among the adult tissues studied, the messenger RNA (mRNA) level of *AccTpx4* in the head and thorax was higher than that in the abdomen. However, the mRNA level of *AccTpx4* in the muscle and midgut was higher than that in the epidermis (Fig. 4b). These results showed that *AccTpx4* was expressed at a higher level in the head and muscle than in other tissues.

Expression patterns of *AccTpx4* under environmental stresses

Prxs are induced by various stimuli such as temperature stress (Shi et al. 2012; Wang et al. 2008) and multiple other environmental stresses (Yu et al. 2011). To characterize the transcript levels of *AccTpx4* under various environmental stresses, adult bees were exposed to an artificial hostile environment or were fed reagents that cause oxidative stress and then Q-PCR was performed. As shown in Fig. 5a, the transcript levels of *AccTpx4* were increased 2.22-fold when the bees were exposed to 4 °C for 1 h and were 5.38-fold higher when the bees were exposed to 25 °C treatments for 1.5 h (Fig. 5b). However, heat treatment (42 °C) reached a peak after 1.5 h with an approximately 2.07-fold relative mRNA increase, followed by a sharp decrease (Fig. 5c), which is possibly due to the bees being nearly dead. When the bees were fed insecticide, the expression patterns of *AccTpx4* were different. The mRNA level of *AccTpx4* changed slightly under acetamiprid treatment (Fig. 5d). For cyhalothrin and phoxime treatments, the mRNA levels of *AccTpx4* were increased 3.06- and 1.74-fold at 2 and 1 h (Fig. 5e-f), respectively. When the bees treated

Fig. 1 Multiple amino acid alignments. A comparison of the amino acid sequences of AccTpx4 (GenBank accession number: KJ551847) from *A. cerana cerana*, AmTpx4 (NP_001164444.1) from *Apis mellifera*, AePrx6 (EG164430.1) from *Acromyrmex echinator*, BiPrx (ACP44068.1) from *Bombus ignitus*, and GoPrx (AAX18657.1) from

Gryllotalpa orientalis. Identical amino acid residues are shaded in dark color. The conserved 1-Cys Prx signature (FTPVCTTE) is underlined. The peroxidatic cysteine (C43) is marked with a black quadrangle, and the serine at position 28 (S28) of motif GX SXG is marked with a black triangle

with H_2O_2 or $HgCl_2$, the expression of *AccTpx4* was increased 1.69-fold after 1.5 h of H_2O_2 treatment (Fig. 5h). In addition, the transcription level of *AccTpx4* was induced within 3 h under $HgCl_2$ treatment (Fig. 5g).

Western blot analysis

To further understand the expression pattern of the AccTpx4 protein in response to environmental stresses, the recombinant AccTpx4 protein containing a 6× His tag was produced. After induction with IPTG, the proteins were separated by SDS-PAGE, which showed that the AccTpx4 fusion protein had a

molecular mass of approximately 31 kDa (Fig. 7a), and the prediction was confirmed using DNAMAN. The recombinant protein was then purified using Ni-nitrilotriacetic acid (NTA) spin columns to test the antioxidant activity, and then, the purified protein used to make the antibody for AccTpx4. Proteins from bees that exposed to acetamiprid, cyhalothrin,

Fig. 2 A phylogenetic tree generated by MEGA version 4.0 with Tpxs from various species. The numbers above and below the branches are the bootstrap values from 500 replicates. AccTpx4 is boxed. The GenBank accession numbers or NCBI references are indicated in parentheses

Fig. 3 The tertiary structure of AccTpx4. The locations of S28 and C43 are labeled respectively

Fig. 4 Expression patterns of *AccTpx4*. **a** The transcription level of *AccTpx4* at different developmental stages. L4–L6 (the fourth to the sixth day instar larvae), Pw (white-eyed pupae), Pp (pink-eyed pupae), Pb (brown-eyed pupae), Pd (dark-eyed pupa), A1 (1-day post-emergence adult), A15 (15-day post-emergence adult), and A30 (30-day post-

emergence adult). **b** The relative expression of *AccTpx4* in different body regions and tissues. Each value is given as the mean (SD) of three replicates. Letters on the bar represent significant difference at $P < 0.001$ as determined by Duncan's multiple range tests using SAS version 9.1 software

