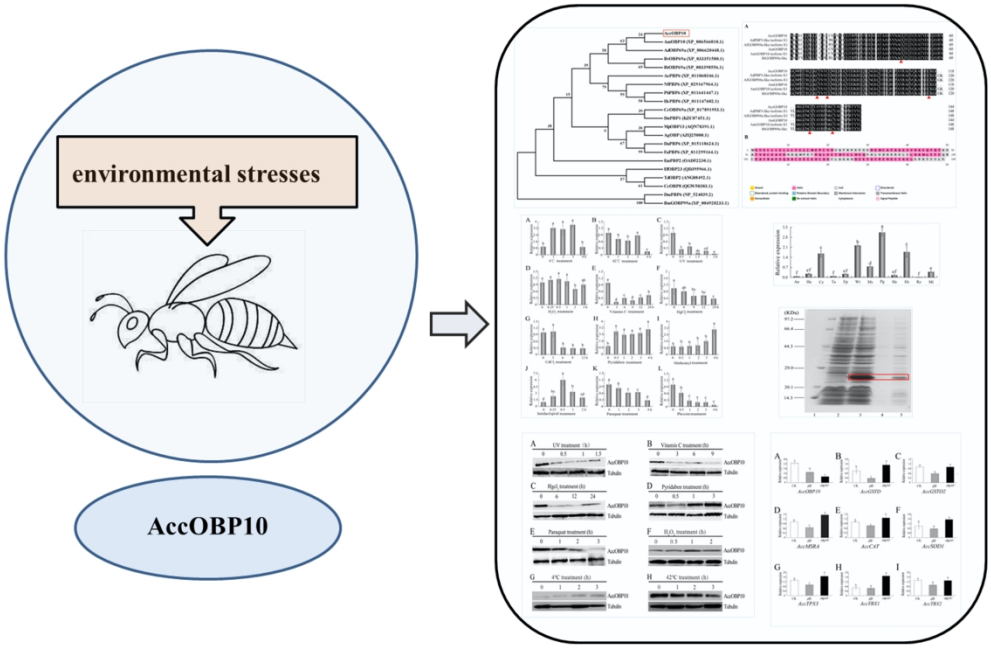

Honeybees are faced with various adverse environmental pressures. In this study, an OBP gene (AccOBP10) was isolated from bees and its function was explored through experiments.
The results indicate that AccOBP10 may be involved in The response to stress conditions.

179x120mm (600 x 600 DPI)

Molecular and functional characterization of the novel odorant-binding protein gene *AccOBP10* from *Apis cerana cerana*

Dezheng Guo [a], Cuihong Hao[a], Xuepei Cui[b], Ying Wang[b], Zhenguo Liu[b], Baohua Xu[b*], Xingqi Guo[a*]

[a]*State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong, 271018, P. R. China*

[b]*College of Animal Science and Technology, Shandong Agricultural University, Taian, Shandong, 271018, P. R. China*

Running title : the role of AccOBP10 in response to stress

*Corresponding author. Tel.: +86 538 8245679, Fax: +86 538 8226399. E-mail addresses: xqguo@sdau.edu.cn (X. Guo), and bhxu@sdau.edu.cn (B. Xu)

Abbreviations

OBPs, Odorant-binding proteins; qRT-PCR, Quantitative real-time PCR; H_2O_2, hydrogen peroxide; UV, ultraviolet; $HgCl_2$, mercuric chloride; $CdCl_2$, cadmium chloride; RNAi, RNA interference; PBPs, pheromone-binding proteins; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GFP, green fluorescent protein.

Summary

Odorant-binding proteins (OBPs) play an important role in odor perception and transport in insects. However, little is known about whether OBPs perform other functions in insects, particularly in *Apis cerana cerana*. Within this study, an OBP gene (*AccOBP10*) was isolated and identified from *Apis cerana cerana*. Both homology and phylogenetic relationship analyses indicated that the amino acid sequence of AccOBP10 had a high degree of sequence identity with other members of the gene family. Analysis of real-time quantitative PCR (qRT-PCR) showed that *AccOBP10* mRNA was expressed at higher levels in the venom gland than in other tissues. The mRNA transcript expression of *AccOBP10* was upregulated by low temperature (4°C), hydrogen peroxide (H_2O_2), pyridaben, methomyl and imidacloprid but downregulated by heat (42°C), ultraviolet (UV) light, vitamin C, mercuric chloride ($HgCl_2$), cadmium chloride ($CdCl_2$), paraquat and phoxim. Expression of AccOBP10 under abiotic stress was analyzed by Western blotting, and the results were consistent with those of qRT-PCR. And as a further study of AccOBP10 function, we demonstrated that knockdown of *AccOBP10* by RNA interference (RNAi) could slightly increase the expression levels of some stress-related genes. Collectively, these results suggest that *AccOBP10* is mainly involved in the response to stress conditions.

Keywords: Odorant-binding protein, Molecular characterization, *Apis cerana cerana*, Abiotic stress, Expression analysis

Insect odorant-binding proteins (OBPs) constitute a class of highly soluble acidic proteins. The first OBP identified in insects was found in the antennae of male *Antheraea polyphemus* moths *(1)*, and this discovery began the study of OBPs.

Studies have found that this protein plays a major role as a soluble protein carrier in a variety of organisms, including bacteria and mammals *(2)*. A specific subgroup of OBPs includes general OBPs (GOBPs) and PBPs *(3)*. OBPs are small soluble polypeptides that detect and release chemical stimuli in the sensory organs of vertebrates and the secretory glands of insects *(4)*, and they have an important function in the recognition of volatiles and sex pheromones *(5)*. The molecular weights of OBPs range from 17 to 22 kDa. Despite the poor sequence similarity between OBPs, their tertiary structure is highly conserved and consists of eight antiparallel *β*-sheets and a short α-helix segment near the C-terminus. *(6-11)*. Typical features of OBPs include six positionally conserved cysteines that form three disulfide bonds that maintain the tertiary protein structure.

Since an OBP was first identified as a female sex pheromone carrier from *Antheraea polyphemus (1)*, many OBPs have been observed in diverse insect species. Previous studies on the functions of insect OBPs have focused on their role as carriers. OBPs promote the reception of odor molecules or pheromones to a large extent and bind odor molecules and then transport them to specialized receptors. OBPs can also act as carriers by binding hydrophobic chemical pheromones, which are then delivered to an olfactory receptor or a sensory neuron membrane protein *(12,13)*. However, the expression and functional properties of most bioinformatically identified OBPs require further study.

Surprisingly, emerging evidence has shown that OBPs participate in responses to adverse environmental conditions *(14)*. ROS (reactive oxygen species) can be produced as a by-product of mitochondrial oxidative metabolism *(15)* in response to cellular invasion by foreign organisms, cytokines, and bacteria *(16)*, hydrogen peroxide (H_2O_2), hydroxyl radicals (HO^-), and superoxide anions (O^{2-}). Excessive

ROS lead to macromolecular damage to nucleic acids, proteins and lipids in various disease states such as atherosclerosis, carcinogenesis, cancer, aging and diabetes. *(16)*.

The OBPs act as scavengers of a number of small hydrophobic molecules, including odor compounds, insect kairomones, and cytotoxic compounds due to oxidative stress, such as 4-hydroxy-2-nonenal, which represent the end products of lipid peroxidation and are involved in the pathogenesis of a number of acute and chronic diseases, including cancer *(14,17)*. Overexpression of GCC-bOBP, a bovine redesigned monomeric mutants of OBP provide protection against chemically induced oxidative stress in *Escherichia coli (18)*. More than 177 OBP genes have been identified in bees, and four, OBP10, OBP13, OBP14 and OBP17, are highly expressed in areas with low chemosensory receptor expression *(19)*. We suspect that the proteins encoded by these genes may have other potential functions. A recent study also found that *Apis mellifera* OBP14 binding pocket interacts with the hydroxy group of eugenol by forming two hydrogen bonds. AmOBP14 can also strongly interact with ligands, such as homovanillic acid, eugenol, and methyl vanillate, all of which contain a hydroxy group *(20)*. There are several hypotheses regarding the roles of OBPs as versatile carriers/scavengers that require further investigation. OBPs, as carriers of odorous compounds or as scavengers of excess odorants, might participate in olfactory prereceptor events and defend against parasitism and infectious diseases. In addition, OBPs might be involved in the removal of toxic compounds produced locally by lipid peroxidation or inhalation *(14)*; therefore, we hypothesized that AccOBP might function in the abiotic stress response.

