Molecular Characterization and Oxidative Stress Response of a Cytochrome P450 Gene (*CYP4G11*) from *Apis cerana cerana*

Weina Shi[a], Jing Sun[a], Baohua Xu[b,*], and Han Li[a,*]

[a] State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong, 271018, P. R. China. Fax: +86–538–8242217. E-mail: lihan@sdau.edu.cn

[b] College of Animal Science and Technology, Shandong Agricultural University, Taian, Shandong, 271018, P. R. China. E-mail: bhxu@sdau.edu.cn

* Authors for correspondence and reprint requests

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Cytochrome P450 proteins, widely distributed multifunctional enzymes, are mainly involved in biosynthetic and degradative pathways of endogenous compounds and the detoxification of xenobiotics in insects. Moreover, these enzymes exhibit peroxidase-like activity, therefore they may be involved in protecting organisms against the toxicity of reactive oxygen species (ROS). In the present study, we cloned a *CYP4G11* gene – *AccCYP4G11* – from the Chinese honey-bee (*Apis cerana cerana*). The open reading frame of the cDNA was 1656 bp long and encoded a 551 amino acids polypeptide, which shared high sequence identity with homologous cytochrome P450 proteins. In the genomic DNA sequence, a 5'-flanking region consisting of 1168 bp was obtained, and some putative transcription factor binding sites were predicted. Quantitative polymerase chain reaction (Q-PCR) revealed that the level of *AccCYP4G11* was higher in the epidermis than in other tissues, and *AccCYP4G11* was expressed in all stages with the highest level in two-week-old adult worker honey-bees. Moreover, the expression patterns under oxidative stress indicated that *AccCYP4G11* transcription was significantly influenced by external factors, such as temperature challenges, ultraviolet (UV) light, and insecticide treatment. *AccCYP4G11* was regulated differentially in response to oxidative stress and may be involved in protecting honey-bees from oxidative injury.

Key words: Apis cerana cerana, *CYP4G11*, Q-PCR, Oxidative Stress

Introduction

The Chinese honey-bee (*Apis cerana cerana* Fabricius 1793), as a social species and a pollinator of flowering plants, is sensitively affected by the balance between the regional ecology and agricultural economic development (Honeybee Genome Sequencing Consortium, 2006). During their foraging on plants, it is difficult for honeybees to survive under various environmental challenges, such as large changes in temperature, intense ultraviolet (UV) radiation, and various synthetic insecticides which may cause oxidative stress following generation of reactive oxygen species (ROS). Thus, understanding the antioxidant system of the honey-bee and its mechanism of defence against ROS has become a primary issue for this research.

ROS, which are produced naturally during oxidative metabolism, include the superoxide radical anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$) (Livingstone, 2001). Under normal conditions, there is a balance between the generation of ROS and antioxidant processes. However, under various types of environmental stresses, *e.g.* relatively low or high temperatures, increased levels of oxidative damage can occur in organisms and lead to oxidative stress (Lopez-Martinez *et al.*, 2008). Excessive ROS can lead to lipid peroxidation which destroys the cell membrane fluidity and results in apoptosis (Green and Reed, 1998). ROS also affect the sperm storage of *Apis mellifera* (Collins *et al.*, 2004). Oxidative damage to proteins includes specific amino acid modifications and peptide rupture**,** thus causing loss of enzymatic activity (Stadtman and Levine, 2003). Redundant ROS can also bring about DNA damage in the form of base deletions, degradation, single-strand breaks, and rearrangements, giving rise to mutations (Imlay and Linn, 1988). To prevent damage mediated by ROS, complex

enzymatic and non-enzymatic defence systems have evolved. In most cases, organisms protect themselves by a set of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POX), glutathione-*S*-transferases (GST) (Dubovskiy *et al*., 2008; Felton and Summers, 1995; Wang *et al*., 2001), and glutathione peroxidase (GPX) (Wang *et al*., 2001). Cytochrome P450 proteins share 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 P450 proteins, a diverse family of heme-containing enzymes, are widely distributed in most organisms. In insects, cytochrome P450 proteins can be induced by a diverse array of lipophilic substrates of endogenous and exogenous origin, and catalyze the initial step in the substrates' metabolism. Thus, cytochrome P450 proteins are crucial to insect biology and physiology in many aspects (Feyereisen *et al*., 1999). Four distinct clades can be distinguished in the phylogeny of the cytochrome P450 proteins: the mitochondrial P450s, and the CYP2, CYP3 (most CYP6 and CYP9 members are included), and CYP4 clades, which are named after the prominent vertebrate members of these clades (Claudianos *et al*., 2006). With particular regard to the CYP4 family, a wide spectrum of such genes was found in a number of insect groups, including *Anopheles* (Scott *et al*., 1994), *Manduca* (Snyder *et al*., 1995), and *Drosophila* (Dunkov *et al*., 1996).

The CYP4 clade is a highly diversified group of enzymes in insects, and CYP4 s in insects are implicated in both pesticide metabolism and chemical communication (Claudianos *et al*., 2006). CYP4G8 is overexpressed in pyrethroid-resistant strains of *Helicoverpa armigera* (Pittendrigh *et al*., 1997), and several CYP4 genes are overexpressed in pesticide-resistant *Diabrotica virgifera* and *Culex pipiens* (Scharf *et al*., 2001; Shen *et al*., 2003). Expression of CYP4AW1 in the scarabaeid beetle *Phyllopertha diversa* is antenna-specific, and its inhibition interferes with pheromone perception, suggesting a role in pheromone degradation (Maibeche-Coisne *et al*., 2004). A very limited number of studies have reported on antioxidant functions of cytochrome P450 proteins which are of significant relevance to an organism. Therefore, an investigation of the possible involvement of the *AccCYP4G11* gene in antioxidative processes appeared promising.

In *Apis mellifera*, proteins involved in oxidative stress, such as vitellogenin and juvenile hormone, have been reported by Corona *et al*. (2007), but little information is available on *A. cerana cerana*. Considering the significant roles of antioxidative processes in insects, we decided to study the effect of the oxidative stress response on the expression of *AccCYP4G11* in *A. cerana cerana.* Our work provides a better understanding of the role of CYP4 in the defence against oxidative stress in *A. cerana cerana* and contributes to our knowledge of this versatile gene superfamily.

