Original article

Molecular cloning, expression and oxidative stress response of the vitellogenin Gene (*AccVg*) from *Apis cerana cerana*

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Abstract – Vitellogenin (Vg) is a yolk precursor protein in most oviparous females. However, Vg has not been studied in the *Apis cerana cerana*. In this work, the Vg gene of the *A. cerana cerana* has been cloned and sequenced. The gene codes for a protein consisting of 1770 amino acids in seven exons with a predicted molecular mass and isoelectric point of 200 kDa and 6.46, respectively. Additionally, an 807-bp 5′-flanking region was isolated, and potential transcription factor binding sites associated with development and stress response were identified. Quantitative real-time PCR analysis showed that AccVg is highly expressed in pupae during different developmental stages. In addition, the expression of AccVg could be induced by cold (4 and 16 °C), CdCl₂, and pesticide treatment. Real-time quantitative PCR (RT-PCR) and western blot analyses indicated that *AccVg* transcription was induced by various abiotic stresses. Western blot was used to measure the expression levels of AccVg protein. Taken together, these results suggest that AccVg most likely plays essential roles in antioxidant defence and that it may be of critical importance to honey bee survival.

Vitellogenin / *Apis cerana cerana* / RNA interference / Oxidative stress response / RT-PCR

1. INTRODUCTION

In China, *Apis cerana cerana* is commonly found and plays an important role in agricultural economics and in the balance of regional ecologies as a pollinator of flowering plants. In honeybees, worker-worker division of labour is based on a process of behavioural maturation; for the first 2–3 weeks of adult life, workers perform tasks inside the hive, such as brood care and food storage, and as they become older, they progress to tasks outside, including foraging for pollen and nectar (Winston, 1987).

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Organisms are exposed to a series of unfavourable environmental stressors on a nearly constant basis, including temperature change, heavy metals, pesticides, and ultraviolet (UV) radiation, all of which are believed to induce the formation of reactive oxygen species (ROS) (Lushchak, 2011; Kottuparambil et al., 2012). The generation of increased ROS and the production of oxidative damage are associated with induced thermal stress (Yang et al., 2010). ROS can be generated by all oxygenic organisms during aerobic metabolism (Dröge, 2002; Balaban et al., 2005). Therefore, understanding the antioxidant system of the honeybee and its ROS defence mechanisms has become a crucial issue for this industry.

Large lipid transfer proteins (LLTP)[2] have multiple roles in animals, such as lipid transporters, inflammation suppressors, immunomodulators and blood coagulators. Vg is one of the

ancient forms of these proteins, with an estimated 700 million-year history (Hayward et al., 2010).Vgs are mostly produced in the liver (vertebrates), hepatopancreas (crustaceans) or fat body (invertebrates); secreted into the blood and taken up by targets via receptor-mediated endocytosis (Spieth et al., 1991; Tufail and Takeda, 2008; Tseng et al., 2001). Honeybee Vg, known as a conserved yolk precursor protein, is a 180-kDa glycolipoprotein synthesized in fat body cells and released to the haemolymph (the insect blood) (Wheeler and Kawooya, 1990; Fleig, 1995). In egg-laying queens, Vg constitutes over 50% of total haemolymph proteins and is taken up by developing oocytes (Hartfelder and Engels, 1998). In the haemolymph of nurse bees, Vg comprise 30–50% of total proteins and impacts both antioxidant and immune function (Amdam et al., 2004; Seehuus et al., 2006; Corona et al., 2007). Additionally, Vg is present in the haemolymph of young worker bees, as well as in drones where it may account for up to 5% of total haemolymph proteins (Trenczek et al., 1989). Vg has an antioxidant function in workers (Seehuus et al., 2006). Knockdown of Vg expression in workers causes decreased resistance to oxidative stress. The Vg titre positively correlates with the honeybee's oxidative stress tolerance because the antioxidant property of Vg shields other haemolymph molecules from reactive oxygen species (ROS) (Seehuus et al., 2006). Additionally, a causal link between honeybee vitellogenin activity and lifespan is supported by the zinc-binding capacity of the protein product (Amdam et al., 2004), which also suggests an antioxidant function (Amdam and Omholt, 2002; Goto et al., 1999; Quinlan et al., 2005).

The vitellogenins have been studied extensively in a wide variety of animals, both vertebrates and invertebrates, including insects. In the present study, we isolated and characterised the Vg in *A. cerana* cerana (AccVg). Additionally, we analysed the expression of Vg during different developmental stages and under various abiotic stress conditions. Finally, we investigated the activity of AccVg under various stress conditions, including cold, insecticides and heavy metal exposure.

2. MATERIALS AND METHODS

2.1. Biological specimens and treatments

The Chinese honeybees, *A. cerana cerana*, maintained at the experimental apiary of Shandong Agricultural University (Tai'an, Shandong Province, China), were used in the experiments following different treatments. Newly emerged workers within an 8-h period were tagged with numbered Opalith plates (Graze, Germany) at emergence from hives. When the tagged workers were 15 days old, these were called foragers. They were captured and randomly divided into 6 groups, each group with 3 independent biological samples of 150 individuals, caged in wooden cage during the treatment which were kept in an incubator at a constant temperature (31 °C) and humidity (60%) and fed with pollen-and-sucrose solutions (Pham-Delègue et al., 2000).

