Identification and antioxidant characterisation of thioredoxin-like1 from *Apis cerana cerana*

Wenjing Lu, Mingjiang Kang, Xiufang Liu, Xiaocui Zhao, Xingqi Guo,
Baohua Xu

► To cite this version:

Wenjing Lu, Mingjiang Kang, Xiufang Liu, Xiaocui Zhao, Xingqi Guo, et al.. Identification and antioxidant characterisation of thioredoxin-like1 from *Apis cerana cerana*. *Apidologie*, 2012, 43 (6), pp.737-752. 10.1007/s13592-012-0148-7 . hal-01003669

HAL Id: hal-01003669

<https://hal.science/hal-01003669>

Submitted on 1 Jan 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Identification and antioxidant characterisation of thioredoxin-like1 from *Apis cerana cerana*

Wenjing LU¹, Mingjiang KANG², Xiufang LIU¹, Xiaocui ZHAO¹, Xingqi GUO¹,
Baohua XU²

¹State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong 271018, People's Republic of China

²College of Animal Science and Technology, Shandong Agricultural University, Taian, Shandong 271018, People's Republic of China

Received 12 January 2012 – Revised 3 May 2012 – Accepted 24 May 2012

Abstract – Thioredoxins (Trxs) play a crucial role in maintaining redox homeostasis and protecting organisms against toxic reactive oxygen species. Compared with the well-studied Trxs in Mammalia, little is reported in insects. In this study, we isolated a *Trx-like1* gene from *Apis cerana cerana* (*AccTrx-like1*), a single copy gene, and characterised its antioxidant activity. Quantitative real-time PCR and immunohistochemical assays revealed that *AccTrx-like1* was most highly expressed in larvae and was localised primarily in the epidermis and brain tissue of adults. Expression analyses indicated that *AccTrx-like1* expression is induced by both H₂O₂ and low temperatures. Moreover, catalase activity and malondialdehyde levels were inversely related to the expression levels of *AccTrx-like1* in honeybee injected with H₂O₂ at 30 min. Taken together, these results suggest that *AccTrx-like1* is an important antioxidant gene that is likely to play a role in preventing oxidative stress in *A. cerana cerana*.

Trx-like1 / *Apis cerana cerana* / quantitative real-time PCR / oxidative stress

1. INTRODUCTION

“Reactive oxygen species (ROS)” is a collective term for H₂O₂ and oxygen radicals, including superoxide (O₂^{•−}), hydroxyl (OH[•]) and hydroperoxyl (HO₂[•]) radicals. ROS actively participate in a diverse array of biological processes that include programmed cell death and the induction and maintenance of the transformed state (Finkel 2003). Excessive ROS lead to oxidative stress, which may be induced by external or internal factors and can damage cellular macromolecules, such as nucleic acids, proteins and lipids. This damage interferes with normal cellular functions and

can ultimately lead to cell death (Tsuda et al. 2010). Of the many biological targets for oxidative stress, lipids are the most often affected (Del et al. 2005). Lipid oxidation results in the generation of many harmful secondary products, such as malondialdehyde (MDA), which can interfere with the sensitive mechanisms involved in cell functionality. Relatively low or high environmental temperatures have also been shown to induce oxidative stress accompanied by a high level of lipid peroxidation (Yang et al. 2010). In addition, oxidative stress generates a number of superoxide radicals, which act as oxidants or reductants and result in the production of hydroxyl radicals (Lopez-Martinez et al. 2008). Typically, superoxide radicals are converted to H₂O₂, and catalase (CAT) then reduces H₂O₂ to water and molecular oxygen (Schwarze et al. 1998). Hydroxyl radicals, which

Corresponding author: X. Guo, xqguo@sdau.edu.cn;
B. Xu, bhxu@sdau.edu.cn
Manuscript editor: Klaus Hartfelder

are believed to be the main species responsible for oxidative damage, are formed if H_2O_2 cannot be eliminated (Fridovich 1978; Halliwell and Gutteridge 1984). In order to defend against the miscellaneous environmental oxidative stresses, social insects have evolved an autologous antioxidant system that includes a series of enzymes and non-enzymatic molecules (Evans et al. 2006). This endogenous antioxidant system (Tanaka et al. 2002) includes thioredoxin (Trx), thioredoxin peroxidase and thioredoxin reductase. Trx-dependent peroxidase, also known as peroxiredoxin, belongs to the Trx family.

Trxs are small, highly conserved oxidoreductase proteins required to maintain the redox homeostasis in many physiological cellular processes (Zhang et al. 2008; Gromer et al. 2004). Trx was first identified as an electron donor for ribonucleotide reductase in *Escherichia coli* (Laurent et al. 1964). An amino acid sequence (Cys-Gly-Pro-Cys) was later found to be linked to the conserved Trx active site, wherein the cysteine residues undergo reversible oxidation between the dithiol and disulfide forms (Jiménez et al. 2006). Seven Trx or Trx-like proteins have been identified in mammals, in which both tissue-specific and ubiquitously expressed forms have been characterised. Spyrou et al. (1997) reported the localisation of Trx2 within mitochondria in rat heart tissue. Sptrx (sperm-specific Trx) was the first Trx with a tissue-specific distribution to be studied in humans (Miranda-Vizuete et al. 2001), where it is expressed exclusively in spermatozoa. In addition, thioredoxin-like (Trx-like) proteins, which have similar structures and spatial topology to Trxs, belong to a group of thioredoxin-related proteins (TRPs) that is itself included in the Trx superfamily (Carvalho et al. 2006). Trx-like1, also known as TRP32, contains a Trx domain with a conserved CXXC active site (Lee et al. 1998), and human Trx-like1 is localised in the cytoplasm (Jiménez et al. 2006). Trxs serve an antioxidant function and have been studied extensively through both genetic and molecular approaches. For example, Trx-like1 participates in various redox reactions via the reversible oxidation and reduction of the two cysteine residues in

its active site (Lee et al. 1998). In mice, overexpression of Trx protects against miscellaneous disease phenotypes associated with oxidative stress (Zhou et al. 2009; Widder et al. 2009). Similarly, the loss of *Trx-2* in *Drosophila* was recently shown to exacerbate oxidative stress-dependent phenotypes (Tsuda et al. 2010).