Being an important native species, *Apis cerana cerana* has a significant impact on regional ecology and agriculture. However, bees are exposed to a variety of adverse environmental factors when foraging on plants, including temperature

changes, heavy metals, pesticides, and ultraviolet (UV) light. As a result, honeybees, which are considered important agricultural insects, are decreasing in number. Thus, a study examining oxidative stress in honeybees would be of great significance. To the best of our knowledge, this study is the first report of OBP involvement in oxidative stress defense in *Apis cerana cerana.*

Materials and methods

Insects and treatments

Honey bees used in the following experiments were routinely reared in experimental apiaries at Shandong Agricultural University, Taian, China. Adult bees were reared in a constant temperature (34°C) and humidity (70%) incubator and fed 30% honey, 70% powdered sugar and water 24 hours before treatment *(21).* In this study, we selected 15-day-old workers for the expression and functional analyses of *AccOBP10*. Various tissues of 15-day-old workers, including the antenna (An), head (Ha), chest (Ce), total abdomen (Ta), epidermis (Ep), wing (Wi), muscle (Ms), poison gland (Pg), hemolymph (He), honey sac (Hs), rectum (Re) and midgut (Mi). were dissected on ice, which was followed by RNA extraction. A15 bees (15-day-old worker bees) collected at the entrance of the hive were randomly divided into 12 groups and exposed to various types of environmental stress. Groups 1, 2 and 3 were exposed to severe cold (0, 1, 2, 3, and 4 hours at 4°C), high heat (0, 1, 2, 3, and 4 h at 42°C) and UV radiation (0, 0.5, 1, 1.5, 2, and 3 h at 254 nm and 30 mJ/cm^2), respectively. Bees from group 4 were injected with H_2O_2 (20 μl, 50 mM) between the first and second abdominal segments and euthanized after 0.25, 0.5, 1, 2, 3 and 4 hours. Group 5 was treated with vitamin C (20,000 mg/kg) and euthanized after 3, 6, 9, 12 and 24 hours. Group 6 was treated with mercury ($HgCl_2$, 3 mg/ml) and

euthanized after 6, 9, 12 and 24 hours. Group 7 was treated with chromic chloride ($CdCl_2$, 3 mg/ml) and euthanized after 3, 6, 9 and 12 hours. Groups 8, 9, 10, 11 and 12 were treated with pesticides (2.0 mg/L pyridaben, methomyl, imidacloprid, paraquat and phoxim, respectively) and euthanized at the appropriate time point. All treatments from group 5 to group 12 were added to the basic adult diet. All honeybees were flash-frozen in liquid nitrogen at the appropriate time points and stored at -80°C.

RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and treated with RNase-free DNase I (Promega, Madison, WI, USA) to prevent potential genomic DNA contamination. First-strand cDNA was then synthesized using the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China).

Primers and amplification conditions

The sequences of primers used in this study are listed in Table 1.

Isolation of AccOBP10 cDNA

The primers CF and CR were designed and synthesized based on the *AccOBP10* sequence according to the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The PCR product was purified, ligated into the pEASY-T3 vector (TransGen Biotech, Beijing, China) and transformed into competent *E. coli* cells (DH5α) for sequencing.

Bioinformatics analysis

Using the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to select

homologous AccOBP sequences, conserved OBP domains of different species were predicted. DNAman software 7.0.2 (Lynnon Biosoft) was used to analyze the physics of AccOBP10 through multiple alignment and chemical properties, and these analyses included calculations of theoretical isoelectric points and predictions of molecular weight. Phylogenetic trees were generated using Molecular Evolutionary Genetic Analysis (MEGA version 4.1) software.

Fluorescent real-time quantitative PCR (qRT-PCR)

Fluorescence qRT-PCR was performed in a CFX96TM real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with SYBR Premix Ex Taq (TaKaRa) to determine the expression pattern of *AccOBP10.* The *AccOBP10* primers QF and QR were designed based on the *AccOBP10* cDNA sequence. The housekeeping gene *β-actin* (GenBank accession no. XM640276) was used as a reference gene, and its primers (*β*-F/*β*-R) were designed according to Wang et al. (2010). All experiments were performed in triplicate. The following cycle conditions were used for qRT-PCR analysis: initial denaturation at 95°C for 30 s; 40 cycles of 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 data were analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification method *(22)*. Duncan's multiple range tests were performed using Statistical Analysis System software (version 9.1) to determine differences among multiple groups. The means ± SE from three independent experiments are denoted by the error bars. Different letters above the error bars indicate significant differences, and the same letters indicate nonsignificant differences.

Protein preparation and purification

The *AccOBP10* open reading frame (ORF) with a stop codon and no signal peptide was amplified using a pair of primers containing *BamH*I and *Sal*I restriction sites to obtain the OBP10 protein. The open reading frame (ORF) of *AccOBP10* was amplified with a pair of primers containing the *BamHI* and *SalI* restriction sites to obtain the OBP10 protein. After ligation of the purified PCR product into the expression vector pET30a(+), the resulting recombinant plasmid pET30a(+)-AccOBP10 was transformed into BL21(DE3) Escherichia coli cells for protein expression. The predicted molecular weight of OBP10 was 14.88 kDa, but the recombinant protein also contained an 8 kDa molecular weight sequence with a His-tag. Therefore, the molecular weight of the recombinant protein was greater than 20 kDa. The cells were grown in 400 ml of Luria-Bertani (LB) broth supplemented with 30 μg/ml kanamycin until the cell density reached 0.4-0.6 OD_{600}. The expression was induced by adding 0.2 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 28°C for 6 hours. The recombinant protein was then purified using the MagneHis™ Protein Purification System (Promega, Madison, WI, USA) and analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Anti-AccOBP10 serum preparation and western blot analysis

Anti-AccOBP10 serum was obtained by subcutaneous injection of purified protein (100 μg) into five pathogen-free female mice as described by Yan et al. (2013). After the various stress treatments, total proteins were extracted using a Tissue Protein Extraction Kit (CoWin Bioscience Co., Beijing, China) and quantified with a BCA Protein Assay Kit (Thermo Scientific Pierce, IL, USA). Proteins from each sample were separated by 12% SDS-PAGE and electro transferred onto polyvinylidene fluoride (PVDF) membranes (Whatman, UK). Western blotting was

performed by wet transfer according to the procedure described by Meng et al.(2010).

RNA interference (RNAi)

The primer sequences RP/RR with a T7 polymerase promoter at their 5'-ends were used to synthesize linear DNA templates. The dsRNAs were generated as described previously (Elias et al. 2010). Newly emerged honeybees were divided into three groups for the RNA interference (RNAi) experiments. A synthetic green fluorescent protein (GFP) gene construct (GenBank accession no. U87974) was used as a control that would not trigger the RNAi response because *Apis cerana cerana* genes do not share homology with dsRNA-GFP. Six micrograms of dsRNA-AccOBP10 or dsRNA-GFP was injected into each adult between the first and second abdominal segments. The last group was not treated and used as the control group (CK). All honeybees were maintained in an incubator for 24 hours in the dark. Healthy honeybees were sampled each day, and the samples were stored at -70°C. The transcriptional levels of the *AccGSTD*, *AccGSTO2*, *AccTRX1*, *AccTRX2*, *AccTPX3*, *AccMSRA*, *AccSOD1*, and *AccCAT* genes, which are up- or downregulated in response to oxidative stress, were detected by qRT-PCR. Each of these experiments was repeated three times.

Results

Characterization of AccOBP10

The cDNA sequence (ORF) of *AccOBP10* (GenBank accession no. KP717060) is 450 bp in length and encodes 149 amino acids with a 20-amino-acid signal peptide. The mature AccOBP10 protein is a secretory protein that contains 129 amino acids. The predicted molecular weight and theoretical isoelectric point of mature AccOBP10

are 14.88 kDa and 7.68, respectively. Full-length nucleotide fragments and deduced amino acid sequences are shown in Fig. 1, with the six position-conserved cysteines indicated by black circles.

Multiple sequence alignments revealed that AccOBP10 has several typical amino acid sequences with high sequence similarity among various species, including the six positionally conserved cysteine residues (Fig. 2A), and the secondary structural analysis revealed that AccOBP10 has typical helix structures. We also used MEGA 4.1 software to construct a neighbor-joining phylogenetic tree that illustrates the evolutionary relationships among various species (Fig. 3). The phylogenetic tree indicated that the protein amino acid sequence is conserved and has the closest evolutionary relationships with AmOBP10 (XP_006566010.1) (Fig. 3).

