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Molecular identification and stress response of the apoptosis-inducing factor gene 3 (*AccAIF3*) from *Apis cerana cerana*

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Abstract – Apoptosis-inducing factors (AIFs) play vital roles in bioenergetic and redox metabolism. Compared with studies focused on the apoptogenic function of AIF, few studies have reported on its oxidoreductase function, especially in insects. In this study, we identified a novel AIF gene *AccAIF3* that was isolated from *Apis cerana cerana*, and investigated its expression and structural features. Quantitative real-time PCR (qRT-PCR) revealed the highest mRNA level of *AccAIF3* in adult bees at 15 days. qRT-PCR results revealed that *AccAIF3* transcripts were induced by cold, CdCl₂, HgCl₂, UV, pyriproxyfen and cyhalothrin, but were downregulated by ecdysone. We also compared the expression of *AIF3* from *Apis cerana cerana* and *Apis mellifera ligustica* Spinola to *Ascosphaera apis*, and found that *AIF3s* may respond to *Ascosphaera apis* in a species-specific manner. These data suggested that *AccAIF3* may play vital roles in the response to abiotic and biotic stresses and contribute to the adaptability of honeybees to adversities.

Apis cerana cerana / biotic and abiotic stresses / quantitative real-time PCR / *AccAIF3*

1. INTRODUCTION

Apoptosis-inducing factor (AIF) is a phylogenetically conserved redox-active flavoprotein that is confined to mitochondria, where it exerts a vital function in bioenergetic and redox metabolism (Klein et al. 2002; Urbano et al. 2005). The first

AIF was identified and named by Guido Kroemer and colleagues (Susin et al. 1999; reviewed in Kroemer and Reed 2000). The crystal structures of mouse and human AIF revealed two important regions: a domain with homology to a bacterial NADH-dependent ferredoxin reductase and a domain required for DNA fragmentation (a putative DNA-binding site) (Maté et al. 2002; Ye et al. 2002). In most mammals, AIF also contains a mitochondrial localisation sequence (MLS) in its N-terminal (Lorenzo et al. 1999) and harbours two nuclear localisation signals (NLS) (Norberg et al. 2010). Two additional members are found in humans, apoptosis-inducing factor-like mitochondrion-associated inducer of death (AMID), which has also been found in the cytosol

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and has no MLS (Wu et al. 2002; Ohiro et al. 2002), and apoptosis-inducing factor-like (AIFL), which lacks a clear MLS (Xie et al. 2005). In addition to these homologues, some alternative splice forms of AIF are known: AIFexon2a, AIFexon2b, Ash, Ash2 and AIFsh3. The alternative usage of exon 2 (exon 2a or 2b) allows for the production of AIFexon2a (AIF1) and AIFexon2b (Loeffler et al. 2001; Otera et al. 2005). Ash is a short variant that contains NLS and the pro-apoptotic segment, but lacks the MLS and redox-active domain (Delettre et al. 2006a). AIFsh2 localises to mitochondria and has redox function, but lacks the pro-apoptotic domain. AIFsh3 resembles AIFsh2, although it lacks MLS (Delettre et al. 2006b). Among all the forms, AIF is most abundant, so we chose AIF as our experimental subject. As its name indicates, AIF may function in the life of the cell.

Confronted with environmental stresses, in principle, cells can react in two distinct ways. To repair damage or resume normal cellular functions, some of the stressed cells can activate defence mechanisms to adapt to stressful conditions. On the other hand, programmed cell death can be activated, leading to apoptosis. The deciding factors in the choice between these two pathways are the stress intensity and cell-intrinsic parameters (Garrido et al. 2003; Mosser and Morimoto 2004; Thompson 1995). Previous studies have identified that mitochondria have a dual role in cells. Mitochondria generate ATP by oxidative phosphorylation and play a central role in metabolic pathways. In addition, mitochondria play an important role in the regulation of cell death (Kroemer 1997; Green and Reed 1998). As a mitochondrial protein, AIF also functions to activate these two opposing ways.

Initial studies concerning AIFs were focused on its caspase-independent apoptotic function. AIF appears to be a caspase-independent death factor (Susin et al. 1999). AIF^{-Y} ES cells are sensitive to various apoptotic stimuli, such as ultraviolet irradiation (UV) and anisomycin (Joza et al. 2001). AIF is essential for the first wave of cell death during mouse morphogenesis. As discussed previously, the addition of

recombinant AIF to purified nuclei causes chromatin condensation (Susin et al. 1999).

