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Research paper

Characterization of an *Apis cerana cerana* cytochrome P450 gene (*AccCYP336A1*) and its roles in oxidative stresses responses

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ABSTRACT

Cytochrome P450 monooxygenases (P450), widely distributed multifunctional enzymes, that play an important role in the oxidative metabolism of endogenous compounds and xenobiotics. Studies have found that these enzymes show peroxidase-like activity and may thus be involved in protecting organisms against reactive oxygen species (ROS). In this work, *Apis cerana cerana* was used to investigate the molecular mechanisms of P450 family genes in resisting ROS damage. A cytochrome P450 gene was isolated, *AccCYP336A1*. The open reading frame (ORF) of *AccCYP336A1* is 1491 bp in length and encodes a predicted protein of 496 amino acids. The obtained amino acid sequence of AccCYP336A1 shared a high sequence identity with homologous proteins and contained the highly conserved features of this protein family. Quantitative real-time PCR (qRT-PCR) analysis showed that *AccCYP336A1* was present in some fast developmental stages and had a higher expression in the epidermis than in other tissues. Additionally, the expression levels of *AccCYP336A1* were up-regulated by cold (4 °C), heat (42 °C), ultraviolet (UV) radiation, H_2O_2 and pesticide (thiamethoxam, deltamethrin, methomyl and phoxim) treatments. These results were confirmed by the western blot assays. Furthermore, the recombinant AccCYP336A1 protein acted as an antioxidant that resisted paraquat-induced oxidative stress. Taken together, these results suggest that AccCYP336A1 may play a very significant role in antioxidant defense against ROS damage.

1. Introduction

The Chinese honeybee (*Apis cerana cerana*) plays a critical role in the balance between agricultural economic development and regional ecology as a pollinator of flowering plants (Weinstock et al., 2006). During their lifespan, honeybees face a variety of adverse environmental stressors, including temperature swings, ultraviolet (UV) radiation, H_2O_2 and pesticides, all of which are considered to produce reactive oxygen species (ROS) (Narendra et al., 2007). High ROS concentrations may cause serious oxidative damage to DNA, proteins and lipids (Halliwell and Gutterridge, 1989). High ROS levels can cause DNA damage in the form of base deletions, degradation, single-strand breaks, and rearrangements, giving rise to mutations (Imlay and Linn, 1988). High ROS damage to proteins can lead to particular amino acid modifications and peptide rupture, leading in turn to a loss of enzymatic activity (Stadtman and Levine, 2003). High ROS levels can also lead to lipid peroxidation which destroys cell membrane fluidity and results in apoptosis (Green and Reed, 1998). Moreover, the sperm storage of *Apis mellifera* may be affected by high ROS concentrations (Collins et al., 2004). Due to increasing ROS damage, the bee population is facing serious survival problems. Thus, it is important to understand the antioxidant system and its mechanism of defense against ROS to prevent further damage to the bee population from ROS.

Organisms have developed complex antioxidant mechanisms to avoid oxidative damage. In most cases, organisms protect themselves by varieties of antioxidant enzymes, such as peroxidases (POX), superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferases (GST) and glutathione peroxidase (GPX) (Dubovskiy et al., 2008; Felton and Summers, 1995; Wang et al., 2001). Cytochrome P450 proteins share semblable characteristics with the antioxidant enzymes, as an uncoupled catalytic cycle of cytochrome P450 possesses properties resembling those of a peroxidase (Matteis et al., 2012).

Cytochrome P450s (P450) are a large superfamily of enzymes found in almost all living organisms (Nelson, 1998). Their ancient origin and ubiquity may reflect their physiological importance. These enzymes constitute an extremely important metabolic system because of their involvement in regulating the titers of endogenous compounds

Abbreviations: AccCYP336A1, *Apis cerana cerana* RNA Cytochrome P450 monooxygenases 336A1; *E. coli*, *Escherichia coli*; qRT-PCR, fluorescent real-time quantitative PCR; L1, one day larval instar; Pr, pre-pupal; Pw, white-eyed pupae; Pp, pink-eyed pupae; Pb, brown-eyed pupae; Pd, dark-eyed pupae; A1, 1-day worker bees; A15, 15-day worker bees; A30, 30-day worker bees; ROS, reactive oxidative stress; SAS, Statistical Analysis System; IPTG, opropyl-1-thio-β-D-galactopyranoside; UV, ultraviolet; H_2O_2, hydrogen peroxide.

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such as hormones, fatty acids, and steroids (Li et al., 2007; Feyereisen, 2005). Additionally, this enzyme system plays a central role in the metabolism of xenobiotics, such as drugs, pesticides, and plant toxins by catalyzing oxidation reactions (Nelson et al., 1993; Anzenbacher and Anzenbacherova, 2001; Nebert and Russell, 2002; Thomas, 2007). Besides, those P450s may be involved in the receipt and transmission of semiochemical signals.

In insects, more than 1000 cytochrome P450 genes have been identified, and this number is rapidly increasing due to the increasing number of insect genome sequences. Most insect cytochrome P450 genes belong to four clades: the mitochondrial P450s and CYP2, CYP3 and CYP4 clades. However, the specific functions of the four clades have not been determined. The CYP3 and CYP4 clades were once thought to be largely responsible for the environmental response and detoxifying functions in insects (Berenbaum, 2002; Feyereisen, 2005). Particularly, CYP4 has been implicated in both pesticide metabolism and chemical communication in insects (Claudianos et al., 2006). However, there is emerging evidence that CYP4 is involved in the response to oxidative stress and may have a role in protecting honeybees from oxidative injury in *A. cerana cerana* (Shi et al., 2013). Meanwhile, several members of the mitochondrial CYP and CYP2 families have been implicated for essential roles in hormone biosynthesis (Gilbert, 2004). Earlier studies on the function of CYP3 from insects focused on their roles on environmental response and detoxifying functions. For example, *CYP6Z1* (belongs to CYP3 clade) is overexpressed in pyrethroid resistant in *An. Gambiae* (Nikou et al., 2003). Similarly, *CYP321A1* of *Helicoverpa zea*, a member of the CYP3 clade, metabolizes Cypermethrin when produced in a baculovirus expression system (Sasabe et al., 2004). However, limited studies have reported the antioxidant functions of CYP3 clades, which are of significant relevance to the organisms.

A. cerana cerana is the main honeybee species in China and has enjoyed exceptional advantages over other species, such as a longer period of collecting honey, an increased disease resistance, and a lower food cost. However, with the increasing trend of ROS damage in China, the honeybee is facing increasing survival problems. Although proteins involved in oxidative stress, such as vitellogenin and juvenile hormone, have been researched in *A. mellifera* (Corona et al., 2007), little information is available on these proteins in *A. cerana cerana*. Considering the significant roles of antioxidative processes in insects, we isolated the *AccCYP336A1* gene from *A. cerana cerana* to further study its role in resisting oxidative stress. We evaluated its expression patterns at different developmental stages and in different tissues. Moreover, the honeybees were exposed to cold (4 °C), heat (42 °C), ultraviolet (UV) radiation, H_2O_2 and pesticides (thiamethoxam, deltamethrin, methomyl, and phoxime) to evaluate the expression patterns in response to oxidative stress. Moreover, the western blot assays confirmed the results. Based on these results, we speculate that *AccCYP336A1* might play an important role in the response to oxidative stress.