Spatial expression patterns of AccOBP10

To determine the *AccOBP10* expression profiles in different tissues, we performed a qRT-PCR analysis of RNA extracted from the antenna, head, chest, total abdomen, epidermis, wing, muscle, poison gland, hemolymph, honey sac, rectum and midgut; *β*-actin was used as a reference for gene expression. As shown in Fig. 4, in all selected bee tissues, *AccOBP10* was expressed, although the highest levels were detected in the poison gland, followed by the wings.

AccOBP10 expression profiles under adverse abiotic stress conditions

To determine whether this OBP plays a role in oxidative stress as a soluble protein carrier, we explored the expression dynamics of *AccOBP10* under a variety of stress conditions. Because adult workers are responsible for outwork when exposed to worsening abiotic stress, the A15 bees were treated with low temperature (4°C), high

temperature (42°C), UV light, H_2O_2, vitamin C, $HgCl_2$, $CdCl_2$, pyridaben, methomyl, imidacloprid, paraquat and phoxim. All adult bees were exposed to artificially simulated environments or fed reagents. After various treatments, RNA of the whole bees was extracted for qRT-PCR, and *β*-actin was used as a reference for gene expression. Levels of gene targets were normalized to those observed in untreated controls. Temperature changes can induce stress associated with enhanced ROS generation and oxidative stress. As shown in Fig. 5A, exposure to low-temperature (4°C) stress increased and then decreased *AccOBP10* transcription, which was primarily because the honeybees died after 4 hours of treatment. The high-temperature (42°C) treatment reduced *AccOBP10* transcription (Fig. 5B), which suggested that *AccOBP10* plays an important role in temperature-induced oxidative stress. However, exposure of the bees to UV light sharply decreased expression (Fig. 5C). We also found that H_2O_2 injection slightly induced *AccOBP10* expression (Fig. 5D). The antioxidant vitamin C notably reduced the *AccOBP10* transcript levels (Fig. 5E), and the $HgCl_2$ and $CdCl_2$ treatments caused sharp decreases in the transcript levels (Fig. 5F, G). Interestingly, treatment with different pesticides yielded different expression patterns of *AccOBP10*, with pyridaben, imidacloprid and methomyl causing substantial increases in expression (Fig. 5H, I, J) and paraquat and phoxim reducing *AccOBP10* expression (Fig. 5K, L). These findings suggest a role of *AccOBP10* in protecting honeybees against pesticides. Taken together, these results suggest that *AccOBP10* plays a role in the oxidative stress response.

Western blot analysis

Gene functions primarily occur at the protein level; thus, the results obtained for the expression levels of *AccOBP10* transcripts by qRT-PCR were confirmed by

western blot analyses. The complete AccOBP10 ORF, which contains a cleavable N-terminal His tag, was cloned. After protein expression was induced with IPTG, the proteins were purified using HisTrap™ FF columns and separated by SDS-PAGE to yield a recombinant AccOBP10 protein with a molecular mass of approximately 21.8 kDa (Fig. 6). This purified protein was then used to generate antibodies for the detection of AccOBP10. As shown in Fig. 7A, the UV treatment sharply decreased AccOBP10 expression. Access to AccOBP10 protein levels was also reduced by vitamin C and $HgCl_2$ treatments (Fig. 7B, C). In response to the pyridaben treatment, AccOBP10 expression was induced and peaked at 3 hours (Fig. 7D). The stress induced by paraquat gradually decreased the AccOBP10 expression level (Fig. 7E). These experimental results are all consistent with the qRT-PCR results.

Expression profiles of AccOBP10 RNAi and oxidative stress-related genes after AccOBP10 silencing.

RNAi is an experimental technique for gene functional analysis (Bellés X, 2010) that is particularly useful when transgenesis is not available for insects (Huvenne, Smagghe, 2010). To determine whether *AccOBP10* participates in oxidative stress, we knocked down the *AccOBP10* gene (Fig. 8A) and then detected the genes closely related to the stress response to further confirm the results. The qRT-PCR results showed that the expression levels of *AccGSTD*, *AccGSTO2*, *AccTRX1*, *AccTRX2*, *AccTPX3*, *AccMSRA*, *AccSOD1*, and *AccCAT* were all elevated when *AccOBP10* was silenced (Fig. 8B-I).

Discussion

Previous studies have demonstrated that OBPs aid the sense of smell by assisting

in the mass transfer of many different sensory compounds that make up the olfactory landscape *(23)*. OBPs play an important role in the detection and recognition of olfactory stimuli, although their full function has not yet been fully clarified *(24-27)*.There may be initial applications for OBPs in the design of new drugs and the removal of environmental pollutants *(28)*. AmOBP14 strongly interacts with ligands that contain a hydroxy group. Nonetheless, there is a lack of information on the role of OBPs in response to oxidative stress *(20)*.

We aimed to confirm whether an OBP plays a role in the ROS response in insects. These findings have potential implications for the roles of OBPs in oxidative stress. In this study, we first cloned and characterized the *AccOBP10* gene from *Apis cerana cerana*. The amino acid sequence alignment and phylogenetic analysis of *AccOBP10* suggest that this gene is a typical OBP family member, indicating that *AccOBP10* might participate in the oxidative stress response.

To further study the potential functions of *AccOBP10*, we examined its transcript expression in various tissues. Previous studies have demonstrated that OBPs are expressed not only in chemosensory organs *(29)* but also in non-chemosensory organs *(30,31)*. The qRT-PCR analysis showed that *AccOBP10* was significantly induced in all the tissues examined, including the antenna, head, poison gland, midgut, wing, and epidermis.

AccOBP10 mRNA expression was detected at the highest levels in the poison gland, followed by the wings. *(32)*. The observed expression pattern strongly suggests that *AccOBP10* might have protective activity against abiotic stress impairment, particularly because all of the tissues tested act as barriers and are closely related to self-defense, protection from exogenous substance detoxification *(33)*, and maintenance of the physical stress response *(34)*.

The above-described results prompted us to further analyze the role of *AccOBP10* during abiotic stress. Disruption of dynamic equilibrium increases ROS levels *(35)*, and temperature critically influences the mechanisms responsible for the induction of oxidative stress *(36)*. A temperature decrease might weaken the ROS elimination systems and/or enhance ROS production, whereas an increase in temperature stimulates all metabolic processes and might increase ROS production. Changes in antioxidative and metabolic systems after exposure to temperature stress have been confirmed in many animals. Real-time qPCR analyses were performed to determine the transcriptional expression level of *AccOBP10*, and we found that the expression level was significantly increased at 4°C but decreased at 42°C (Fig. 5A, B), which suggests that cold and heat cause oxidative stress in cells and regulate *AccOBP10* expression through distinct pathways. Consistent with these results, the transcriptional expression levels were significantly decreased after the honeybees were exposed to UV light, vitamin C, $HgCl_2$ and $CdCl_2$. UV radiation leads to the activation of complex signaling pathways before resulting in cellular death *(37)* and plays a prominent role in the induction of cutaneous oxidative stress. Although it is not a radical, H_2O_2 is a major cellular source of ROS damage *(38)* because it has higher activity than molecular oxygen; furthermore, H_2O_2 can modify purines and pyrimidines and cause strand breaks that result in DNA damage. Vitamin C has been proposed as a suitable source for antioxidant therapy due to its capacity to scavenge ROS *(39)*, and it also has potential pro-oxidant activity in the presence of transition metal ions *(40)*. Experimental evidence has revealed that mercury plays a role in the generation of oxygen radicals *(41,42)*. Cadmium affects cell proliferation, differentiation and apoptosis and other cellular activities and can cause oxidative lesions and mutations of DNA *(43)*. However, protein expression was significantly

enhanced after the honeybees were exposed to UV light, vitamin C, $HgCl_2$ and $CdCl_2$ (Fig. 5C, E, F, and G). Treatment of the honeybees with H_2O_2 only slightly increased the *AccOBP10* expression level compared with that in the control group (Fig. 5D). Such findings support the hypothesis that *AccOBP10* plays an important role in oxidative damage. Pesticides pose a significant threat to insects, and pesticide-induced oxidative stress leads to an imbalance between pro-oxidant and antioxidant defense mechanisms *(44)*, resulting in impaired biochemical and physiological functions *(45)*, such as lipid peroxidation, proteomic damage, changes in DNA and RNA structure, and disruption of total antioxidant capacity in cells *(46)*. Insecticides can be divided into organophosphorus (OP)-, organochlorine-, and carbamate-containing compounds. Paraquat generates mitochondrial superoxide anions and affects early amphibian embryonic development *(47)*. The addition of pesticides to *Saccharomyces cerevisiae* cultures can result in cellular aging *(46)*. Treatment with the pesticides pyridaben, methomyl, and imidacloprid increased *AccOBP10* transcript levels (Fig. 5H, I, J). With these results, it was shown that pesticide treatment affected the expression profile of AccOBP10.