However, recent in vivo data indicate that in addition to its lethal activity, AIF plays a vital role in the mitochondria of healthy cells by regulating the activity of the mitochondrial respiratory chain complex I; this form of AIF is dispensable for apoptosis and has an NADH oxidase domain (Hangen et al. 2009, 2010). Other studies have shown that AIF has life-supporting mitochondrial activity (Joza et al. 2001; Miramar et al. 2001). AIF has been proposed to act as a putative reactive oxygen species (ROS) scavenger, but this hypothesis remains to be confirmed (Klein et al. 2002). Another subsequent work showed that the loss of AIF leads to an increase in ROS, and exogenous antioxidants can alleviate the complex I deficiency caused by AIF depletion (Apostolova et al. 2006). Compared with studies in mammals, few studies have focused on insects. *D. melanogaster* AIF (*DmAIF*) is highly conserved in the regions of mouse AIF (*mAIF*) which was critical for its oxidoreductase activity (Joza et al. 2008).

Chinese honeybees (*Apis cerana cerana*) play a vital role in the balance of native ecologies and economic crops. Compared with *Apis mellifera ligustica* Spinola (*A. mellifera* L.), *A. cerana cerana* has unique characteristics, such as heat hardiness and disease resistance, as well as the pollination of flowering crops at low temperature (Li et al. 2008). Environmental pollution threatens the existence of *A. cerana cerana*. In order to protect *A. cerana cerana*, we chose it as experimental insect to investigate its defences against oxidant stresses. In this study, we cloned and characterised *AIF3* from *A. cerana cerana* and detected its levels of expression at various developmental stages. We also cloned the 5' flanking region of *AccAIF3* and predicted some putative cis-acting elements. qRT-PCR results revealed that the *AccAIF3* transcript was upregulated in response to various environmental stresses. Our study may contribute to the knowledge of AIF in insects and protect Chinese honeybees from injury.

2. MATERIALS AND METHODS

2.1. Biological specimens and treatment

A. cerana cerana and *A. mellifera* L. were maintained at the experimental apiary of Shandong Agricultural University, Taian, China. Eggs were collected from the honeycomb. Using age, eye colour and shape, we divided worker honeybees into ten groups: larvae from the first to fifth instars (L1–L5), prepupae (PP) and pupae with white eyes (Pw), pink eyes (Pp), brown eyes (Pb) and dark eyes (Pd) (Michelette and Soares 1993). We labeled bees to distinguish their ages. The one-day-old adult worker honeybees (A1) were collected soon after they emerged from the honeycomb, while 15-day post-emergence (A15) and 30-day post-emergence (A30) adults were collected at the entrance of the hive (Yan et al. 2012). The number of collected A30 groups was 10, and the number of collected A15 groups was 300 to do experiments. Worker bees collected were maintained in wooden cages (20×15×10 cm) covered with transparent plastic, and the number of bees per cage was 50. The adults were fed basic adult food containing 30 % honey from the source colonies, 70 % powdered sugar and water (Alaux et al. 2010) in an incubator with 60 % relative humidity at 34 °C under a 24-h dark regimen. A15 adults were divided into nine groups (n=25). Groups 1–3 were treated at 4 °C, 25 °C and 42 °C. The controls for groups 1–3 were incubated at 34 °C. Groups 4–6 were treated with pesticides (pyriproxyfen, cyhalothrin and phoxime) as food. The pesticides were dissolved in food at a final

concentration of 20 mg/L and 0.5 µL were delivered to each bee in the thoracic notum. All insecticides were purchased from Huayang Technology Co., Ltd. (Shandong, China). The controls for groups 4–6 were fed normal food. Group 7 was subjected to ultraviolet (UV) light (30 mj/cm²). Groups 8 and 9 were fed a pollen and sucrose solution containing HgCl₂ (3 mg/mL) and CdCl₂ (3 mg/mL), and each bee was fed a mixture containing pollen and HgCl₂ or CdCl₂ (3 µg) with a micropipette. The controls for groups 8 and 9 were fed the basic adult diet. For ecdysone treatment, L3 were placed in 24-well plates and fed food dissolved in a pollen and sucrose solution at a final concentration of 1.0 µg/mL and 0.001 µg/mL. *A. cerana cerana* L3 and *A. mellifera* L. L3 were placed in 24-well plates and fed brood food containing *Ascospaera apis* (spore numbers to 10⁶/mL). The L3 control group was fed normal food. Three honeybees were harvested at the appropriate time (Table I) for each condition and were stored at –80 °C until they were analysed. Each treatment had three replicates.

2.2. Primers

Primers used for PCR amplification are listed in Table II.