2. Materials and methods

2.1. Insects and treatments

The Chinese honeybees used in this study were obtained from the experimental apiary of Shandong Agricultural University (Taian, China). Based on their shape, age and eye color, the honeybees were divided into egg, larvae, pupae, and adults, which were collected from the hive. The 15 days post-emergence adult workers were fed a basic adult diet of water and powdered sugar for two days before treatments and were placed in the dark, and divided into 13 groups. Adult workers in groups 1–4 were placed in a cold (4 °C) or hot (42 °C) environment for 0.25, 0.5, 1, 2, 3 and 4 h. Group 5 of the adult worker Chinese honeybees was treated by ultraviolet (UV)-light (30 mJ/cm^2) for 0.25, 0.5, 1, 2, 3 and 4 h. Groups 6–9 were treated with four types of pesticide (thiamethoxam, deltamethrin, methomyl, and phoxime) that were diluted into 1 μg/mL for 0.25, 0.5, 1, 2, 3 and 4 h. Group 10 honeybees were injected with 20 μL of H_2O_2 for 0.25, 0.5, 1, 2, 3 and 4 h. Group 11 honeybees were fed normal food as a control. The tissues of the brain, epidermis, muscle, hemolymph, rectum and midgut were collected from groups 12 and 13, the 15 days post-emergence adult workers Chinese honeybees. All bees and tissues were frozen in liquid nitrogen and stored at −80 °C.

2.2. Primers

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

2.3. RNA extraction, cDNA synthesis

Total RNA was extracted with Trizol (TransGen Biotench, Beijing, China), and first-strand cDNA was synthesized using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China) according to the manufacturer's instructions. The cDNA was used as the PCR template in the gene cloning procedures and qRT-PCR transcriptional analysis.

2.4. The full-length cDNA isolation of AccCYP336A1

Reverse transcription-PCR (RT-PCR) was used to amplify full-length *AccCYP336A1*. To obtain the internal fragment of the *AccCYP336A1* cDNA, primers TP1 and TP2 were designed based on conserved regions of the P450 from *A. mellifera*, *Nasonia vitripennis*, and *Drosophila melanogaster* and synthesized (Sangon Biotechnological Company, Shanghai, China). Specific primers (5P1/5P2 and 3P1/3P2) were generated for the 5′ RACE and 3′ RACE, respectively. Based on the deduced cDNA, specific primers QP1 and QP2 were designed, and the complete coding sequence of *AccCYP336A1* was obtained by RT-PCR.

2.5. Bioinformatic analyses and phylogenetic analyses

Conserved domains of AccCYP336A1 were retrieved using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ORF of *AccCYP336A1* was retrieved from the DNAman version 5.2.2 (Lynnon

Table 1
The primers in this study.

Abbreviation	Primer sequence (5′–3′)	Description
TP1	AATGGGATTCCGTACAGCAA	cDNA sequence primer, forward
TP2	GTAGTTGCATTTTCGGCGAAACCTC	cDNA sequence primer, reverse
5P1	CCGGTTTCATTCCTTTGTACAT	5′RACE reverse primer, outer
5P2	CTTCATTATCAGAGGTAGAAAATG	5′RACE reverse primer, inner
3P1	CCGAATTATTGATACCGATCTGTG	3′RACE forward primer, outer
3P2	CAGCGATGAGAATAAGCAAAGAA	3′RACE forward primer, inner
AAP	GGCCACGCGTCGACTAGTAC$(G)_{14}$	Abridged Anchor Primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged Universal Amplification Primer
B25	GACTCTAGACGACATCGA	3′RACE universal primer, outer
B26	GACTCTAGACGACATCGA$(T)_{18}$	3′RACE universal primer, inner
QP1	GTTCAGTTTTTTTTTCTCGGCG	Full-length cDNA sequence primer, forward
QP2	CAACTCGAACTTTGACTGCAC	Full-length cDNA sequence primer, reverse
SQ1	TGTTCGGTTATTTGCCATTCC	Real-time PCR primer, forward
SQ2	GGTCTGCCAGTAAACTCTTCC	Real-time PCR primer, reverse
β-s	TTATATGCCAACACTGTCCTTT	Standard control primer, forward
β-x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
PRS	CGCCATATGCTGCCTACGAAACACGAC	Protein expression primer, forward
PRX	TTGCGGCCGCTTTCATCAATGTAGCCAAAC	Protein expression primer, reverse

Biosoft, Quebec, Canada). The online tool PeptideMass (http://web.expasy.org/peptide_mass/) was used to forecast the molecular weight and theoretical isoelectric point of AccCYP336A1. The phylogenetic analysis was conducted using the neighbor-joining method with the Molecular Evolutionary Genetics Analysis (MEGA version 4.1). Hydrophilicity was analyzed on the web site of ProtScale (http://web.expasy.org/protscale/). The transmembrane segments were analyzed using TMHMM Server (http://www.cbs.dtu.dk/services/TMHMM/).

2.6. Fluorescent real-time quantitative PCR (qRT-PCR) for transcriptional analysis

To confirm the expression pattern of the *AccCYP336A1* gene at different developmental stages and in different tissues under different environmental stresses, real-time quantitative PCR (qRT-PCR) was carried out using SYBR Premix Ex Taq (TaKaRa, Dalian, China) and a CFX96TM Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Two specific primers (SQ1/SQ2) were used. The β-actin (β-s/β-x) gene (GenBank accession number: HM640276) was used as a reference gene. The primers are listed in Table 1. Three individual samples were prepared for each sample, and each sample was analyzed three times. The linear relationship, amplification efficiency and data analysis were conducted using CFX Manager Software version 1.1. An analysis of the significant differences was performed using Duncan' s multiple range tests with Statistical Analysis System (SAS) software version 9.1.

2.7. Expression and purification of recombinant AccCYP336A1

The 240–460 amino acid sequence of the *AccCYP336A1* ORF, flanked by a Nde *I* site and a Not *I* restriction site, was amplified and subcloned into the expression vector pET-21a (+) with a His-tag. The recombinant plasmid pET-21a (+)-AccCYP336A1 was then transformed into *Escherichia coli* BL21, and a transformed colony was cultured in Luria–Bertani (LB) broth with 40 μg/mL ampicillin (37 °C) until the cell density reached OD_{600} 0.4–0.6. Expression of the recombinant AccCYP336A1 was induced by adding a final concentration of 0.4 mM opropyl-1-thio-β-D-galactopyranoside (IPTG) for 10 h (24 °C). Subsequently, the recombinant AccCYP336A1 protein was purified via the C-term His tag following the manufacturer's instructions. The expression of the purified protein was evaluated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining.