For the environmental stress analysis, the worker bees were treated with cold (4 °C) or heat (42 °C) for the indicated times. For the pesticide treatments, cyfluthrin, phoxim and paraquat were diluted to a final concentration of 20 mg/L. The diluted liquid (200 μL) was sprayed into gauze and the gauze was put in the wooden cage. For the heavy metal treatment, worker bees were fed with pollen-and-sucrose solutions containing CdCl2 (1.0 μg/mL). The control group was fed only the pollen-and-sucrose solution. The bees were kept in the cages and exposed to the different treatments for up to 4 h. For different developmental stages and tissues, untreated larvae and adult workers were harvested for gene expression analysis. A total of 60 15-day-old workers were anaesthetised on ice, and the brains, thoraces and guts were dissected. Three individual bees were dissected for each stage. All of the bees were flash-frozen in liquid nitrogen at an appropriate time and stored at −80 °C for RNA or DNA extraction.

2.2. RNA extraction, cDNA synthesis and genomic DNA isolation

Total RNA was extracted using the RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's instructions, followed by RNase-

free DNase I treatment. Single-stranded cDNA was synthesized using Transcript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, Beijing, China) at 42 °C for 15 min, followed by 85 °C for 15 s. Genomic DNA was isolated from newly emerged workers using an EasyPure Genomic DNA Extraction Kit according to the manufacturer's instructions (TransGen Biotech).

2.3. Primers

The primers used in the present study were listed in Table I.

2.4. Isolation of the full-length cDNA and the genomic sequence of *AccVG*

To obtain the fragment of *AccVg* cDNA, special primers (VG1/VG2, VG3/VG4, VG5/VG6) were designed and synthesized (Shanghai Sangon Biotechnological Company, Shanghai, China) by the conserved regions of the Vg from *A. mellifera*, *Nasonia vitripennis* and *Locusta migratoria.* Supplementary Fig. 1 shows the position of primers in Vg genes. The cDNA of newly emerged workers was used for cloning the full-length cDNA. On the basis of the sequence of the cloned conservative fragments, specific primers (3N1, 3N2, 5N1, 5N2) were designed and synthesized for 3′- and 5′- rapid amplification of cDNA ends (RACE). All the PCR products were purified using a gel extraction kit (Solarbio, Beijing, China). The PCR products were ligated into pEasy-T3 vector (TransGen Biotech) and transformed into *Escherichia coli* strain DH5α for sequencing.

To isolate the AccVg gene, three pairs of specific primers (N1/N2, N3/N4 and N5/N6) were designed based on the AccVg cDNA sequence. The PCR reaction uses 1 μL genomic DNA as template, 0.25 μL Tap DNA polymerase, 2.5 μL PCR buffer, 1 μL dNTP, 1 μL forward primer and reverse primer and 18.25 μL ddH$_2$O in a 25 μL volume with the following cycling conditions: initial denaturation program (94 °C for 10 min), followed by 35 cycles of 94 °C for 40 s and 72 °C for 120 s. Finally, the PCR products were separated, purified, ligated into pEasy-T3 vector and transformed into *E.coli* DH5α for sequencing.

2.5. Amplification of the 5′-flanking region of *AccVg*

The 5′-flanking region of *AccVg* was obtained using the inverse polymerase chain reaction (IPCR). The genomic DNA was digested with the restriction endonuclease *Nsi* I at 37 °C overnight, self-ligated to form circles using T4 DNA ligase (TaKaRa, Dalian, China) and used as a template for IPCR. Two pairs of primers (IF1/IR1, IF2/IR2) were designed and synthesised based on the genomic DNA sequence. Finally, the sequence of the 5′-flanking region of *AccVg* was used to design specific primers (P1/P2) for direct PCR amplification from genomic DNA. The PCR reaction uses 1 μL genomic DNA as template, 1 μL dNTP, 1 μL forward primer and reverse primer, 0.25 μL Tap DNA polymerase, 2.5 μL PCR buffer and 18.25 μL ddH$_2$O in a 25 μL volume with the following cycling conditions: initial denaturation program (94 °C for 10 min), followed by 35 cycles of 94 °C for 40 s, 50 °C for 40 s and 72 °C for 90 s. All of the PCR products were purified, ligated into the pMD19-T vector and sequenced.

2.6. Bioinformatic and phylogenetic analyses

The theoretical isoelectric point and molecular weight of AccVg were predicted using ExPASy online software (http://web.expasy.org/compute_pi/). The sequences of *AccVg* homologous with other species were retrieved using the NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using DNAMAN version 5.2.2 (Lynnon Biosoft, Quebec, Canada). The phylogenetic analysis was carried out using the Molecular Evolutionary Genetics Analysis (MEGA version 4.1) (http://www.megasoftware.net/) based upon the neighbour-joining method. The SWISS-MODEL (http://swissmodel.expasy.org/) online software was used to predict the tertiary structure of AccVg. The TFSEARCH website (http://www.cbrc.jp/research/db/TFSEARCH.html) was used to predict the transcription factor binding sites.

