Identification and Characterization of Three New Cytochrome P450 Genes and the Use of RNA Interference to Evaluate Their Roles in Antioxidant Defense in *Apis cerana cerana* Fabricius

Weixing Zhang*, Wenfeng Chen, Zhenfang Li, Lanting Ma, Jing Yu, Hongfang Wang, Zhenguo Liu and Baohua Xu*

College of Animal Science and Technology, Shandong Agricultural University, Tai’an, China

Cytochrome P450s play critical roles in maintaining redox homeostasis and protecting organisms from the accumulation of toxic reactive oxygen species (ROS). The biochemical functions of the P450 family have essentially been associated with the metabolism of xenobiotics. Here, we sequenced and characterized three P450 genes, *AccCYP314A1*, *AccCYP4AZ1*, and *AccCYP6AS5*, from *Apis cerana cerana* Fabricius; these genes play a critical role in maintaining biodiversity. Quantitative PCR (qPCR) analysis indicated that the three genes were all predominantly expressed in the epidermis (EP), followed by the brain (BR) and midgut (MG). In addition, the highest expression levels were detected in the dark-eyed pupae and adult stages. The three genes were induced by temperature (4°C and 44°C), heavy metals (CdCl$_2$ and HgCl$_2$), pesticides (DDV, deltamethrin, and paraquat) and UV treatments. Furthermore, Western blot analysis indicated that the protein expression levels could be induced by some abiotic stressors, a result that complements the qPCR results. We analyzed the silencing of these three genes and found that silencing these genes enhanced the enzymatic activities of peroxidase (POD) and catalase (CAT). Additionally, we investigated the expression of other antioxidant genes and found that some were upregulated, while others were downregulated, suggesting that the upregulated genes may be involved in compensating for the silencing of *AccCYP314A1*, *AccCYP4AZ1*, and *AccCYP6AS5*. Our findings suggest that *AccCYP314A1*, *AccCYP4AZ1*, and *AccCYP6AS5* may play very significant roles in the antioxidant defense against damage caused by ROS.

**Keywords:** *Apis cerana cerana* Fabricius, cytochrome P450s, RNA interference, abiotic stresses, antioxidant enzyme
INTRODUCTION

As social insects and pollinators of flowering plants, Chinese honeybees (Apis cerana cerana Fabricius, A. c. cerana) play an important role in the balance between agricultural economic development and regional ecology. Compared with Apis mellifera (A. mellifera), A. c. cerana has acquired some incomparable advantages through the history of evolution. A. c. cerana has a strong resistance to mites, an acute sense of smell, and can forage more widely of pollens, which are irreplaceable (Oldroyd and Wongsiri, 2006). Nevertheless, the population of A. c. cerana has been reported to have severely declined, which has been referred to as colony collapse disorder (CCD), likely due to various abiotic stresses that exist in the environment, such as extreme changes in temperature and widely used insecticides that may generate reactive oxygen species (ROS). Therefore, understanding the antioxidant system and its molecular mechanisms of defense against ROS damage has become a primary focus of research.

Excessive ROS can lead to lipid peroxidation and protein damage, which cause cell apoptosis and the loss of enzymatic activity, respectively (Green and Reed, 1998; Stadtman and Levine, 2003). Sperm storage was also affected by ROS in A. mellifera (Collins et al., 2004). Organisms protect themselves through a range of antioxidant enzymes, such as catalase (CAT), glutathione-S-transferases (GSTs), superoxide dismutase (SOD), peroxidases (POD), glutathione peroxidase (GPX), and cytochrome P450s (P450s) (Dubovsky et al., 2008; Matteis et al., 2012).

P450s, a supergene family, are widely distributed in almost all living organisms and play crucial roles in insect biology and physiology (Feyereisen, 1999). P450s are classified into different clades on the basis of their evolutionary relationships (Claudianos et al., 2006; Moktali et al., 2012). In insects, P450s belong to four clades: the mitochondrial P450s (Mit P450s), CYP2 clades, CYP3 clades (including CYP6 and CYP9), and CYP4 clades. The Mit P450s and CYP2 clades have been implicated in essential roles in hormone biosynthesis (Gilbert, 2004; Semak et al., 2008; Sangar et al., 2010). Meanwhile, the CYP3 and CYP4 clades are largely responsible for abiotic stresses, such as pesticide metabolism, detoxifying functions, and chemical communication (Yu et al., 1984; Claudianos et al., 2006; Musasia et al., 2013).

Previous studies concerning the roles of CYP3 P450s have focused on their functions in the environmental response and detoxification in insects. In the pyrethroid-resistant Anopheles gambiae, CYP6Z1 (belonging to the CYP3 clade) is overexpressed (Nikou et al., 2003). Similarly, in Helicoverpa zea, CYP321A1, a member of the CYP3 clade, can metabolize cypermethrin (Sasabe et al., 2004). Meanwhile, CYP3A enzymes play a crucial role in preventing the bioaccumulation of xenobiotics substances (drugs, environmental pollutants, and fungal products) that enter the body and metabolize endogenous substrates, such as sex hormones, in steroidogenic tissues (Celander et al., 2000; Danielson, 2002; Hegelund and Celander, 2003).

In insects, the CYP4 clade, a highly diversified group of enzymes, are involved in both pesticide metabolism and chemical communication (Scott et al., 1994; Claudianos et al., 2006; Kirischian and Wilson, 2012). For example, some CYP4 clade genes are over expressed in several pesticide-resistant insects (Culex pipiens and Diabrotica virgifera virgifera), and CYP4G8 is over expressed in pyrethroid-resistant strains of Helicoverpa armigera (Pittendrigh et al., 1997; Scharf et al., 2001; Shen et al., 2003). Moreover, the expression of CYP4G11 was significantly induced by external factors such as temperature challenges, UV radiation, insecticide treatment, and the expression patterns under oxidative stress, suggesting that CYP4G11 may be involved in protecting honeybees from oxidative injury (Shi et al., 2013). These studies demonstrate that the CYP3 and CYP4 clades play key roles in protecting organisms from ROS damage.