2.3. Extraction of total RNA, isolation of genomic DNA and cDNA synthesis

Total RNA was extracted from worker honeybees using Trizol reagent (Invitrogen, Carlsbad, CA) and

Table I. The collection time (post treatment) for each experimental group

Experiment condition	Collection time after treatment
4 °C	0.5, 1, 1.5, 2, and 2.5 h
25 °C	0.5, 1.5, 2.5, 3.5, and 4.5 h
42 °C	0.5, 1, 1.5, 2, and 2.5 h
pyriproxyfen	1, 2, 3, 4, 5 and 6 h
cyhalothrin	1, 2, 3, and 4 h
phoxime	0.5, 1, 1.5, 2 and 2.5 h
UV	1, 2, 3, 4 and 5 h
HgCl ₂	3, 6, 9 and 12 h
CdCl ₂	3, 6, 9 and 12 h
ecdysone	3, 6, 9 and 12 h
<i>Ascospaera apis</i>	1, 2 and 3 d

Table II. The primers used in this study

Abbreviation	Primer sequence(5' - 3')	Description
PU	CCACATGCAATTCGAGGTCAATC	Internal fragment primer, forward
PD	GGATCTGCCGCAACACTACTCAT	Internal fragment primer, reverse
5P1	GCTTTCACGCGTACAAAACCAGATG	5' RACE reverse primer, primary
5P2	CCTGTGTGAAGTAAAGCCCCAT	5' RACE reverse primer, nested
AAP	GGCCACGCGTCGACTAGTAC (G) ₁₄	Abridged anchor primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged universal amplification primer
3P1	GCAGTCTCCTATCGAAATGCTACC	3' RACE reverse primer, primary
3P2	TTTCAGCTGCAATAGGTCATTATCC	3' RACE reverse primer, nested
B25	GACTCGAGTCGACATCGAT	Universal primer, nested
B26	GACTCGAGTCGACATCGA (T) ₁₈	Universal primer, primary
QCU	CCACATGCAATTCGAGGTCAATC	Full length primer, forward
QCD	GCAATTTTCATTCAATTTTCCACTTG	Full length primer, reverse
N1	GGGCGGAAAAAATTGTAAAGGACT	Genomic sequence primer, forward
N2	GCTTTCACGCGTACAAAACCAGAT	Genomic sequence primer, reverse
N3	CATCTGGTTTTGTACGCGTGAAAG	Genomic sequence primer, forward
N4	GGATCTGCTGCAACACTACTCATAGC	Genomic sequence primer, reverse
RTU	TGGGGCTTTACTTCACACAGG	qRT-PCR perimer, forward
RTD	GCTTTCACGCGTACAAAACCAGATG	qRT-PCR perimer, reverse
QDU	GCTGGACCTTCTTGTGTGAT	5' flanking region primer, forward
QDD	GCCTGGTAGAGTATTAGAATTTGGT	5' flanking region primer, nested
RTU	TGGGGCTTTACTTCACACAGG	qRT-PCR perimer, forward
RTD	GCTTTCACGCGTACAAAACCAGATG	qRT-PCR perimer, reverse
<i>ARPI-s</i>	GTTTTCCCATCTATCGTCGG	Standard control primer, forward
<i>ARPI-x</i>	TTTTCTCCATATCATCCAG	Standard control primer, reverse
<i>ARPI-s-y</i>	TTGTATGCCAACACTGTCCTTT	<i>A. mellifera</i> standard control primer, forward
<i>ARPI-x-y</i>	AGAATTGACCCACCAATCCA	<i>A. mellifera</i> standard control primer, reverse
RTU-y	TGGGGCTTTACTTCATACAGG	<i>A. mellifera AIF3</i> , qRT-PCR primer, forward
RTD-y	CGCTTTCACGTGTACAAAACCAGATG	<i>A. mellifera AIF3</i> , qRT-PCR primer, reverse

digested with RNase-free DNaseI (Promega, Madison, WI). The first stand cDNA was synthesised using a reverse transcriptase system (TransGen Biotech, Beijing, China). Genomic DNA was isolated from worker bees using the EasyPure Genomic DNA Extraction Kit (TransGen Biotech, Beijing, China).

2.4. Full-length cDNA isolation of *AccAIF3*

The full-length cDNA of *AccAIF3* was isolated by reverse transcription-PCR (RT-PCR) and rapid amplification of cDNA ends-PCR (RACE-PCR).