Materials and Methods

Materials and treatment

The Chinese honey-bees (*A. cerana cerana*) used in the experiment were maintained at Shandong Agricultural University (Tai'an, China). The worker honey-bees were generally classified into larvae, pupae, and adults according to age, shape, and eye colour. Three-day-old and six-day-old larvae, and pupae with white eyes (Pw), pink eyes (Pp), or brown eyes (Pb) were obtained from the hive. The two-week-old adult worker honey-bees were collected once they had emerged from combs in outdoor beehives. Then they were caged in groups of 40 individuals at constant temperature (34 °C) and humidity (70%). Before treatment, they were fed a mixture of pollen and sucrose for 2 d.

To examine tissue-specific gene expression, the head, thorax, abdomen, epidermis, muscle, and midgut of two-week-old adult worker honey-bees were dissected on ice and immediately collected. Groups 1–3 were placed at 4, 25, and 42 °C, respectively. Group 4 was exposed to UV light (30 mJ/cm^2), while group 5 was treated with H_2O_2 at a final concentration of 2 mM. The H_2O_2 (0.5 ml) was delivered to the thoracic notum of the bees. Groups 6–9 were treated with four different pesticides, *i.e.* phoxime, acaricide, paraquat, and decamethrin, which were all diluted to a final concentration of 20 mg/l. Control bees in group 8 were fed only the pollen-and-sucrose solution. All honey-bees used in the research were collected at the appropriate time and stored at –80 °C.

Primers and polymerase chain reaction (PCR) amplification

The sequences of primers and PCR amplification conditions used are listed in Tables I and II, respectively.

Table I. Primers used for PCR amplification.

Primer	Primer sequence (5'→ 3')	Description
For cloning of full-length cDNA		
CP1	CTTCTCATTCCTGCTCTCATTC	Gene special primer, forward
CP2	GATAAATATGTGGCTGTCGGTG	Gene special primer, reverse
5P1	GCATCTTCTCGACGACACTTC	5' RACE reverse primer, reverse
5P2	CCAGTGGAAATAAGAAGACCGTC	5' RACE reverse primer, nested
AAP	GGCGACGCGTCGACTAGTAC$(G)_{14}$	Abridged anchor primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged universal amplification primer
3P1	CCGGTCAAAATGGTGTTCTCC	3' RACE reverse primer, reverse
3P2	GGTGACAGTGATAGGCCAGC	3' RACE reverse primer, nested
B26	GACTCTAGACGACATCGA$(T)_{18}$	Universal primer, primary
B25	GACTCTAGACGACATCGA	Universal primer, nested
QC1	GATCGAACAATGTCCACGGC	Full-length cDNA primer, forward
QC2	CACCGCATGAAAAGCCCAC	Full-length cDNA primer, reverse
For cloning of genomic DNA and 5'-flanking region		
N1	GTCACTTCTCATTCCCGCTC	Genomic sequence primer, forward
N2	GGATTATCTCAACGTCACG	Genomic sequence primer, reverse
N3	CGTGACGTTGAGATAATCC	Genomic sequence primer, forward
N4	CAGAAGGATGTCAACAGTCAG	Genomic sequence primer, reverse
N5	CTGACTGTTGACATCCTTC	Genomic sequence primer, forward
N6	CCTACACATGATCGCGGTC	Genomic sequence primer, reverse
QDP1	CGTTCGTTAATGCATAATATCAGCC	Inverse PCR forward primer, outer
QDP2	CTACTTGCCATCGTGACTTCC	Inverse PCR reverse primer, outer
QDP3	GGTCAGAAATGGCGTAATCACCG	Inverse PCR forward primer, inner
QDP4	GAGAGCAGGAATGAGAAGTGACAAG	Inverse PCR reverse primer, inner
CS1	GGCAATGATACGATATTCTGGC	Promoter-specific primer, forward
CS2	GATGTAATGTCACGATGCTT	Promoter-specific primer, reverse
For fluorescence real-time Q-PCR		
RTP1	CGCAAAGAGAATGGGAAGG	Real-time PCR primer, forward
RTP2	CTTTTGTGTGACGGAGGTGC	Real-time PCR primer, reverse
β-Actin-s	TTATATGCCAACACTGTCCTTT	Standard control, forward
β-Actin-x	AGAATTGATCCACCAATCCA	Standard control, reverse

Table II. PCR amplification conditions.

Primer pair	Amplification conditions
CP1/CP2	10 min at 94 °C; 40 s at 94 °C, 40 s at 49 °C, and 50 s at 72 °C for 35 cycles; 10 min at 72 °C
5P1/AAP	10 min at 94 °C; 40 s at 94 °C, 40 s at 51 °C, and 40 s at 72 °C for 35 cycles; 10 min at 72 °C
5P2/AUAP	10 min at 94 °C; 40 s at 94 °C, 40 s at 52 °C, and 40 s at 72 °C for 35 cycles; 10 min at 72 °C
3P1/B26	10 min at 94 °C; 40 s at 94 °C, 40 s at 49 °C, and 40 s at 72 °C for 35 cycles; 5 min at 72 °C
3P2/B25	10 min at 94 °C; 40 s at 94 °C, 40 s at 49 °C, and 40 s at 72 °C for 35 cycles; 5 min at 72 °C
QC1/QC2	10 min at 94 °C; 40 s at 94 °C, 40 s at 49 °C, and 2 min at 72 °C for 35 cycles; 5 min at 72 °C
N1/N2	10 min at 94 °C; 40 s at 94 °C, 40 s at 48 °C, and 1 min at 72 °C for 35 cycles; 5 min at 72 °C
N3/N4	10 min at 94 °C; 40 s at 94 °C, 40 s at 48 °C, and 2 min at 72 °C for 35 cycles; 5 min at 72 °C
N5/N6	10 min at 94 °C; 40 s at 94 °C, 40 s at 48 °C, and 1 min at 72 °C for 35 cycles; 5 min at 72 °C
CS1/CS2	10 min at 94 °C; 40 s at 94 °C, 40 s at 50 °C, and 1 min at 72 °C for 35 cycles; 5 min at 72 °C

RNA extraction, cDNA synthesis, and DNA preparation

On the basis of the manufacturer's protocol, trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from honeybees after various treatments. The total RNA obtained was digested by RNase-free DNase I. Then the first-strand cDNA was obtained by reverse transcriptase (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Genomic DNA was isolated from the bees using the EasyPure genomic DNA extraction kit according to the manufacturer's instructions (TransGen Biotech).