Although the functions of the P450s have been investigated in other species, there is limited knowledge regarding the P450s in honeybees, particularly in A. c. cerana. The above discussion demonstrates that the P450s play key roles in the response to oxidative stress in most species, and we predict that the P450s may also be involved in antioxidant defense in A. c. cerana. To gain insight into the roles of the P450s, we isolated and characterized three genes (AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5) from A. c. cerana and analyzed the expression patterns of these genes in different tissues and different developmental stages. We also investigated the transcripts of these three genes when exposed to various oxidative stresses, including different temperatures (4 and 44°C), heavy metals (HgCl2 and CdCl2), pesticides (DDV, deltamethrin, and paraquat), and ultraviolet light (UV). Moreover, we used RNAi technology to knockdown AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5. We also investigated the activity of two antioxidant enzymes (POD and CAT) and examined the expression levels of other antioxidant genes. A very broad conclusion can be drawn from our results, with some considerable degree of certainty: AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 might play crucial roles in the response to oxidative stress in A. c. cerana.

MATERIALS AND METHODS

Laboratory Feeding of Honeybees and Experimental Design

Animal housing facilities and handling protocols were approved by the Animal Welfare and Health Committee of Shandong Agricultural University. Honeybees were collected from the experimental apiary of Shandong Agricultural University (Tai’an, China). Honeybees of different development stages, including larvae (L1–L5), pupae [pupa (Pp), white-eyed pupae (Pw), brown-eyed pupae (Pb), and dark-eyed, dark pigmented thorax pupae (Pbd)] (de F Michelette and Soares, 1993), and newly emerged worker bees (less than 12 h of age), were obtained from five healthy hives. Newly emerged workers were collected at 15 days after being labeled with paint. The brain (BR), midgut (MG), muscle (MS), and epidermis (EP) of 15-day post-emergence adult bees (n = 90; 15/group) were dissected on ice, frozen immediately in liquid nitrogen, and stored at −80°C until ready for use. Adult bees (post-emergence age: 15 days) were collected in wooden cages (dimensions of 10 cm × 7 cm × 8 cm), which were maintained in an incubator (33 ± 1°C, 60 ± 10% relative humidity, darkness). Cages were divided into nine groups.
Groups 1 and 2 were fed with a sucrose solution containing HgCl$_2$ and CdCl$_2$, respectively. Group 3 was subjected to UV light. Groups 4 and 5 were treated with different temperatures. In addition, groups 6–8 were treated with one of three different pesticides (DDV, deltamethrin, and paraquat). Group 9 served as the negative control. The abiotic stress conditions for each experimental group are shown in Supplementary Table S1. The final effective concentrations of the pesticides were determined according to the manufacturer's instructions. All bee samples were immediately frozen in liquid nitrogen at the appropriate time points and were stored at $-80^\circ C$ until ready for use. All experiments were performed with at least six biological replicates.

### Primers and PCR Amplification Conditions

All sequences of the PCR primers and the amplification conditions are listed in Tables 1, 2, respectively. In this study, all primers used for quantitative PCR (qPCR) were designed based on the principle of quantitative primer design. The $E$ value and $R^2$ value of each qPCR primer pair are listed in Table 1. All primer pairs were synthesized by the Sangon Biotechnological Company (Shanghai, China).

| Gene   | Amplification | Primer sequence (5′–3′) | Product size (bp) | Coefficient ($R^2$) |
|--------|---------------|--------------------------|-------------------|---------------------|
| CYP314A1 | ORF amplification | F: CGAGATGTTACTCTCGAGTG 1,506 | R: AGATGAACGATCTCAAGAAC |
|         | qPCR          | F: CTTCGGGTAGGCTCTACGCAGTC 125 | R: ACTTTGTATCCGACCCGTTG |
|         | dsRNA         | F: TAAATACGACTCACTATAGGGCTAACATATTAATTTCATGCCTCAG 475 | R: TAAATACGACTCACTATAGGGCTAACATATTAATTTCATGCCTCAG |
| CYP4AZ1 | ORF amplification | F: ATGGATTCTGCCAATATTGTTTCTTTTA 1,548 | R: CATGATCAAAATATATATCTCCTTAACACGG |
|         | qPCR          | F: TTGGCGCAGATCCAAATAATG 123 | R: GCAATGCTGAATCTCAAGGC |
|         | dsRNA         | F: TAAATACGACTCACTATAGGGCTATACATATTAATTTCATGCCTCAG 526 | R: TAAATACGACTCACTATAGGGCTATACATATTAATTTCATGCCTCAG |
| CYP6AS5 | ORF amplification | F: ATGGCGAGCAGTTCCTCAGA 1,500 | R: TCATATTTGTATATTTCATATATATTT |
|         | qPCR          | F: AATGGGCAGAGAAGTTCTCAGC 127 | R: AAAGAATGGTGAGAGCTAGCATAGTG |
|         | dsRNA         | F: TAAATACGACTCACTATAGGGCTAGAGAGATATGTTTCTTGCTCAAG 507 | R: TAAATACGACTCACTATAGGGCTAGAGAGATATGTTTCTTGCTCAAG |
| GFP    | dsGFP        | F: TAAATACGACTCACTATAGGGCTAGAGAGATATGTTTCTTGCTCAAG 546 | R: TAAATACGACTCACTATAGGGCTAGAGAGATATGTTTCTTGCTCAAG |
| AccTrx1 | qPCR          | F: GGTTGAGAAATATACACGTAGC 124 | R: GAGTAAGAGTAATATGAGTACAGGTACAGGT |
| AccsHSP22.6 | qPCR       | F: CGATGAGCGACCTTGTTTCTTCAAGAC 127 | R: GAGTAAGAGTAATATGAGTACAGGTACAGGT |
| AccGSTO1 | qPCR         | F: CATCTCTTTACATTCTCTGTGC 123 | R: TTAATACGACTCACTATAGGGCTAGAGAGATATGTTTCTTGCTCAAG |
| β-acting | qPCR       | F: TTATATGCGAAACACTGGCTTTT 126 | R: AAGAAATGGTGAGAGCTAGCATAGTG |

Underlined sequences in primers for dsRNA production are added adaptors containing T7 polymerase promoters.
TABLE 2 | PCR amplification conditions.