To confirm that the *AccOBP10* protein plays a role in abiotic stress, we performed western blot analyses after treatment with UV light, vitamin C, $HgCl_2$, pyridaben and paraquat. Bees treated with pyridaben showed increased AccOBP10 protein expression, whereas this protein expression was reduced following exposure to UV light, vitamin C, $HgCl_2$, and paraquat (Fig. 7). These findings are consistent with the transcript levels. Taken together, these results suggest that AccOBP10 may play a key role in the oxidative stress process.

We subsequently knocked down the mRNA expression of *AccOBP10* by RNAi (Fig. 8A) at the posttranscriptional level *(48,49)*. Recent studies have shown that

RNAi is an important tool for studying the gene functions of honeybees *(50,51)*. After RNA silencing, the transcript levels of *AccGSTD*, *AccGSTO2*, *AccTRX1*, *AccTRX2*, *AccTPX3*, *AccMSRA*, *AccSOD1*, and *AccCAT*, which play vital roles in different environmental stress responses *(51-56),* were slightly increased (Fig. 8B-I). However, the mRNA expression levels did not increase significantly, which was possibly because *AccOBP10* has functional redundancy with other genes in the complicated stress defense regulatory networks. Thus, knockdown of *AccOBP10* increased ROS levels, resulting in increased expression of stress-related genes to eliminate the increased ROS. These findings provide further evidence that *AccOBP10* interacts with the abovementioned genes to participate in oxidative stress processes.

Collectively, these results represent the first confirmation that *AccOBP10* plays a role in abiotic stress responses. These findings might provide further insights into the detailed mechanisms underlying the response of *Apis cerana cerana* to abiotic stress. Additional work is needed to understand the function of *AccOBP10* in the defense against oxidative stress.

Funding

This work was financially supported by the Shandong Agricultural Fine Varieties Breeding Projects (2017LZN006), the Funds of Shandong Province "Double Tops" Program, the National Natural Science Foundation of China (No. 31572470), and the Earmarked Fund for the China Agriculture Research System (No. CARS-44).

Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

References

1. Vogt, R. G., Riddiford, L. M. (1981) Pheromone binding and inactivation by moth antennae. *Nature*. 293(5828):161-3. doi:10.1038/293161a0

2. Pelosi, P., Maida, R. (1995) Odorant-binding proteins in insects. *Comp Biochem Physiol B Biochem Mol Biol*. 111(3):503-514. doi:10.1016/0305-0491(95)00019-5

3. Vogt, R. G., Große-Wilde, E., and Zhou, J. J. (2015) The lepidoptera odorant binding protein gene family: gene gain and loss within the gobp/pbp complex of moths and butterflies. *Insect Biochem Mol Biol*. 62:142-153. doi: 10.1016/j.ibmb.2015.03.003

4. Pelosi, P., Mastrogiacomo, R., Iovinella, I., Tuccori, E., and Persaud, K. C. (2014) Structure and biotechnological applications of odorant-binding proteins. *Appl Microbiol Biotechnol*. 98(1):61-70. doi: 10.1007/s00253-013-5383-y

5. Chen, X., Li, G., Xu, X., and Wu, J. (2010). Molecular and Functional Characterization of Odorant Binding Protein 7 From the Oriental Fruit Moth Grapholita molesta (Busck) (Lepidoptera: Tortricidae). *Front. Physiol.* 9:1762. doi: 10.3389/fphys.2018.0176

6. Böcskei, Z., Groom, C. R., Flower, D. R., Wright, C. E., Phillips, S. E. V., Cavaggioni, A., et al. (1992) Pheromone binding to two rodent urinary proteins revealed by x-ray crystallography. *Nature*. 360(6400):186-8. doi:10.1038/360186a0

7. Bianchet, M. A., Bains, G., Pelosi, P., Pevsner, J., Snyder, S. H., Monaco, H. L., et al. (1996) The three-dimensional structure of bovine odorant binding protein

and its mechanism of odor recognition. *Nat Struct Biol*. 3(11):934-9.

8. Tegoni, M., Ramoni, R., Bignetti, E., Spinelli, S., and Cambillau, C. (1996) Domain swapping creates a third putative combining site in bovine odorant binding protein dimer. *Nat Struct Biol*. 3(10):863-7. doi:10.1038/nsb1096-863
9. Spinelli, S., Ramoni, R., Grolli, S., Bonicel, J., Cambillau, C., and Tegoni, M. (1998) The structure of the monomeric porcine odorant binding protein sheds light on the domain swapping mechanism. *Biochemistry* 37(22):7913-8. doi:10.1021/bi980179e
10. Spinelli, S., Vincent, F., Pelosi, P., Tegoni, M., and Cambillau, C. (2002) Boar salivary lipocalin three-dimensional x-ray structure and androsterol/androstenone docking simulations. *Eur J Biochem*. 269(10):2449-56. doi:10.1046/j.1432-1033.2002.02901.x
11. Vincent, F., Spinelli, S. R., Grolli, S., Pelosi, P., Cambillau, C., and Tegoni, M. (2000) Complexes of porcine odorant binding protein with odorant molecules belonging to different chemical classes. *J Mol Biol*. 300(1):127-139. doi:10.1006/jmbi.2000.3820
12. Gong, Z. J., Liu, S., Jiang, Y. D., Zhou, W, W., Liang, Q. M., Cheng, J., et al. (2015) Construction and analysis of antennal cdna library from rice striped stem borer, chilo suppressalis, (walker) (lepidoptera: pyralidae), and expression profiles of putative odorant-binding protein and chemosensory protein genes. *Arch Insect Biochem Physiol*. 89(1):35-53. doi: 10.1002/arch.21224
13. March, C. A., Golebiowski, J. (2014) A computational microscope focused on the sense of smell. *Biochimie.* 107:3-10. doi: 10.1016/j.biochi.2014.06.006
14. Grolli, S., Merli, E., Conti, V., Scaltriti, E., and Ramoni, R. (2006) Odorant

binding protein has the biochemical properties of a scavenger for 4-hydroxy-2-nonenal in mammalian nasal mucosa. *FEBS J*. 273(22):5131-5142. doi:10.1111/j.1742-4658.2006.05510.x

15. Balaban, R. S., Nemoto, S., and Finkel, T. (2005) Mitochondria, oxidants, and aging. *Cell.* 120(4):483-95.

16. Ray, P. D., Bo-Wen, H., Yoshiaki, T. (2012) Reactive oxygen species (ros) homeostasis and redox regulation in cellular signaling. *Cell Signal*. 24(5):981-90. doi: 10.1016/j.cellsig.2012.01.008

17. Uchida, K. (2003) 4-hydroxy-2-nonenal: a product and mediator of oxidative stress. *Prog Lipid Res.* 42(4):318-343. doi:10.1016/s0163-7827(03)00014-6

18. Macedo-Márquez, M., Vázquez-Acevedo, L., Ongay-Larios, H., Miranda-Astudillo, R., Hernández-Muñoz, D., González-Halphe, D., et al. (2014) Overexpression of a monomeric form of the bovine odorant-binding protein protects escherichia coli from chemical-induced oxidative stress. *Free Radic Res*. 48(7):814-22. doi: 10.3109/10715762.2014.910867

19. Kulanthaivel, L., Jeyakanthan, J., Richard M., and Saravanan, S. (2018) Insights from the Molecular modeling, docking analysis of illicit drugs and Bomb Compounds with Honey Bee Odorant Binding Proteins (OBPs) *Bioinformation* 14(5): 219-231

20. Spinelli, S., Lagarde, A., Iovinella, I., Legrand, P., Tegoni, M., Pelosi, P., et al. (2012) Crystal structure of *Apis mellifera*, OBP14, a C-minus odorant-binding protein, and its complexes with odorant molecules. *Insect Biochem Molec.* 42(1):41-50. doi: 10.1016/j.ibmb.2011.10.005