Table I. PCR primers in this study

Abbreviation	Primer sequence (5′→3′)	Description
VG1	ATGTTGCTACTTCTAACGCTT	cDNA sequence primer, forward
VG2	CGTAGAGGCTGTTGTCGTGT	cDNA sequence primer, reverse
VG3	CAACACCGCCGCCACCTTAT	cDNA sequence primer, forward
VG4	TTGCTGTTCGCCTGCTTGTCCG	cDNA sequence primer, reverse
VG5	GAGGAAATGTGGGAACTGAT	cDNA sequence primer, forward
VG6	ACGAAAGAAAGCGACAAAGT	cDNA sequence primer, reverse
5P1	CGGGCATCATACAAACCACAT	5′-RACE reverse primer, outer
5P2	TGGTGGTCGGTGAACAAACT	5′-RACE reverse primer, inner
AAP	GGCCACGCGTCGACTAGTAC(G)14	Abridged Anchor Primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged universal amplification primer
3P1	AAGGAGACCGACGACAAGA	3′-RACE forward primer, outer
3P2	AAGAACGAGGCCGCGATGAA	3′-RACE forward primer, inner
B26	GACTCTAGACGACATCGA(T)18	3′-RACE universal primer, outer
B25	GACTCTAGACGACATCGA	3′-RACE universal primer, inner
N1	ATGTTGCTACTTCTAACGCTT	Genomic sequence primer, forward
N2	CGTAGAGGCTGTTGTCGTGT	Genomic sequence primer, reverse
N3	CAACACCGCCGCCACCTTAT	Genomic sequence primer, forward
N4	TTGCTGTTCGCCTGCTTGTCCG	Genomic sequence primer, reverse
N5	GAGGAAATGTGGGAACTGAT	Genomic sequence primer, forward
N6	ACGAAAGAAAGCGACAAAGT	Genomic sequence primer, reverse
IF1	GGTCTGAGCAGTTGCACGCTG	Inverse PCR forward primer, outer
IF2	TCTAAACGAAACAAAGCGGCA	Inverse PCR reverse primer, outer
IR1	TCGCAATCTTCATCGTTGTA	Inverse PCR forward primer, inner
IR2	GTTCAAGTCCTCGGTGATAG	Inverse PCR reverse primer, inner
P1	AAAGCGGGGTACAGGATG	Promoter special primer, forward
P2	GGTGAGCAACGCCTTTA	Promoter special primer, reverse
VG-1	<u>GGATCC</u>TTGGAAAGCTATGACACAG (*Bam* HI)	Protein expression primer, forward
VG-2	<u>CTCGAG</u>CTGGCTCTGGCTAACTTTACC (*Xho* I)	Protein expression primer, reverse
RT1	TCGGTGACTACGAGCAGGAT	Real-time PCR primer, forward
RT2	GGGCAGAGGTTTGGACACT	Real-time PCR primer, reverse
β-s	CCGTGATTTGACTGACTACCT	Standard control primer, forward
β-x	AGTTGCCATTTCCTGTTC	Standard control primer, reverse

2.7. Production of recombinant *AccVg* and antibody preparation

Using the primers VG-1/VG-2 (Table I), a 1128-bp cDNA fragment was obtained (a part of the whole cDNA, 920-1295 amino acids), cloned into the expression vector PET-28a (+), and the recombinant plasmid pET-28a (+)-*AccVg* was transformed into *E. coli* BL21 (ED3), which was grown at 37 °C in 10 mL of Luria-Bertani broth (LB) supplemented with 40 μg/mL kanamycin. When the cell density reached 0.6 OD$_{600}$, isopropyl-1-thio-β-galactopyranoside (IPTG) was added to a 0.5 mM final concentration to induce the expression of the recombinant protein. Cells were centrifuged at 6500×*g* at 4 °C for 10 min, after incubation at 37 °C for 4 h. The pellet was resuspended in 10 mL of lysis buffer,

sonicated and centrifuged again at 4 °C at 13,000×g for 15 min. The recombinant protein was purified using the HisTrap™ FF column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Finally, the purified protein was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% acrylamide gels. The purified protein was more than 95% pure by SDS-PAGE gel analysis. The purified protein was injected subcutaneously into New Zealand White male rabbits weighing 2–3 kg for generation of antibodies (Bitondi and Simoes, 1996).

2.8. Quantitative RT-PCR

To determine the expression patterns of the AccVg in *A. cerana cerana*, quantitative RT-PCR was used. Amplification of the actin transcript (Gene ID: LOC108003299) was used as a sample control (Guidugli et al., 2005). The special primers RT1/RT2 and the actin gene primers actin-f/ actin-r are listed in Table I. The quantitative RT-PCR was carried out using the TransStart® Tip Green qPCR SuperMix (TransGen Biotech) in a 20 μL volume on a CFX96™ Real-time System (Bio-Rad) with the following cycling conditions: denaturation program (94 °C for 30 s), followed by 40 cycles of 94 °C for 5 s, 55 °C for 15 s and 72 °C for 10 s. At the end of the amplification phase, a melting curve analysis was performed with a minimum of 60 °C to a maximum of 94 °C and 0.2 °C increases for every 15 s to test the specificity and identity of the qRT-PCR products. Negative controls without the addition of RT enzyme were run to check for contamination by genomic DNA. To check reproducibility, each SYBR green assay was performed in triplicate, and for each data point, we analysed three independent biological samples. The relative transcript quantities of the Vg and actin genes were calculated using the comparative Ct method (Applied Biosystems, User Bulletin 2; Livak and Schmittgen, 2001; Tang-Feldman et al., 2006). The statistical analysis of the differences was determined by Duncan's multiple range test using Statistical Analysis System (SAS) version 9.1 software.

2.9. Western blot analysis

Whole-body proteins were extracted using RIPA Lysis Buffer (Beyotime, Jingsu, China) according to the manufacturer's instructions and quantified with BCA Protein Assay Kit (Beyotime). Soluble proteins were separated by SDS-PAGE on 7.5% acrylamide gels, transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Immobilon 0.22 μm) and processed for western blot. Membranes were treated with the anti-AccVg serum against AccVg protein raised in rabbits, used at a dilution of 1:500. The Goat Anti-Rabbit lgG, HRP conjugated (CWBIO, China), was used as the secondary antibody at a 1:2000 dilution. Proteins were visualized using chemiluminescent peroxidase substrate (Beyotime Biotechnology, Shanghai, China), and then the blots were quantified using a Fusion Fx system (UVP, America) and the analysis software FusionCapt Advance system (UVP, America).