| Primer pairs | Amplification conditions |
|--------------|-------------------------|
| AccCYP314A1-F/R | 10 min at 94°C, 40 s at 94°C, 40 s at 49°C, 60 s at 72°C for 35 cycles, 10 min at 72°C |
| AccCYP4AZ1-F/R | 10 min at 94°C, 40 s at 94°C, 40 s at 55°C, 70 s at 72°C for 35 cycles, 10 min at 72°C |
| AccCYP6AS5-F/R | 10 min at 94°C, 40 s at 94°C, 40 s at 54°C, 70 s at 72°C for 35 cycles, 10 min at 72°C |
| *AccCYP314A1-F/R | 10 min at 94°C, 40 s at 94°C, 40 s at 56°C, 40 s at 72°C for 35 cycles, 10 min at 72°C |
| *AccCYP4AZ1-F/R | 10 min at 94°C, 40 s at 94°C, 40 s at 57°C, 40 s at 72°C for 35 cycles, 10 min at 72°C |
| *AccCYP6AS5-F/R | 10 min at 94°C, 40 s at 94°C, 40 s at 55°C, 40 s at 72°C for 35 cycles, 10 min at 72°C |
| *GFP-F/R | 10 min at 94°C, 40 s at 94°C, 40 s at 58°C, 40 s at 72°C for 35 cycles, 10 min at 72°C |

*Primers for dsRNA productions.

conditions: initial denaturation program (94°C for 10 min), followed by 35 cycles of 94°C for 40 s, N°C for 40 s, 72°C for M s (N: the advisable annealing temperature of the primers; M: determined by the length of the gene sequences), and 72°C for 10 min. All PCR products were separated by electrophoresis and purified using a Gel Extraction Kit (Solarbio, China), cloned into pEASY-T1 vectors (TransGen, China), and transformed into competent *Escherichia coli* DH5α (*E. coli* DH5α) cells for sequencing (Sangon, China).

Bioinformatics Analysis and Phylogenetic Tree Construction

DNAman version 5.2.2 (Lynnon Biosoft, Canada) was used to search for the ORFs of the three genes and to predict the theoretical isoelectric points (pIs) and molecular weights (MWs) of the protein products. Conserved domains in the three P450 genes were detected using bioinformatics tools available at the NCBI server.1 Phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis 7 software (MEGA version 7), using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the analyzed taxa (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates are collapsed. The evolutionary distances were computed using the Poisson correction method (Felsenstein, 1985) and are expressed as the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There was a total of 507 positions in the final dataset.

Real-Time Relative Quantitative PCR

Real-time relative qPCR was used to analyze the mRNA levels of the three P450 genes using the 7500 Real-time System (ABI, United States) and ABI SDS 1.4 for the 7500 system (ABI, United States). The *A. c. cerana* β-actin gene (GenBank accession no. XM_017065464) was used as a reference gene. The levels of the target genes were compared among the groups of interest. qPCR reactions were performed in a final volume of 20 µL. 7 µL sterile deionized water, 1 µL each primer (10 µmol/L), 10 µL Taq DNA polymerase (5 U/µL, TaKaRa), and 1 µL DNA template (50 ng/µL). All qPCR amplifications were performed using the following conditions: (1) 30 s at 95°C for pre-denaturation; (2) 40 cycles of amplification (5 s at 95°C for denaturation, 35 s at 60°C for annealing and extension). Three technical replicates were performed for each experiment.

Antibody Production

Custom-made polyclonal antibodies were used. The epitopes were predicted using the GenScript OptimumAntigen design tool, and the peptide antigen sequences for AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were PFGAGRRICPGK, EAHRRNKIDDEGIRE, and PNPDSFDPERFDQDAMAS, respectively. The peptide antigens were then synthesized (Sangon, China). After the coupling reaction and mixing with complete adjuvant, the coupled antigen was used for injection. Next, the coupled antigen was mixed with incomplete adjuvant (Sigma, United States) and injected into white mice (Taibang, China), which were 6 weeks old and specific pathogen-free, four times at 4-week intervals. Subsequently, blood was collected by eyeball puncture, incubated at 37°C for 1 h, and centrifuged at 3,000 × g for 10 min. Finally, the anti-serum was prepared. The collected antibody was hybridized to a blot containing the overexpressed proteins to detect the specificity of the anti-serum.

Western Blotting

The honeybee whole bodies were lysed in RIPA buffer (pH 7.5). The lysate was centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was then collected, and the protein concentration was determined using the BCA Protein Assay Kit (Beyotime, China). The extracted protein was separated on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore, Bedford, MA, United States). The membrane was blocked with QuickBlock™ Western buffer (Beyotime, China) for 1.5 h to reduce non-specific binding. Next, the blot was incubated with the primary antibody overnight at 4°C. After washing, the blot was incubated with AP-labeled goat anti-mouse IgG (H + L) secondary antibody (Beyotime, China) for 4 h at 4°C. Finally, the signal was detected using an enhanced BeyECL Plus kit (Beyotime, China) and visualized in Fusion Fx by Vilber Lourmat. The optical density of each band was quantified using Fusion Capt Advance Fx7 software (Beijing Oriental Science, China), using tubulin as an internal control. The antibodies used included anti-AccCYP314A1 (1:100), anti-AccCYP4AZ1 (1:100), anti-AccCYP6AS5 (1:100), and anti-tubulin (1:1,000).

---

1http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml
RNA Interference

To synthesize dsRNA for AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5, gene-specific primers (listed in Table 1), with a T7 polymerase promoter sequence at their 5' ends, were used to amplify the target sequences of the three genes by PCR. The PCR amplification conditions are shown in Table 2. The PCR products were purified using the Gel Extraction Kit (Solarbio, China). Next, the dsRNAs for the three P450 genes were synthesized using RiboMAX T7 large-scale RNA production systems (Promega, United States), according to the manufacturer's instructions. To remove the DNA template, the synthesized dsRNA was digested using DNase I, precipitated with absolute ethyl alcohol, and then redissolved in RNase-free water. dsRNA of the green fluorescent protein gene (GFP) (GFP control; GenBank accession no. U87974) was also synthesized.

Newly emerged workers, divided randomly into six groups \((n = 50/\text{group})\), were used for RNA interference (RNAi) experiments. Four groups were injected with 0.5 \(\mu\)L (9 \(\mu\)g) of either dsAccCYP314A1, dsAccCYP4AZ1, dsAccCYP6AS5 or dsGFP, performed as previously described by Amdam et al. (2003) (Hossain et al., 2008). The fifth group was injected with 0.5 \(\mu\)L of sterile water (\(H_2O\) control), and the sixth group was the negative control group without treatment. Honeybees were maintained in an incubator (60% relative humidity at 34°C) under a 24-h darkness regimen. Healthy workers were subsequently sampled each day and flash frozen in liquid nitrogen and stored at −80°C until ready for use. qPCR was performed to detect AccsHsp22.6 (GenBank accession no. KF150016), AccGSTO1 (GenBank accession no. KF496073), and AccTrx1 (GenBank accession no. JX844652) expression profiles when AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were knocked down. Six independent biological replicates were performed in each experiment.