Amplification of full-length cDNA of AccCYP4G11

By using reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends PCR (RACE-PCR) technology described by Guo *et al.* (2010), full-length cDNA of *AccCYP4G11* was obtained. An internal conserved fragment was obtained by the primers CP1 and CP2 designed according to conserved amino acid sequences of CYP4 s in several insects. Based on the internal sequence of *AccCYP4G11*, two pairs of specific primers – 5P1/5P2 and 3P1/3P2 – were designed and synthesized for 5' and 3' RACE, respectively. As described by Guo *et al*. (2010), two rounds of PCR were performed. Primer 3P1 and the universal primer B26 were used in the first round, the product of which was subjected to nested PCR with the special primers 3P2 and B25. For 5' RACE, the primary-round template was purified and then a poly-C tail added. Additional primers used in the RACE were: primer 5P1 and the abridged anchor primer (AAP) in the first-round PCR, and then 5P2 and the abridged universal amplification primer (AUAP) for the second round. Finally, the accurate sequence of the full-length cDNA was obtained by PCR using primers QC1 and QC2, which were designed based on the putative full-length *AccCYP4G11* cDNA deduced from comparison and alignment of the fragments of 5'-untranslated region (UTR), 3'-UTR, and internally conserved region with DNAman software 5.2.2 (Lynnon Biosoft, Quebec, Canada). All products were purified, cloned into the pEASY-T3 vector (TransGen Biotech), and then transformed into competent *E. coli* DH5α cells for sequencing.

Isolation of the genomic sequence and the 5'-flanking region of AccCYP4G11

Since the *AccCYP4G11* genomic sequence is extremely large, we decided to subdivide it into three segments prior to amplification. Three pairs of primers – N1/N2, N3/N4, and N5/N6 – were designed and synthesized in accordance with the full-length cDNA of *AccCYP4G11* using *A. cerana cerana* genomic DNA as the template.

To obtain the 5'-flanking region of *AccCYP4G11*, we designed two pairs of specific primers – QDP1/QDP2 and QDP3/QDP4 – according to the genomic DNA sequence of *AccCYP4G11* and used them in inverse-PCR. Genomic DNA was used as the template, which had previously been digested by restriction endonuclease Hand III and then self-ligated by T4 DNA ligase (TaKaRa, Dalian, China). Then, two specific primers – CS1 and CS2 – were designed to further verify the validity of the 5'-flanking sequence with the PCR amplification conditions shown in Table II. The transcription factor binding sites in the 5'-flanking region of *AccCYP4G11* were predicted by the MatInspector database (http://www.cbrc.jp/research/db/TFSEARCH.html).

Bioinformatic analysis and phylogenetic tree construction

The nucleotide and amino acid sequences were analysed with bioinformatics tools available at the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The deduced amino acid sequence was aligned with homologous CYP4 protein sequences from various species using DNAman software 5.2.2. The phylogenetic tree was constructed using the neighbour-joining method and molecular evolution genetics analysis (MEGA) software, version 4.0, on the basis of the predicted amino acid sequence of a number of CYP4 s of various species.

Fluorescence real-time quantitative PCR (Q-PCR)

To determine the levels of the *AccCYP4G11* transcripts under various stress conditions, fluorescence real-time Q-PCR was performed. Special primers were designed, including RTP1/RTP2, based on the cDNA of *AccCYP4G11*, and β-actin-s/β-actin-x based on the basis of *Apis mellifera* β-actin (GenBank accession no. XM640276). The β-actin transcript was used as an internal control. According to the manufacturer's instructions, fluorescence real-time Q-PCR was performed using the SYBR® PrimeScript™ RT-PCR kit (TaKaRa) on a CFX96TM real-time system (Bio-Rad, Hercules, CA, USA). Q-PCR was carried out in a 25-μl reaction system consisting of 12.5 μl SYBR, 2.0 μl of 1:10 diluted cDNA, 0.5 μl of each primer (10 mM), and 9.5 μl of PCR-grade water. The samples were analysed in triplicate with the following program: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of amplification (95 °C for 10 s, 53 °C for 20 s, and 72 °C for 15 s), and a

melting cycle from 65 °C to 95 °C. CFX Manager Software version 1.1 and $\Delta\Delta$-C_T method (Livak and Schmittgen, 2001) were used to analyse the *AccCYP4G11* transcript levels. On the basis of Statistical Analysis System (SAS), version 9.1, the overall differences in *AccCYP4G11* transcript levels were determined, and the significance was set at $P < 0.05$. At least three independent experiments were performed.

Results

Isolation and characterization of the AccCYP4G11 cDNA sequence

We obtained the full-length cDNA sequence through reverse-transcription PCR (RT-PCR) and rapid amplification of cDNA ends PCR (RACE-PCR). The identified P450 gene was classified by Dr. D. Nelson (University of Tennessee, Memphis,