21. Alaux, C., Ducloz, F., Crauser, D., and Le, C. Y. (2010) Diet effects on honeybee

immunocompetence. *Biol. Lett.* 6:562-565. doi: 10.1098/rsbl.2009.0986

22. Livak, K. J., Schmittgen. T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta C(T)) Method. *Method*. 25(4):402-408. doi:10.1006/meth.2001.1262

23. White, S. A., Bri, L., Scott, D. J., and Borysik, A. J. (2009) Structure of rat odorant-binding protein obp1 at 1.6 a resolution. *Acta Crystallogr D Biol Crystallogr*. 65(Pt 5), 403-10. doi: 10.1107/S090744490900420X

24. Sun, Y. F., De, B. F, Qiao, H. L., Iovinella, I., Yang, S.X., Ling, Y., et al. (2012) Two odorant-binding proteins mediate the behavioural response of aphids to the alarm pheromone (E)-ß-farnesene and structural analogues. *PLoS One*. 7(3):e32759. doi: 10.1371/journal.pone.0032759

25. Xu, P., Atkinson, R., Jones, D. N., and Smith, D.P. (2005) Drosophila OBP LUSH is required for activity of pheromone-sensitive neurons. Neuron 45(2):193-200. doi:10.1016/j.neuron.2004.12.031

26. Matsuo, T., Sugaya, S., Yasukawa, J., Aigaki, T., and Fuyama, Y. (2007) Odorant-Binding Proteins OBP57d and OBP57e Affect Taste Perception and Host-Plant Preference in *Drosophila sechellia*. *Plos Biol*. 5(5):e118-e118. doi:10.1371/journal.pbio.0050118

27. Swarup, S., Williams, T. I., Anholt, R. R. (2011) Functional dissection of *Odorant binding protein genes* in *Drosophila melanogaster*. *Genes Brain Behav*. 10(6):648-57. doi: 10.1111/j.1601-183X.2011.00704.x

28. Bianchi, F., Basini, G., Grolli, S., Conti, V., Bianchi, F., Grasselli, F., et al. (2013) An innovative bovine odorant binding protein-based filtering cartridge for the removal of triazine herbicides from water. Anal Bioanal Chem. 405(2):1067-1075.

doi: 10.1007/s00216-012-6499-0

29. Iovinella, I., Dani, F. R., Niccolini, A., Sagona, S., Michelucci, E., Gazzano, A., et al. (2011) Differential expression of odorant-binding proteins in the mandibular glands of the honey bee according to caste and age. *J Proteome Res*. 10(8):3439-49. doi: 10.1021/pr2000754

30. Ban, L., Napolitano, E., Serra, A., Zhou, X., Iovinella, I., and Pelosi, P. (2013) Identification of pheromone-like compounds in male reproductive organs of the oriental locust locusta migratoria. *Biochem Biophys Res Commun.* 437(4): 620-4. doi: 10.1016/j.bbrc.2013.07.015

31. Sun, Y. L., Huang, L. Q., Pelosi, P., and Wang, C. Z. (2011) Expression in antennae and reproductive organs suggests a dual role of an odorant-binding protein in two sibling, helicoverpa, species. *PLoS One*. 7(1):e30040. doi: 10.1371/journal.pone.0030040

32. Li, G., Jia, H., Wang, H., Yan, Y.,, Guo, X., Sun, Q., et al. (2016) A typical RNA-binding protein gene (*AccRBM11*) in *Apis cerana cerana*: characterization of AccRBM11 and its possible involvement in development and stress responses. *Cell Stress Chaperones.* 21(6):1-15. doi:10.1007/s12192-016-0725-1

33. Enayati, A. A., Ranson, H., and Hemingway, J. (2005) Insect glutathione transferases and insecticide resistance. *Insect Mol Biol.* 14(1):3-8. doi:10.1111/j.1365-2583.2004.00529.x

34. Marionnet, C., Bernerd, F., Dumas, A., Verrecchia, F., Mollier, K., Compan, D., et al. (2003) Modulation of gene expression induced in human epidermis by environmental stress in vivo. J Invest Dermatol 121(6):1447-58. doi:10.1111/j.1523-1747.2003.12629.x

35. Lushchak, V. I. (2011) Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol*. 101(1):13-30. doi: 10.1016/j.aquatox.2010.10.006

36. An, M. I., Choi, C. Y. (2010) Activity of antioxidant enzymes and physiological responses in ark shell, Scapharca broughtonii, exposed to thermal and osmotic stress: effects on hemolymph and biochemical parameters. *Comp Biochem Phys B.* 155(1):34-42. doi: 10.1016/j.cbpb.2009.09.008

37. Jiang, Q., Zhou, C., Healey, S., Chu, W., Kouttab, N., Bi, Z., et al. (2006) UV radiation down-regulates dsg-2 via rac/nadph oxidase-mediated generation of ros in human lens epithelial cells. *Int J Mol Med*. 18(2):381-7

38. Campanini, M. Z., Custódio, D. L., Ivan, A. L. M., Martins, S. M., Paranzini, M. J. R., Martinez, R. M., et al. (2014) Topical formulations containing pimenta pseudocaryophyllus extract: in vitro antioxidant activity and in vivo efficacy against uv-b-induced oxidative stress. *AAPS PharmSciTech.* 15(1):86-95. doi: 10.1208/s12249-013-0049-8

39. Lee, S. H., Oe, T., and Blair, I. A. (2001) Vitamin c-induced decomposition of lipid hydroperoxides to endogenous genotoxins. *Science*. 292(5524):2083-6. doi:10.1126/science.1059501

40. Ar, P., Ca, R. E., Rehman, A. (2000) Potential problems of ascorbate and iron supplementation: Pro-oxidant effect in vivo? *Biochem Biophys Res Commun.* 277(3):535-40. doi:10.1006/bbrc.2000.3711

41. Lee, Y. W., Mi, S. H., and Yong, K. K. (2001) Role of reactive oxygen species and glutathione in inorganic mercury-induced injury in human glioma cells. *Neurochemical Research* 26(11):1187-93.doi:10.1023/a:1013955020515

42. Wiggers, G. A., Peçanha, F. M., Briones, A. M., Pérez-Girón J.V., Miguel,

M., Vassallo, DV., et al. (2008) Low mercury concentrations cause oxidative stress and endothelial dysfunction in conductance and resistance arteries. *Ajp Heart Circulatory Physiology*. 295(295):H1033-H1043. doi: 10.1152/ajpheart.00430.2008

43. Filipic, M., Fatur, T., and Vudrag, M. (2006) Molecular mechanisms of cadmium induced mutagenicity. *Hum Exp Toxicol*. 25(2):67-77. doi:10.1191/0960327106ht590oa

44. Banerjee, B. D., Seth, V., and Ahmed, R. S. (2001) Pesticide-induced oxidative stress: perspectives and trends. *Rev Environ Health*. 16(1):1-40.

45. Narendra, M., Bhatracharyulu, N. C., Padmavathi, P., and Varadacharyulu, N. C. (2007) Prallethrin induced biochemical changes in erythrocyte membrane and red cell osmotic haemolysis in human volunteers. *Chemosphere*. 67(6):1065-1071. doi:10.1016/j.chemosphere.2006.11.064

46. Owsiak, A., Marchel, M., and ŻYracka, E. (2015) Impact of pesticides used in the culture of the vine on the viability of the yeast saccharomyces cerevisiae wine in chronological aging. Journal of Microbiology Biotechnology Food Sciences 04 (Special issue 01), 48-51

47. Rodriguez-Rocha, H., Garcia-Garcia, A., Pickett, C., Li, S., Jones, J., Han, C., et al. (2013) Compartmentalized oxidative stress in dopaminergic cell death induced by pesticides and complex inhibitors: distinct roles of superoxide anion and superoxide dismutases. *Free Radical Biology Medicine*. 61(8):370-383. doi: 10.1016/j.freeradbiomed.2013.04.021

48. Ding, S.W. (2010) RNA-based antiviral immunity. *Nat Rev Immunol*. 10(9):632-644. doi: 10.1038/nri2824

49. Goic, B., Vodovar, N., Mondotte, J.A., Monot, C., Frangeul, L., Blanc, H., et al. (2013) RNA-mediated interference and reverse transcription control the persistence of RNA viruses in the insect model *Drosophila*. *Nat Immunol*. 14(4):396-403. doi: 10.1038/ni.2542

50. Wilson, M.J., Kenny, N.J., and Dearden, P.K. (2014) Components of the dorsalventral pathway also contribute to anterior-posterior patterning in honeybee embryos (*Apis mellifera*). *EvoDevo.* 5(1):1419-1419. doi: 10.1186/2041-9139-5-11.