Although Trx has been widely studied because of its antioxidant properties, reports focusing on the Trx-like proteins are limited, particularly in insects. The Chinese honeybee (*Apis cerana cerana*) is a major subspecies of *A. cerana* that is more resistant to adverse conditions than other subspecies (Yang 2005), making it important for agriculture. Recently, the numbers of *A. cerana cerana* colonies have decreased, possibly due to oxidative stress caused by infectious diseases, UV light or global warming (Xu et al. 2009), and inappropriate management. Here, we cloned and characterised the *Trx-like1* gene from *A. cerana cerana* and evaluated its level of expression in various tissues and at various developmental stages. To determine the expression patterns in various oxidative stresses, we also investigated the oxidative stress response of *AccTrx-like1* at various temperatures and to injection of H_2O_2 , after which we measured the activity of CAT and the concentration of MDA. This work provides valuable insight into the ability of *Trx-like1* to remove peroxides from *A. cerana cerana* under oxidative stress conditions.

2. MATERIALS AND METHODS

2.1. Insect material

A. cerana cerana at various stages of development (larvae, pupae and adult bees) were identified by age (Robinson 1992), eye colour (Rothenbuhler et al. 1952; Kerr and Laldlaw 1956) and cuticle pigmentation (Ziegler 1961). Bees were reared under artificial conditions in the experimental apiary of Shandong Agricultural University, Taian, China. Worker bees were collected in groups and kept in an incubator to induce oxidative stress. Adult bees

were divided into five experimental groups, incubated at various temperatures (4, 15, 25, 34 and 43 °C, respectively) and collected at the indicated time points (0.5, 1, 3, 5 and 7 h). Tissues samples from the brain, muscle, honey sac, epidermis and midgut were quickly isolated from adult bees. All bees and tissues samples were flash-frozen in liquid nitrogen at the indicated time points and stored at -80 °C.

2.2. Cloning and sequencing of *AccTrx-like1* cDNA

Total RNA was extracted from adult bees using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and incubated with RNase-free DNase I (Promega, Madison, WI, USA) to eliminate potential genomic DNA contamination. First-strand cDNA was synthesised from approximately 2 µg of total RNA using reverse transcriptase (TransGen Biotech, Beijing, China). The full-length cDNA of *AccTrx-like1* was obtained using primers Z1/Z2 via reverse transcription-PCR (RT-PCR) and with specific primers 3P1/3P2, B26/B25, 5P1/5P2 and AAP/AUAP via rapid amplification of cDNA ends-PCR (RACE-PCR). All primer sequences are listed in Table I.

2.3. Isolation of genomic DNA and the 5'-flanking region of *AccTrx-like1*

Genomic DNA was prepared from adult bees using the EasyPure Genomic DNA Extraction Kit (TransGen Biotech, Beijing, China) with primers N11/N12, N21/N22 and N31/N32. Overlapping PCR fragments were amplified using specific primers QP1/QP2 to isolate *AccTrx-like1* genomic DNA. The genomic DNA was then digested with *Pst*I at 37 °C overnight, followed by ligation with T4 DNA ligase (TaKaRa, Dalian, China). The ligation product was used as a template to obtain the 5'-flanking region via inverse polymerase chain reaction (IPCR) with the specific primers QDP1/QDP2 and QDP3/QDP4. We confirmed the sequence of the 5'-flanking region using the primers QDJ1/QDJ2 and analysed it using the TFSEARCH database (www.cbrc.jp/research/db/TFSEARCH.html). Putative transcription factor binding sites (TFBS) were predicted in this 5'-flanking region using the web software program MatInspector.

2.4. Southern blot

For Southern blot hybridisation, genomic DNA (30 µg/sample) from adult bees was completely digested with *Eco*RV, *Hind*III and *Xba*I for 48 h at 37 °C and then electrophoresed on a 1 % agarose gel and transferred to Hybond-N⁺ Nylon membrane (Amersham Pharmacia, England) by capillary blotting. The [α^{32} P]-radiolabelled probe was amplified using primers SB1/SB2 and the Primer-a-Gene Labelling System (Promega, Madison, WI, USA). Southern blot hybridisation was performed as described previously (Wang et al. 2010). Membranes were pre-hybridised overnight in blocking reagent and then incubated for 48 h at 65 °C with the gene-specific probe. After hybridisation, the membranes were washed twice in $\times 2$ SSC and 0.2 % SDS followed by $\times 0.2$ SSC and 0.2 % SDS for 10 min each at 42 °C. The radioactive signal was visualised using a FLA-7000 phosphorimager (Fujifilm).

2.5. Production, purification and western blot analysis of recombinant protein

The cDNA fragment containing the full-length open reading frame (ORF) was obtained using primers YH1/YH2, excised using *Bam*HI and *Sac*I and cloned into a pET-30a(+) (Novagen, Darmstadt, Germany) vector that has been digested with the same restriction enzymes. The recombinant plasmid, pET-30a(+)-*AccTrx-like1*, was then transformed into *E. coli* BL21 (DE3). Protein expression was induced with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 8 h at 37 °C at an optical density of $A_{600} = 1.5$, after which cultures were centrifuged at 5,000 rpm for 10 min at 4 °C. Supernatants were analysed by SDS-PAGE. *AccTrx-like1* was purified using the MagneHisTM Protein Purification System (Promega, Madison, WI, USA). After purification, the protein (100 µg mL⁻¹) was mixed with Freund's adjuvant and injected subcutaneously into white mice in order to generate specific antibodies. Western blot was carried out to confirm the specificity of the anti-*AccTrx-like1* antibody. Protein samples were loaded on 12 % SDS-PAGE gels and blotted onto a polyvinylidene difluoride membrane using a semi-dry transfer apparatus. The membrane was blocked with 5 % bovine serum albumin diluted in TBS before incubation with

Table 1. The primers used in this study.