Primers PU and PD were designed to amplify the internal fragment, and primers 5P1/5P2, 3P1/3P2, AAP/AUAP and B26/B25 were designed for 5' RACE and 3' RACE (all primers are listed in Table II). Finally, the full-length cDNA of *AccAIF3* was obtained using primers QCU and QCD (primers are listed in Table II). All products were purified, cloned into the pEASY-T3 vector (TransGen Biotech, China), transformed into the *Escherichia coli* strain DH5α and then sequenced. All sequencing was done by BioSune using ABI 3730 XL sequenator.

2.5. Amplification of the 5' flanking region and amplification of the genomic sequence of *AccAIF3*

The promoter region was amplified by the inverse polymerase chain reaction (I-PCR) method using specific primers (QD51/QD31 and QD52/QD32) (primers are listed in Table II). The template was genomic DNA digested by restriction endonuclease *EcoRI* and then self-ligated by T4 DNA ligase (TakaRa, Dalian, China). Primers QDU and QDD (primers are listed in Table II) were used to obtain the full-length sequence of 5' flanking region. Transcription factor binding sites (TFBS) were searched in the 5' flanking region using the MatInspector database (<http://www.cbrc.jp/research/db/TFSEARCH.html>) and TRANSFAC database (<http://www.gene-regulation.com/pub/databases.html>). To amplify the genomic sequence of *AccAIF3*, two pairs of specific primers (N1/N2 and N3/N4; shown in Table II) were designed and used. All PCR products were purified, cloned into the pEASY-T3 vector (TransGen Biotech, China) and transformed into the *Escherichia coli* strain DH5 α for sequencing. All sequencing was done by BioSune using ABI 3730 XL sequencer.

2.6. Bioinformatic analysis

Conserved domains of *AccAIF3* were analysed and compared using the BLAST algorithm available at NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?RID=54139JC601R&mode=all>). Using DNAMAN software 5.2.2 developed by the American Lynnon Biosoft Company (USA), we identified the open reading frame (ORF) and conducted multiple alignments amongst homologues. A phylogenetic tree was generated using Molecular Evolutionary Genetic Analysis (MEGA version 4.0). The web software program TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>) was used to predict the cis-elements in the promoter region.

2.7. The transcription expression level of *AccAIF3* using quantitative PCR

Total RNA was extracted from samples from different treatments, and the first strand cDNA was

synthesised as described above. qRT-PCR was performed with a modified qRT-PCR volume containing 1.6 μ L of diluted cDNA from different samples, 10.0 μ L of Takara SYBR[®] premix Ex Taq[™], 0.4 μ L of each primer (10 pmol/mL), and 7.6 μ L of ddH₂O from Yao et al. 2013 using primers RTU/RTD designed based on the sequences of *AccAIF3*, primer *ARP1-s/ARP1-x* designed based on the *A. cerana cerana* *ARP1* gene (GenBank accession no. HM640276) and primers *ARP1-s-y/ARP1-x-y* designed based on the *A. mellifera* (GenBank accession no. GB44311). *ARP1* was selected for the normalization of RNA levels (Yao et al. 2014), and we also tested the expression stability of this endogenous control gene (date not show). Primers RTU-y/ RTd-y were designed based on the *A. mellifera* *AIF3* gene (GenBank accession NM_001185146.1). All primers above are listed in Table II. The relative *AccAIF3* gene expression was analysed by the comparative CT method ($2^{-\Delta\Delta CT}$ method). At least three individual samples were prepared for each sample and all samples were analysed in three replicates.

2.8. Statistical analysis

Error bars denote the standard error of the mean (SEM) from three independent experiments. Significant differences were determined by Duncan's multiple range tests using the Statistical Analysis System (SAS) version 9.1 software programs (SAS Institute, Cary, NC, USA).

3. RESULTS

3.1. Isolation and sequence analysis of the full-length cDNA of *AccAIF3*

We cloned the cDNA sequence of *AIF3* from *A. cerana cerana* (GenBank accession number: KF745895). Sequence analysis indicates that the cDNA is 2586 bp long, containing a 1734 bp ORF, a 622 bp 5' untranslated region (UTR) and a 230 bp 3' UTR. The ORF encodes a protein of 577 amino acids. Figure 1a shows that *AccAIF3* is most similar to *A. mellifera* *AIF3* (AmAIF3, 96.37 % identity, GenBank ID: XP_625035.2).