2.10. Statistical analysis

All data are reported as the mean ± standard error (SE), unless otherwise stated. The SAS version 9.1 was used for data analysis. Differences between the treatment groups were analysed using the Duncan's multiple range test and analysis of variance (ANOVA) to identify homogenous groups of treatments. P values <0.05 were considered statistically significant. The different letters above bars denote significant differences between the treatment groups.

3. RESULTS

3.1. Molecular characterization of *AccVg* and features of the recombinant *AccVg* protein

The full-length *AccVg* cDNA sequence (GenBank accession number: kp398512) was 5411 bp, including 5313 bp in the open reading frame, 26 bp in the 5′-untranslated region and 72 bp in the 3′-untranslated region. The ORF encodes a polypeptide of 1770 amino acids with a calculated molecular mass of 200 kDa and a theoretical isoelectric point (PI) of 6.46,

respectively (Fig. S2). Multiple sequence alignment revealed high amino acid identity (89.05%) between *AmVg* and *AccVg* (Fig. S3).

To investigate the evolutionary relationship between *AccVg* and other *Vg*s from different species, a phylogenetic tree of *Vg*s was constructed. Of the eight insect species for which *Vg*s were examined, *AccVg* was categorised into the Hymenoptera order. As shown in Fig. 1, phylogenetic analysis revealed that *AccVg* was more closely related to *AmVg* than to homologues in other species. The SWISS-MODEL was used to predict and reconstruct the three-dimensional structure (Fig. S4).

The molecular mass of *AccVg* protein is so large that preparation of recombinant plasmid PET-28a (+)-*AccVg* was difficult. We expressed a portion of the protein (920–1295 aa) to prepare antibodies against *AccVg*. SDS-PAGE analysis showed that the recombinant protein was soluble and had a molecular mass of approximately 50 kDa (Fig. 2). The entire western blot of the specific antibody against *AccVg* is shown in Supplementary Fig. 5.

### 3.2. Genomic structure and the characterization of the 5′-flanking region of *AccVg*

By cloning and assembling cDNA fragments and intron, we obtained the *AccVg* genomic sequence. To better understand the organization of the regulatory elements, primers (IF1/IF2 and IR1/IR2) were used to obtain the 5′ flanking region of AccERR, using inverse PCR (IPCR). Briefly, total genomic DNA was digested with

(kDa)

| | | | | | | | | 70 |
|----|----|----|----|----|----|----|----| 55 |

1   2   3   4   5   6   7   8
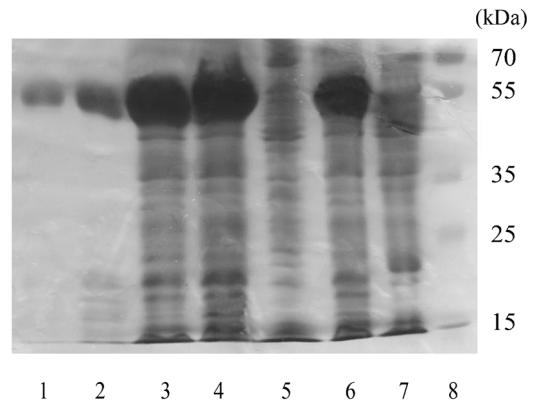
Figure 2 An SDS-PAGE analysis of the recombinant *AccVg* protein. *Lane 8* protein marker. *Lines 1* and *2* purified recombinant protein. *Lines 3* and *4* suspension of recombinant part of *AccVg*, respectively. *Lines 6* and *7* induced and non-induced overexpression of pET-28a (+)-*AccVg* in BL21, respectively. *Line 5* induced overexpression of pET-28a (+) in BL21.

the restriction endonuclease *Nsi* I at 37 °C overnight, self-ligated to form circles using T4 DNA ligase (TaKaRa, Dalian, China) and used as a template for IPCR. The genomic DNA sequence of *AccVg* contains 7064 bp, which includes seven exons that are separated by six introns with high AT content and flanked by the 5′ splice donor GT and the 3′ splice acceptor AG signals. The introns have typical eukaryotic characteristics with high AT content.

To further analyse the relationship of *AccVg* with other *Vg*s, the genomic structures of *E. formosa Vg*, *A. mellifera Vg*, *T. amboinensis Vg* and *A. aegypti Vg* were compared, as shown
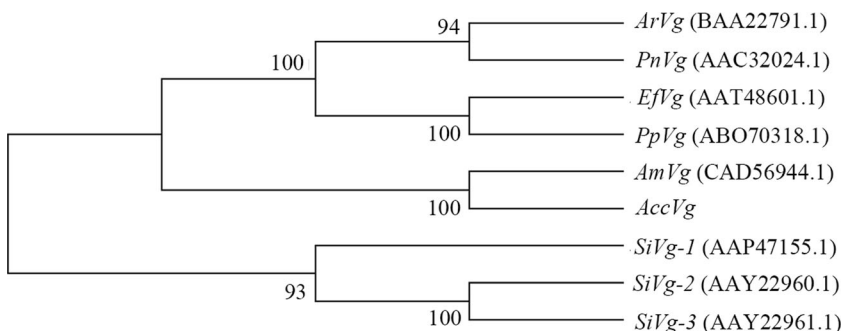
Figure 1 Phylogenetic relationships on the basis of the amino acid sequences between *Vg*s from different insect species.

in Fig. 3. The analysis showed that *EfVg*, *AmVg* and *AccVg* contain seven exons and possess the longest stretch of sequence similarity, whereas *TaVg* and *AaVg* contained only three exons. The numbers, sizes and relative positions of all the introns of *Vg*s in different species are different.