Primer	Primer sequence
For the cloning of the full-length cDNA	
Internal fragment primers	
Z1	5'-GTGCGGGTACAAAATTGGTG-3'
Z2	5'-GTTGTTGAAATTGGTGAGCC-3'
3'-RACE primers	
3P1	5'-CCAATTGCTTTGCGTTATGTG-3'
3P2	5'-CTGGTAGTGAAGTAACAAGGATTG-3'
B26	5'-GACTCGAGTCGACATCGA(T) ₁₈ -3'
B25	5'-GACTCGAGTCGACATCGA-3'
5'-RACE primers	
5P1	5'-GGGCATTGCACTAACTCC-3'
5P2	5'-CACCAATTTTGTACCCGCAC-3'
AAP	5'-GGCCACGCGTCGACTAGTACG(G) ₁₄ -3'
AUAP	5'-GGCCACGCGTCGACTAGTACG-3'
The full-length cDNA primers	
QP1	5'-GCCGCCTTTCATGTTGTATTAAATC-3'
QP2	5'-TTAATGACTTTCTCCTTTCTTACC-3'
For the cloning of the genomic sequence	
Intron 1 primers	
N11	5'-GTGCGGGTACAAAATTGGTG-3'
N12	5'-GGGCATTGCACTAACTCC-3'
Intron 2 primers	
N21	5'-GGAGTTAGTGCAATGCCC-3'
N22	5'-GTTTCATCACATTCACTTTCC-3'
Intron 3 primers	
N31	5'-GGAAAGTGAATGTGATGAAC-3'
N32	5'-CACATAACGCAAAGCAATTGG-3'
For the cloning of the promoter region	
IPCR primers	
QDP1	5'-CACCAATTTTGTACCCGCAC-3'
QDP2	5'-GGAGTTAGTGCAATGCCC-3'
QDP3	5'-CCACCAATTTTGTACCCGC-3'
QDP4	5'-CCAAATGCAGTATTTCTTAAAG-3'
The full-length promoter primers	
QDJ1	5'-CTGCAGCTGACATCTCGCAATTTTCG-3'
QDJ2	5'-CACCAATTTTGTACCCGCAC-3'
Primers used in constructing expression vector	
YH1	5'- <u>GGATCC</u> GCCGCCTTTCATGTTG-3' <i>Bam</i> HI
YH2	5'- <u>GAGCTC</u> GACTTTCTCCTTTCTTACC-3' <i>Sac</i> I

Table 1. (continued).

Primer	Primer sequence
Real-time PCR primers	
D1	5'-GGAGTTAGTGCAATGCCC-3'
D2	5'-GTTTCATCACATTCACTTTCC-3'
β -actin1	5'-GTTTTCCCATCTATCGTCGG-3'
β -actin2	5'-TTTCTCCATATCATCCCAG-3'
Primer used for probe synthesised	
SB1	5'-GTGCGGGTACAAAATTGGTG-3'
SB2	5'-GGGCATTGCACTAACTCC-3'

anti-AccTrx-like1 (1:200) at 4 °C overnight. After washing with phosphate-buffered saline (PBS), the membrane was incubated with goat anti-mouse IgG (Dingguo, Beijing, China) conjugated to horseradish peroxidase (HRP), which was visualised with a chemiluminescent HRP substrate.

2.6. Immunohistochemical analysis

Adult bees were fixed with 4 % paraformaldehyde overnight at 4 °C. After being thoroughly washed in PBS, fixed samples were dehydrated with ethanol in a series of increasing concentrations, cleared in xylene and embedded in paraffin. Transverse sections (10 μ m) were prepared and mounted onto poly-L-lysine-coated glass slides and then dewaxed. The sections were treated with 3 % H_2O_2 in the dark to quench any endogenous peroxidases. After antigen retrieval performed twice with 0.01 M citrate (pH 6.0), the sections were blocked with 10 % normal goat serum and incubated at 4 °C overnight with the anti-AccTrx-like1 serum (1:20) diluted in 10 % normal goat serum. The following day, after washing in PBS, the sections were incubated at 37 °C with HRP-conjugated goat anti-mouse IgG (Boster Biological Technology, Wuhan, China) and then with SABC Immunohistochemistry Kit (Boster Biological Technology, Wuhan, China) at 37 °C. Colour development was performed using a DAB Horseradish Peroxidase Colour Development Kit (Boster Biological Technology, Wuhan, China). Immunolocalisation was observed using a light microscope. Negative controls were obtained by omitting the AccTrx-like1 antibody in the immunocytochemistry protocol.

2.7. Experimental injections

Prior to injection, the worker bees were placed on ice for 8 min. Adult bees (around 5 days after emergence) were injected with 5 μ L of H_2O_2 (50 μ M of H_2O_2 /worker) (Kim et al. 2011) or PBS (5 μ L/worker) for the injection controls between the first and second abdominal segments, using a sterile needle. Untreated bees, H_2O_2 -injected bees and PBS-injected bees were kept in cages, respectively, and they were all kept in an incubator with a controlled 60 % relative humidity at 34 °C until collection at the appropriate time points. They were fed on the basic adult diet containing 30 % honey from the source colonies and 70 % powdered sugar and water.

2.8. CAT activity assay and determination of MDA concentration

CAT activity and MDA concentration were measured using commercially available assay kits (Institute of Biologic Technology of Nanjing Jiancheng, Nanjing, China). CAT activity was measured as the development of a yellow complex produced by the reaction between H_2O_2 and molybdenum-acid-nedymium. The amount of H_2O_2 decomposition in 1 s by 1 mg protein was defined as 1 unit of CAT activity and expressed as units per milligramme protein. The absorbance was read at 405 nm. MDA, a reliable indicator of lipid peroxidation, was measured using thiobarbituric acid (TBA). Stored post-treatment specimens were ground, weighed and immersed in 0.02 mmol/L Tris-HCl at a ratio of 1:10 (in milligrammes per millilitre). Tissue homogenates were then centrifuged at 4,000 rpm for 10 min, and the

supernatants were transferred to a test tube. A standard solution was added to control test tubes, while distilled water was added to a blank test tube. After addition of TBA to each tube, samples were induced at 95 °C for 1 h, cooled to room temperature and centrifuged at 4,000 rpm for 10 min. MDA concentration was expressed as nanomoles of MDA per milligramme protein (Yang et al. 2010), and absorbance was read at 405 nm using a spectrophotometer (Spectrumlab S22pc, China).