Enzymatic Activities of RNAi-Mediated Silencing Samples of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5

Total proteins were extracted from adults injected with dsAccCYP314A1, dsAccCYP4AZ1, dsAccCYP6AS5 or dsGFP at 2 and 3 days post injection. The total proteins were quantified using the BCA Protein Assay Kit (Beyotime, China). Next, a CAT test kit (Jiancheng, China) was used to assay CAT capacity. In addition, a POD assay kit (Jiancheng, China) was used to assay the POD capacity, according to the manufacturer's protocols.

Assessment of Survival Rate

Honeybees were randomly assigned to six experimental groups with three cages per group \((n = 3; 1,080 \text{ bees in total})\). Groups I and II were injected with 0.5 \(\mu\)L (9 \(\mu\)g) dsRNA-AccCYP314A1; groups III and IV were injected with 0.5 \(\mu\)L (9 \(\mu\)g) dsRNA-AccCYP4AZ1; groups V and VI were injected with 0.5 \(\mu\)L (9 \(\mu\)g) dsRNA-AccCYP6AS5. Groups I, III, and V were fed with a 50% sucrose diet (control group, CK); groups

![FIGURE 1](Image)
RESULTS

Isolation of the Sequences for AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 and Analysis

The ORF sequence of AccCYP314A1 (GenBank accession no. MH748719) was 1,554 bp. The ORF of AccCYP314A1 encodes a polypeptide of 517 amino acids, with a calculated molecular mass of 58.6 kDa and a theoretical pI of 8.48. The length of the AccCYP4AZ1 ORF sequence (GenBank accession no. MH748718) was 1,548 bp, encoding a polypeptide of 515 amino acids, with a calculated molecular mass of 61 kDa and a theoretical pI of 9.07. The AccCYP6AS5 ORF (GenBank accession no. MH748720) was 1,500 bp long. The ORF encoded a polypeptide of 499 amino acids, with a predicted molecular mass of 58.57 kDa and an pI of 8.57.

As shown in Supplementary Figures S1–S3, multiple sequence alignments also revealed that the deduced amino acid sequences of each P450 (AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5) have a high similarity with those of other insect genes. The deduced amino acid sequence of AccCYP314A1 was closely related to those of A. mellifera 314A1 (GenBank accession no. XP_006610075, 96.36% protein sequence identity), A. florea 314A1 (GenBank accession no. XP_012348791, 94.88% protein sequence identity), and A. dorsata 314A1 (GenBank accession no. XP_006619840, 94.5% protein sequence identity). The deduced amino acid sequence of AccCYP4AZ1 was closely related to those of A. mellifera 4AZ1 (GenBank accession no. XP_006610075, 100% protein sequence identity), A. florea 4AZ1 (GenBank accession no. XP_012348791, 82.14% protein sequence identity), and A. dorsata 4AZ1 (GenBank accession no. XP_006619840, 93.98% protein sequence identity). The deduced amino acid sequence of AccCYP6AS5 had 99.4 and 74.75% similarity with A. mellifera 6AS5 (GenBank accession no. NP_001035324), and Habropoda laboriosa 6a2-like (GenBank accession no. XP_017796836), respectively. The characteristic active-site motifs Helix-K (EXXR) (Grahamlorence et al., 1995) and Helix-C (WXXXR) (Nelson, 1998) of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were highly conserved among P450 genes (Supplementary Figures S1–S3). In addition, the predicted AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 proteins share several characteristics with other members of the P450 supergene family, such as the amino acid residues of FXXGXRXCXG in the haem-binding domain (Kasai and Scott, 2000) (Supplementary Figures S1–S3). As shown in the above results, we named the three P450 genes AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5.

Multiple sequence alignment revealed the nucleotide identity (54.8%) among the three P450 genes (Supplementary Figure S4). A phylogenetic tree was also built to investigate the evolutionary relationships among AccCYP314A1, AccCYP4AZ1, AccCYP6AS5 and their homologs in insects. As shown in Figure 1, the phylogenetic tree revealed that AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 belong to the mitochondrial P450 clade, the CYP4 clade and the CYP3 clade, respectively.
Tissue-Specific Distribution and Developmental Expression of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5

To investigate the expression patterns of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 in various tissues and in different developmental stages of *A. c. cerana*, qPCR was performed using total RNA extracted from various tissues from larvae, pupae, and adults. The three P450 genes were expressed differently among various tissues (Figure 2B). Specifically, Acc314A1 was expressed predominantly in the EP, followed by the BR, and little expression was detected in MS. The relative expression level of AccCYP4AZ1 showed the highest level in the

![Graphs showing expression levels of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 under various treatments.](image-url)

**FIGURE 3** | qPCR analysis of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 expression levels after treatment with various abiotic stresses. Total mRNA was extracted from adult bees (15 days post emergence) treated at the indicated times with (A) 4 °C, (B) 44 °C, (C) HgCl₂, (D) CdCl₂, (E) DDV, (F) paraquat, (G) deltamethrin, and (H) UV and qPCR was then performed. Each value is given as the mean + SEM. Different letters above the bars indicate significant differences (*P* < 0.05).
Expression Pattern of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 Under Different Oxidative Stresses

Fifteen-day post-emergence workers were exposed to 4°C, 44°C, HgCl₂, CdCl₂, UV light, and pesticides (DDV, paraquat, and deltamethrin). The relative expression levels were normalized to those of the control workers (CK). As shown in Figure 3A, under 4°C treatment, AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were all induced and reached their highest levels at 3, 4, and 1 h, respectively. Acc314A1 was obviously induced, while AccCYP4AZ1 and AccCYP6AS5 were hardly induced, when exposed to 44°C (Figure 3B). Under CdCl₂ treatment, the transcript levels of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were all upregulated and reached the maximum levels at 1, 6, and 1 h, respectively (Figure 3C). Under HgCl₂ stress (Figure 3D), AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were all upregulated and reached their highest transcripts at 6, 1, and 3 h, respectively. As shown in Figure 3E, AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were upregulated when exposed to DDV and reached their peaks at 0.5 h. Under deltamethrin and paraquat treatments, AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were all induced (Figures 3F,G). As shown in Figure 3H, AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 reached their peaks at 2 h, whereas AccCYP4AZ1 reached its peak at 0.25 h. As shown in Figure 3H, the levels AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were upregulated after treatment with UV and reached maximums at 2, 0.5, and 0.5 h, respectively.