To better understand the molecular mechanism of the transcriptional regulation of *AccVg*, an 833-bp 5′-flanking fragment was isolated. A series of putative *cis*-acting elements was predicted using TFSEARCH software. A series of putative *cis*-acting elements was also predicted after analysing the promoter sequence of *AccVg*. Some heat shock elements, which are the binding sites for heat shock factor (HSF), were also found (Ding et al., 2005). Several important *cis*-acting elements involved in embryo or organ development, including broad-complex (BR-C), NIT 2 and Cdx A were identified (Zhang et al., 2013). Additionally, the Ovo homologue-like transcription factor (TF) binding site involved in epidermis differentiation was predicted. The CAAT-box, which is critical for transcription, was found in the 5′-flanking region of the promoter. Other putative transcription factors are shown in Fig. 4. These putative transcription factor binding sites imply that AccVg may be subject to developmental regulation.

3.3. Developmental and tissue expression profiles of *AccVg*

Vg expression has been discovered not only in adults but also in younger stages and late pupae in *A. mellifera* (Engels et al., 1990; Hartfelder and Engels, 1998; Barchuk et al., 2002; Piulachs et al., 2003; Guidugli et al., 2005). Thus, we were interested in studying whether *Vg* is expressed in the developmental stages (larvae, pupae, adults) and in various tissues of *A. cerana cerana*, using total RNA for qPCR analysis and protein isolates in western blots. In workers, *AccVg* mRNA expression slowly rose until reaching a maximum at the third larval instar, decreased in pre-pupae, became practically undetectable in pupae and increased again in newly emerged workers (Fig. 5a) (F = 99.16; $P < 0.05$). The expression of *AccVg* in different tissues is shown in Fig. 5b. In terms of various tissues, the highest amount of *AccVg* was detected in the abdominal epidermis; because, the epidermis contains more fat body cells that are known to produce abundant vitellogenin, more than do other tissues. The lowest quantity transcript was detected in the brain (F = 101.13; $P < 0.05$).

3.4. Expression patterns of *AccVg* under different environmental stresses

Previous studies have indicated that the expression of *Vg* could be induced by abiotic stress. To better understand whether *AccVg* is involved in various abiotic stress responses, the relative expression of *AccVg* under cold (4 °C), heat (42 °C), heavy metal and insecticide (cyfluthrin, phoxim, paraquat) exposure was determined by qPCR. Following the cold (4 °C) treatment, the *AccVg* transcript level was slightly elevated (Fig. 6a) (F = 6.10; $P < 0.05$), but a significant alteration of *AccVg* expression was observed after the heat (42 °C) treatment (Fig. 6b) (F = 155.88;
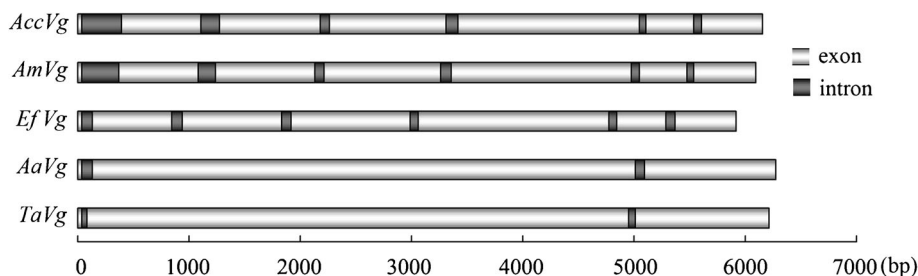
Figure 3 The DNA structures of Vgs. The length extrons and introns of genomic DNA of AccVg, AmVg (NC_007073), EfVg (AY553878), AaVg (AY691327) and TaVg (AY691326) are shown according to the scale below.

AAAGCGGGGTACAGGATGTAAATATCTAGAATTCAAAGTTTAACATTGAAAATATCATTGCCGTGAACT   -739

Ovo homolog-like transcription factor   Ovo homolog-like transcription factor

CATGACAC\[TCGT\]GTTATTTTATATATCATATTTTTTAAT\[CAAT\]CGTAATATTTACGAATGAAAATC\[TCG\]   -670

CAAT box   Ovo homolog-like transcription factor

\[T\]GTTTACTTGC\[ATTTATA\]TTAACGATAAATTTTTTTTTATATCATATTCATCATATTGTATCATTATCAC   -601

Cdx A   NIT 2   CAAT box

ATTACGAATATTTCGATGATTTTG\[TATCAA\]GATACTGAT\[CAAT\]GTATCAGAGAGATTTTTTTTTTTTTA   -532

ATTATTAAAAGTAATCATAAAATTAAAATAAAATCTTAATTGTATCTTAATCATAGTAATTATTAAAAA   -463

TTATAAAATATTTTAAATTAAAATCTAGGGGTGAAAATTCAAAGTGGTAAGCAAAGATTAACTTTAAGA   -394

TTAAAAATGCAACTTTTAAACTATTTGCTAAAAATAAATTTTTCACTTTTTTATGTAAAATTATGTTAT   -325

CAAT box   HSF

GTAATTTTATACTTCTCGATTATATATCGACCTTGTAATCGCATCTTAAAAAT\[CAAT\]TTAA\[AGAAA\]ACA   -256

CAAT box   Cdx A

GATATTATTATCACGCATTCGAAGA\[CAAT\]AATATTGAATC\[ATTTATA\]GTTACATGTTTTTTTCAAAAGA   -187

HSF

TATTATTATTAGCTGTTGTTACGCCAAACACAGATTATAGACATGCT\[AGAAA\]CGAGCGGAGAGATAAAA   -118

BR-C   CAAT box   HSF

TAAAAAAGATAAAATAAAAAAA\[AATAG\]ACATAATAACGTGTTT\[CAAT\]CTAGCAAAAG\[AGAAA\]AATGCTG   -49

+1

ATTTAGCATAAATAAGATATAAATTGCTAAGCCGAATCAAATGCATCGTTACTTTCTCGAAAGTTGTCT   +21

└──► Transcription start

TCAACATGTTGCT   +34

└──► Translation start

Figure 4 The characterization of 5′-flanking region in AccVg. Predicted transcription factor binding sites are *boxed*. The transcription start site and translation start site are marked with an *arrowhead*.