2.9. Quantitative real-time PCR

AccTrx-like1-specific primers D1/D2 were used to amplify a PCR product of 274 bp and β -actin1/ β -actin2 primers were used to amplify an internal control gene fragment of 170 bp from *A. cerana cerana* β -actin (GenBank ID: XM640276). The quantitative real-time PCR (qRT-PCR) reactions were carried out in triplicate in a total volume of 25 μ L using a SYBR®PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. qRT-PCR was performed using a CFX96™ Real-time System (Bio-Rad) with the following parameters: 95 °C for 30 s, followed by 40 amplification cycles (95 °C for 5 s, 53 °C for 15 s and 72 °C for 15 s) and a melt cycle from 65 to 95 °C. All analyses were based on the CT values of the PCR products. The qRT-PCR data were analysed with CFX Manager Software (version 1.1) to estimate the transcript copy numbers for each sample. *AccTrx-like1* expression levels were calculated using the $2^{-\Delta\Delta C_t}$ comparative CT method (Livak and Schmittgen 2001). Data were represented as the triplicate mean $\pm$ SD (standard deviation) and presented as the *n*-fold difference relative to β -actin gene expression. Statistical significance was determined by one-way ANOVA and a post-hoc Tukey's test using the Statistical Analysis System (SAS), version 9.1 (SAS Institute, Cary, NC, USA). Significance was defined as $P < 0.05$. Three independent experiments were performed.

3. RESULTS

3.1. Isolation and molecular characterisation of *AccTrx-like1*

To explore the oxidative stress response of *Trx-like1* in *A. cerana cerana*, we isolated the

gene, designated *AccTrx-like1* (GenBank ID: JN699056), using RT-PCR and RACE-PCR. The full-length cDNA sequence of *AccTrx-like1* consists of a 157 bp 5'-untranslated region (UTR) and a 600-bp ORF that encodes 287-amino acid residues (a 3' UTR has not been isolated). The mature protein has a theoretical molecular mass of 31.5 kDa and a deduced pI of 4.94, as calculated using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html). Figure 1a shows that the protein sequence is closely related to the sequences from *Apis mellifera* (99.30 % protein sequence identity), *Drosophila melanogaster* (64.93 % protein sequence identity), *Bombyx mori* (67.36 % protein sequence identity) and *Nasonia vitripennis* (82.23 % protein sequence identity). Furthermore, *AccTrx-like1* contains a Trx domain with the conserved Cys- X_1X_2 -Cys active site and four typical C-terminal proteasome-interacting domains, characteristic of Trx-like (Figure 1a).

A phylogenetic tree of Trxs from various species was generated using a neighbour-joining method implemented in MEGA 4 to analyse the possible evolutionary relationships among Trxs. *AccTrx-like1* clearly belongs to the Trx-like1 superfamily and it displays obvious differences from other characteristic Trx superfamilies, including Trx-1, Trx-2, Trx-3, Trx-like2 and Trx-like3 (Figure 1b). *AccTrx-like1* is closely related to Trx-like1 from *A. mellifera*.

3.2. Structural analysis of the genomic sequence of *AccTrx-like1*

The full-length genomic sequence (no 3' UTR included; GenBank ID: JN699057) of *AccTrx-like1* is 1,987 bp and contains four exons separated by three introns, which is surprisingly similar to the structure of *AmTrx-like1* (Figure 2a). Southern blot analysis was performed to determine the copy number of *AccTrx-like1* in the *A. cerana cerana* genome. Genomic DNA was extracted from adult *A. cerana cerana* and individually digested with *EcoRV*, *HindIII* and *XbaI*. A specific region from the 5' end of the *AccTrx-like1* cDNA was

Figure 1. Characterisation of thioredoxins (*Trxs*) from various species. **a** Multiple amino acid sequence alignment of AccTrx-like1 from *A. cerana cerana*, AmTrx-like1 (GenBank ID: XP_623128.1) from *A. mellifera*, DmTrx-like1 (AAF66635.1) from *D. melanogaster*, BmTrx (NP_001040348.1) from *B. mori* and NvTrx-like1 (XP_001608075.1) from *N. vitripennis*. The conserved Trx-like1 sequence motifs are boxed, and conserved sites are marked with a dot. **b** A phylogenetic tree of Trxs from several species was constructed using the neighbour-joining method. Six classes of proteins (Trx-like1, Trx-2, Trx-like3, Trx-like2, Trx-3 and Trx-1) and five categories (Mammalia, Reptilia, Pisces, Insecta and Prokaryotea) are shown on the tree. The scale bar represents 0.02-amino acid substitutions per site. Bootstrap values, provided at each node, are an indication of the reliability of each branch within the phylogenetic tree. *A. cerana cerana* is highlighted in grey. Each gene name is followed by the protein ID. The species of origin for each Trx is displayed by the abbreviation before the gene name: *Mm* *Mus musculus*, *Hs* *Homo sapiens*, *Ac* *Anolis carolinensis*, *El* *Esox lucius*, *Ss* *Salmo salar*, *Am* *A. mellifera*, *Acc* *A. cerana cerana*, *Dm* *D. melanogaster*, *Mb* *Monosiga brevicollis*, *As* *Ascaris suum*, *Cb* *Caenorhabditis briggsae*, *Bt* *Bombus terrestris*, *Ag* *Anopheles gambiae*, *Cf* *Camponotus floridanus*.

Figure 2. Schematic representation of *Trx-like1* DNA structures and copy number analysis of the genomic sequence of *AccTrx-like1*. **a** Intron–exon structure of the *Trx-like1* gene from *C. briggsae* (*CbTrx-like1*, GenBank ID: XP_002639152.1), *N. vitripennis* (*NvTrx-like1*, GenBank ID: XP_001608075.1), *A. mellifera* (*AmTrx-like1*, GenBank ID: XP_623128.1) and *A. cerana cerana* (*AccTrx-like1*, GenBank ID: JN699057). Exons are indicated by grey boxes and introns by light grey boxes. UTRs are displayed as striped boxes in this alignment. A 3' UTR has not been isolated from *AccTrx-like1*. The translation initiation (ATG) and terminal codons are marked by inverted triangles and reference marks, respectively. **b** Southern blot analysis of *AccTrx-like1* from *A. cerana cerana*. Genomic DNA was completely digested with three restriction enzymes, *EcoRV*, *HindIII* and *XbaI*. A single discrete band was detected in each lane using hybridisation with a specific probe.

isolated for use as a probe. After hybridisation, a single discrete band was detected in each lane of the Southern blot containing the three restriction digests (Figure 2b), indicating that there is a single copy of *Trx-like1* in the *A. cerana cerana* genome.