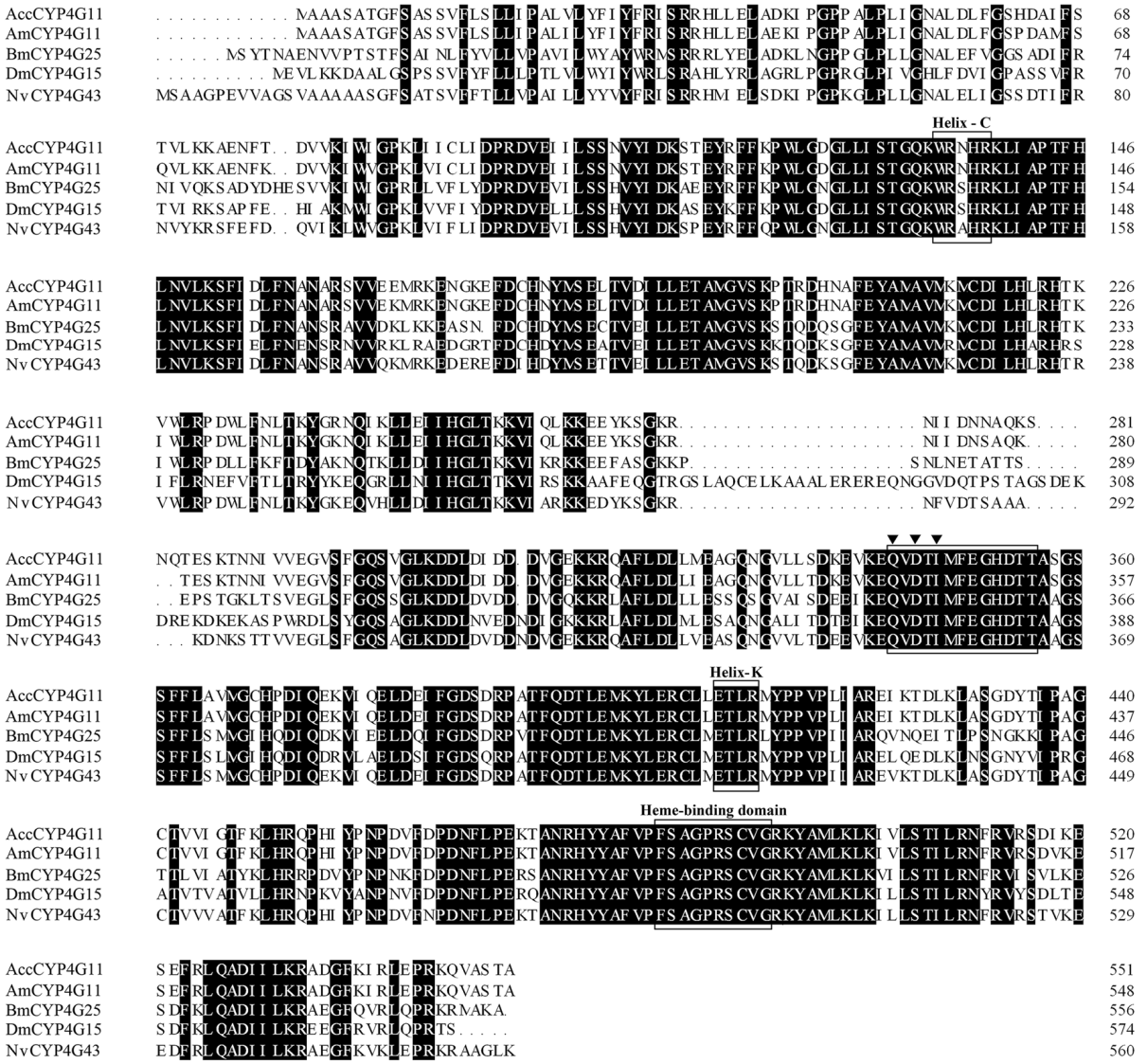

Fig. 1. Deduced amino acid sequence of AccCYP4G11 and alignment with other CYP4 s. Identical amino acid residues are shaded in black. Characteristic regions are boxed including Helix-C (WxxxR), Helix-K (ExxR), and heme-binding domain (FxxGxRxCxG) as well as a 12-residue sequence (QVDTIMFEGHDTT) (344–356) with three residues marked by arrowheads (▼) indicating highly conserved sites in the region.

TN, USA) and is available from the GenBank database under the accession number *AccCYP4G11* (KC243984). The *AccCYP4G11* cDNA sequence is 2041 bp in length including a 99-bp 5'-untranslated region (UTR), a 286-bp 3'-UTR, and an 1656-bp open reading frame (ORF). The ORF encodes a polypeptide of 551 amino acids with a theoretical molecular mass of 62,517 Da and an isoelectric point (pI) of 8.27.

Multiple sequence alignment revealed that the deduced amino acid sequence of AccCYP4G11 has significant similarity (61.49% – 96.73%) with sequences from other species, including *Apis mellifera* (NP_001035323), *Bombyx mori* (NP_001106223), *Drosophila melanogaster* (NP_572721), and *Nasonia vitripennis* (NP_001165992) (Fig. 1). As is evident from Fig. 1, CYP4G11 of *A. cerana cerana* contains important domains that are conserved among P450s, such as the amino acid residues of FxxGxRxCxG at position 484 – 493, which represent the heme-binding region (Kasai *et al*., 2000). In addition, the predicted AccCYP4G11 has a number of characteristics in common with other members of the P450 superfamily, such as the charge pair consensus (ExxR) (Grahamlorence *et al*., 1995) in the Helix-K, the consensus (WxxxR) within the Helix-C, and the consensus sequence [(A/G/E) GxxT] (Nelson, 1998). Moreover, a partial amino acid sequence alignment of P450 s reveals a highly conserved amino acid sequence consisting of 12 residues (QVDTIMFEGHDTT) (344 – 356). This conserved region is in contrast to the 13-residues motif (EVDTFMFEGHDTT) previously reported as invariant among CYP4 family members (Liu and Zhang, 2004; He *et al*., 2002).

Phylogenetic analysis at the protein level

In order to investigate the evolutionary relationship of CYP4 s in different spices, a phylogenetic tree was generated. The deduced amino acid sequence of AccCYP4G11 and other representative sequences of the CYP4 family from various species were used to construct an evolutionary tree by the neighbour-joining method using MEGA 4.0 software (Fig. 2). It is evident that CYP4G11 of *A. cerana cerana* shares higher similarity with the homologous protein of *Apis mellifera* than with those of any other organism.

Structure analysis of the genomic sequence of AccCYP4G11

In order to further investigate the characteristics of *AccCYP4G11*, the genomic sequence was obtained by PCR amplification. The full-length *AccCYP4G11* genomic sequence (GenBank accession no. KC243983) is 3849 bp in length with seven exons and six introns which are all located in the ORF sequences. Moreover, the introns have a high AT content, typical for eukaryotes, and a typical 5'-GT splice donor and 3'-AG splice acceptor.