$P < 0.05$). As shown in Fig. 6c, expression of *AccVg* was enhanced by heavy metal ($CdCl_2$) treatment and reached the highest transcript level at 9 h of exposure (F = 42.39; $P < 0.05$). With regard to the insecticide treatments, *AccVg* was induced by cyfluthrin, phoxim, and paraquat and reached the highest transcript level at 4, 2 and 2 h, respectively. The results shown are relative to control levels at time 0; nonetheless, we also checked the levels of AccVg expression in control bees during the duration of the treatment period to make sure that there was no change other than the one induced by the treatments. These results suggested that *AccVg* may be involved in abiotic stress response.

3.5. Western blot analysis

To gain a comprehensive understanding of the molecular mechanisms underlying the effects of abiotic stress, the protein levels of AccVg were examined by Western blot analysis. An anti-AccVg antibody was generated and used to detect AccVg. As shown in Fig. 7, when workers were exposed to heat (42 °C), $CdCl_2$, phoxim or paraquat, the expression of AccVg was significantly increased. In terms of mRNA, the high mRNA levels seen in the figure correspond to the low Vg amounts detected in the western blots, which suggests the existence of mechanisms operating on the translatability and/or stability of Vg mRNA.

4. DISCUSSION

Previous studies demonstrated that Vg plays an important role in energy metabolism and contributes to growth and development in invertebrates. A growing number of studies have been conducted on the roles of Vg in response to adverse stress. However, no studies had systematically imitated the variety of stresses that animals may experience during their life. In this study, the honeybees were foragers obtained from the comb, which were always subjected to the outside environment. Several stresses that honeybees may encounter during their lifespan were imitated, and the resulting changes were explored.

The phylogenetic tree constructed in this study indicated that the Vg amino acid sequence has been conserved throughout evolution and that *AccVg* is in the typical *Vg* cluster, sharing the highest degree of homology with Vg of
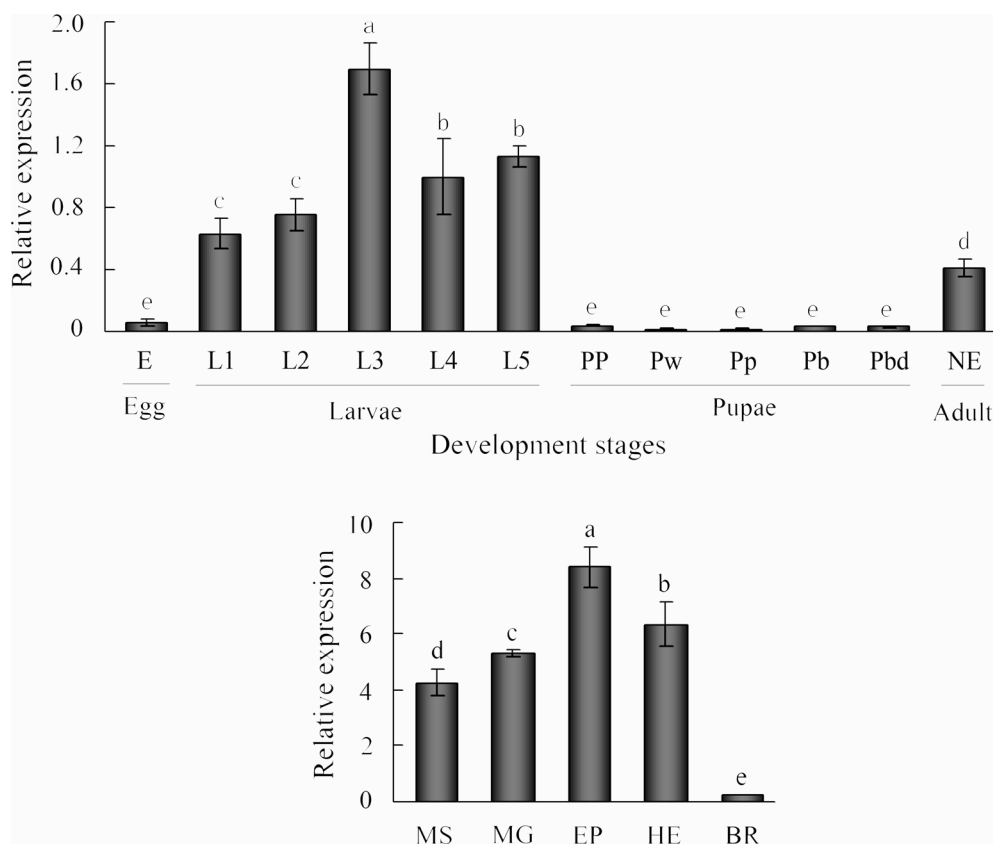
Figure 5 *Vg* expression during different developmental stages and in tissues of *A. cerana cerana* workers. **a** Quantitative RT-PCR analysis of *Vg*. L1, L2, L3, L4, L5 = larval stage; *PP*, Pw, Pp, Pb, Pbd = pupal phase (*PP* pre-pupae, *Pw* white-eyed pupae, unpigmented cuticle, *Pp* pink-eyed pupae, unpigmented cuticle, *Pb* brown-eyed pupae, unpigmented cuticle, *Pbd* brown-eyed pupae, dark pigmented cuticle), and adults (*NE* newly emerged workers) according to Michelette and Soares (1993). **b** *AccVg* expression profiles in the brain (*BR*), epidermis (*EP*), muscle (*MS*), haemolymph (*HP*), midgut (*MG*).