The conserved domains of *AccAIF3* were identified by NCBI Conserved Domain Database Search, and included a Rieske domain

Figure 1 a Characterisation of apoptosis-inducing factors (AIFs) from various species. An alignment of the identified AccAIF3 protein sequence (GenBank ID: KF745895) with other known insect AIF3s. The amino acid sequences used in the analysis were from *A. mellifera* (AmAIF3, GenBank ID: XP_625035.2), *Bombus terrestris* (BtAIF3-like, XP_003695310.1), *Camponotus floridanus* (CfAif3, EFN71795.1) and *Megachile rotundata* (Mraif3-like, XP_003703654.1). Identical amino acid residues are shaded in black. A triangle was used to show the specific amino acid that exists in *A. mellifera* but does not exist in *A. cerana cerana*. Circles were used to show the [2Fe-2S] cluster binding site. **b** The conserved domains of AccAIF3

and a pyridine nucleotide-disulfide oxidoreductase domain (Pyr_redox) (Fig. 1b). The Rieske domain, a [2Fe-2S] cluster binding domain, is involved in electron transfer. The Pyr_redox domain is a small NADH binding domain within a larger FAD binding domain. A phylogenetic tree of AIFs from different species was constructed using a neighbour-joining method implemented in MEGA, and showed the possible evolutionary relationship among AIFs (Fig. 2). Phylogenetic protein sequence analysis revealed that *AccAIF3* was more closely related to *AmAIF3*, respectively, than other homologues. In addition, this result was in agreement with the relationship predicted from the multiple sequence alignment.

3.2. The genetic structure of *AccAIF3*

The full-length DNA sequence of *AccAIF3* is 4051 bp long, containing six introns inserted within seven exons (GenBank accession number: KF745896). All introns have classical characteristics: they are flanked by the 5'-GT splice donor and 3'-AG splice donor and exhibit high AT content. To further investigate the features of *AccAIF3*, a comparison was performed. According to Table III and Figure 3, the results indicated that the AT content of the introns was really much higher than that in exons. In addition, the length of exons or introns in *AIF3s* from different species was also highly conserved; especially in *A. cerana*.

Figure 2 A phylogenetic tree of AIF3s was constructed from different species using the neighbour-joining method and MEGA 4.0 software. The amino acid sequences of the AIF3s obtained from NCBI are as follows: NvAIF3 (*Nasonia vitripennis*, XP_001607016.1), MdAIF3-like (*Musca domestica*, XP_005183902.1), Si SINV_02158 (*Solenopsis invicta*, EFZ18689.1), CfAIF3 (*Camponotus floridanus*, EFN71795.1), ApAIF3-like (*Acyrtosiphon pisum*, XP_003241906.1), XtAIF3-like (*Xenopus (Silurana) tropicalis*, XP_002936104.1), CmSIF3 (*Chelonia mydas*, EMP37830.1), CcAIF3-like (*Ceratitidis capitata*, XP_004518444.1), AeAIF3 (*Acromyrmex echinator*, EGI65010.1), HsAIF3 (*Harpegnathos saltator*, EFN79971.1), BmAIF3-like (*Bombyx mori*, XP_004926894.1), AfAIF3-like (*Apis florea*, XP_003695310.1), PcAIF3-like (*Pomacea canaliculata*, AFQ23946.1), BtAIF3-like (*Bombus terrestris*, XP_003695310.1), BiAIF3-like (*Bombus impatiens*, XP_003489952.1), MrAIF3-like (*Megachile rotundata*, XP_003703654.1), AmAIF3 (*A. mellifera*, GenBank ID: XP_625035.2) and AdAIF3-like (*Apis dorsata*, XP_006620108.1)

Table III. The lengths of the exons and introns and the AT content in *AccAIF3*, *AmAIF3* and *MrAIF3-like*

Exon	Gene	Length (bp)	AC content (%)	Intron	Gene	Length (bp)	AT content (%)
1	<i>AccAIF3</i>	70	63	1	<i>AccAIF3</i>	1062	84
	<i>AmAIF3</i>	73	64		<i>AmAIF3</i>	1153	84
	<i>MrAIF3-like</i>	73	54		<i>MrAIF3-like</i>	1012	72
2	<i>AccAIF3</i>	292	66	2	<i>AccAIF3</i>	61	87
	<i>AmAIF3</i>	293	66		<i>AmAIF3</i>	62	89
	<i>MrAIF3-like</i>	293	64		<i>MrAIF3-like</i>	70	79
3	<i>AccAIF3</i>	337	69	3	<i>AccAIF3</i>	66	90
	<i>AmAIF3</i>	337	68		<i>AmAIF3</i>	65	93
	<i>MrAIF3-like</i>	337	61		<i>MrAIF3-like</i>	59	75
4	<i>AccAIF3</i>	351	73	4	<i>AccAIF3</i>	87	90
	<i>AmAIF3</i>	351	74		<i>AmAIF3</i>	106	90
	<i>MrAIF3-like</i>	351	64		<i>MrAIF3-like</i>	69	76
5	<i>AccAIF3</i>	206	68	5	<i>AccAIF3</i>	99	87
	<i>AmAIF3</i>	206	68		<i>AmAIF3</i>	96	85
	<i>MrAIF3-like</i>	206	61		<i>MrAIF3-like</i>	61	76
6	<i>AccAIF3</i>	217	69	6	<i>AccAIF3</i>	90	88
	<i>AmAIF3</i>	217	69		<i>AmAIF3</i>	100	88
	<i>MrAIF3-like</i>	217	64		<i>MrAIF3-like</i>	69	76
7	<i>AccAIF3</i>	260	72				
	<i>AmAIF3</i>	260	73				
	<i>MrAIF3-like</i>	254	63				

