Isolation of arginine kinase from *Apis cerana cerana* and its possible involvement in response to adverse stress

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Abstract Arginine kinases (AK) in invertebrates play the same role as creatine kinases in vertebrates. Both proteins are important for energy metabolism, and previous studies on AK focused on this attribute. In this study, the arginine kinase gene was isolated from *Apis cerana cerana* and was named *AccAK*. A 5'-flanking region was also cloned and shown to contain abundant putative binding sites for transcription factors related to development and response to adverse stress. We imitated several abiotic and biotic stresses suffered by *A. cerana cerana* during their life, including heavy metals, pesticides, herbicides, heat, cold, oxidants, antioxidants, ecdysone, and *Ascospaera apis* and then studied the expression patterns of *AccAK* after these treatments. *AccAK* was upregulated under all conditions, and, in some conditions, this response was very pronounced. Western blot and *AccAK* enzyme activity assays confirmed the results. In addition, a disc diffusion assay showed that overexpression of *AccAK* reduced the resistance of *Escherichia coli* cells to multiple adverse stresses. Taken together, our results indicated that *AccAK* may be involved of great significance in response to adverse abiotic and biotic stresses.

Keywords *Apis cerana cerana* · Arginine kinase · Abiotic and biotic stress · Expression patterns · Enzyme activity

Introduction

Arginine kinases (AK) in invertebrates and creatine kinases in vertebrates play major roles in energy metabolism by functioning as phosphagen kinases (Uda et al. 2006). AK catalyses a reversible phosphorylation reaction as follows:

Arginine can be phosphorylated by MgATP to form phosphoarginine and MgADP, and during bursts of cellular activity, the reaction can be reversed to regenerate ATP (Ellington 1989). This system for ATP turnover could provide a large pool of “high-energy phosphates” to refresh ATP levels during periods of high energy demand of the host organisms through temporal and spatial energy buffering (Kammermeier and Seymour 1993; Sauer and Schlattner 2004).

AKs are ubiquitous in invertebrates, and some cDNA sequences have been obtained from a variety of invertebrates. Notably, more than 40 AK cDNAs have been cloned from insects, either in their full-length forms or as fragments. Among these sequences, the AKs from the American cockroach (Brown et al. 2004; Sookrung et al. 2006), *Apis mellifera* (Kucharski and Maleszka 1998), *Bombyx mori* (Liu et al. 2009b), and locusts (Li et al. 2006; Wu et al. 2007) have been well studied. Carlson et al. (1971) isolated the first insect AK from *A. mellifera*, drawing attention to AK purification, enzyme activity analysis, and tissue localization.

Earlier studies on the function of AKs from insects focused on their roles in energy metabolism and development. Schneider et al. (1989) study on *Locusta migratoria* speculated that the phospho-L-arginine/L-arginine kinase system acted as a shuttle mechanism for high-energy phosphate between the mitochondria and myofibrils, as well as a high-energy phosphate buffer system. The highest AK mRNA expression

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in various *A. mellifera* tissues was found in the compound eye, while the levels of mRNA encoding α -GPDH, another metabolically important enzyme, were quite low. Together, these results suggested that AK contributed to the energy releasing mechanism in the visual system, which has high and fluctuating energy demands (Kucharski and Maleszka 1998). The expression of the AK gene in the red imported fire ant *Solenopsis invicta* and its AK protein activity were developmentally and tissue specifically regulated. This regulation was dependent on the different demands for energy-consumption and production in the different castes and was linked to their different labor and physiological activities in the colonies (Wang et al. 2009).

However, there is emerging evidence that AK might participate in responses to adverse environmental conditions and during innate immune responses. In the gills of *Callinectes sapidus*, acclimation to salinity had no impact on AK activities, but exposure to low salinity resulted in a 1.7-fold increase in expression (Kinsey and Lee 2003; Holt and Kinsey 2002). In *Penaeus monodon*, increased AK activity was detected in different tissues after exposure to low salinity (Shekhar et al. 2013). *Trypanosoma cruzi* treated with hydrogen peroxide produced an up to tenfold increase in AK expression, while trypanosomes treated with nifurtimox, an oxidative-stress generating compound, exhibited more than twofold increase in expression (Miranda et al. 2006). After exposure to copper sulfate (CuSO_4), AK was shown to be downregulated in a proteomic study of *Artemia sinica* larvae (Zhou et al. 2010). After chronic cadmium exposure, AK was identified as a downregulated protein using a proteomic approach in the anterior gills of the Chinese mitten crab, *Eriocheir sinensis* (Silvestre et al. 2006). These reports provided clues that AKs may be involved in the response to abiotic stress, even though its expression patterns may differ depending on the particular stressor. In the context of immune responses, using two-dimensional electrophoresis and electrospray ionization mass spectrometry, AK was found to be significantly decreased in the plasma of *Fenneropenaeus chinensis* 45 min after laminarin injection, but its expression level recovered after 3 h (Yao et al. 2005). It was reported that laminarin could act as an immunostimulant that can enhance resistance of many animals against bacterial and viral infections and could be also a source of energy to crustacea (Johansson et al. 2000). In many studies, laminarin was used as an immunostimulant (Awad and Osman 2002; Vargas-Albores and Yepiz-Plascencia 2000). Furthermore, it was reported that shrimp AK could act as an allergen that caused allergic reactions in hypersensitive individuals (Leung and Chu 2001; Yu et al. 2003). These findings suggested that AK might play a role in the process of immunisation.