2.8. Characterization of recombinant AccCYP336A1 protein by disc diffusion assay

The recombinant AccCYP336A1 was expressed in *E. coli* BL21. Cells transformed with the empty pET-21a (+) vector was used as the control. All the cells were cultivated to the same OD_{600} ($OD_{600} = 0.6$). Then, all the cells were induced by 0.4 mM opropyl-1-thio-β-D-galactopyranoside (IPTG) for 8 h (24 °C). An initial bacterial culture with a density of 10^7 cells/mL was plated on LB-ampicillin agar plates and incubated at 37 °C (1 h). Then, filter discs (8-mm diameter) soaked in different concentrations (0 mM, 10 mM, 20 mM, 40 mM, and 80 mM) of paraquat were placed on the surface of the agar. The cells were then grown for 24 h (37 °C).

2.9. Anti-AccCYP336A1 preparation and western blot analysis

The purified protein was injected subcutaneously into white mice for generation of antibodies as described by Yan et al. (2013). The total proteins were extracted from entire adult bees A BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to quantify these proteins. Western blotting was performed according to the procedure described by Meng et al. (2010) with some modifications. The anti-AccCYP336A1 serum was used as the primary antibody at a 1:600 (*v*/*v*) dilution. Peroxidase-conjugated goat anti-mouse immunoglobulin G (Dingguo, Beijing, China) was used as the secondary antibody at a 1:2000 (*v*/*v*) dilution. A SuperSignal® West Pico Trial Kit (Thermo Scientific Pierce, IL, USA) was used to visualize the visualized. The housekeeping gene tubulin was used as the control. The tubulin antibody (Beyotime, Jiangsu, China) was used as the housekeeping gene antibody.

3. Results

3.1. Sequence analysis of AccCYP336A1

Using RT-PCR and RACE-PCR, a P450 gene was cloned from *A. cerana cerana* and named *AccCYP336A1*. The open reading frame (ORF) of *AccCYP336A1* is 1491 bp in length and encodes a predicted protein of 496 amino acids with a predicted molecular mass of 57 kDa and an isoelectric point of 8.8. Genes encoding cytochrome P450 enzymes metabolizing endogenous compounds are generally well conserved (Feyereisen, 2005; Rewitz et al., 2007; Rewitz and Gilbert, 2008). A BLAST search showed that the putative AccCYP336A1 protein is present in many bees. An alignment of the AccCYP336A1 amino acid sequence with the P450 reference sequences from other species demonstrated that the predicted protein is highly homologous to AmCYP336A1 from *A. mellifera*, AfCYP336A1 from *A. florea*, and AdCYP336A1 from *A. dorsata* (Fig. 1A). The identity ranged from 58.52% to 99.26%, suggesting that the P450 family is highly conserved across species.

3.2. Phylogenetic analysis

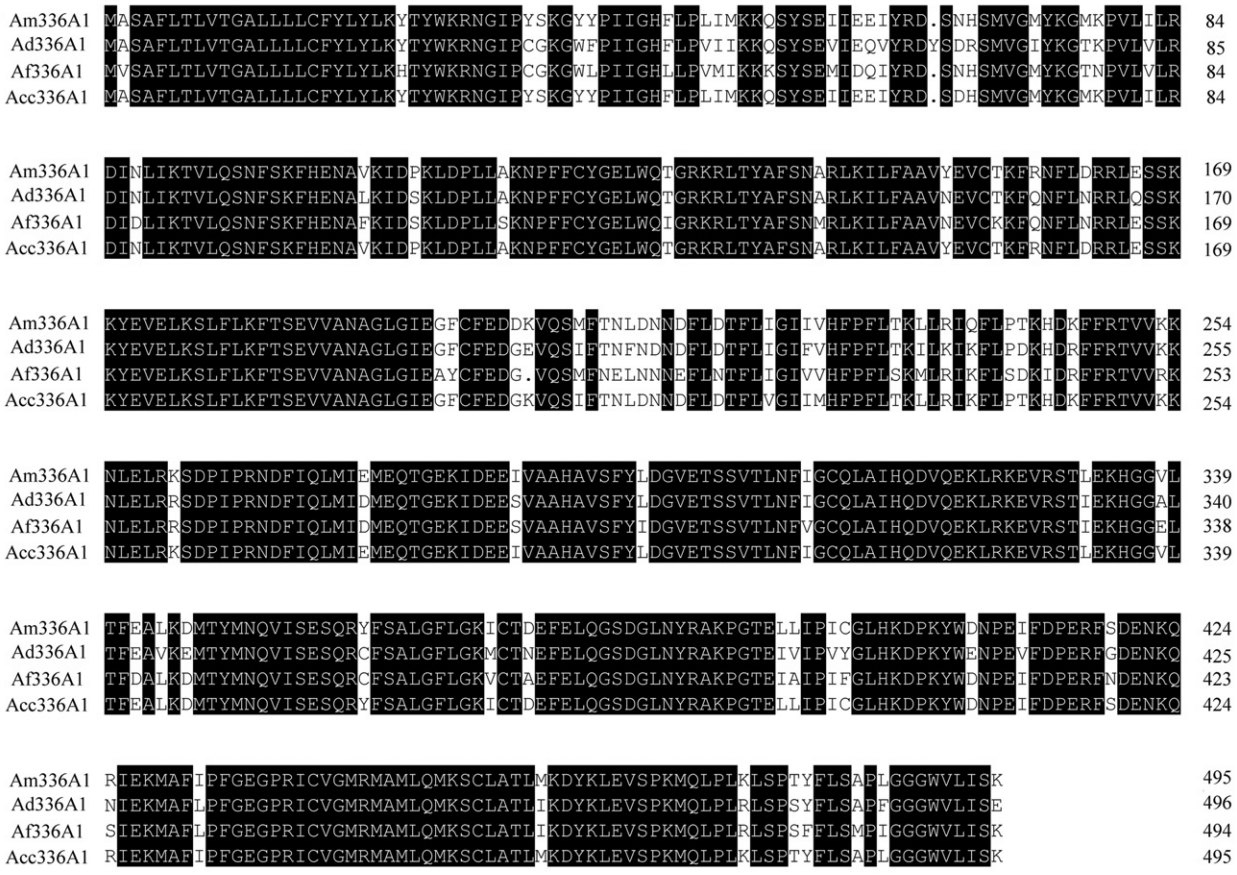
A phylogenetic tree was produced to determine the evolutionary relationship between *CYP336A1* in different species. The neighbor-joining method with MEGA 4.0 software was used to build an evolutionary tree containing the predicted amino acid sequence of *AccCYP336A1* and other similar sequences from various species (Fig. 1B). In this analysis, AccCYP336A1 and AmCYP336A1 were found to be more closely related to each other than to homologues in other species and they all belong to CYP3 clade. We may safely draw the conclusion that the homologous protein AmCYP336A1 of *A. mellifera* shares a higher similarity with the AccCYP336A1 of *A. cerana cerana* than with those of any other species.