Fig. 5 Expression patterns of *AccTpx4* under different environmental stresses. The relative expression of *AccTpx4* under different stress conditions, such as cold (4, 25 °C) (**a**, **b**), heat (42 °C) (**c**), acetaminophen (**d**), cyhalothrin (**e**), phoxime (**f**), HgCl_2 (**g**) (3 mg/mL), and H_2O_2 (**h**) is shown. The expression levels were normalized to that of untreated bees (0 h), and each value is given as the mean (SD) of three replicates. Letters on the bar represent significant differences at $P < 0.001$ as determined by Duncan's multiple range tests using SAS version 9.1 software

Fig. 6 The Western blot assays of AccTpx4 recombinant protein exposed to acetamiprid, cyhalothrin, H_2O_2 , $HgCl_2$, phoxime, and 42 °C treatments (a–f). The total protein was immunoblotted with anti-AccTpx4. The signal of the binding reactions was visualized with HRP substrates

phoxime, H_2O_2 , $HgCl_2$, and heat at 42 °C were extracted and subjected to Western blot analysis. These results revealed that changes in the AccTpx4 protein levels were consistent with the changes in the transcription levels (Fig. 6).

Peroxidase activity of AccTpx4 recombinant protein

Prx can maintain proper cellular levels of H_2O_2 by removing unnecessary H_2O_2 (Greetham and Grant 2009; Poole et al. 2011; Rhee et al. 2001; Wood et al. 2003b). To examine the H_2O_2 peroxidase activity of the AccTpx4 fusion protein, the ability to remove H_2O_2 was measured in vitro. Purified recombinant AccTpx4 protein degraded H_2O_2 in a concentration-dependent manner in the presence of DTT, and the H_2O_2 content barely changed in the absence of DTT (Fig. 7b). Thus, AccTpx4 possesses the thiol-dependent antioxidant activity of removing H_2O_2 .

Fig. 7 **a** Expression of AccTpx4 recombinant protein. Recombinant AccTpx4 protein was separated by SDS-PAGE analysis, lane 1: molecular weight marker; lane 2: non-induced overexpression of pET-30a (+)-AccTpx4 in BL21; lane 3 and lane 4: induced overexpression of pET-30a (+)-AccTpx4 in BL21. The AccTpx4 recombinant protein was marked by

Disc fusion assays for oxidative stress

To further investigate the protective function of AccTpx4 toward oxidative stresses, disc fusion assays were performed in the presence of oxidative stresses. Cumene hydroperoxide and t-butylhydroperoxide were chosen as intracellular ROS because they are more stable than H_2O_2 when applied under cultivation conditions (Burmeister et al. 2008). After overnight cultivation, the zone diameters were measured. Figure 8 showed that the killing zones around the drug-soaked filters were smaller on the plates containing control bacteria than on those containing bacteria overexpressing AccTpx4.

Discussion

Peroxioredoxin (also called thioredoxin peroxidase) is a ubiquitous peroxidase that plays important roles in the

an arrow. **b** The peroxidase activity of the AccTpx4 recombinant protein. The H_2O_2 degradation activity with or without DTT for different concentrations of AccTpx4 protein. Letters on the bar represent significant differences at $P < 0.001$ as determined by Duncan's multiple range tests using SAS version 9.1 software

Fig. 8 Disc fusion assay using cumene hydroperoxide and t-butylhydroperoxide, and the zone diameters of the killing zones were showed in the histograms. **a** The cumene hydroperoxide concentrations of discs 1–5 are 0, 10, 20, 50, and 100 mM, respectively. **b** The t-butylhydroperoxide concentrations of discs 1–5 are 0, 10, 30, 50, and 100 mM, respectively

antioxidative response (Poole et al. 2011), signal transduction (Jarvis et al. 2012; Wood et al. 2003a), and the regulation of phospholipid metabolism (Chen et al. 2000; Manevich and Fisher 2005). 1-Cys Prx, also classified as Prx6 (Mizohata et al. 2005; Nelson et al. 2011), is a special type of peroxidase in which the resolving Cys is missing and the sulfenic acid of the peroxidatic Cys can be reduced by a heterologous thiol-containing reductant (Loumaye et al. 2008). In this study, we isolated a 1-Cys Prx (*AccTpx4*) from *A. cerana cerana*. As Fig. 1 showed, *AccTpx4* was predicted to have the PVCTTE motif, similar to TgPrx2 of *T. gondii* (Deponete and Becker 2005), AmTpx4 (Corona and Robinson 2006), and 1-Cys peroxiredoxin of yeast (Greetham and Grant 2009). The PVCTTE motif may act as a peroxidase to form a specific conformation similar to that of hORF6 (Mizohata et al. 2005) and is conserved in 1-Cys Prx (Trujillo et al. 2006; Wood et al. 2003b). In addition, the tertiary structure of *AccTpx4* (Fig. 3) indicated that it was a 1-Cys Prx that might have common functions and might participate in similar physiological processes, including cellular stress and antioxidative processes. However, it was noted that a GXSSG motif was located 14 amino acids before the PVCTTE motif and could be responsible for the phospholipase A₂ activities of 1-cysteine peroxiredoxin (Nevalainen 2010).