Apis cerana cerana is the main honeybee species in China, which has enjoyed exceptional advantages over other species, such as the longer period of collecting honey, the more

resistance to disease, and the lesser cost of food. As a flowering plant pollinator, *A. cerana cerana* greatly contributes to the balance of regional ecologies and agricultural development (Yang 2005). The environment in China is getting worse, which is a threat for raising the honeybees. Thus, a study examining the resistance of *A. cerana cerana* to adverse stress is of great significance, as raising this species is becoming more and more difficult. Though some work has been carried out on AKs in other insects, little is known about the role of AK in response to environment stress and pathogen stimuli, especially in *A. cerana cerana*. To increase this knowledge, the AK of *A. cerana cerana* was isolated in our study, and the common stresses suffered by the honeybees were imitated in order to thoroughly assess the role AK may play in response to adverse stress.

Materials and methods

Specimens and treatments

Chinese honeybees (*A. cerana cerana*) were obtained from the artificial beehives in Shandong Agricultural University (Taian, China). The honeybees were raised for collecting honey. For analysis of *AccAK* expression during different developmental stages, the eggs; the first (L1), second (L2), third (L3), fourth (L4), and fifth (L5) day instar larvae; prepupal phase pupa; white-eyed (Pw), pink-eyed (Pp), brown-eyed (Pb), and dark-eyed (Pd) pupae; and 1-day-old worker adults were collected from the hive, while 15-day-old adults and 30-day-old adults were collected from the hive entrance. The larvae and pupae were classified according to the criteria of Michelette and Soares (1993). The newly emerged bees were marked and identified as 1-day-old adults. The 15- and 30-day-old adults were collected after 15 and 30 days, respectively. The 15-day-old adults were divided into several groups for expression analysis under various environmental stresses. The adults were maintained in an incubator at constant temperature (34 °C) and humidity (70 %) under a 24-h dark regimen and fed with a pollen-and-sucrose solution (Yao et al. 2014). Group 1 was fed with CdCl_2 (0.5, 5, or 10 mg/L) that was added into their food for 1 day, 4, 5, and 6 days. Group 2 was fed with CdCl_2 (1 mg/mL) that was added into their food. Groups 3–6 were treated with pesticides (acaricide and pyriproxyfen at a final concentration of 20 mg/mL) and herbicides (phoxim and paraquat at a final concentration of 20 mg/mL). Groups 7–8 were placed at high (42 °C) and low (4 °C) temperatures. Groups 9–10 were subjected to hydrogen peroxide (H_2O_2) and vitamin C. H_2O_2 was diluted to a concentration of 2 mM, and 0.5 μL of this solution was applied to the thoracic notum of the adult bees. Vitamin C was diluted to a final concentration of 20,000 mg/kg in the food. The fourth instar larvae were divided into two groups for biotic

treatments. Group 1 was treated with ecdysone diluted in the larval food at a final concentration of 0.01, 0.1, or 1 µg/mL. Group 2 was treated with *Ascosphaera apis*, a main fungal parasite of *A. cerana cerana*, for 1, 2, or 3 days, which was cultured in the larval food at a concentration of 1×10^6 CFU/mL. Three samples were used for each treatment.

Primers and PCR procedure

The primers and polymerase chain reaction (PCR) procedure used in these experiments are listed in Tables 1 and 2, respectively.