3.3. Developmental and tissue-specific expression patterns of AccCYP336A1

We used qRT-PCR to determine the expression patterns of *AccCYP336A1* at different developmental stages and in various tissues. As shown in Fig. 2A, *AccCYP336A1* expression was on average the highest during the egg stage and the lowest during the adult stages. In the larval stage, the highest level of expression appeared at the first larval instar stage; in the pupal stage, the expression levels in the brown eyes pupae were higher than the other phases. The spatial expression patterns showed that *AccCYP336A1* transcripts were most abundant in the epidermis (Fig. 2B). Although the expression levels in the brain, midgut and muscle were nearly the same, none of the expression levels in these tissues were higher than that in the epidermis (Fig. 2B). Total RNA was collected from the head, epidermis, muscle, hemolymph, rectum and midgut.

Fig. 1. Characterization of cytochrome P450 monooxygenases (P450) from various species. A, multiple alignment analysis of cytochrome P450 monooxygenases (P450) from various species. A multiple amino acid sequence alignment of AccCYP336A1 from *A. cerana cerana*, AmCYP336A1 from *A. mellifera*, AfCYP336A1 from *A. florea*, and AdCYP336A1 from *A. dorsata*. The sequences were obtained from the NCBI database. B, Phylogenetic tree created by the neighbor-joining method using MEGA 4.0 software with sequences from other species.

A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Ad336A1 MASAFLTLVTGALLLLCFYLYLKYTYWKRNGIPCGKGWFPIIGHFLPVIIKKQSYSEVIEQVYRDYSDRSMVGIYKGTKPVLVLR 85
Af336A1 MVSAFLTLVTGALLLLCFYLYLKHTYWKRNGIPCGKGWLPIIGHLLPVMIKKKSYSEMIDQIYRD.SNHSMVGMYKGTNPVLVLR 84
Acc336A1 MASAFLTLVTGALLLLCFYLYLKYTYWKRNGIPYSKGYYPIIGHFLPLIMKKQSYSEIIEEIYRD.SDHSMVGMYKGMKPVLILR 84
Am336A1 DINLIKTVLQSNFSKFHENAVKIDPKLDPLLAKNPFFCYGELWQTGRKRLTYAFSNARLKILFAAVYEVCTKFRNFLDRRLESSK 169
Ad336A1 DINLIKTVLQSNFSKFHENALKIDSKLDPLLAKNPFFCYGELWQTGRKRLTYAFSNARLKILFAAVNEVCTKFQNFLNRRLQSSK 170
Af336A1 DIDLIKTVLQSNFSKFHENAFKIDSKLDPLLSKNPFFCYGELWQIGRKRLTYAFSNMRLKILFAAVNEVCKKFQNFLNRRLESSK 169
Acc336A1 DINLIKTVLQSNFSKFHENAVKIDPKLDPLLAKNPFFCYGELWQTGRKRLTYAFSNARLKILFAAVYEVCTKFRNFLDRRLESSK 169
Am336A1 KYEVELKSLFLKFTSEVVANAGLGIEGFCFEDDKVQSMFTNLDNNDFLDTFLIGIIVHFPFLTKLLRIQFLPTKHDKFFRTVVKK 254
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Af336A1 KYEVELKSLFLKFTSEVVANAGLGIEAYCFEDG.VQSMFNELNNNEFLNTFLIGIVVHFPFLSKMLRIKFLSDKIDRFFRTVVRK 253
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Af336A1 NLELRRSDPIPRNDFIQLMIDMEQTGEKIDEESVAAHAVSFYIDGVETSSVTLNFVGCQLAIHQDVQEKLRKEVRSTIEKHGGEL 338
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Ad336A1 TFEAVKEMTYMNQVISESQRCFSALGFLGKMCTNEFELQGSDGLNYRAKPGTEIVIPVYGLHKDPKYWENPEVFDPERFGDENKQ 425
Af336A1 TFDALKDMTYMNQVISESQRCFSALGFLGKVCTAEFELQGSDGLNYRAKPGTEIAIPIFGLHKDPKYWDNPEIFDPERFNDENKQ 423
Acc336A1 TFEALKDMTYMNQVISESQRYFSALGFLGKICTDEFELQGSDGLNYRAKPGTELLIPICGLHKDPKYWDNPEIFDPERFSDENKQ 424
Am336A1 RIEKMAFIPFGEGPRICVGMRMAMLQMKSCLATLMKDYKLEVSPKMQLPLKLSPTYFLSAPLGGGWVLISK 495
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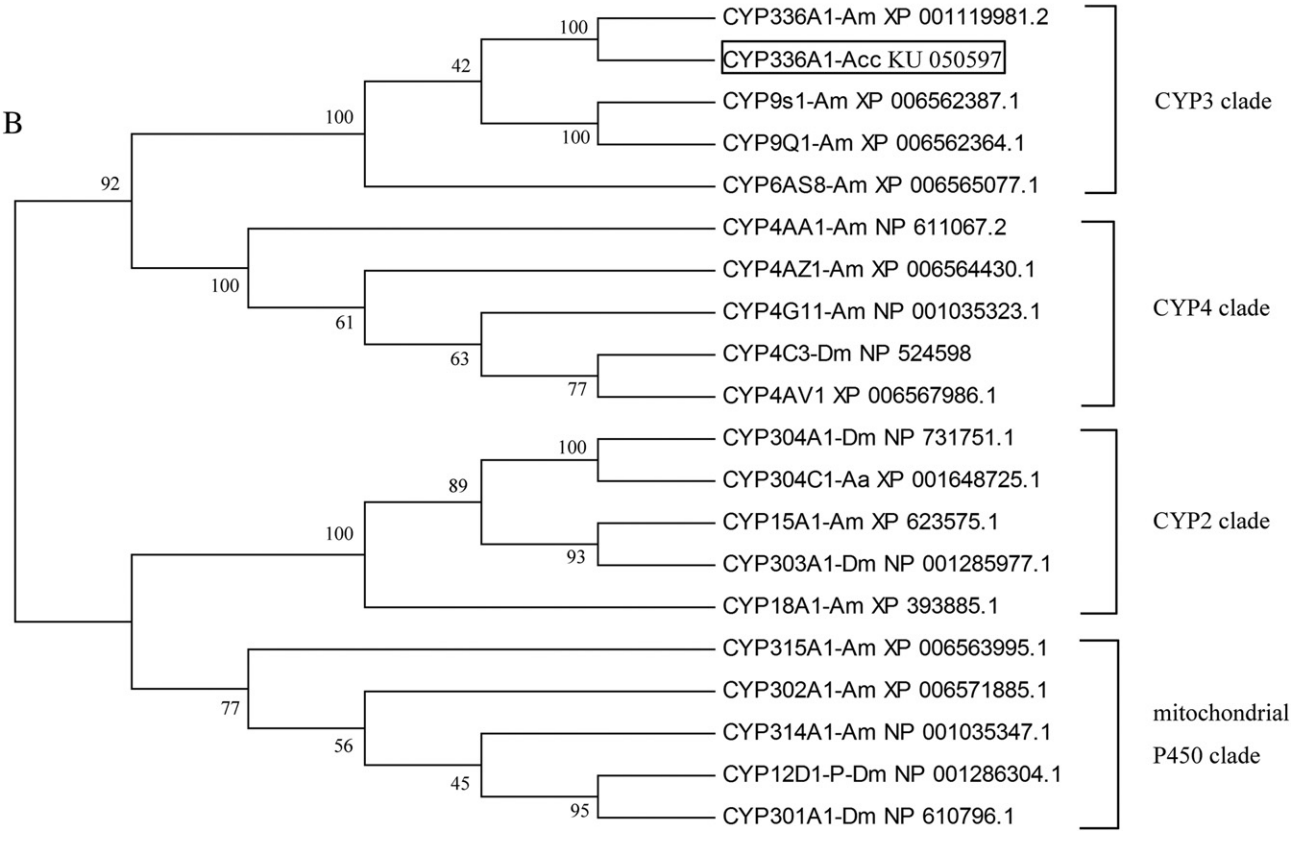
B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CYP9s1-Am XP 006562387.1
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CYP6AS8-Am XP 006565077.1
CYP3 clade
CYP4AA1-Am NP 611067.2
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CYP4G11-Am NP 001035323.1
CYP4C3-Dm NP 524598
CYP4AV1 XP 006567986.1
CYP4 clade
CYP304A1-Dm NP 731751.1
CYP304C1-Aa XP 001648725.1
CYP15A1-Am XP 623575.1
CYP303A1-Dm NP 001285977.1
CYP18A1-Am XP 393885.1
CYP2 clade
CYP315A1-Am XP 006563995.1
CYP302A1-Am XP 006571885.1
CYP314A1-Am NP 001035347.1
CYP12D1-P-Dm NP 001286304.1
CYP301A1-Dm NP 610796.1
mitochondrial
P450 clade
100
42
100
100
92
100
61
63
77
100
89
100
93
77
56
45
95