51. Yao, P., Chen, X., Yan, Y., Feng, L., Zhang, Y., Guo, X., et al. (2014) Glutaredoxin 1, glutaredoxin 2, thioredoxin 1, and thioredoxin peroxidase 3 play important roles in antioxidant defense in *Apis cerana cerana*. *Free Radic Biol Med.* 68(3):335-346. doi: 10.1016/j.freeradbiomed.2013.12.020

52. Yu, F., Kang, M., Meng, F., Guo, X., and Xu, B. (2011) Molecular cloning and characterization of a thioredoxin peroxidase gene from *Apis cerana cerana*. *Insect Mol Biol.* 20(3):367-378. doi: 10.1111/j.1365-2583.2011.01071.x

53. Jia, H., Sun, R., Shi, W., Yan, Y., Han, L., Guo, X., et al. (2014) Characterization of amitochondrial manganese superoxide dismutase gene from *Apis cerana cerana*, and its role in oxidative stress. *Insect Physiol*. 60(1): 68-79. doi: 10.1016/j.jinsphys.2013.11.004

54. Meng, F., Zhang, Y., Liu, F., Guo, X., Xu, B. (2014) Characterization and mutational analysis of omega-class GST (GSTO1) from *Apis cerana cerana*, a gene involved in response to oxidative stress. *PLoS One.* 9(3):e93100. doi: 10.1371/journal.pone.0093100

55. Chen, X., Yao, P., Chu, X., Hao, L., Guo, X., Xu, B. (2015) Isolation of arginine

kinase from Apis cerana cerana and its possible involvement in response to adverse stress. *Cell Stress Chaperones.* 20(1):169-83. doi: 10.1007/s12192-014-0535-2

56. Huaxia, Y., Fang, W., Yan, Y., Feng, L., Wang, H., Guo, X., et al. (2015) Anovel 1-Cys thioredoxin peroxidase gene in *Apis cerana cerana*: characterization of AccTpx4 and its role in oxidative stresses. *Cell Stress Chaperones*. 20(4):1-10. doi: 10.1007/s12192-015-0594-z

Fig. 1. ORF nucleotide sequence (top) of AccOBP10 and deduced amino acid (bottom) sequence. The stop codon is indicated with an asterisk (★). The start codon (ATG), stop codon (TAG), and deduced peptide signal sequence are indicated with boxes. The six highly conserved cysteine residues are indicated by black circles.

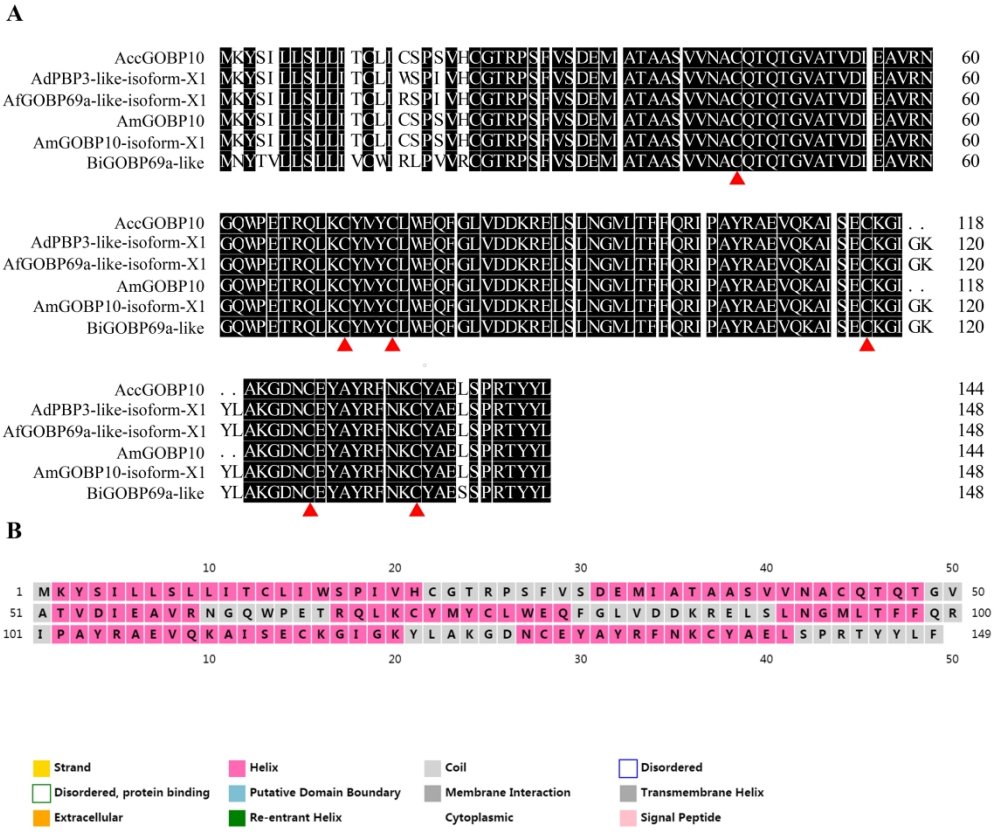

Fig. 2. Amino acid sequence alignment of AccOBP10 among various species and the secondary structures of amino acid sequences.
A: Alignment of the amino acid sequences of AccOBP10 and known insect OBPs. The amino acid sequences used in the analysis are from Apis dorsata (GenBank Accession No. XP_006620449.1), Apis florea (GenBank Accession No. XP_003692791.1), Apis mellifera (GenBank Accession Nos. NP_001035294.1 and XP_006566010.1), and Bombus impatiens (GenBank Accession No. XP 003493474.1). Identical amino acid residues are shaded in black, and the six conserved cysteine residues are indicated by a triangle (▲).
B: The secondary structures of the amino acid sequences.

161x139mm (600 x 600 DPI)

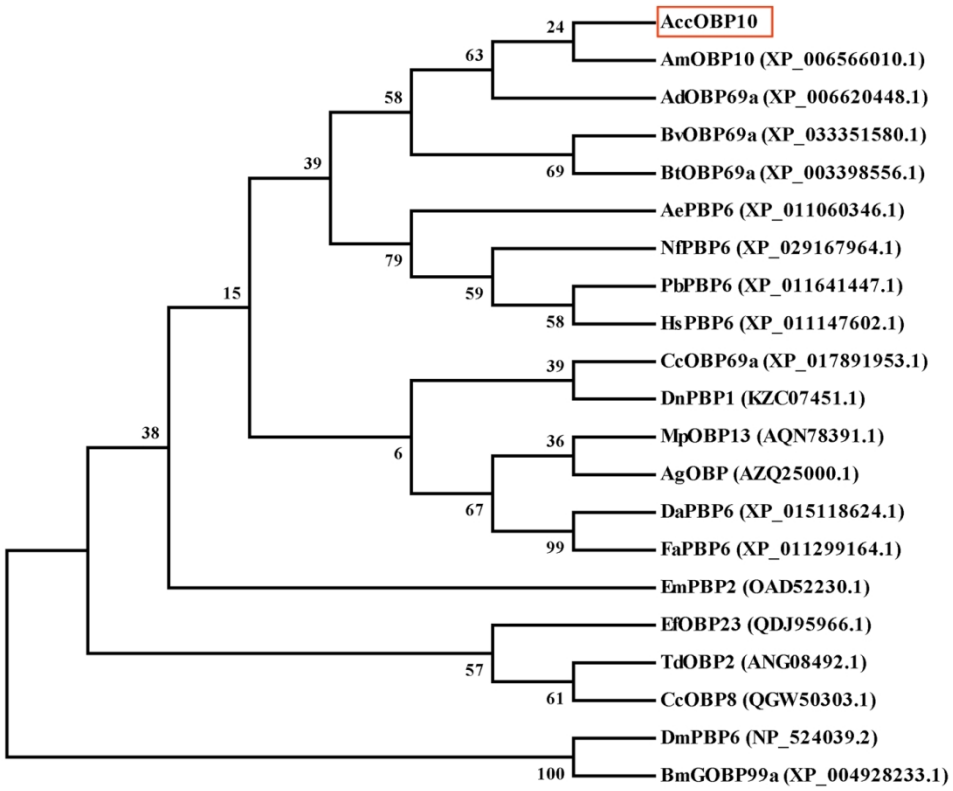

Fig. 3. Phylogenetic analysis of AccOBP10 from different species.
Phylogenetic analysis of the homologous sequences from various species were obtained using the neighbor-joining (NJ) method with bootstrap values of 1000 replicates. AccOBP10 is shown in the box. The sequences were obtained from the NCBI database.