An alignment of the genomic sequence across the CYP450 sequences from *A. cerana cerana*, *Apis mellifera* (NM_001040233.1), *Culex quinquefasciatus* (NW_0001888017), and *Nasonia vitripennis* (NM_001172521.1) revealed variation in the intron numbers and gene lengths (Fig. 3). Notably, *AccCYP4G11* and *AmCYP4G11* contain six introns, while *NvCYP4G43* and *CqCYP4G15* contain four and two, respectively. In addition, a comparison of the intron positions showed that the honey-bees share a common feature which is different from *Culex quinquefasciatus* and *Nasonia vitripennis*.

Characterization of some putative cis-acting elements in the 5'-flanking region of AccCYP4G11

To further understand the *AccCYP4G11* gene, we obtained an 1168-bp (GenBank accession no. KC243985) fragment, located in the 5'-flanking region, by inverse PCR (I-PCR). Several putative transcription factor binding sites were predicted using the web-based software program TFSEARCH. In Fig. 4, parts of the 5'-flanking region are displayed and several significant transcription factor binding sites have been annotated. Three binding sites for CdxA, which was reported to play important roles in gut closure and intestinal epithelial differentiation in chicken (Frumkin *et al*., 1994), were identified in the 5'-flanking region of *AccCYP4G11* (–248 to –256, –656 to –662, –898 to –904). Three heat shock elements (–320 to –324, –758 to –762, –1106 to –1110), being well known as the binding sites for heat shock factor (HSF) in *Drosophila* and *Saccharomyces*, as well as two Dfd elements (–281 to –296, –919 to –928), which play important roles in determining the specificity of homeotic gene action (Fernandes *et al*., 1994; Ekker *et al*., 1992), were discovered in the pro-

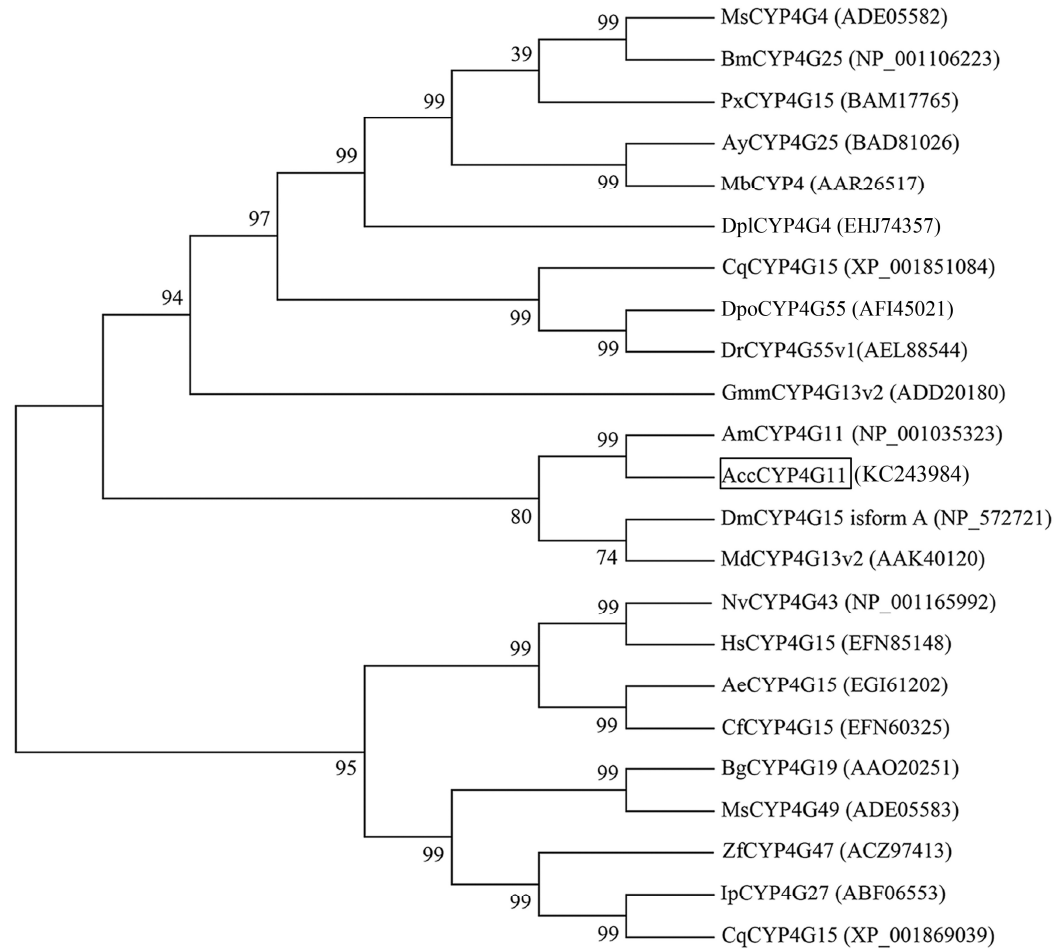

Fig. 2. Phylogenetic tree created by the neighbour-joining method using MEGA 4.0 software with sequences from other species. The species are: Am, *Apis mellifera*; Ae, *Acromyrmex echinatior*; Ay, *Antheraea yamamai*; Bg, *Blattella germanica*; Bm, *Bombyx mori*; Cf, *Camponotus floridanus*; Cq, *Culex quinquefasciatus*; Dm, *Drosophila melanogaster*; Dpl, *Danaus plexippus*; Dpo, *Dendroctonus ponderosae*; Dr, *Dendroctonus rhizophagus*; Gmm, *Glossina morsitans morsitans*; Hs, *Harpegnathos saltator*; Ip, *Ips paraconfusus*; Mb, *Mamestra brassicae*; Md, *Musca domestica*; Ms, *Manduca sexta*; Nv, *Nasonia vitripennis*; Px, *Papilio xuthus*; Zf, *Zygaena filipendulae*.