cerana and *A. mellifera*, which agrees with the relationship predicted from the multiple sequence alignment.

3.3. Characterisation of the 5' flanking region of *AccAIF3*

To investigate the regulatory region of *AccAIF3*, a 663 bp fragment upstream of the

transcription start site was cloned by I-PCR. Some putative TFBS were predicted and are shown in Figure 4. In addition, the basic TATA-box and CAAT-box, which are critical for transcription, were also found in the 5' flanking region (data not shown). All the TFBS indicated that *AccAIF3* might be involved in the regulation of stress and development at the level of transcription.

Figure 3 Comparison of Genomic structures among the *AIF3s* gene family. Lengths of genomic DNA sequences of *AccAIF3* (KF745896) and its homologues, including *AmAIF3* (NC_007076.3) and *MrAIF3-like* (NW_003797177.1), are shown. The exons are indicated with black boxes, and the introns are indicated with grey boxes

Figure 4 Characterisation of the 5' flanking region in *AccAIF3*. The transcription start site is marked with an arrowhead. The predicted transcription factor binding sites mentioned in the text are marked as follows: HSF, CF2-II, Dfd, SKY, SRK, Oct-1, NIT2 and BR-C

Figure 5 The expression patterns of *AccAIF3*. mRNA expression of *AccAIF3* at different developmental stages: eggs, larvae (L1–L5), pupae (PP, Pw, Pp, Pb and Pd) and adults (A1, A10 and A30). Each value is presented as the mean (SD) of three replicates. Vertical bars represent the mean $\pm$ SEM ($n=3$). Letters above the bars indicate significant differences ($P<0.001$) identified by SAS software version 9.1

3.4. Developmental expression pattern of *AccAIF3*

Total RNA from the larvae, pupae and adults was extracted to investigate the expression pattern of *AccAIF3* at different developmental stages using qRT-PCR. In Figure 5, the results indicate that *AccAIF3* is rarely expressed in eggs. High expression levels of *AccAIF3* were detected in the Pd, A1 and A30 stages, and the expression of *AccAIF3* peaked at the A15 stage.

3.5. The expression profile of *AccAIF3* under environmental stresses, and exposure to hormones and fungus

To characterise the influence of temperature on the *AccAIF3* transcriptional level, qRT-PCR was performed. The expression levels were normalised to those of control worker honeybees. Under cold (4 °C) treatments, the expression of *AccAIF3* reached a peak after 1.0 h, followed by a sharp decrease (Fig. 6a), while heat treatment (42 °C)

Figure 6 The expression profiles of *AccAIF3*. **a–c** The expression of *AccAIF3* under various temperature stress treatments, with the *β-actin* gene shown for comparison at the appropriate time. **d** and **e** The transcription level of *AccAIF3* under heavy metal treatment with CdCl₂ and HgCl₂. **f** *AccAIF3* mRNA expression level after exposure to UV treatment at the appropriate time. CK is the abbreviation for control check. Vertical bars represent the mean ± SEM (n=3). Letters above the bars indicate significant differences ($P<0.001$) identified by SAS software version 9.1.

had slight significant influence on the *AccAIF3* transcriptional level (Fig. 6c). Under 25 °C treatment, the level of *AccAIF3* transcription was induced within 0.5 h (Fig. 6b).

Under CdCl_2 treatment, the transcriptional rates of *AccAIF3* were induced with a peak at 6 h (Fig. 6d). Different from CdCl_2 treatment, HgCl_2 treatment caused a nearly 6.0-fold relative expression increase in *AccAIF3* (Fig. 6e). In addition, UV treatment caused a drastic upregulation of *AccAIF3* expression (Fig. 6f), which indicated that *AccAIF3* might be associated with UV-related activation.