3.3. Identification of the 5'-flanking region of *AccTrx-like1*

To study the organisation of the *AccTrx-like1* regulatory region, a 1,147-bp fragment upstream of the transcription start site was isolated by IPCR. Putative transcription factor binding sites (TFBS) were predicted in this 5'-flanking region. Several interesting TFBS that have been studied in *Drosophila* are shown in Figure 3. There were 11 heat shock factor (HSF) binding sites related to heat-induced transcriptional activation (Fernandes et al. 1994) and four CF2-II sites related to the zinc finger DNA-binding domain protein family (Gogos et al. 1992). Three homeobox sites were found to be related to gene expression (Stanojević et al. 1989). Three sites were also found for Broad-Complex (BR-C), which is an ecdysone-responsive key regulator of metamorphosis (von Kalm et al. 1994). We identified one deformed (Dfd) binding site, which is related to

the specificity of homeotic gene action (Egger et al. 1992) and two crocodile (Croc) binding sites, which are related to brain development (Jeffrey et al. 2000). In addition, basic TATA and CAAT boxes were identified in the 5'-flanking region of the promoter (data not shown). The above listed transcription factor binding sites are, so far, only computationally predicted ones, based on sequence similarity. They have neither been validated statistically, nor experimentally.

3.4. Expression patterns of *AccTrx-like1* at various development stages and in various tissues

To examine the stage- and tissue-specific expression of *Trx-like1* in *A. cerana cerana*, qRT-PCR assays were performed. Stage-specific expression analysis showed that the amount of *AccTrx-like1* mRNA increased rapidly from days 3 to 6 of larval development and then decreased from white- to pink-eyed pupae, reaching the lowest expression level in the adult stages. *AccTrx-like1* is therefore expressed at the highest level in larvae (Figure 4a). Various tissues were next extracted from adult bees; tissue-specific analyses indicated that high transcript levels are present in the epidermis and

Figure 3. Characterisation of the 5'-flanking region in *AccTrx-like1*. The transcription start site is indicated with an arrowhead. The predicted transcription factor-binding sites mentioned in the text are marked as follows: HSF, CF2-II, Dfd and BR-C sites are boxed, Hb sites are highlighted in light grey and Croc sites are highlighted in grey.

the brain. These results suggest that *AccTrx-like1* functions in early development and that it is primarily expressed in epidermis and brain.

3.5. Localisation of *AccTrx-like1*

To provide further insight into the role of *AccTrx-like1*, we next measured protein expression. SDS-PAGE analysis revealed a molecular mass of approximately 31 kDa for the recombinant protein, which was purified using the MagneHis™ Protein Purification System (Figure 5a). We used western blot analysis to

determine the specificity of the anti-*AccTrx-like1* antibody and observed a single band at approximately 31 kDa (Figure 5b), indicating that the anti-*AccTrx-like1* antibody is reasonably specific.

To determine the localisation of *AccTrx-like1* in epidermis and brain, immunohistochemistry was performed on paraffin sections from adult bees, using the anti-*AccTrx-like1* antibody. The results revealed that expression of *AccTrx-like1* is primarily localised to the median calyx of the mushroom bodies, the centre body, the surrounding regions of the antennal lobe (al) and

Figure 4. Expression patterns of *AccTrx-like1* at various developmental stages and tissues. **a** The distribution of *AccTrx-like1* transcripts at various developmental stages: larvae on days 3, 4 and 6; successive pupal stages including pupae with white (*Pw*) and pink (*Pp*) eyes; adults on day 1 (*A1*), nurses and foragers. **b** *AccTrx-like1* expression patterns in the brain, muscle, honey sac, epidermis and midgut, with the β -actin gene shown for comparison. Vertical bars represent the means $\pm$ SEM ($n=3$). Letters above the bars designate significant differences ($P<0.05$).

the lobula (lo) and medulla (me) of the optic lobes. Epidermis contained broadly expressed *AccTrx-like1* (Figure 5c).

3.6. Expression of *AccTrx-like1* at various temperatures

To assess the influence of temperature on the expression of *AccTrx-like1*, we divided the insects into five groups. We analysed the effect of temperature, with 34 °C representing hive tem-

perature as the control setting. Heat shock (43 °C) and indirect chilling injuries (4 and 15 °C) are considered to be main functional injuries (Yocum et al. 1994). As shown in Figure 6, *AccTrx-like1* expression was induced by shifts to temperatures between 4 or 25 °C within 0.5 h and peaked at 15 °C. Extended incubation at low temperatures increased the expression of *AccTrx-like1*, which was up-regulated by 3- or 7-h incubation at 4 °C and 1-, 5- or 7-h incubation at 15 °C. Interestingly, high temperatures (43 °C) did not result in

Figure 5. Overexpression, purification, western blot and immunohistochemistry analysis of AccTrx-like1. **a** Expression and purification of AccTrx-like1 protein, as shown by SDS-PAGE. *Lane 1*, non-induced expression of recombinant AccTrx-like1 protein; *lane 2*, overexpression of recombinant AccTrx-like1 protein after IPTG induction; *lane 3*, purified recombinant AccTrx-like1 protein and *lane M*, protein molecular weight marker. **b** Western blot analysis showing specificity of the anti-AccTrx-like1 antibody. *Lane 1*, the specific anti-AccTrx-like1. *Lane M*, pre-stained protein molecular weight marker. **c** Immunohistochemistry analysis of AccTrx-like1 expression in the brain and epidermis of *A. cerana cerana*. *Top*, the negative control staining; *bottom*, the tissues reacted with the AccTrx-like1 antibody. The major regions of the brain are indicated with arrows as follows: *me* medulla, *cb* centre body, *al* antennal lobe, *lo* lobula, *mc* median calyx of the mushroom body. Scale bars, 500 μ m.

up-regulation of *AccTrx-like1* expression. We conclude that *AccTrx-like1* expression is induced by low temperatures, while high temperatures inhibit its expression.

3.7. Expression profiles of *AccTrx-like1* in response to oxidative stress

To investigate the transcriptional expression profile of *AccTrx-like1* after H_2O_2 injection, *A.*

cerana cerana worker bees were injected with H_2O_2 , and RNA samples were prepared at various time points. We used qRT-PCR to analyse changes in the transcription of *AccTrx-like1* induced by H_2O_2 injection. As shown in Figure 7a, the mRNA levels of the *AccTrx-like1* were increased 30 min after H_2O_2 injecting compared with control. Expression then decreased from 45 to 180 min, with the lowest level of expression occurring at 120 min.