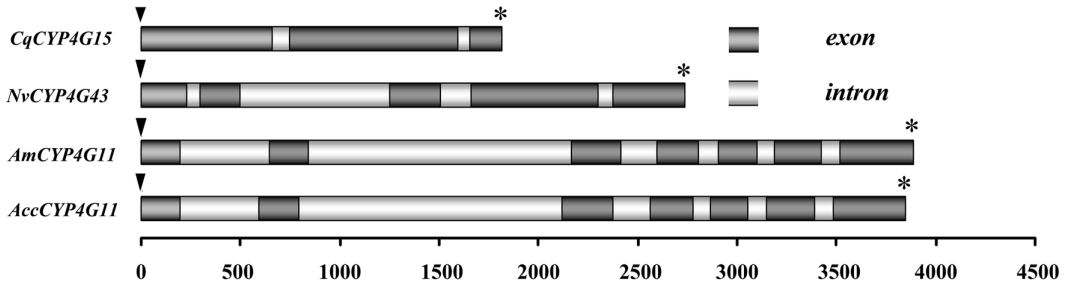

Fig. 3. Schematic representation of *CYP4* gene organization. Exons and introns in the genomic DNA of *Culex quinquefasciatus*, *Nasonia vitripennis*, *Apis mellifera*, and *Apis cerana cerana* are indicated by different grey levels, respectively. The translational initiation codon (ATG) and termination codons are marked by (▼) and (*), respectively. The numbers indicate bp.

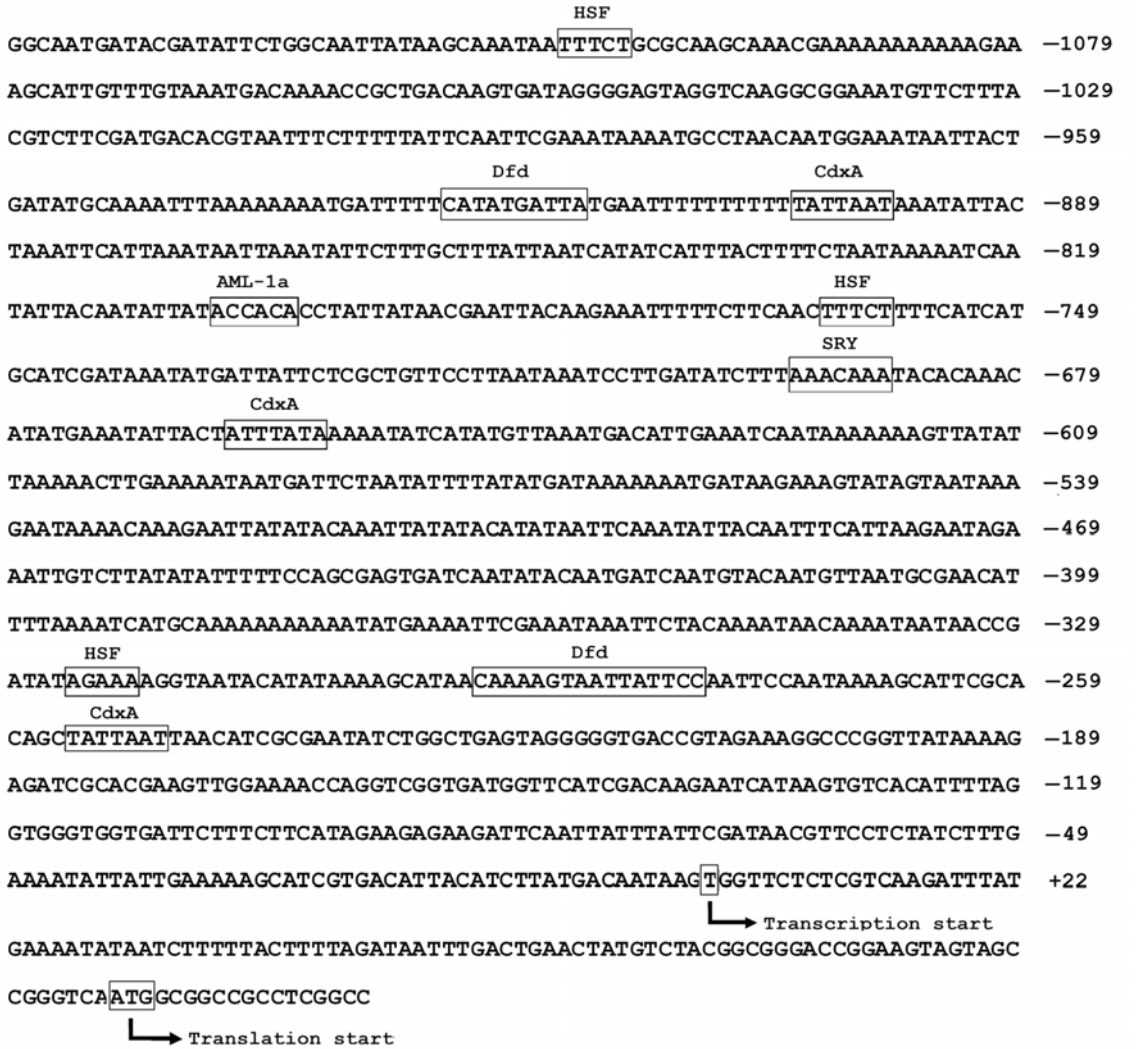

Fig. 4. Partial nucleotide sequences and putative *cis*-acting elements of the 5'-flanking region of *AccCYP4G11*. The transcription start site and translation start site are both marked with arrows, and the transcription factor binding sites mentioned in the text are boxed.

moter region of the *AccCYP4G11*. Apart from the elements mentioned above, we also putatively identified several other *cis*-acting elements: AML-1a, which was identified as sequence-specific DNA-binding protein (Meyers *et al*., 1993), and SRY (sex-determining region Y gene product), which combined with DNA determines the sex (Pontiggia *et al*., 1994).

Tissue distribution and developmental regulation of AccCYP4G11

In order to determine the spatial distribution of *AccCYP4G11* transcripts, quantitative PCR (Q-PCR) analysis was performed using adult worker bees on day 14. Expression levels were normalized to that in the head. As shown in Fig. 5A, there was a significant 11.8- and 1.7-fold accumulation of transcripts in the epidermis and abdomen, respectively, in comparison to the head.