3.4. Expression profiles of AccCYP336A1 under conditions of oxidative stresses

qRT-PCR was used to characterize the transcriptional expression patterns of *AccCYP336A1* after exposure to several types of oxidative stress. Surprisingly, *AccCYP336A1* was induced by all of the tested treatments (Fig. 3). *AccCYP336A1* robustly responded to cold temperatures (4 °C) treatment, which dramatically induced the *AccCYP336A1* transcripts to a maximum level at 3 h (Fig. 3A). Similarly, after heat (42 °C) treatment, the expression level of *AccCYP336A1* was enhanced slightly compared to the control, reaching its maximum at 0.25 h (Fig. 3B). Furthermore, with the ultraviolet and H_2O_2 treatments, the expression of *AccCYP336A1* was dramatically induced and quickly climbed to a maximum value. Moreover, the results also show that the expression of *AccCYP336A1* was enhanced more drastically after exposure to ultraviolet treatment (Fig. 3C and D). Although *AccCYP336A1* expression changed after treatment with all tested pesticides, it seemed that the induction varied by treatment type, as thiamethoxam and methomyl caused a much more rapid increase in expression than deltamethrin and phoxim (Fig. 3E, F, G and H). The above results suggest that *AccCYP336A1* may play an important part in the response to various reactive oxidative stresses.

3.5. Expression, purification and characterization of recombinant AccCYP336A1 protein

To further characterize the AccCYP336A1 protein, we obtained a part of the *AccCYP336A1* ORF. Through the analysis of the AccCYP336A1 amino acid sequence, the hydrophobicity is too high to support the expression of a recombinant protein in the N-terminal sequence (Fig. 4A). Through the analysis of the AccCYP336A1 transmembrane segments, potential transmembrane segments were identified in the N-terminal sequence (Fig. 4B). According to the results of the above analysis, the 240–460 amino acid sequence of the *AccCYP336A1* ORF was cloned into the pET-21a (+) vector, which was digested with Nde *I* and Not *I*. The recombinant plasmid was then transformed into BL21 *E. coli* cells. The result of SDS-PAGE showed that the target protein was induced by IPTG at 28 °C for 10 h (Fig. 5). The target protein contained the AccCYP336A1 protein and a C-term His tag. We characterized the activity of the recombinant AccCYP336A1 protein using the disc diffusion method. We cultured the bacteria with part of AccCYP336A1 and the control bacteria to achieve the same cell density. The AccSOD2 was proved that may play an important role in protecting Chinese honeybees from oxidative stress by Jia et al. (2014). The death zones around the paraquat filters were smaller in diameter on the plates containing *E. coli* overexpressing AccSOD2 compared with plates containing control bacteria, which we used as the positive control. The killing zones of *E. coli* overexpressing the target AccCYP336A1 exposed to paraquat were smaller than those of the bacteria with empty pET-21a (+) vector (Fig. 6), which definitively demonstrated that AccCYP336A1 contributes to cellular resistance to oxidative stress.

3.6. Western blot analysis

To further understand the expression patterns of AccCYP336A1 in response to various types of ROS damage, western blot analysis was used to assess the AccCYP336A1 changes after 4 °C (A), UV (B), Phixom (C) and Methomyl (D) treatments (Fig. 6). Anti-AccCYP336A1 was used to detect AccCYP336A1. Following 4 °C treatment for 1 h, the protein level of AccCYP336A1 was clearly induced (Fig. 7A). After exposure to UV for 3 h, the AccCYP336A1 expressions were also induced (Fig. 7B). After exposure to phoxim and methomyl, the AccCYP336A1 was obviously induced rapidly (Fig. 7C and D). Although the western blot data for UV treatment and pesticide (phoxim and methomyl) treatment does not correlate with qRT-PCR data (Fig 3) with respect to time points,