Western Blot Analysis

The above qPCR results showed that the AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 genes were induced in A. c. cerana in response to various types of oxidative stress. The entire Western blot using the three specific antibodies against the AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 proteins is shown in Supplementary Figure S5. Western blot was used to analyze the changes in AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 protein levels after 4°C (A), CdCl₂ (B), paraquat (C), and deltamethrin (D) treatments (Figure 4). In our Western blot results, the protein expression levels of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 are basically consistent with the qPCR results (Figure 4), although there were small differences in the time points and degree of expression.

Knockdown of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 and the Expression Profiles of Other Antioxidant Genes

To investigate the functions of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 in the antioxidant defense of honeybee workers, RNAi experiments were performed to ascertain their functions. Newly emerged adult workers were injected with H₂O, dsRNA-GFP, dsRNA-AccCYP314A1, dsRNA-AccCYP4AZ1, or dsRNA-AccCYP6AS5. The transcripts of the control groups, the H₂O and dsRNA-GFP groups, were almost equal, showing that injection with dsRNA-GFP did not influence the expression of the above three genes in A. c. cerana (Figure 5). qPCR results showed that the AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 genes were successfully silenced compared with the control groups.
AccCYP314A1 was silenced, no differences in the transcription levels of AccCYP4AZ1 and AccCYP6AS5 were apparent among the treatment groups 3 days after injection. When AccCYP4AZ1 was silenced, the transcription level of AccCYP314A1 increased, but the transcription level of AccCYP6AS5 in the H2O-injected group, the GFP-injected group and the dsRNA-AccCYP4AZ1-injected group were all downregulated compared with the uninjected group after 2 days (Figure 7B). When AccCYP6AS5 was knocked down for 3 days, the expression level of AccCYP314A1 was upregulated, while the expression level of AccCYP4AZ was downregulated (Figure 7C).

Quantitative PCR was performed to further evaluate the responses of several genes that have been reported to be involved in oxidative stress responses after AccCYP314A1, AccCYP4AZ1, and AccCP6AS5 were silenced (Meng et al., 2014; Yao et al., 2014; Zhang et al., 2014). When AccCYP314A1 was knocked down, AccGSTO1 was induced (Figure 8A). As shown in Figure 8B, AccGSTO1 and AccsHsp22.6 were upregulated compared with the control groups. The qPCR results showed that AccGSTO1, AccsHsp22.6, and AccTrx1 were all suppressed when AccCYP6AS5 was silenced (Figure 8C). All of the above results suggest that the induced genes participate in the compensation for the knockdown of AccCYP314A1, AccCYP4AZ1, and AccCP6AS5 in A. c. cerana.

**Determination of Enzymatic Activities After Knockdown of AccCYP314A1, AccCYP4AZ1, and AccCP6AS5**

When the AccCYP314A1, AccCYP4AZ1, and AccCP6AS5 were knock down, the enzymatic activities were all higher compared with control groups, respectively (Figure 9). As shown in Figures 9A,B, the CAT and POD capacities of A. c. cerana after the silencing of AccCYP314A1 were all higher than those of the control groups. Overall, for CAT, the activity increased at 3-day time point significantly higher than AccCYP4AZ1 was silenced after 2 days (Figure 9C). In A. c. cerana, silenced AccCYP4AZ1 had a significant effect on the activity of POD. The enzymatic activity of POD were significantly higher compared with control bees (Figure 9D). Both CAT and POD activity dose significantly enhanced compared with the control groups when AccCP6AS5 was silenced after 2 or 3 days (Figures 9E,F).

**Survival Rate of Artificially Rearing Bees**

The three different pesticides treatment groups were all survived shorter compared with control groups, respectively (Figure 10). For AccCYP314A1, there was no significant difference between control group and DDV treatment until day 1. By day 2, the control group had significantly higher survival rate compared with the DDV treatment (Figure 10A).

For AccCYP4AZ1, there was no significant difference between control group and deltamethrin treatment until day 1. By day 2, honeybees fed the control diet had significantly higher survival rate compared with honeybees fed deltamethrin (Figure 10B). For AccCP6AS5, honeybees fed the control diet had significantly higher survival rate compared with paraquat treatment starting on day 2, and the trend continued until day 4 (Figure 10C).

and the lowest transcript levels were discovered at 2 or 3 days post-injection. Additionally, the Western blot results showed the protein expression levels of the three P450 genes at 2 or 3 days post-injection (Figure 6). As shown in Figures 6A,C, 3 days after injection, the protein expression level of AccCYP314A1 and AccCP6AS5 were obviously silenced; the protein expression level of AccCYP4AZ1 was downregulated 2 days after injection (Figure 6B). The protein expression levels of AccCYP314A1, AccCYP4AZ1, and AccCP6AS5 after injection are consistent with the qPCR results.

To evaluate the response of the other two P450 genes after one was silenced, qPCR was used. As shown in Figure 7A, when AccCYP314A1 was silenced, no differences in the transcription

**FIGURE 5 | Effects of RNAi on mRNA levels of newly emergence honeybees, as induced by injecting 9 μg dsRNAs. The mRNA levels of (A) AccCYP314A1, (B) AccCYP4AZ1, and (C) AccCP6AS5 are shown. The β-actin gene was used as an internal control. Each value is given as the mean + SEM. Different letters above the bars indicate significant differences (P < 0.05).**
DISCUSSION