Figure 6. Expression patterns of *AccTrx-like1* after incubation at various temperatures (4, 15, 25, 34 and 43 °C) for various lengths of time (0.5, 1, 3, 5 and 7 h) as measured by qRT-PCR. Expression of the endogenous control gene β -actin was used for normalisation. Note that 34 °C is the normal temperature for *A. cerana cerana*; therefore, it was considered the control temperature for each treatment. Vertical bars represent the means $\pm$ SEM ($n=3$). Various letters above the bars designate significant differences ($P<0.05$).

In order to determine whether H_2O_2 influences CAT activity in *A. cerana cerana* worker bees or whether *AccTrx-like1* possesses the ability to remove H_2O_2 , we measured traditional oxidative stress parameters, including the activity of the antioxidant scavenging enzyme CAT and a highly toxic marker for lipid peroxidation, MDA (Yang et al. 2010; Del et al. 2005). MDA was assayed using TBA, and we measured CAT activity and MDA levels in vitro. As shown in Figure 7b, the MDA content increased within 15 min after H_2O_2 injection, declined at 30 min, and then consistently increased until 180 min. Figure 7c shows that the activity of CAT increased within 15 min after H_2O_2 injection and subsequently declined. We interpret these results to mean that MDA levels and CAT activity were both low at 30 min, when the expression of *AccTrx-like1* was relatively high, indicating that *AccTrx-like1* plays an antioxidant role at 30 min post-injection.

4. DISCUSSION

Oxidative stress plays a role in many different disease processes and is characterised by depletion of general antioxidant systems, leading to alterations in the cellular redox state (Jiménez et al. 2006). The balance between ROS production and antioxidants determines the degree of oxidative stress (Finkel and

Holbrook 2000). Previous studies have demonstrated that members of the Trxs family are essential proteins directly related to the cellular antioxidant network (Gromer et al. 2004). In this work, we have described the sequence features and expression profile of *AccTrx-like1*. Clearly, *AccTrx-like1* plays an important role during short periods of oxidative stress, particularly those related to low environmental temperatures.

Stage-specific expression analysis revealed that *AccTrx-like1* expression in the early larval stage is higher than that observed in adults. One explanation for this pattern is that other defensive systems are not yet fully developed in the larvae, making them more susceptible to diseases and other stressors (Figure 4a). Both biotic and abiotic factors can result in elevated levels of ROS, and insects have evolved a sophisticated response to oxidative stress. It is reasonable to assume that this defensive system takes time to mature and functions imperfectly at larval stages. Accordingly, there are many antioxidative genes that have been shown to respond to oxidative stress in vivo (Corona and Robinson 2006). When an organism is exposed to certain stimuli such as disease, the expression of these antioxidative genes may increase as a protective mechanism. Nevertheless, the adult bees are mainly subjected to abiotic stress especially the oxidative stress. Our findings imply that *AccTrx-like1* may function earlier

Figure 7. Expression patterns of *AccTrx-like1* induced by H_2O_2 overload and detection of MDA content and CAT activity. In all experiments, untreated *A. cerana cerana* worker bees were used as controls (CK). *A. cerana cerana* worker bees injected with PBS were used as injection controls. **a** Induction of *AccTrx-like1* in worker bees was analysed by quantitative PCR, with the β -actin gene used for normalisation. **b** Effects of H_2O_2 -induced stress on MDA concentration (in nanomoles per milligramme protein) in *A. cerana cerana*. **c** Effects of H_2O_2 -induced oxidative stress on CAT activity (in units per milligramme protein) in *A. cerana cerana*. Vertical bars represent the means $\pm$ SEM ($n=3$). Various letters above the bars designate significant differences ($P<0.05$).

than other antioxidative genes. The expression of *AccTrx-like1* was relative low in adults, which could be due to the mature defensive system and the sufficient protection provided by the adult epidermis. Although the overall expression of *AccTrx-like1* in adults was low, tissue-specific analysis revealed specific, local-

ised *AccTrx-like1* expression (Figure 4b). The honeybee brain, which is important for organised social and behavioural patterns, is very sensitive to ROS (Ament et al. 2008; Rival et al. 2004). The epidermis bears the brunt of external attacks and thus could be important in resistance to external stressors, including oxidative stress

(Marionnet et al. 2003). High expression of *AccTrx-like1* in these two tissues may further defend the honeybee against oxidative stress.

Many social insects maintain precise environmental conditions within their nests by controlling such parameters as relative humidity, temperature and carbon dioxide levels (Jones et al. 2005). These conditions are thought to be those that are optimal for normal development of the brood (McMullan and Brown 2006). Temperature has been identified as the dominant abiotic factor directly affecting herbivorous insects, and honeybee colonies maintain their brood nests between 33 and 36 °C (Kleinhenz et al. 2003). When living organisms are subjected to environmental stresses such as extreme temperatures, excessive ROS are generated. As shown in Figure 6, we observed that high temperatures suppressed expression at nearly every time point. The inhibition of expression by high temperatures may be due to the inactivation of *AccTrx-like1* (Ikemoto 2005) and other antioxidant gene products. Because relatively low temperatures induced high expression of *AccTrx-like1*, we conclude that it is a cold-inducible gene. Low temperatures are harmful to insects (Michaud and Denlinger 2004) because they suppress flight metabolic rates and stimulate oxygen radical formation (Harrison and Fewell 2002). Therefore, *A. cerana cerana* benefits from being cold tolerant, and *AccTrx-like1* likely plays an important role in the antioxidant response to cold stress.