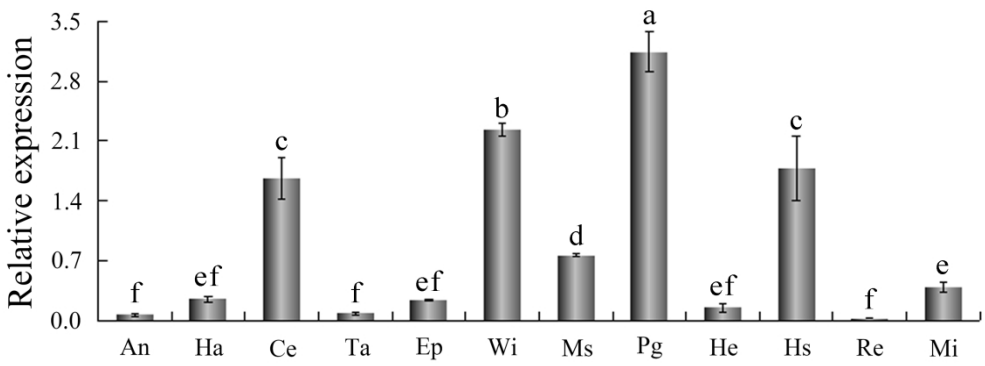

Fig. 4. Relative AccOBP10 expression in different tissues.
AccOBP10 mRNA levels in different tissues: antenna (An), head (Ha), chest (Ce), total abdomen (Ta), epidermis (Ep), wing (Wi), muscle (Ms), poison gland (Pg), hemolymph (He), honey sac (Hs), rectum (Re) and midgut (Mi).

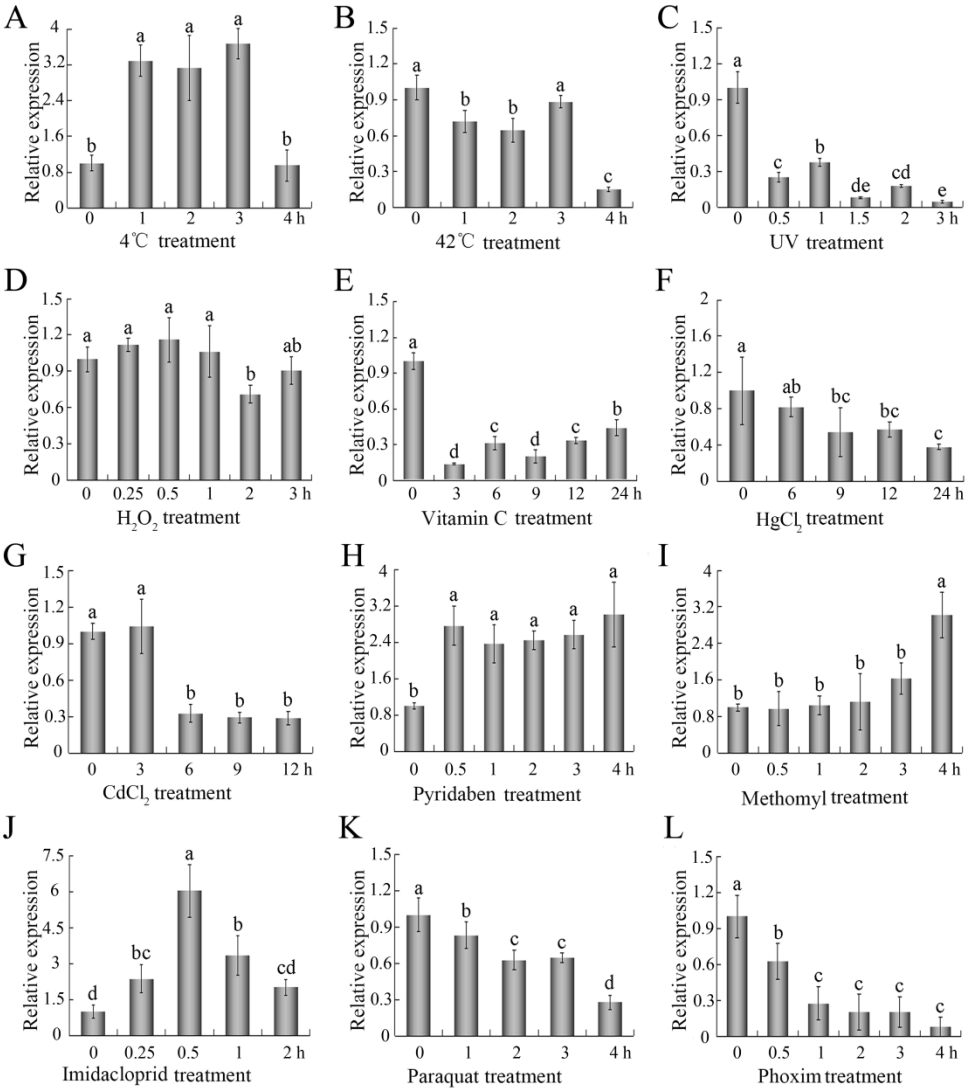

Fig. 5. AccOBP10 expression patterns under different abiotic stress conditions.
The treatments are as follows: 4°C (A), 42°C (B), UV (C), H2O2 (D), vitamin C (E), HgCl2 (F), CdCl2 (G), pyridaben (H), methomyl (I), imidacloprid (J), paraquat (K), and phoxim (L). The β-actin gene was employed as an internal control. Each value is presented as the mean (SD) of three replicates.

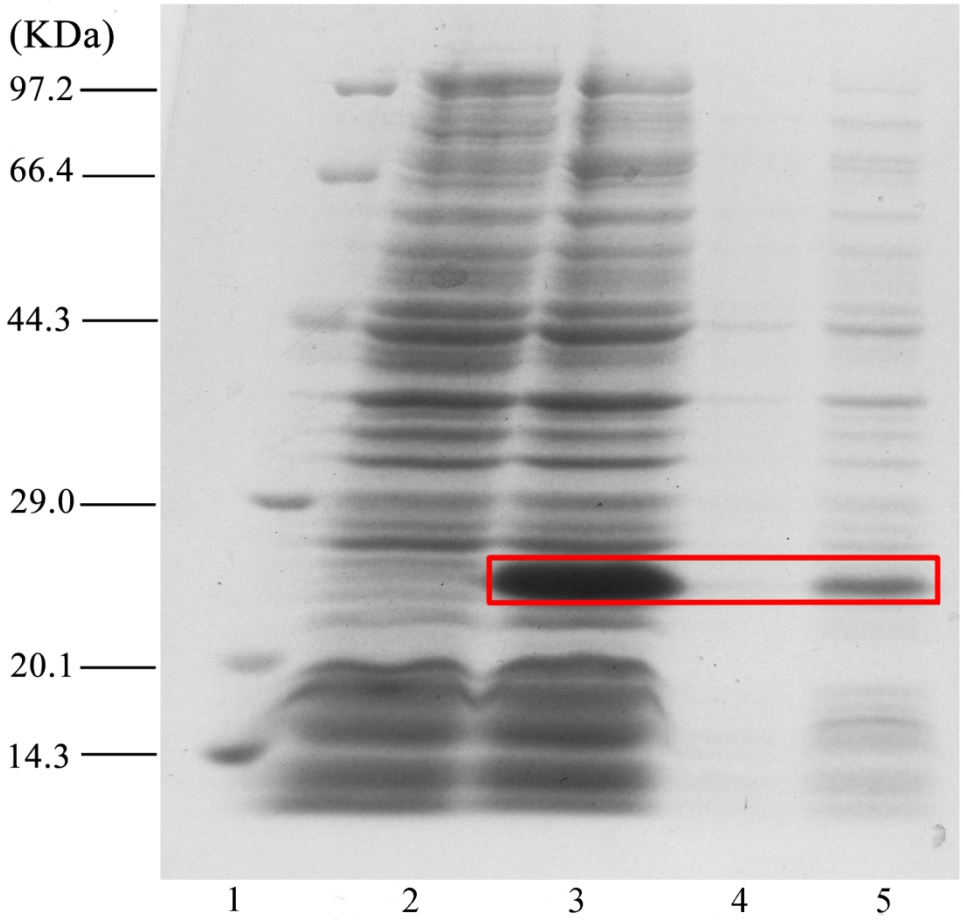

Fig. 6. Expression of the recombinant AccOBP10 protein.
Recombinant AccOBP10 expressed after induction with IPTG was purified and separated by SDS-PAGE. Lane 1, protein molecular weight marker; lane 2, expression of AccOBP10 without IPTG induction; lane 3, expression of AccOBP10 after IPTG (0.2 mM) induction; lane 4, empty lane; and lane 5, purified recombinant AccOBP10 protein. The recombinant AccOBP10 protein produced from the pET-30a (+) vector is shown with a black box.