In many insects, P450 genes are known to play crucial roles in the metabolism of chemicals from host plants and the degradation of diverse insecticides (pyrethroids, organophosphates, and carbamates), resulting in the bioactivation or detoxification of these compounds (Feyereisen, 1999; Scott, 1999; David et al., 2013). However, only a few P450 genes from A. c. cerana have been reported. Therefore, the identification and characterization of new P450 genes have become very attractive research areas. In this study, we obtained the ORF sequences of three new P450 genes (AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5). P450 genes are classified into four clades based on their amino acid sequence similarities. Within the same clades, P450s share ≥55% identities among amino acid (AA) sequences, whereas individual P450 enzymes can have up to 97% identity with any other P450 enzyme (Nebert et al., 1987). As shown in Figure 1, AccCYP314A1 and AccCYP4AZ1 were clustered in the mitochondrial P450 and CYP4 clades, respectively. Phylogenetic analysis showed that AccCYP6AS5 was clustered in the CYP3 clade (Figure 1). The CYP3 and CYP4 clades were once thought to be largely responsible for xenobiotic metabolism and insecticide resistance and are induced by phenobarbital, pesticides, and natural products (Berenbaum, 2002). Meanwhile, the CYP4 clade has been reported to be involved in chemical communication and pesticide metabolism in several insects (Claudianos et al., 2006). Additionally, previous research reported that the CYP4 clade is implicated in the oxidative response to oxidative stress and may play crucial roles in research reported that the CYP4 clade is implicated in the xenobiotic metabolism and insecticide resistance and are induced in several insects (Claudianos et al., 2006). Additionally, previous research reported that the CYP4 clade has been reported to be diversified in different developmental stages of A. c. cerana. For example, CYP4G11 was expressed at higher levels in the 14-day post-emergence stage than in other developmental stages, a finding that has been previously reported (Shi et al., 2013). As shown in Figure 2A, AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were all observed to be expressed in all developmental stages. Additionally, AccCYP314A1, and AccCYP4AZ1 were expressed at higher levels of transcript accumulation in Pd, and AccCYP6AS5 showed significantly higher expression in NE than in the other developmental stages, for which stage AccCYP314A1 showed the lowest expression (Figure 2A). The above results suggest that the three genes could be involved in specific functions in the Pd and NE stages. Previous studies also found similar results for P450s in L. migratoria, where CYP408B1 was detected at a higher expression level in adults (Guo Y. et al., 2012). However, the expression profiles of these three P450 genes were different from those of AccCYP336A1 in A. c. cerana and CYP409A1 in L. migratoria (Guo Y.Q. et al., 2012; Zhu et al., 2016). In addition, the differences in the expression patterns of the P450 genes during different developmental stages and in different tissues imply their specific functions in insects (Chung et al., 2009).

Previous research findings have revealed that environmental conditions, such as temperature (cold or heat), heavy metals, insecticides, and UV exposure, can induce oxidative stress
Deltamethrin and pyrethroid are still being widely used as major insecticides. In insects, a previous study reported that P450 genes were involved in the process of insecticide resistance. AccCYP336A1, a stress-inducible gene, could be induced by various stresses, such as deltamethrin, heat, H$_2$O$_2$, and UV (Zhu et al., 2016). CYP409A1 and CYP408B1 from L. migratoria were upregulated after deltamethrin treatment, revealing that CYP409A1 and CYP408B1 play key roles in reducing harmful ROS (Guo Y.Q. et al., 2012). Previous research showed that AccCYP4G11 was upregulated by treatments with 4°C temperatures, H$_2$O$_2$, several insecticides (cyhalothrin, acaricide, parquet, phoxime) and HgCl$_2$ (Shi et al., 2013). Previous results have revealed that some P450 genes (CYP6FD2, CYP6FF1, and CYP6FG2) showed higher expression levels when treated with the LD10 of deltamethrin but were not induced at the LD30 and LD50 levels of deltamethrin.
These differences could be due to deltamethrin affecting the expression profiles of different P450 genes differently and in a dose-dependent manner. As a previous report described, temperature (heat or cold stress) is one of the key mediators of ROS generation, which could cause physiological changes in organisms (An and Choi, 2010). For example, research has shown that heat stress can induce polyamine oxidation due to ROS damage; cold stress can result in the apoptosis of hepatocytes and liver endothelial cells and the generation of ROS (Rauen et al., 1999). As shown in Figure 3, after treatment with cold (4 °C) or heat (44 °C), the expression levels of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were increased in this study. These results suggest that AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 may be involved in regulating body temperature and the heat ceiling, thus protecting A. c. cerana from ROS damage. It was also demonstrated that heavy metals can damage normal development and enhance the endogenous ROS levels in insects (Narendra et al., 2007). Mercury (Hg) and cadmium (Cd), the most poisonous heavy metals in nature, can directly bind to metal ion sites on enzymes, resulting in the inactivation of enzymes (Rashed, 2001). When foragers forage for pollen and nectar, foragers may come into greater contact with heavy metals in the environment. qPCR results also proved that the expression levels of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were enhanced by CdCl\textsubscript{2} (Figure 3C) and HgCl\textsubscript{2} (Figure 3D) treatment. These results suggest that AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 are involved in avoiding injury under conditions of HgCl\textsubscript{2} and CdCl\textsubscript{2} stress. Insecticides, which are the primary threat to honeybees in the environment, could destroy physiological and biochemical functions due to lipid biomembrane oxidation damage (Narendra et al., 2007). In insects, P450 genes have been reported to be involved in insecticide resistance. Several insecticides, such as deltamethrin and pyrethroid, have been widely used and are still being used. In this study, we investigated the effect of three insecticides (deltamethrin, paraquat, and DDV) on AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 expression at the mRNA and protein levels, to determine whether the three P450 genes are involved in the process of insecticide metabolism. As shown in Figure 3, the expression levels of AccCYP314A1,
AccCYP4AZ1, and AccCYP6AS5 were all induced compared with the control samples, although the time to reach peak expression were different for all three (Figures 3E–G). It is a complex manner in which an insecticide affects the expression pattern of different P450 genes differently and which is a time-dependent manner. The differences could be due to the more pronounced toxic effects of pesticides over the long term. UV radiation, a typical oxidant, causes oxidative damage (Schauen et al., 2007; Nguyen et al., 2009). Here, our data showed that AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 can be induced by UV radiation treatment (Figure 3H). These findings support the hypothesis that AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 play crucial roles in protecting A. c. cerana against ROS damage. The regulation of molecular mechanisms by different P450 genes in honeybees is not well understood. If the gene are involved in the process of insecticide resistance, the expression patterns of P450 genes may affect the susceptibility of insects to insecticides.

Western blot analysis was performed to explore the protein expression patterns of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 after A. c. cerana were treated with 4 °C, CdCl₂, paraquat and deltamethrin. As a whole, the protein expression patterns of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were consistent with the qPCR data. Concerning the mRNA and protein levels of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5, there were certain differences in time points and degree of change (Figure 4). There are some explanations for these differences: first, the increased protein levels of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 could be the result of accumulation; second, the inconsistent mRNA and protein levels could be due to post-transcriptional regulation. Indeed, Hfq (RNA-binding protein) was shown to regulate the expression of invE through post-transcriptional regulation in S. sonnei; although the mRNA expression of invE was easy to detect, the protein of invE was barely detected (Mitobe et al., 2009). The above results support the hypothesis that AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 play important roles in protecting A. c. cerana against ROS damage.