To further characterise the *AccTrx-like1* response to oxidative stress, H_2O_2 was used to stimulate oxidative stress-induced apoptosis (Hasnain et al. 1999). We examined the expression profiles of *AccTrx-like1* after H_2O_2 injection in worker bees. The results demonstrate that after H_2O_2 overload, *AccTrx-like1* expression is induced at early time points and then decreases over time. This result differs from those observed in response to temperature changes. *AccTrx-like1* appears to play an immediate antioxidant role as a result of short-time oxidative stress caused by H_2O_2 , particularly at 30 min (Figure 7). In this study, because of the low CAT activity observed at 30 min, MDA levels would be expected to

increase. However, our results show that the MDA content was also low, suggesting that *AccTrx-like1* might be playing a critical role in the response to oxidative stress. At 30 min, the activity of CAT was low due to inactivation of the enzyme. After bees were injected with H_2O_2 , the antioxidant response was presumably activated resulting in the production many other enzymes designed to protect against oxidative stress and maintain the MDA concentration at a low level. Eventually, these antioxidative mechanisms become depleted as free radicals accumulate, lipid peroxides continue to degrade, and the level of MDA increases. OH^- is one example of a ROS with active chemical properties, whose accumulation could result in oxidative injury. CAT serves to protect the organism by breaking down H_2O_2 into H_2O and O_2 . After injection of H_2O_2 , it is possible that OH^- levels were not high enough to activate CAT but still caused damage to cells. In other words, OH^- may inhibit CAT activity until the accumulation of ROS reaches a level sufficient to induce expression of CAT (Breusegem et al. 2001). In contrast, the expression of *AccTrx-like1* was inversely related to CAT activity at early time points, suggesting that *AccTrx-like1* might play a critical role in the response to short-time oxidative stress. Thus, when Chinese honeybees were exposed to H_2O_2 for short periods of time, the expression of *AccTrx-like1* was induced, presumably to protect against oxidative stress (Figure 7).

Taken together, the results obtained in the present study indicate that *AccTrx-like1* plays an antioxidant role in response to oxidative stress caused by short-term H_2O_2 exposure and low temperatures. These findings provide evidence for the importance of *AccTrx-like1* in the Chinese honeybee and should encourage additional work aimed at understanding this antioxidant gene.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation (No. 31172275) of China, China Agriculture Research System (No. CARS-45) and National Department Public Benefit Research Foundation (No. 200903006).

Identification de la TXNL1 d'*Apis cerana cerana* et caractérisation de son activité anti-oxydante.

Apidae / PCR quantitative en temps réel / stress oxydatif / thiorédoxine-like protéine1

Identifizierung des thioredoxin-like1 Gens von *Apis cerana cerana* und Charakterisierung seiner antioxidanten Wirkung.

Trx-like1 / *Apis cerana cerana* / quantitative Real-time PCR / oxidativer Stress

REFERENCES

- Ament, S.A., Corona, M., Pollock, H.S., Robinson, G.E. (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc. Natl. Acad. Sci. USA* **105**, 4226–4231
- Breusegem, F.V., Vranová, E., Dat, J.F., Inzé, D. (2001) The role of active oxygen species in plant signal transduction. *Plant Sci.* **161**, 405–414
- Carvalho, A.P., Fernandes, P.A., Ramos, M.J. (2006) Similarities and differences in the thioredoxin superfamily. *Prog. Biophys. Mol. Biol.* **91**, 229–248
- Corona, M., Robinson, G.E. (2006) Genes of the antioxidant system of the honey bee: annotation and phylogeny. *Insect Mol. Biol.* **15**, 687–701
- Del, R.D., Stewart, A.J., Pellegrini, N. (2005) A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* **15**, 316–328
- Ekker, S.C., von Kessler, D.P., Beachy, P.A. (1992) Differential DNA sequence recognition is a determinant of specificity in homeotic gene action. *EMBO J.* **11**, 4059–4072
- Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imtru, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D. (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol.* **15**, 645–656
- Fernandes, M., Xiao, H., Lis, J.T. (1994) Fine structure analyses of the *Drosophila* and *Saccharomyces* heat shock factor–heat shock element interactions. *Nucleic Acids Res.* **22**, 167–173
- Finkel, T. (2003) Oxidant signals and oxidative stress. *Curr. Opin. Cell Biol.* **15**, 247–254
- Finkel, T., Holbrook, N.J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239–247
- Fridovich, I. (1978) The biology of oxygen radicals. *Science* **201**, 875–880
- Gogos, J.A., Hsu, T., Bolton, J., Kafatos, F.C. (1992) Sequence discrimination by alternatively spliced isoforms of a DNA binding zinc finger domain. *Science* **257**, 1951–1954
- Gromer, S., Urig, S., Becker, K. (2004) The thioredoxin system—from science to clinic. *Med. Res. Rev.* **24**, 40–89
- Halliwell, B., Gutteridge, J.M.C. (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219**, 1–14
- Harrison, J.F., Fewell, J.H. (2002) Environmental and genetic influences on flight metabolic rate in the honey bee, *Apis mellifera*. *Comp. Biochem. Physiol. A Mol. Integr.* **133**, 323–333
- Hasnain, S.E., Taneja, T.K., Sah, N.K., Mohan, M., Pathak, N., Sahdev, S., Athar, M., Totey, S.M., Begum, R. (1999) *In vitro* cultured *Spodoptera frugiperda* insect cells: model for oxidative stress-induced apoptosis. *J. Biosci.* **24**, 13–19
- Ikemoto, T. (2005) Intrinsic optimum temperature for development of insects and mites. *Environ. Entomol.* **34**, 1377–1387
- Jeffrey, P.L., Capes-Davis, A., Dunn, J.M., Tolhurst, O., Seeto, G., Hannan, A.J., Lin, S.L. (2000) CROC-4: a novel brain specific transcriptional activator of c-fos expressed from proliferation through to maturation of multiple neuronal cell types. *Mol. Cell. Neurosci.* **16**, 185–196
- Jiménez, A., Peltó-Huikko, M., Gustafsson, J.A., Miranda-Vizuete, A. (2006) Characterization of human thioredoxin-like-1: potential involvement in the cellular response against glucose deprivation. *FEBS Lett.* **580**, 960–967
- Jones, W.D., Nguyen, T.A., Kloss, B., Lee, K.J., Vossall, L.B. (2005) Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr. Biol.* **15**, R119–R121
- Kerr, W.E., Laldlaw, H.H. (1956) General genetics of bees. *Adv. Genet.* **8**, 109–153
- Kim, B.Y., Hiu, W.L., Lee, K.S., Wan, H., Yoon, H.J., Gui, Z.Z., Chen, S., Jin, B.R. (2011) Molecular cloning and oxidative stress response of a sigma-class glutathione S-transferase of the bumblebee *Bombus ignites*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **158**, 83–89
- Kleinhenz, M., Bujok, B., Fuchs, S., Tautz, J. (2003) Hot bees in empty broodnest cells: heating from within. *J. Exp. Biol.* **206**, 4217–4231
- Laurent, T.C., Moore, E.C., Reichard, P. (1964) Enzymatic synthesis of deoxyribonucleotides. *J. Biol. Chem.* **239**, 3436–3444
- Lee, K.K., Murakawa, M., Takahashi, S., Tsubuki, S., Kawashima, S., Sakamaki, K., Yonehara, S. (1998) Purification, molecular cloning, and characterization of TRP32, a novel thioredoxin-related mammalian protein of 32 kDa. *J. Biol. Chem.* **273**, 19160–19166
- Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods* **25**, 402–408