A. mellifera. Taken together, these results indicate that the gene we isolated is a bona fide *Vg* homologue.

The timing and level of gene expression are determined by the promoter region, both of which depended on the role the gene plays. To predict the putative roles of *AccVg*, an 807-bp 5′-flanking region was cloned and putative binding sites for transcription factors were predicted. The region contained a large number of putative binding sites for transcription factors involved in development and the response to environmental stress, such as a BR-C binding site and an Ovo homologue-like TF binding site, which are involved in the early stages of tissue development and growth. This result

prompted us to study the putative roles of *AccVg*. To achieve this goal, we analysed the transcript levels of the gene encoding *AccVg* during all development stages, in different tissues and under various abiotic stresses. Stage-specific expression analysis indicated that the *AccVg* gene is expressed throughout the lifecycle of bees and that the highest accumulation of *AccVg* was detected in the third larval instar (Fig. 5a). *AccVg* expression was still very high in adults, although lower than in the third larval instar. At the egg and pupal stages, *AccVg* expression was the lowest. This finding suggests that during the larval stages, the function of *AccVg* is to control the rate of development, whereas during the pupal stages,
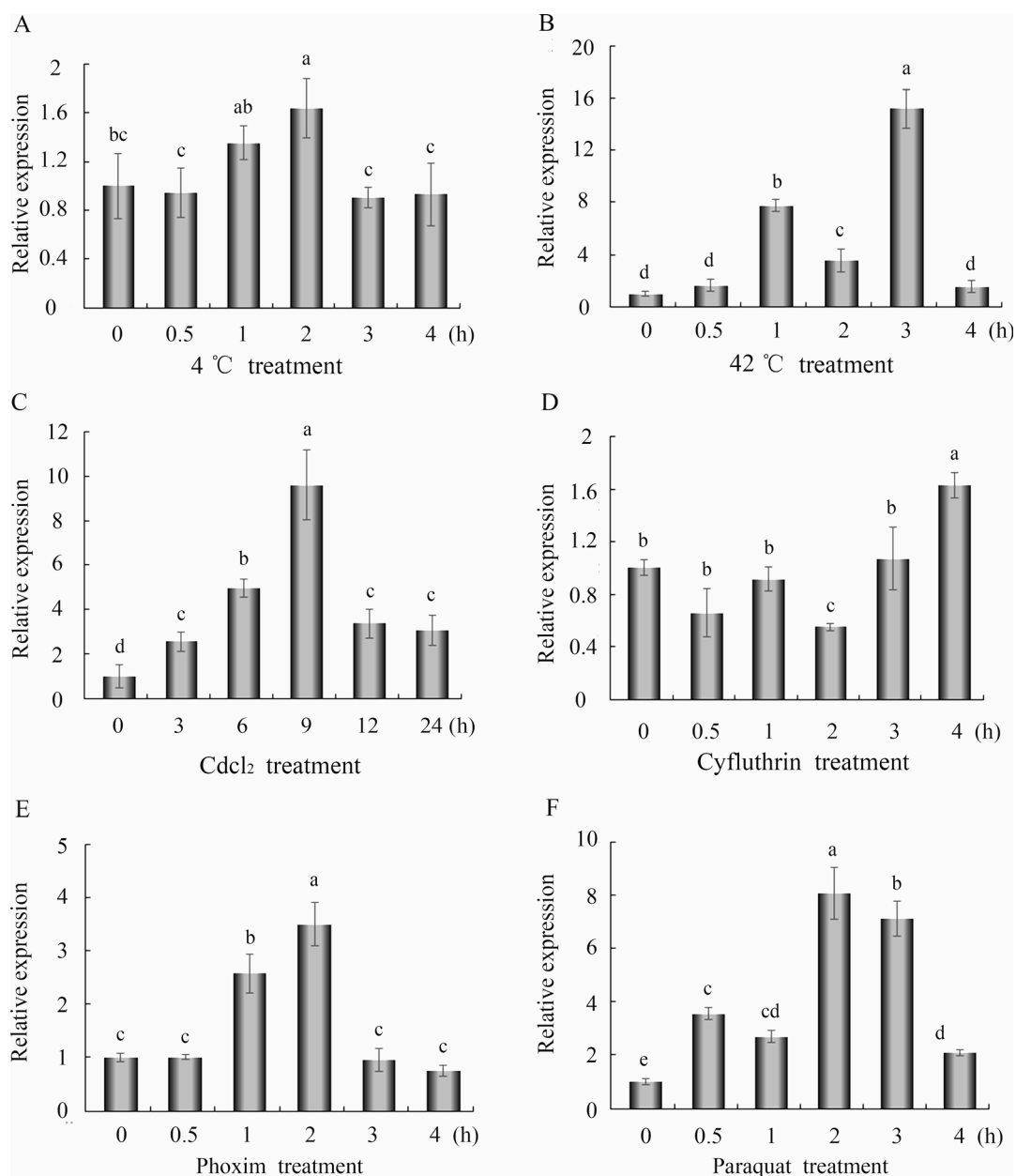
Figure 6 *AccVg* transcript levels under different stress condition. These conditions include cold (4 °C) (**a**), heat (42 °C) (**b**), Cdcl₂ (**c**), cyfluthrin (**d**), phoxim (**e**) and paraquat (**f**). The RT-qPCR results for the treatments were normalized to bees without any stress treatment (0 h) for **a**–**f**. The *different letters above the columns* indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests.

the expression of *AccVg* decreased. The stage-specific expression of *AccVg* indicated that *AccVg* might play roles when the bees were exposed to adverse stress.