As worker honeybees work within the environment, they contact unavoidable pesticides, which may lead to death. To study the potential role of *AccAIF3* against pesticides, qRT-PCR was performed. Treatment with pyriproxyfen persistently induced a sharply increase in the level of *AccAIF3* transcription, except at 4 h (Fig. 7a). The transcript level of *AccAIF3* was highest at 2 h following cyhalothrin treatment (Fig. 7b). In contrast to pyriproxyfen and cyhalothrin, no significant alteration was observed after phoxime treatment (Fig. 7c). To study the response to ecdysone, which is a

Figure 7 The transcript level of *AccAIF3* under pesticide stress. **a–c** The expression level of *AccAIF3* under pyriproxyfen, cyhalothrin and phoxime treatment. The ecdysone treatments were 1.0 µg/mL (**d**) and 0.001 µg/mL (**e**). CK is the abbreviation for control check. Each value is presented as the mean (SD) of three replicates. Letters above the bars indicate significant differences ($P < 0.001$) identified by SAS software version 9.1

hormone that influences the development of larvae to pupae, 1.0 $\mu\text{g/mL}$ and 0.001 $\mu\text{g/mL}$ of ecdysone were used to treat larvae separately. Figure 7d and e showed that the transcriptions of *AccAIF3* were noticeably inhibited.

Ascosphaera apis, a primary fungus, threatens the life of the honeybee. To detect the differences in *AccAIF3* and *AmAIF3* in response to *Ascosphaera apis*, qRT-PCR was performed. Figure 8 showed that the transcript rate of *AccAIF3* reached a peak at approximately 5.0-fold relative to the normal, while that of *AIF3* in *A. mellifera* L. was not significantly altered, which showed that *AccAIF3* might play an important role in resistance to *Ascosphaera apis*.

4. DISCUSSION

Apoptosis is pivotal for the development and maintenance of healthy tissues by removing damaged and mutated cells and preventing the induction of cancer. The deregulation of apoptosis leads to the progression of tumours and malignancy due to an accumulation of gene mutations and genetic instability (Johnstone et al. 2002; Kitada et al. 2002). AIFs are factors

involved in apoptosis. Although many studies have focused on the mammals AIFs, few reports have investigated AIFs in insects. In this study, we reported the first cloning and characterisation of the *AIF3* gene from *A. cerana cerana*, and we have studied its expression level in response to various stresses.

AmAIF3 has another name in NCBI: *A. mellifera* thioredoxin reductase 3 (*AmTrxR3*) (GenBank accession number: XM_625032.4). TrxRs is a flavoprotein that contains an FAD prosthetic group, an NADPH binding site and an active site (Mustacich and Powis 2000). Studies have demonstrated that TrxRs in their oxidised form can protect organisms against oxidant injury (Yang et al. 2010a). In addition, TrxRs also play roles in cell growth and transformation, and the recycling of ascorbate (Mustacich and Powis 2000). Among all known enzymatic functions of eukaryotic proteins, AIF has the strongest homology with plant semidehydroascorbate and ascorbate reductases (Susin et al. 1999). In this respect, *AccAIF3* may have a potential function such as *AmTrxR3* in maintaining the redox homeostasis of a cell.

To better understand the potential roles of *AccAIF3*, the 5' flanking region was isolated. Cell factor 2-II (CF2-II), related to development and growth (Stanojević et al. 1989), was identified. Therefore, we studied the expression pattern of *AccAIF3* throughout development. Adult worker honeybees have to forage for pollen and nectar at 2–3 weeks of age (Ament et al. 2008). We hypothesised that when worker honeybees fly to forage, the expression of *AccAIF3* is needed to protect organisms against various environmental stresses. Interestingly, the qRT-PCR results demonstrated that the transcript level of *AccAIF3* was highest at A15 (Fig. 5). This stage is approximately when worker honeybees begin to forage outside.