- Lopez-Martinez, G., Elnitsky, M.A., Benoit, J.B., Lee Jr., R.E., Denlinger, D.L. (2008) High resistance to oxidative damage in the Antarctic midge *Belgica antarctica*, and developmentally linked expression of genes encoding superoxide dismutase, catalase and heat shock proteins. *Insect Biochem. Mol. Biol.* **38**, 796–804
- Marionnet, C., Bernerd, F., Dumas, A., Verrecchia, F., Mollier, K., Compan, D., Bernard, B., Lahfa, M., Leclaire, J., Medaisko, C., Mehul, B., Seité, S., Mauviel, A., Dubertret, L. (2003) Modulation of gene expression induced in human epidermis by environmental stress in vivo. *J. Invest. Dermatol.* **121**, 1447–1458
- McMullan, J.B., Brown, M.J. (2006) Brood-cell size dose not influence the susceptibility of honey bees (*Apis mellifera*) to infestation by tracheal mites (*Acarapis woodi*). *Exp. Appl. Acarol.* **39**, 273–280
- Michaud, M.R., Denlinger, D.L. (2004) Molecular modalities of insect cold survival: current understanding and future trends. *Int. Congr. Ser.* **1275**, 32–46
- Miranda-Vizuete, A., Ljung, J., Damdimopoulos, A.E., Gustafsson, J.A., Oko, R., Pelto-Huikko, M., Spyrou, G. (2001) Characterization of Sptx, a novel member of the thioredoxin family, specifically expressed in human spermatozoa. *J. Biol. Chem.* **276**, 31567–31574
- Rival, T., Soustelle, L., Strambi, C., Besson, M.T., Iche, M., Birman, S. (2004) Decreasing glutamate buffering capacity triggers oxidative stress and neuropil degeneration in *Drosophila* brain. *Curr. Biol.* **17**, 599–605
- Robinson, G.E. (1992) Regulation of division of labor in insect societies. *Annu. Rev. Entomol.* **37**, 637–665
- Rothenbuhler, W.C., Gowen, J.W., Park, O.W. (1952) Androgenesis with zygogenesis in gynandromorphic honeybees (*Apis mellifera* L.). *Science* **115**, 637–638
- Schwarze, S.R., Weindrich, R., Aiken, J.M. (1998) Oxidative stress and aging reduce COX I RNA and cytochrome oxidase activity in *Drosophila*. *Free Radic. Biol. Med.* **25**, 740–747
- Spyrou, G., Enmark, E., Miranda-Vizuete, A., Gustafsson, J. (1997) Cloning and expression of a novel mammalian thioredoxin. *J. Biol. Chem.* **272**, 2936–2941
- Stanojević, D., Hoey, T., Levine, M. (1989) Sequence specific DNA-binding activities of the gap proteins encoded by hunchback and Krüppel in *Drosophila*. *Nature* **341**, 331–335
- Tanaka, T., Hosoi, F., Yamaguchi-Iwai, Y., Nakamura, H., Masutani, H., Ueda, S., Nishiyama, A., Takeda, S., Wada, H., Spyrou, G., Yodoi, J. (2002) Thioredoxin-2(TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO J.* **21**, 1695–1703
- Tsuda, M., Ootaka, R., Ohkura, C., Kishita, Y., Seong, K.H., Matsuo, T., Aigaki, T. (2010) Loss of Trx-2 enhances oxidative stress-dependent phenotypes in *Drosophila*. *FEBS Lett.* **584**, 3398–3401
- von Kalm, L., Crossgrove, K., Von Seggern, D., Guild, G.M., Beckendorf, S.K. (1994) The Broad-Complex directly controls a tissue-specific response to the steroid hormone ecdysone at the onset of *Drosophila metamorphosis*. *EMBO J.* **13**, 3505–3516
- Wang, M., Kang, M., Guo, X., Xu, B. (2010) Identification and characterization of two phospholipids hydroperoxide glutathione peroxidase genes from *Apis cerana cerana*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **152**, 75–83
- Widder, J.D., Fraccarollo, D., Galuppo, P., Hansen, J.M., Jones, D.P., Ertl, G., Bauersachs, J. (2009) Attenuation of angiotensin II-induced vascular dysfunction and hypertension by overexpression of thioredoxin 2. *Hypertension* **54**, 338–344
- Xu, P., Shi, M., Chen, X.X. (2009) Antimicrobial peptide evolution in the Asiatic honey bee *Apis cerana*. *PLoS One* **4**, e4239
- Yang, G.B. (2005) Harm of introducing the western honeybee *Apis mellifera* L. to the Chinese honeybee *Apis cerana* F. and its ecological impact. *Acta Entomol. Sin.* **3**, 401–406
- Yang, L.H., Huang, H., Wang, J.J. (2010) Antioxidant responses of citrus red mite, *Panonychus citri* (McGregor) (Acari: Tetranychidae), exposed to thermal stress. *J. Insect Physiol.* **56**, 1871–1876
- Yocum, G.D., Zdárek, J., Joplin, J.H., Lee Jr., R.E., Smith, C.D., Manter, K.D., Denlinger, D.L. (1994) Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *J. Insect Physiol.* **40**, 13–21
- Zhang, Y., Bao, R., Zhou, C.Z., Chen, Y. (2008) Expression, purification, crystallization and preliminary X-ray diffraction analysis of thioredoxin Trx1 from *Saccharomyces cerevisiae*. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **64**, 323–325
- Zhou, F., Gomi, M., Fujimoto, M., Hayase, M., Marumo, T., Masutani, H., Yodoi, J., Hashimoto, N., Nozaki, K., Takagi, Y. (2009) Attenuation of neuronal degeneration in thioredoxin-1 overexpressing mice after mild focal ischemia. *Brain Res.* **1272**, 62–70
- Ziegler, I. (1961) Genetic aspects of ommochrome and pterin pigments. *Adv. Genet.* **10**, 349–403