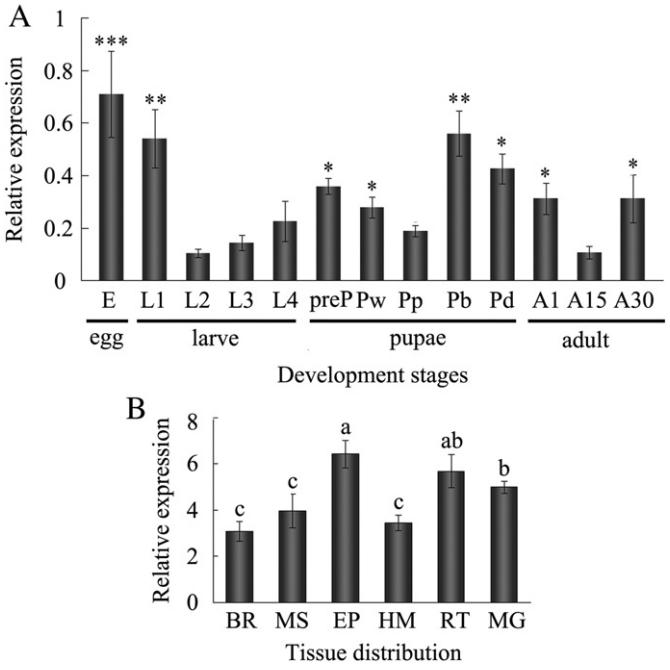

Fig. 2. The relative expression of AccCYP336A1 at different stages and in several tissues. A, the different developmental stages: egg, larvae (L1–L5, from the first to fifth instars), pupae (preP prepupae, Pw white-eyed, pupae, Pp pink-eyed pupae, Pb brown-eyed pupae and Pd dark-eyed pupae), and adult workers (A1, 1 day post-emergence; A15, 15 days post-emergence; A30, 30 days post-emergence). B, expression analysis of *AccCYP336A1* in various tissues: brain (BR), muscle (MS), epidermis (EP), hemolymph (HM), rectum (RT) and midgut (MG). The tissues were collected from 15 days post-emergence adult workers Chinese honeybees. The data are given as the mean ± SE of three replicates. The tissue letters above the bar indicate significant differences ($P < 0.0001$) as determined by Duncan's multiple range tests using SAS software version 9.1 and the one-way analysis of variance (ANOVA) and the least significant difference (LSD) test were used to analyze the differences at developmental stages.

the total tendency was induced. The reason for this discrepancy might because that there is a certain hysteresis and intervention by other factors at protein level.

4. Discussion

The cytochrome P450 proteins form an important body detoxification enzyme system, and are involved in pathways of the biosynthesis and the degradation of endogenous metabolites. They are known to play a vital role in protecting organisms against environmental stress (Gilbert and Wilkinson, 1975; Yu et al., 1984). Studies on the insect P450 have mainly focused on their roles in insecticide resistance (Pilling et al., 1995; Suchail et al., 2004). However, a few studies have systematically studied the antioxidative stress that insects experience during their lives.

To achieve this goal, we imitated several types of ROS damage that *A. cerana cerana* may encounter during their lifespan and explored the resulting changes in P450 expression at the mRNA and protein levels to look for evidence of their antioxidant functionality. We first isolated a predicted P450 gene from *A. cerana cerana* and named it *AccCYP336A1*. Sequence analysis revealed that *AccCYP336A1* contained highly considerable conservation believed to determine substrate specificity. Meanwhile, the phylogenetic tree indicated that *CYP336A1* orthologs are present in many insects and crustaceans and AccCYP336A1 belongs to CYP3 clade, sharing the highest degree of homology with AmCYP336A1 of *A. mellifera*. Taken together, these results indicate that *AccCYP336A1* is a member of the P450 family.

Knowledge on the stages and tissue distributions of *AccCYP336A1* mRNA could be useful to better understand their functional mechanisms. To achieve this goal, we investigated the expression patterns of

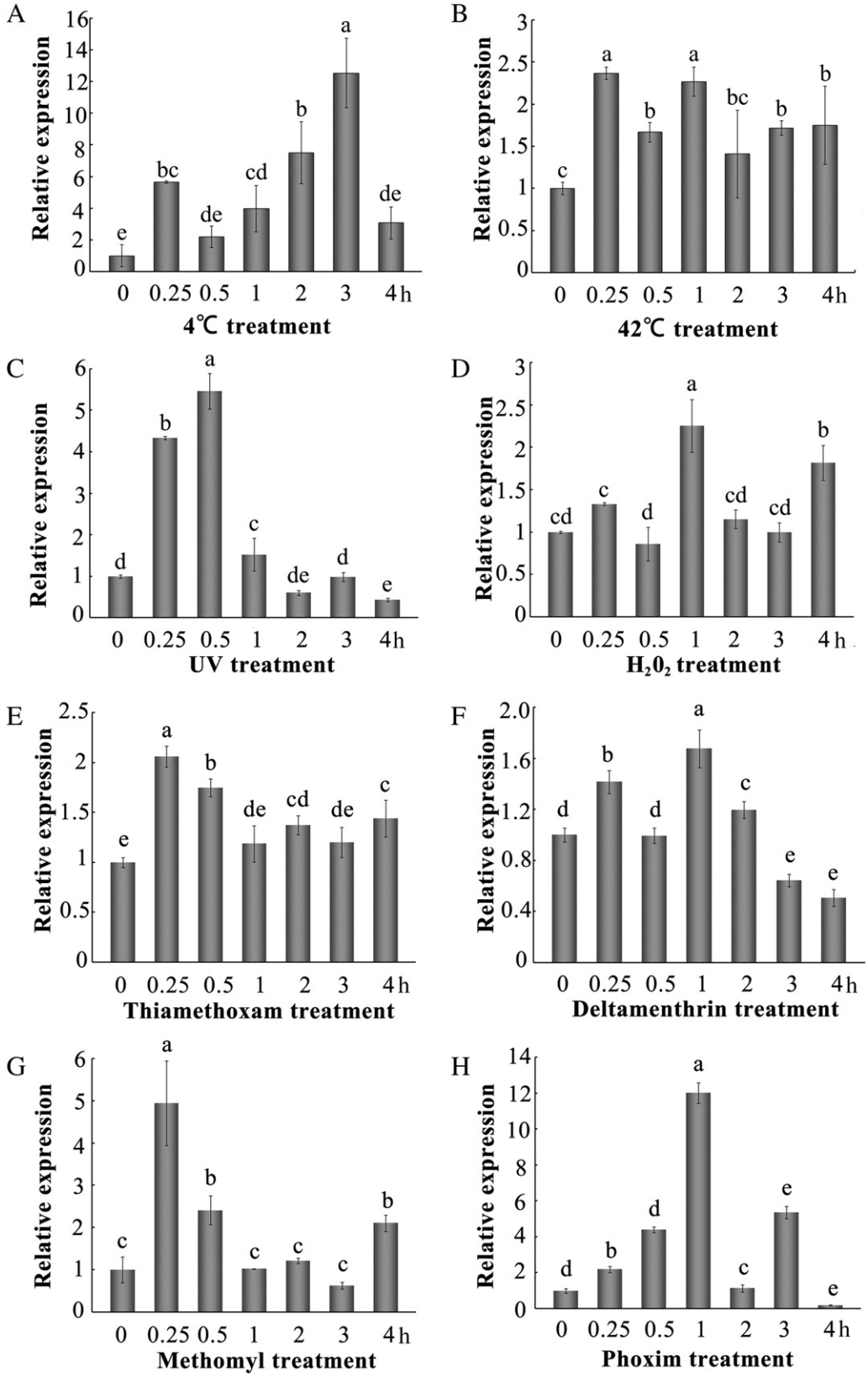

Fig. 3. Expression profiles of AccCYP336A1 under antioxidant stresses. qPCR was performed on total RNA extracted from 15 days post-emergence adult bees. These stresses are as follows: 4 °C (A), 42 °C (B), UV (C), H_2O_2 (D), thiamethoxam (E), deltamethrin (F), methomyl (G), and phoxim (H). The data are given as the mean ± SE of three replicates. The letters above the bar indicate significant differences ($P < 0.0001$) as determined by Duncan's multiple range tests using SAS software version 9.1.