To further understand gene roles, RNAi technology has been used in many insects. To examine the potential roles of these three genes, we first established gene-silencing procedures, in which H₂O, dsRNA-GFP, dsRNA-AccCYP314A1, dsRNA-AccCYP4AZ1, or dsRNA-AccCYP6AS5 was microinjected into A. c. cerana. The controllable dose and minimal invasiveness make the microinjection method widely useful for diverse insect types. The results showed that the effect of dsGFP was less obvious; there were no off-target effects because there is no GFP target in A. c. cerana (Elias-Neto et al., 2010). As shown in Figures 5, 6, the gene silencing efficiency was different for the three genes. In this study, 40–70% gene silencing efficiency was achieved for the different genes. Above results showed that the three P450 genes of AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 might be involved in environmental response and detoxification in insects. The qPCR method was performed to elucidate the relationship among the three P450 genes. As shown in Figure 7, the transcription levels of the other two genes were significant changed compared with control groups when one of the three genes was silenced. These results should be useful for understanding the relationship and the functions of the three P450 genes.

The RNAi technology provides a valuable research tool for future research into the functions of P450s in A. c. cerana. Compared with the control groups, AccCYP314A1 silencing markedly down-regulated AccsHSP22.6 and AccTrx1 on the
second day post injection, but AccGSTO1 expression was upregulated on the third day post injection, suggesting that AccGSTO1 may be involved in the process of reducing the production of ROS after AccCYP314A1 is silenced (Figure 8A). The qPCR results also showed that, when AccCYP4AZ1 was silenced, the expression levels of AccGSTO1, AccTrx1, and AccHSP22.6 were induced (Figure 8B). In addition, when AccCYP6AS5 was knocked down, the expression levels of AccGSTO1, AccTrx1, and AccHSP22.6 were lower than those of the control groups (Figure 8C). Among these genes, AccGSTO1 and AccTrx1 were demonstrated to be involved in different abiotic stress responses (Meng et al., 2014; Yao et al., 2014). Previous research showed that AccHSP22.6 could not only be involved in the abiotic stress response but also in development (Zhang et al., 2014). Thus, we speculate that AccCYP314A1 and AccCYP6AS5 might also be involved in the process of abiotic stress defense and development in A. c. cerana.

The antioxidant enzymes CAT and POD play crucial roles in scavenging ROS, which can cause oxidative damage to DNA, proteins, and lipids in an organism (Lee et al., 2005; Corona and Robinson, 2006). As shown in our results, the activities of CAT and POD were all enhanced when AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were knocked down, suggesting that A. c. cerana was exposed to a high level of oxidative stress when AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were silenced, and the two antioxidant enzymes might be involved in scavenging ROS (Figures 9A–C). When AccCYP314A1, AccCYP4AZ1, and AccCYP6AS5 were silenced, the DDV, deltamethrin, and paraquat treatments significantly decreased survival rate, respectively (Figure 10). This results further confirmed that enzymes encoded by the three P450 genes might contribute to the detoxification of the three pesticides in A. c. cerana (Figure 10).

In this study, we identified and characterized the expression patterns of three novel P450 genes from the major agricultural insect A. c. cerana. The expression patterns of the three novel genes showed developmental stage-specific and tissue-specific expression in A. c. cerana. Our findings on the three antioxidant genes in A. c. cerana reveal that there may be more extensive diversification within the P450 supergene family in insects (Sasabe et al., 2004). Our results also imply that the three genes were involved in antioxidant activities, based on the results of using RNAi knockdown for each of the three genes in A. c. cerana. The knowledge gained from this study may help us to better understand the roles of insect P450s and their interactions with pesticides at the mRNA and protein levels. Thus, further functional research on these P450s is essential for the assessment of their functions in abiotic stress and in agriculturally important insects.

REFERENCES

Amdam, G. V., Simões, Z. L., and Guidugli, K. R. (2003). Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. BMC Biotechnol. 3:1. doi: 10.1186/1472-6750-3-1

An, M. I., and Choi, C. Y. (2010). Activity of antioxidant enzymes and physiological responses in ark shell, Scapharca bicornis, exposed to thermal and osmotic stress: effects on hemolymph and biochemical parameters. Comp. Biochem. Physiol. 155B, 34–40. doi: 10.1016/j.cbpb.2009.09.008

Berenbaum, M. (2002). Postgenomic chemical ecology: from genetic code to ecological interactions. J. Chem. Ecol. 28, 873–896. doi: 10.1023/A:1015260931034

Celander, M. C., Moore, M. J., and Stegeman, J. J. (2000). Cellular localization of CYP3A proteins in various tissues from pilot whale ( Globicephala

AUTHOR CONTRIBUTIONS

WZ carried out the experimental work and wrote the paper. BX designed the experiments. WC participated in the SDS-PAGE and Western blot work. ZL designed the primers and analyzed the data of Western blot. LM analyzed the data of RT-qPCR. JY carried out the breeding of Apis cerana cerana. HW assisted with transcript expression assessments. ZL carried out RNA extraction and cDNA synthesis. All authors read and approved the final manuscript.

FUNDING

This work was financially supported by Funds of Shandong Province “Double Tops” Program (2016–2020), the National Natural Science Foundation of China (No. 31572470), and the earmarked fund for the China Agriculture Research System (No. CARS-44).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01608/full#supplementary-material

FIGURE S1 | The alignment of the deduced AccCYP314A1 amino acid sequence with other species known AccCYP314A1. Characteristic regions are underlined including Helix-C (WXXXR), Helix-K (EXXR), and the consensus sequence FXXGXRXCXG are marked with two triangle ▲.

FIGURE S2 | The alignment of the deduced AccCYP4AZ1 amino acid sequence with other species known AccCYP4AZ1. Characteristic regions are underlined including Helix-C (WXXXR), Helix-K (EXXR), and the consensus sequence FXXGXRXCXG are marked with two triangle ▲.

FIGURE S3 | The alignment of the deduced AccCYP6AS5 amino acid sequence with other species known AccCYP6AS5. Characteristic regions are underlined including Helix-C (WXXXR), Helix-K (EXXR), and the consensus sequence FXXGXRXCXG are marked with two triangle ▲.