Total RNA was also extracted from larvae, pupae, and adults of the honey-bee, and Q-PCR was

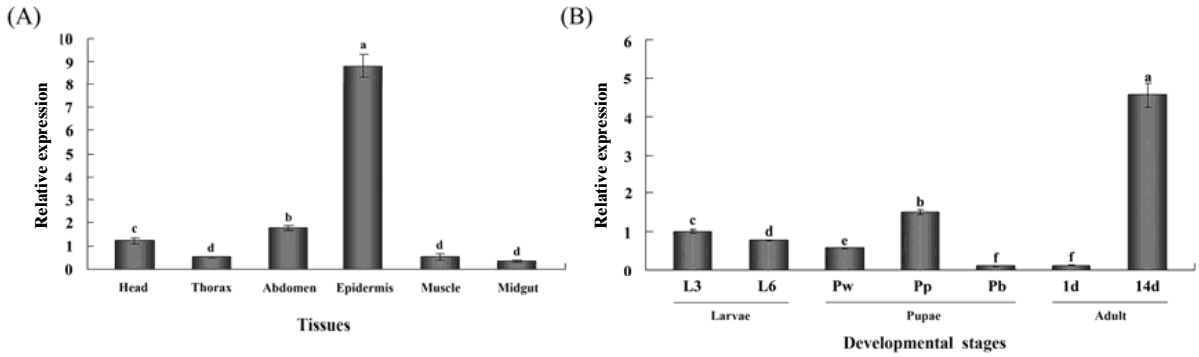

Fig. 5. Transcript levels of *AccCYP4G11* in different tissues and developmental stages determined by Q-PCR. (A) Head, thorax, abdomen, epidermis, muscle, and midgut. (B) Developmental stages: larvae on days 3 and 6; pupae with white, pink, or brown eyes; and adults on days 1 and 14. Vertical bars represent the mean ± S.E.M. ($n = 3$). Different letters above the bars indicate significant difference as determined by one-way ANOVA analysis ($P < 0.05$).

performed to examine the expression of *AccCYP4G11* during different developmental stages. The transcript level in day-3 larvae was regarded as the standard. As shown in Fig. 5B, *AccCYP4G11* transcripts are present throughout the whole life of the worker bees and there were, respectively, 1.5- and 4.5-fold higher levels of *AccCYP4G11* transcripts in pupae with pink eyes and adults on day 14 compared with day-3 larvae. There was no significant difference in *AccCYP4G11* expression during other stages. These results suggest that the expression of *AccCYP4G11* at the transcript level is specific both in tissues and developmental stages.

Expression levels of AccCYP4G11 in response to environmental stresses

In order to examine whether the expression of *AccCYP4G11* is affected by different environmental stresses, bees were first exposed to different temperatures (4, 25, and 42 °C, respectively). The transcript levels, normalized to those in control bees (34 °C), are presented in Figs. 6A–C. Expression of *AccCYP4G11* was significantly affected by all temperatures compared with the controls. Notably, when the honey-bees were exposed to 42 °C for 1 h, the level of *AccCYP4G11* transcripts increased sharply (57.3-fold) and reached a peak at 3 h (108.5-fold). A drastic (45.3-fold) up-regulation of *AccCYP4G11* expression was observed after 2.5 h at 25 °C, and the transcript level reached a maximum at 3.5 h (96.7-fold) after which it sharply decreased. Similarly, during treatment at 4 °C, *AccCYP4G11* expression increased between 0.5 h and 1 h (3.6-fold) and then decreased gradually. We conclude that *AccCYP4G11* may be involved in temperature-induced oxidative stress in the bees. H_2O_2 treatment caused a 0.9-fold relative transcript level of *AccCYP4G11*. In addition to temperature shock and H_2O_2 exposure, honey-bees often suffer from environmental stresses such as UV irradiation and pesticides. Q-PCR assays revealed that *AccCYP4G11* expression responds to all of these treatments. Transcript levels of *AccCYP4G11* reached their maximum at 2 h following UV and paraquat treatment, after an initial decline. Phoxime treatment caused an 1.4-fold up-regulation from 0.5 to 1.5 h. While acaricide caused an 1.2-fold up-regulation after an initial decline, and decamethrin caused a continuous increase up to 16-fold at 2.5 h.

Discussion

Insect cytochrome P450 proteins, being involved in pathways of the biosynthesis and degradation of endogenous metabolites and in the detoxification of exogenous compounds, play important roles in insect growth, development, and reproduction (Liu and Zhang, 2004). Previous studies mainly focused on the involvement of P450 proteins in the insecticide metabolism of honey-bees (Gilbert and Wilkinson, 1974; Yu *et al.*, 1984; Pilling *et al.*, 1995; Suchail *et al.*, 2004), while few studies have referred to an antioxidative function. In this work, we have characterized a P450 gene from *A. cerana cerana*, determined