AccCYP336A1. The transcript level of *AccCYP336A1* was strikingly higher in the egg and pupal stages than that in the adult stages. Corona and Robinson (2006) demonstrated that ROS can cause oxidant damage, especially in fast-growing organisms, which is caused by high oxygen levels. Thus, we infer that *AccCYP336A1* may play an essential role in the early developmental stages and may be essential for the

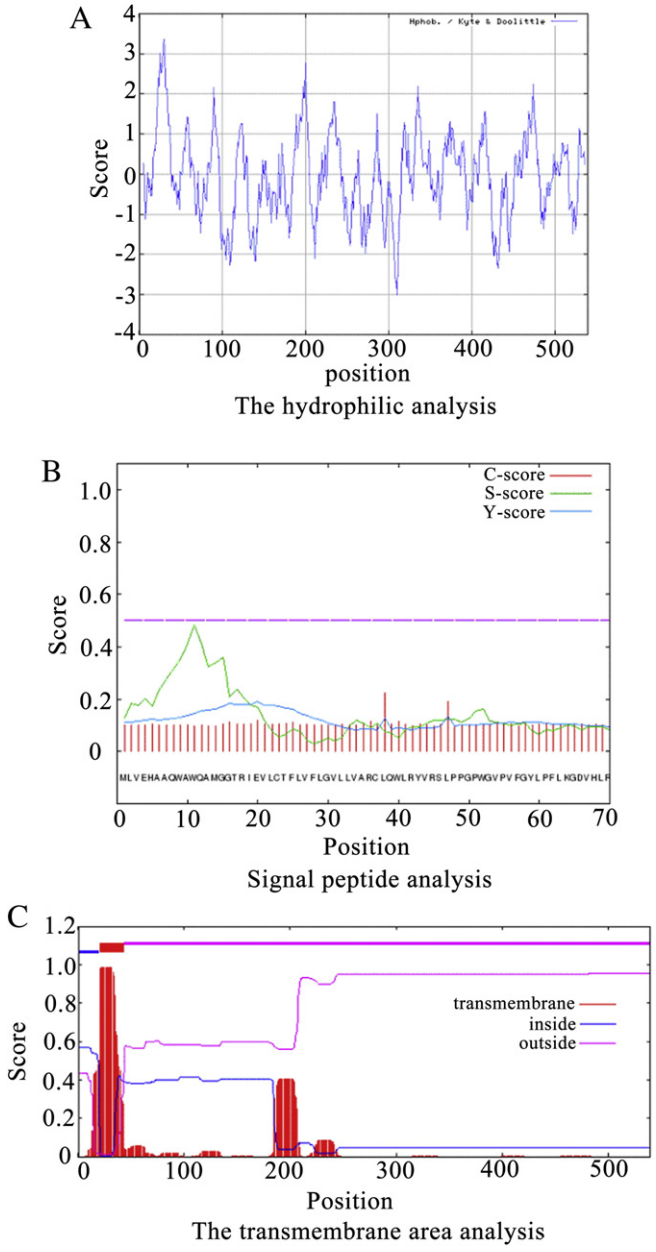

Fig. 4. The hydrophilic analysis, signal peptide analysis and transmembrane analysis. The higher the ordinate value is, the higher the hydrophobicity. It is generally believed that if the value is greater than 2, the hydrophobicity is high to support the expression of a recombinant protein (A); The red area denotes the transmembrane segments and the strong hydrophobic area (B).

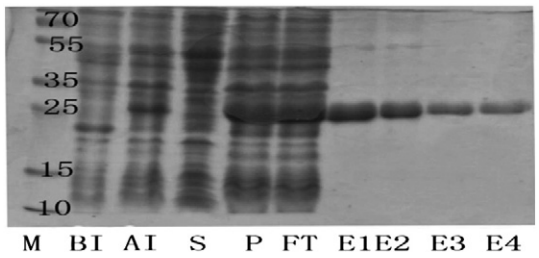

Fig. 5. The expression of the recombinant AccCYP336A1 protein. Lane M, protein molecular weight marker; lane BI, expression of AccCYP336A1 without IPTG induction; lane AI, expression of AccCYP336A1 after IPTG induction. Lane S, supernatant. Lane P-FT, inclusion body of AccCYP336A1 after IPTG induction. E1–E4 purified recombinant AccCYP336A1.

development of the honeybee or a greater exposure of stages to ROS damage. Furthermore, the tissue-specific expression analysis of the *AccCYP336A1* gene showed greater mRNA accumulation in the epidermis. It was believed that the influence of *AccCYP336A1* might play an important role in preventing damage due to ROS in the epidermis.

Previous research has revealed that ROS can be induced by temperature, H_2O_2, UV radiation, and insecticides (Lushchak, 2011; Kottuparambil et al., 2012). Temperature is an abiotic environmental factor that causes physiological changes in organisms (An and Choi, 2010). Indeed, temperature has been described as one of the key mediators of ROS generation. Studies have shown that heat-shock stress can result in polyamine oxidation and the generation of H_2O_2 and that cold stress can induce hepatocytes and liver endothelial cells to undergo apoptosis due to ROS damage (Harari et al., 1989; Rauen et al., 1999). In this study, we found that after cold (4 °C) or heat (42 °C) treatment, the *AccCYP336A1* transcript levels increased before decreasing. These results suggest that *AccCYP336A1* may involve in the process that regulates body temperature and the heating ceiling, thus protecting honeybees from ROS damage. UV radiation and H_2O_2 treatment, a typical oxidant and antioxidant, both cause oxidative damage (Goldshmit et al., 2001; Casini et al., 1986; Schauen et al., 2007; Nguyen et al., 2009). Here, the qRT-PCR results showed that *AccCYP336A1* can be induced by UV radiation and H_2O_2 treatment, and the western blot assays further confirmed the results. These findings support the hypothesis that *AccCYP336A1* plays an important role in protecting cells against oxidative damage. Pesticides are the main threat to a honeybee's life. Pesticides destroy the biochemical and physiological functions of erythrocytes and lymphocytes by causing lipid biomembrane oxidation (Narendra et al., 2007). For many years, the available data on P450 suggested that oxidative metabolism was not a major process in parasitic nematodes and that P450 activity was generally absent, or present only at a low level, in parasite extracts (Pemberton and Barrett, 1989; Yadav et al., 2010). However, there is now strong evidence that this is not the case. The increased expression of *AccCYP336A1* observed here was in agreement with previous studies in which researchers observed the enhanced activity of metabolic detoxification as a major mechanism for pesticide resistance (Eziah et al., 2009; Zhu et al., 2012; Johnson et al., 2013). Our data also showed that the expression of *AccCYP336A1* was up-regulated after exposure to pesticide treatment at the mRNA and protein levels. Taken together, all the results indicated that the *AccCYP336A1* would be induced after a series of oxidative stress, which indicates that *AccCYP336A1* quite possibly plays a significant role in the response of *A. cerana cerana* to ROS. Previous studies have identified many genes involved in the response to adverse reactive oxidative stress in *A. cerana cerana*. The several stressors could lead to the transformation of these genes in different expression patterns. For example, cold temperature (4 °C) treatment induced the expression of *AccSOD2* (Jia et al., 2014). UV treatment inhibited the expression of *Acctpx-3* (Yao et al., 2013). Pyriproxyfen treatment inhibited the expression of *Acctpx5* (Yan et al., 2014). Although part of these stressors caused an accumulation of gene expressions, the cumulates and patterns are significantly different from those of *AccCYP336A1*. This phenomenon showed that the several stressors would not lead to an accumulation of all other genes and that the *AccCYP336A1* mRNA is specifically up-regulated in response to ROS damage in *A. cerana cerana*.