FIGURE S4 | The nucleotide sequences multiple alignment among the three target genes.

FIGURE S5 | The specific antibodies against the three target proteins. Protein marker molecular weights are given in kDa. (A) the specific antibodies anti-AccCYP314A1 against AccCYP314A1; M, protein marker; lines 1–4: the bands of AccCYP314A1. (B) The specific antibodies anti-AccCYP4AZ1 against AccCYP4AZ1; M, protein marker; lines 1–4: the bands of AccCYP4AZ1. (C) The specific antibodies anti-AccCYP6AS against AccCYP6AS5; M, protein marker; line 1: the negative sample (only SDS-PAGE loading buffer and RIPA buffer); lines 2–4: the bands of AccCYP6AS5.

TABLE S1 | Abiotic stress conditions for each experimental group.

Frontiers in Physiology | www.frontiersin.org 14 November 2018 | Volume 9 | Article 1608
Hossain, M., Shimizu, S., Matsuki, M., Imamura, M., Sakurai, S., and Iwami, M. (2007). Identification of two new cytochrome P450 genes associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. *Pestic. Biochem. Physiol.* 88, 34–41. doi: 10.1016/j.pestbio.2006.07.001

Kasai, S., and Scott, J. G. (2000). Overexpression of cytochrome P450 CYP6D1 is associated with monooxygenase-mediated pyrethroid resistance in house flies from Georgia. *Pestic. Biochem. Physiol.* 68, 34–41. doi: 10.1006/pest.2000.2492

Kirschstein, N. L., and Wilson, J. Y. (2012). Phylogenetic and functional analyses of the cytochrome P450 family 4. *Mol. Phylogen. Evol.* 62, 458–471. doi: 10.1016/j.ympev.2011.10.016

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

Malone, L. A., and Burgess, E. P. J. (2001). Effects of ingestion of a *Bacillus thuringiensis* toxin and a trypsin inhibitor on honey bee flight activity and longevity. *Apidologie* 32, 57–68. doi: 10.1051/apido:2001111

Marionnet, C., Bernard, F., Dumas, A., Verrecchia, F., Mollié, K., Coman, D., et al. (2003). Modulation of gene expression induced in human epithelium by environmental stress *In vivo*. *J. Invest. Dermatol.* 121, 1447–1458. doi: 10.1111/j.1523-1747.2003.12629.x

Mattei, F. D., Ballou, D. P., Coon, M. J., Estabrook, R. W., and Haines, D. C. (2012). Peroxidase-like activity of uncoupled cytochrome P450 studies with bilirubin and toxicological implications of uncoupling. *Biochem. Pharmacol.* 84, 374–382. doi: 10.1016/j.bcp.2012.04.016

Meng, F., Zhang, Y., and Liu, F. (2014). Characterization and mutational analysis of omega-class GST (GSTO1) from *Apis cerana cerana*, a gene involved in response to oxidative stress. *PLoS One* 108, 927–939. doi: 10.1371/journal.pone.0093510

Mitobe, J., Morita-Ishihara, T., Ishihara, A., and Watanabe, H. (2009). Involvement of RNA-binding protein HEF1 in the osmotic-response regulation of invG gene expression in *Shigella sonnei*. *Microbiol. Res.* 164, 110. doi: 10.1016/j.micres.2009.02.009

Moktali, V., Park, J., Fedorova-Abrams, N., Park, B., Choi, J., and Lee, Y. H. (2012). Systematic and searchable classification of cytochrome P450 proteins encoded by fungal and oomycte genomes. *BMCMicrobiol.* 12, 97. doi: 10.1186/1471-2180-12-97

Musasia, F. K., Masiga, D. K., Omedo, I. A., Musanga, D. K., Mwakubambanya, R., Ochieng, R., et al. (2013). Sex-specific induction of CYP6 cytochrome P450 genes in cadmium and lead tolerant *Anopheles gambiae*. *Malar. J.* 12, 97. doi: 10.1186/1475-2875-12-97

Narendra, M., Bhattaracharyula, N. C., Padnavath, P., and Varadacharyulu, N. C. (2007). Peralph12 induced biochemical changes in erythrocyte membrane and red cell osmotic haemolysis in human volunteers. *Chemosteril* 67, 1085–1071. doi: 10.1016/j.chemosphere.2006.11.064

Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., and Guengerich, F. P. (1987). The P450 gene superfamily: recommended nomenclature. DNA 6, 1–11. doi: 10.1089/dna.1987.6.1

Nelson, D. R. (1998). Metazoan cytochrome P450 evolution. *Comp. Physiol. C Pharmacol. Toxicol.* 121, 15–22. doi: 10.1007/bf03146431

Nguyen, T. T., Michaud, D., and Cloutier, C. (2009). A proteomic analysis of the aphid *Macrosiphum euphorbiae* under heat and radiation stress. *Insect Biochem. Mol. Biol.* 39, 20–30. doi: 10.1016/j.ibmb.2008.09.014

Nikou, D., Ranson, H., and Hemingway, J. (2003). An adult-specific CYP6 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, *Anopheles gambiae*. *Gene* 318, 91–102. doi: 10.1016/s0378-1119(03)00763-7

Oldroyd, B., and Wongtiri, S. (2006). *Asian Honey Bees: Biology, Conservation, and Human Interactions*. Cambridge, MA: Harvard University Press.

Pfendringer, B., Aronstein, K., Zinkovsky, E., Andrews, O., Campbell, B., Daly, J., et al. (1997). Cytochrome P450 genes from *Helicoverpa armigera*: expression in a pyrethroid-susceptible and -resistant strain. *Insect Biochem. Mol. Biol.* 27, 507–512. doi: 10.1016/s0965-1748(97)00025-8
Zhang, Y., Liu, Y., and Guo, X. (2014). sHsp22.6, an intronless small heat shock protein gene, is involved in stress defence and development in *Apis cerana cerana*. *Insect Biochem. Mol. Biol.* 53, 1–12. doi: 10.1016/j.ibmb.2014.06.007

Zhu, M., Zhang, W., and Liu, F. (2016). Characterization of an *Apis cerana cerana* cytochrome P450 gene (AccCYP336A1) and its roles in oxidative stresses responses. *Gene* 584, 120–128. doi: 10.1016/j.gene.2016.02.016

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Copyright © 2018 Zhang, Chen, Li, Ma, Yu, Wang, Liu and Xu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.*