Other TFBS heat shock factors (HSFs) related to heat-induced transcriptional activation (Fernandes et al. 1994) were also found in the region. Temperature can cause physiological changes in organisms and is one of the most important abiotic environmental factors (An and

Figure 8 Functional comparison of *AccAIF3* and *AmAIF3* after exposure *Ascosphaera apis* for 1 d, 2 d and 3 d. CK is the abbreviation for control check. The expression levels of *AccAIF3* are indicated with grey boxes, and the transcription of *AmAIF3* is indicated with black boxes. Vertical bars represent the mean $\pm$ SEM (n=3). Letters above the bars indicate significant differences ($P < 0.001$) identified by SAS software version 9.1

Choi 2010). To avoid harm caused by temperature, organisms have evolved many strategies, such as changing the cell membrane fluidity, expressing some proteins and seeking shelter (Yang et al. 2010b). To detect whether *AccAIF3* was associated with temperature stress, the expression levels of *AccAIF3* were measured. The data showed that the *AccAIF3* transcription levels were significantly increased in a short time when bees exposed to 4 °C and 25 °C, while no significant alteration was observed at 42 °C (Fig. 6a–c), indicating that *A. cerana cerana* may adapt to heat conditions. These data indicated that *AccAIF3* might be associated with temperature-induced transcriptional activation. In addition, binding sites for Broad-Complex (BR-C), which contributes to the tissue-specific response to ecdysone (von Kalm et al. 1994), were identified in the promoter. To study the responses of *AccAIF3* to ecdysone, qRT-PCR was performed. Results indicated that the transcription of *AccAIF3* was inhibited in response to different concentrations of ecdysone (Fig. 7d–e). The reasons that the results were opposite to the function of the putative BR-C may be due to the material, the treatment method and the collection time.

In addition to the TFBS mentioned above, CdxAs were also identified. Frumkin et al. (1994) demonstrated that CdxAs play roles in gut closure, indicating that *AccAIF3* may be involved in gut protection. Some diseases initially occur in the gut. For example, *Ascosphaera apis* is a filamentous fungus that causes Chalkbrood (Williams 2000; Hornitzky 2001). After ingestion, the fungal spores germinate in the larval gut, and then mycelia cross the gut lining and proliferate through the body cavity. Chalkbrood can lead to heavy losses in affected honeybee colonies (Cornman et al. 2012). At present, very few studies have focused on the molecular mechanism of honeybee immune responses to Chalkbrood (Aronstein and Saldivar 2005). In this study, a comparison was performed to investigate the changes in *AccAIF3* and *AmAIF3* after exposure to *Ascosphaera apis*. Our data indicated that despite the high homology between *AccAIF3*

and *AmAIF3*, the responses were different (Fig. 8). *AccAIF3* may be involved in the protection against *Ascosphaera apis*, while *AmAIF3* may not be relevant. This phenomenon perhaps resulted from the cis-acting factors Dfd, which determine the specificity of homeotic gene action (Ekker et al. 1992).

Furthermore, we also detected changes in the expression levels of *AccAIF3* with exposure to various environmental stresses, such as heavy metals, UV, and pesticides. Heavy metal contamination remains a critical environmental problem (Nagajyoti et al. 2010). Worker honeybees foraging for nectar and pollen outside may contact heavy metals. qRT-PCR results revealed that both CdCl₂ and HgCl₂ induce the transcript level of *AccAIF3* in a time-dependent manner (Fig. 6d–e), and *AccAIF3* may function in avoiding injury. UV is harmful to organisms and can destroy the structure of DNA, inhibit DNA repair pathways and interfere with the functions of proteins (Park et al. 2000). The transcript levels of *AccAIF3* were dramatically enhanced by UV treatment (Fig. 6f), suggesting a role for *AccAIF3* in the enzymatic protection against oxidative stress. The excessive use of pesticides has a negative impact on the normal development of the digestive system and glands in insects (Franco et al. 2009). Figure 7a–c showed that *AccAIF3* might protect bees against pesticides. Murakami and Johnson (1996) revealed that increased resistance to environmental stresses is necessary for protection, and enhanced resistance to ROS may be efficient for the extension of life in the fruit fly.

In conclusion, we cloned the *AIF3* gene from *A. cerana cerana*, and the expression of *AccAIF3* peaked at the A15 stage during development. The transcript levels of *AccAIF3* were significantly increased by exposure to cold, HgCl₂, CdCl₂, UV, pesticides and *Ascosphaera apis*. The increased expression level perhaps partially corresponds to the increased levels of ROS. Taken together, *AccAIF3* may have an important influence on the response to environmental stresses and result in the elevated resistance of honeybees to adversities.

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Identification moléculaire et réponse au stress du gène 3 codant pour le facteur inducteur d'apoptose (*AccAIF3*) chez *Apis cerana cerana*

Stress biotique / stress abiotique / PCR en temps réel / oxydo-réductase / *Ascosphaera apis*

Molekulare Charakterisierung und Stressantwort des Apoptose-induzierenden Faktor Gens 3 (*AccAIF3*) von *Apis cerana cerana*

Apis cerana cerana / Biotischer und abiotischer Stress / Quantitative Real-Time PCR / *AccAIF3*

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