Because several environmental factors lead to an accumulation of ROS, cells react to ROS threat with a variety of enzymatic and non-enzymatic protection mechanisms (Ahmad, 1995). In this study, after exposure to paraquat treatment, the killing zones of *E. coli* overexpressing AccCYP336A1 were smaller than those for the control bacteria. This result showed that the overexpression of AccCYP336A1 increased the resistance of the bacterial cells to ROS.

In conclusion, we identified and characterized a P450 gene from *A. cerana cerana* named *AccCYP336A1*. We also imitated several stresses that *A. cerana cerana* may experience during their lifetime and demonstrated corresponding expression changes in *AccCYP336A1* at

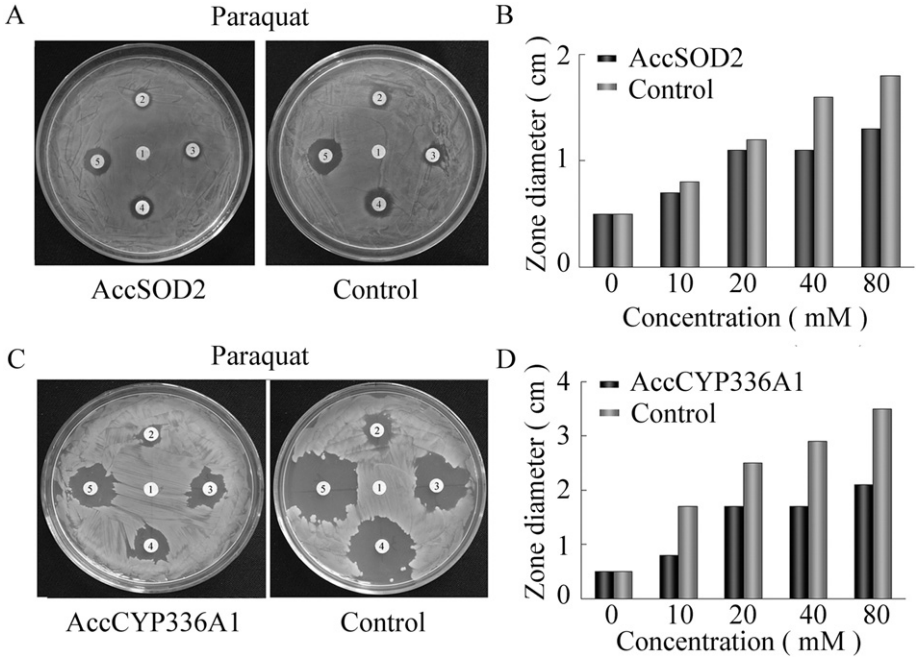

Fig. 6. The resistance of bacterial cells overexpressing AccCYP336A1 to paraquat. A, *E. coli* cells were transformed with a plasmid to overexpress AccCYP336A1 by IPTG inducing. *E. coli* BL21 bacteria transformed with pET-21a (+) (vector only) without IPTG inducing were used as the control. B, The halo diameters of the killing zones are compared in the histograms. The paraquat concentrations of discs 1–5 are 0, 10, 20, 40, and 80 mM, respectively. The data are the mean ± SE of three replicates.

the mRNA and protein levels. In addition, we explored the resistance of bacteria overexpressing AccCYP336A1 to determine the role that *AccCYP336A1* may play. The experimental results provided evidence for the functionality of AccCYP336A1 in antioxidation. The results of this study are helpful in further revealing antioxidant mechanisms and physiological responses in insects exposed to reactive oxidative stress and may help facilitate the identification of the functional mechanisms of antioxidation.

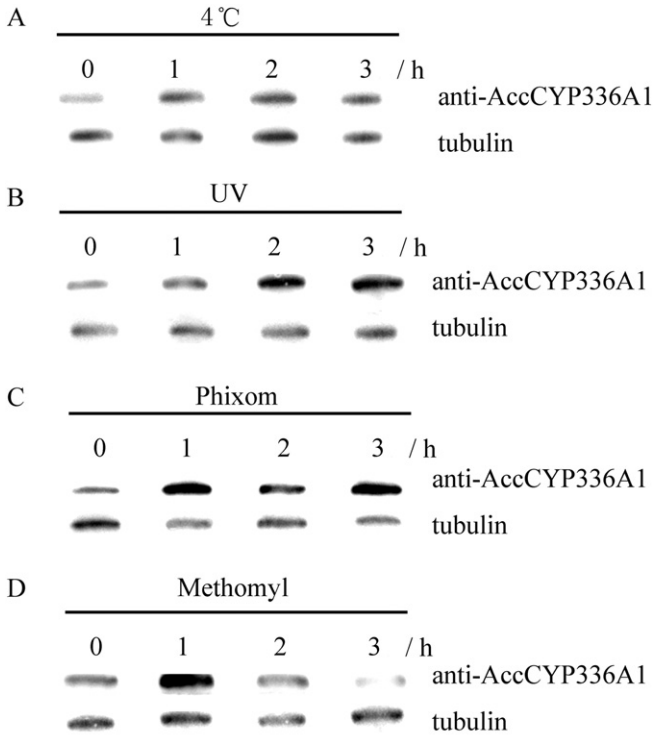

Fig. 7. Western blot analysis of AccCYP336A1 changes after 4 °C (A), UV (B), phixom (C) and methomyl treatment (D). A part of the AccCYP336A1 protein was immunoblotted with anti-AccCYP336A1. The signal of the binding reactions was visualized with HRP substrates.

Acknowledgments

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