Additionally, the mRNA level of *AccVg* was also characterized after various abiotic stress treatments (Fig. 6). When the honeybees were treated with CdCl₂, *AccVg* expression was slightly
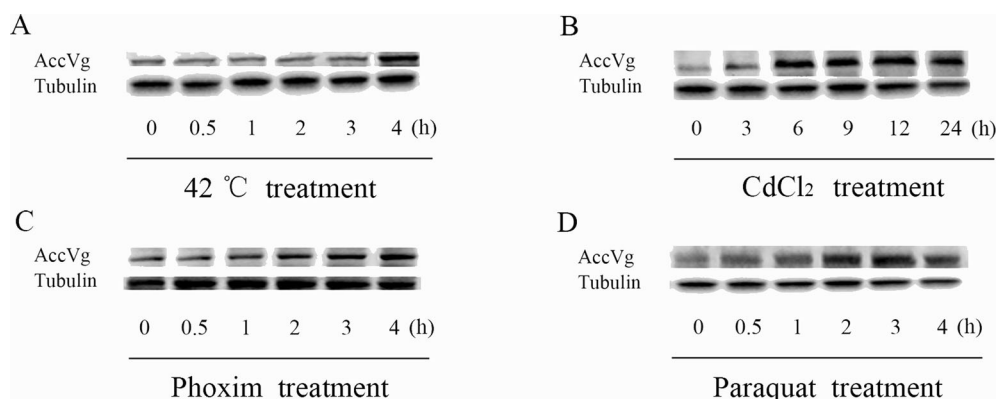
Figure 7 Western blot analysis of *Vg* in different treatments of *A. cerana cerana* workers. SDS-PAGE (7.5%) was performed with 80 ng of total proteins from workers. *Images* are representative of three replicates.

increased and reached the highest level at 9 h; extension of the treatment time resulted in decreased expression. Many heavy metals, including CdCl$_2$ (Liu et al., 2009), can induce the formation of endogenous ROS, which include superoxide anions, hydrogen peroxide and hydroxyl radicals. Endogenously generated ROS should be kept in balance, as high ROS concentration can damage DNA, protein and lipids (Narendra et al., 2007). In this study, *AccVg* expression increased rapidly, peaking at 9 h, and then decreased in response to heavy metal exposure. The increased *AccVg* expression following CdCl$_2$ exposure could enhance the tolerance of *A. cerana cerana* to oxidative stress. Considering our result, we hypothesised that heavy metals (such as CdCl2) could cause stress and induce the expression of *AccVg* through distinct pathways.

A large number of putative HSF binding sites were identified in the *AccVg* 5′-flanking region that are likely to participate in heat-induced transcriptional activation. Western blot analysis of AccVg after the 42 °C treatment confirmed the result, suggesting that AccVg might play a role in response to extreme temperature. Pesticides were the main threat to a honeybee's life. Pesticide treatments, which induce the formation of reactive oxygen species, resulted in an increase in the expression of *AccVg* (Fig. 6) (Day et al., 1999). Bus et al. (1974) noted that paraquat induced oxidative stress by redox cycling with various cellular diaphorases and oxygen to produce superoxide radicals. Our data suggest that Vg activity protects the bee against oxidative damage induced by pesticides. AccVg is preferentially oxidized, which is a property that is indicative of an antioxidant function (Quinlan et al., 2005; Salo et al., 1988; Andrus et al., 1998). With regard to protein expression, the western blot results also indicated the response of AccVg to various oxidative stresses (Fig. 7). All the results demonstrated that AccVg would be induced and activated after abiotic stress, which indicated that AccVg quite possibly plays a significant role in the response of *A. cerana cerana* to adverse environmental stresses. Though some of these stressors also cause increases of gene expression in *A. cerana cerana*, the transcript levels and patterns are quite different from those of the AccVg protein levels.

In conclusion, we identified that the *Vg* gene in *A. cerana cerana* cloned the 5′-flanking region and predicted putative binding sites for transcription factors. Next, the patterns of *Vg* expression during different developmental stages and under different adverse stresses were examined at the transcriptional level. Then, the expression patterns at the protein level were examined to confirm these results. All of these results raise the possibility that AccVg plays a role in the response to adverse stress. To further understand the characteristics of AccVg, additional studies that directly address the functional significance of this gene and its products should be carried out.

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Author's Contributions

Baohua Xu and Xingqi Guo designed the experiments; Hongfang Wang and Ying Wang participated in the design and Lanting Ma, Zhenguo Liu and Ming Zhu performed experiments.

Clonage moléculaire, expression et réponse au stress oxydatif du gène de la vitellogénine (*AccVg*) d'*Apis cerana cerana*

Apidae / stade de développement / stress abiotique / PCR quantitative en temps réel / interférence ARN

Klonierung, Expression und oxidative Stressantwort des *Apis cerana cerana* Vitellogenin-Gens (*AccVg*)

Vitellogenin / *Apis cerana cerana* / RNA-Interferenz / oxidative Stressantwort / RT-PCR

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