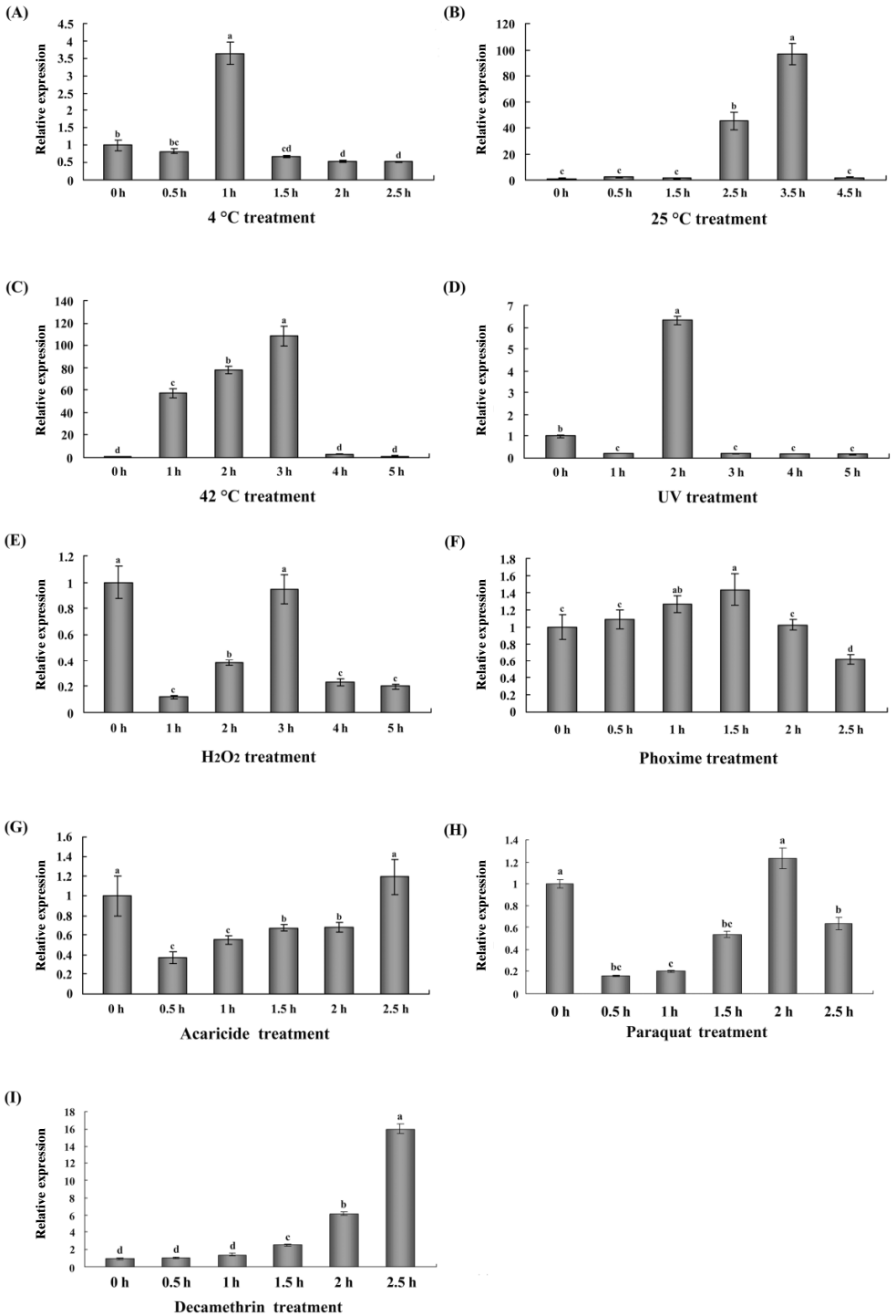

Fig. 6. Expression profiles of *AccCYP4G11* in adult worker bees under various abiotic stresses. (A) 4 °C; (B) 25 °C; (C) 42 °C; (D) UV light; (E) H_2O_2; (F) phoxime; (G) acaricide; (H) paraquat; (I) decamethrin. Vertical bars represent the mean ± S.E.M. ($n = 3$). Different letters above the bars indicate significant difference as determined by one-way ANOVA analysis ($P < 0.05$).

its expression profile, and found evidence of its functionality in antioxidation.

Sequence analysis revealed that the protein encoded by the gene contains not only the classical conserved domains (Helix-C, Helix-K, and heme-binding domain) but also the characteristic sequence QVDTIMFEGHDTT (344–356) of the CYP4 family. So it clearly belongs to the CYP4 clade, and the gene was therefore named *AccCYP4G11*. Genomic analysis revealed that the numbers, sizes, and relative positions of the introns of *CYP4* genes in different species are variable, suggesting that the *CYP4G11* gene in *A. cerana cerana* evolved specifically after divergence of the insect orders.

Previous studies had shown that *AmCYP4G11* is expressed to a higher degree in workers as compared to queens (Evans and Wheeler, 2000). In our study, the transcript level in two-week-old adult worker bees, which are foragers, was strikingly higher than in one-day-old worker bees. The highest expression level of *AccCYP4G11* at the adult stage was in accordance with the presumed role in the protection against reactive oxygen species (ROS) generated by various oxidative stimulation from environmental stressors. Lower transcript levels of *AccCYP4G11* were found in the larvae and pupae stages, indicating that the expression of *AccCYP4G11* is stage-specific. In addition, the highest transcript accumulation in the epidermis revealed that the expression of *AccCYP4G11* is tissue-specific. However, *DmCYP4G15* was found to be specifically expressed in the brain cortex and thoracic ganglia (Ma-Coisne *et al*., 2000). A plausible explanation for this may be that epidermis is most exposed to external attack and thus plays an important role in resistance to external stressors, oxidative stress included (Marionnet *et al*., 2003).

Insect P450 proteins are generally expressed at low levels, their natural substrates are frequently unknown and their activities are unstable in cell-free extracts. Molecular approaches can provide accurate and specific information on insect P450 expression (Feyereisen, 1999), and Q-PCR is a highly sensitive detection method to analyse the expression patterns of specific P450 proteins. Temperature, as an important abiotic environmental factor, is responsible for a variety of physiological changes in organisms (An and Choi, 2010). Increased levels of oxidative damage can occur in organisms under relatively low or high temperatures and result in oxidative stress (Lopez-Martinez *et al*., 2008). Generally, thermal shock contributes to stimulating polyamine oxidation to generate H_2O_2 (Hariari *et al*., 1989), and cold-induced apoptosis is related to the formation of ROS (Rauen *et al*., 1999). To prevent damage by ROS, antioxidant defence systems have evolved consisting of both enzymatic and non-enzymatic components (Felton and Summers, 1995). Our results indicated that temperature changes dramatically enhance mRNA accumulation of *AccCYP4G11* in a time-related manner (Figs. 6A–C), which may be accompanied by higher activity of P450 that reduces ROS. In agreement with the temperature sensitivity of *AccCYP4G11*, three heat shock-responsive elements were found in the promoter of *AccCYP4G11*. Moreover, Matteis *et al*. (2012) have found that the uncoupled catalytic cycle of cytochrome P450 confers peroxidase-like properties, which is consistent with our conclusion.

Additionally, transcript abundance of *AccCYP4G11* was affected by UV irradiation and H_2O_2 treatment, which both cause the generation of more endogenous ROS (Yasui and Sakurai, 2000; Wang *et al*., 2010). Furthermore, insecticide treatment was performed to examine the mRNA level of *AccCYP4G11*. Pesticides may generate oxidative stress and elicit changes in antioxidant levels and ROS scavenging enzyme systems (Agrawal *et al*., 1991; El-Sharkawy *et al*., 1994; Almeida *et al*., 1997) thus preventing lipid peroxidation of biomembranes (Narendra *et al.*, 2007). Therefore, we propose that *AccCYP4G11* contributes to the antioxidative defence system.

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