Peroxiredoxins are a fascinating group of thiol-dependent peroxidases that are ubiquitously expressed, with multiple isoforms present in most organisms (Poole et al. 2011). Each of these isoforms is unique in that they exhibit different expression patterns during development, distribute differently in

organelles, and produce different reaction intermediates during catalysis (Rhee et al. 2001). Moreover, Prxs can scavenge reactive oxygen species to protect organisms against oxidative damage. Figure 4b showed that *AccTpx4* was expressed in the head than in other tissues, a finding that may be attributed to the head sensing a hostile environment early. This result was different from that of *AccTpx1*, which was expressed at a higher level in the thorax (Yu et al. 2011). It has been reported that Prxs may act as a housekeeping-type gene to regulate the intracellular ROS level because its expression can be found in all stages of the bee life cycle (Wang et al. 2008). In addition, the accumulation of oxidized Prxs may indicate the disruption of cellular redox homeostasis (Poynton and Hampton 2014). Q-PCR analysis showed that *AccTpx4* was expressed throughout the life cycle of bees (Fig. 4a), in keeping with the results of previous studies. All of these results indicated that *AccTpx4* might play a positive role in the cellular redox homeostasis of bees.

The role of Prx enzymes as peroxidases has been demonstrated by increasing or decreasing their expression levels in various cell lines and by evaluating the sensitivity of the cells to oxidative insults (Rhee et al. 2012). Some members of the Prx family are upregulated in cells under stress conditions. The upregulation of the Prxs in cells and tissues under oxidative stress conditions is one of the cellular recovery responses that occur after oxidative damage (Ishii and Yanagawa 2007). The mRNA level of *AccTpx4* was induced by various stress conditions, such as cold and heat treatment (Fig. 5a–c). In addition, *AccTpx4* was more sensitive to cyhalothrin and

phoxime treatment to acetamiprid treatment (Fig. 5d–f). Western blot results also demonstrated the response of AccTpx4 to various oxidative stresses at the protein level (Fig. 6). These results provide insight to the role of *AccTpx4* in the oxidative stress responses. Additionally, the increased expression of *AccTpx4* exposure to various environmental stresses may be involved in the increased protection of oxidative stress. However, this finding needs to be further explored.

To further test the peroxidase activity of the AccTpx4 protein, its ability to remove H₂O₂ was measured in vitro. The recombinant protein existed mostly as a soluble protein (Fig. 7a), the same as BmPrx4 (Shi et al. 2012). Furthermore, the purified recombinant AccTpx4 protein could efficiently degrade H₂O₂ in the presence of DTT (Fig. 7b), similar to *Arenicola marina* PRDX6 (Loumaye et al. 2008) and AbTPx2 (Pushpamali et al. 2008) but different from the *AccTpx4* induced by H₂O₂ (Fig. 5h). In addition, the disc diffusion assay also proved that overexpressing AccTpx4 in *E. coli* cells could protect cells from oxidative stresses (cumene hydroperoxide and t-butylhydroperoxide) (Fig. 8). These results suggest that AccTpx4 can function as a peroxidase to remove H₂O₂. However, the regulation of the *AccTpx4* mRNA was more complex and requires more research.

Peroxiredoxins are a family of multifunctional antioxidant, thioredoxin-dependent peroxidases that have been identified in numerous organisms. The major functions of Prxs comprise cellular protection against oxidative stress, modulation of intracellular signaling cascades that apply H₂O₂ as a second messenger molecule, and regulation of cell proliferation (Immenschun and Baumgart-Vogt 2005). In conclusion, we have isolated and characterized a 1-Cys Prx from *A. cerana cerana* that is abundantly expressed in body regions and tissues. Additionally, *AccTpx4* responds to temperature change as well as insecticide, H₂O₂, and HgCl₂ treatments. Furthermore, the AccTpx4 protein expressed in *E. coli* could efficiently degrade H₂O₂, indicating that *AccTpx4* might play important roles in responses to environmental stress.

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