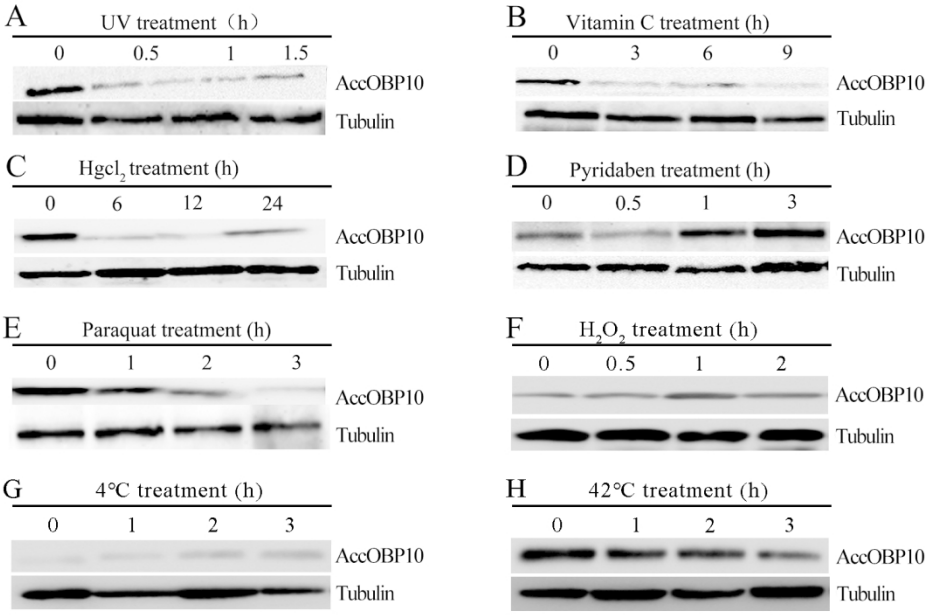

Fig. 7. Western blot analysis of AccOBP10.
Western blot analysis of AccOBP10 levels in 15-day-old adult bees under abiotic stress conditions, including UV (A), vitamin C (B), HgCl2 (C), pyridaben (D), and paraquat (E) treatments. The molecular weight of the AccOBP10 recombinant protein is 21.8 kDa. β-actin served as the loading control.

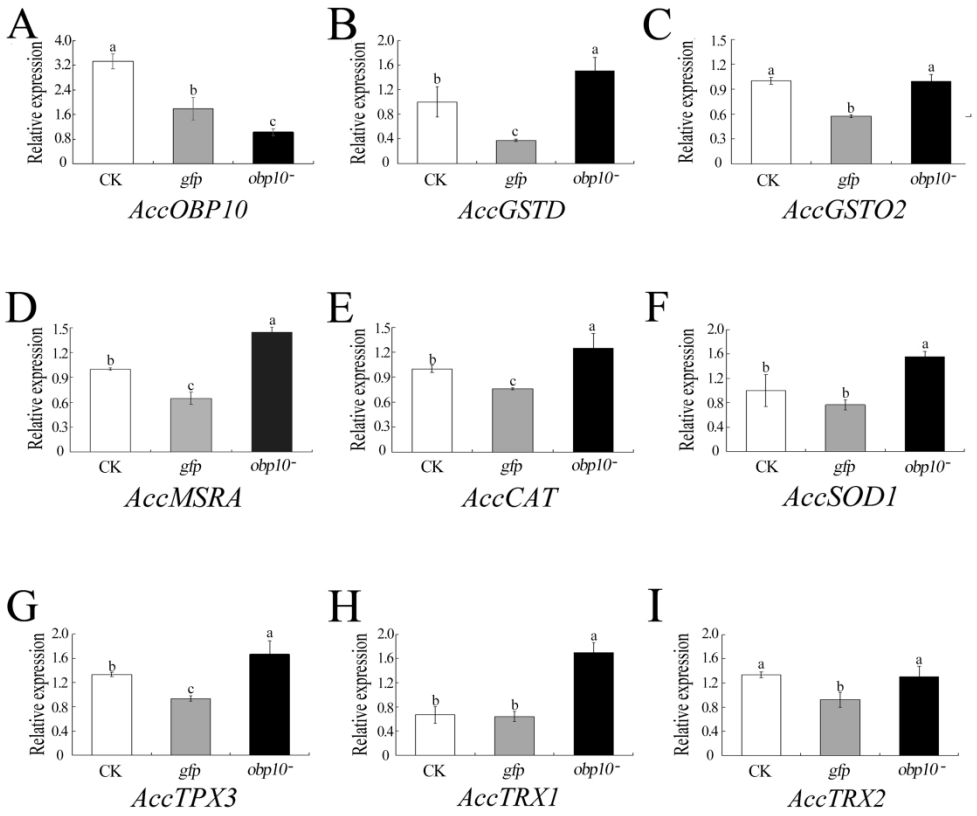

Fig. 8. Effect of AccOBP10 silencing by RNAi on AccOBP10 transcript levels and the expression profiles of oxidative stress-related genes in adult honeybees.
(A) AccOBP10 mRNA levels after RNAi treatment. dsRNA-GFP was injected and used as a negative control, and untreated adults were used as a control group. (B-I) qRT-PCR results showing the expression profiles of stress response-related genes. The β-actin gene was used as an internal control. All the data are presented as the means±SEs from three independent experiments.

Table

Table 1 Primers used in this study

	Primer sequence (5'-3')	Description
CF	CGGACCACATTATCTAACTCAACTTCG	cDNA sequence primer, forward
CR	GGCTCAGATGAAACTCGATTAAAACAG	cDNA sequence primer, reverse
QF	GCTTGCCAGACACAGACAGGAGT	Real-time PCR primer, forward
QR	GTCATCCACCAATCCGAATTGTTCCC	Real-time PCR primer, reverse
β-s	TTATATGCCAACACTGTCCTTT	Standard control primer, forward
β-x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
PF	GGATCCATGCATTGTGGGACACGACCGAGTTTT	Protein expression primer, forward
PR	GTCGACAAACAGATAATACGTCCGTGGAGACAATTC	Protein expression primer, reverse
RF	TAATACGACTCACTATAGGGCGAATGAAATATTCGATAC TATTGAGC	RNA inference primer, forward
RR	TAATACGACTCACTATAGGGCGACAGATAATACGTCCGT GGAGAC	RNA inference primer, reverse
GSTDF	CGAAGGAGAAAACTATGTGGCAG	Real-time PCR primer, forward
GSTDR	CGTAATCCACCACCTCTATCG	Real-time PCR primer, reverse
Trx1F	GGTTTGAGAATTATACGCACTGC	Real-time PCR primer, forward
Trx1R	GAGTAAGCATGCGACAAGGAT	Real-time PCR primer, reverse
Tpx3F	CCTGCACCTGAATTTTCCGG	Real-time PCR primer, forward
Tpx3R	CTCGGTGTATTAGTCCATGC	Real-time PCR primer, reverse
Trx2F	GGTTCGGTAGTACTTGTGGAC	Real-time PCR primer, forward
Trx2R	GGACCACACCACATAGCAAAG	Real-time PCR primer, reverse
MsrAF	GGTGATTGTTTATTTGGCG	Real-time PCR primer, forward
MsrAR	TTTGTATTGCTCTTGTTCACG	Real-time PCR primer, reverse
SOD1F	AAACTATTCAACTTCAAGGACC	Real-time PCR primer, forward
SOD1R	CACAAGCAAGACGAGCACC	Real-time PCR primer, reverse
CATF	GTCTTGGCCCGAACAATTTG	Real-time PCR primer, forward
CATR	CATTCTCTAGGCCCACCAAA	Real-time PCR primer, reverse
GSTO2F	GTTAGATATATTGATGTGGC	Real-time PCR primer, forward
GSTO2R	CCTGTTTTCAAATCTTTGAC	Real-time PCR primer, reverse