RNA extraction, cDNA synthesis, and DNA preparation

TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA according to the manufacturer's instructions, and RNA was treated with RNase-free DNaseI to remove any potential genomic DNA. First-strand cDNA was obtained using the EasyScript cDNA Synthetic SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. The EasyPure Genomic DNA Extraction Kit (TransGen Biotech, Beijing, China) was used to isolate genomic DNA according to the manufacturer's instructions.

cDNA isolation of AK

The specific primers AKF and AKR were designed and synthesized (Sangon Biotechnological Company, Shanghai, China) based on the *AK* sequence of *A. mellifera*, an organism that

shares high similarity with *A. cerana cerana*, to obtain the internal region of the *AccAK* gene. Then, the specific primers AK5RO and AK5RI were designed for the 5' rapid amplification of cDNA ends (RACE) based on the obtained sequence, while the AK3RO and AK3RI primers were used for 3' RACE. The Abridged Anchor Primer (AAP) and the Abridged Universal Amplification Primer (AUAP) were used in the first and second rounds of 5' RACE, respectively, whereas the B26 and B25 primers were used in the first and second rounds of 3' RACE, respectively. The resulting PCR products were ligated into the pEASY-T3 vectors and transformed into *Escherichia coli* strain DH5α for sequencing.

Amplification of the 5'-flanking region of AccAK

The *AccAK* 5'-flanking region was amplified using specific primers AKQDZF and AKQDZR, which were designed against the *A. mellifera* promoter region sequence. The resulting PCR products were cloned into the pEASY-T3 vectors and transformed into *E. coli* strain DH5α for sequencing. The TFSEARCH database (<http://www.cbrc.jp/research/db/TFSEARCH.html>) was used to predict the putative transcription factor binding sites in the 5'-flanking region of *AccAK*.

Bioinformatic analysis

Bioinformatics tools available on the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) were used to retrieve the homologous AccAK protein sequence from *A. mellifera*.

Table 1 Primer sequences used in this research

Abbreviation	Primer sequence (5'–3')	Description
AKF	GACGTGCCTCGAGGAGTAACT	cDNA sequence primer, forward
AKR	GGATCCTCGATGTATCAAGAACGT	cDNA sequence primer, reverse
AK3RO	GGCAATCTTGATCCAGCTAAT	3'RACE forward primer, outer
AK3RI	TCGTGTAAGATGCGGTCGCT	3'RACE forward primer, inner
B26	GACTCTAGACGACATCGA(T)18	3'RACE universal primer, outer
B25	GACTCTAGACGACATCGA	3'RACE universal primer, inner
AK5RO	GTAAATACCAACGCCAGAAT	5'RACE reverse primer, outer
AK5RI	GAAGAGTAGAATCGAAGGAAGTT	5'RACE reverse primer, inner
AAP	GGCCACGCGTCGACTAGTAC(G)14	Abridged anchor primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged universal amplification primer
AKQDZF	GTTGCATTTTGTTTATTAACT	Promoter-specific primer, forward
AKQDZR	GAGTTACTCTCGAGGCACGT	Promoter-specific primer, reverse
AKRTF	GAAGACTGACGAGCACCCG	Real-time PCR primer, forward
AKRTR	GAAGGTCTTATCATCGTTGTGGT	Real-time PCR primer, reverse
β-s	AGAATTGATCCACCAATCCA	Standard control primer, forward
β-x	GGTACCATGCAGCACATATTATTG	Standard control primer, reverse
AKPETF	GGATCCATGGTTGACCAAGCTGTTT	Primers of constructing vector, forward
AKPETR	AAGCTTAAGTTCCTTTTCGAGTTTAAT	Primers of constructing vector, reverse

Table 2 Procedures used in this research

Primers pair	Amplification conditions
AKF/AKR	5 min at 94 °C, 40 s at 94 °C, 40 s at 50 °C, 1 min at 72 °C for 35 cycles, 5 min at 72 °C
AK3R1 B26	5 min at 94 °C, 40 s at 94 °C, 40 s at 45 °C, 30 s at 72 °C for 35 cycles, 5 min at 72 °C
AK3R2 B25	5 min at 94 °C, 40 s at 94 °C, 40 s at 52 °C, 30 s at 72 °C for 35 cycles, 5 min at 72 °C
AK5R1 AAP	5 min at 94 °C, 40 s at 94 °C, 40 s at 47 °C, 40 s at 72 °C for 35 cycles, 5 min at 72 °C
AK5R2 AUAP	5 min at 94 °C, 40 s at 94 °C, 40 s at 52 °C, 40 s at 72 °C for 35 cycles, 5 min at 72 °C
QDZF/QDZR	5 min at 94 °C, 40 s at 94 °C, 40 s at 53 °C, 40 s at 72 °C for 35 cycles, 5 min at 72 °C
PETF/PETR	5 min at 94 °C, 40 s at 94 °C, 40 s at 53 °C, 1 min at 72 °C for 35 cycles, 5 min at 72 °C

The open reading frame (ORF) was identified and aligned with multiple homologues using DNAMAN software 5.2.2; additionally, the molecular mass and isoelectric point were calculated. Phylogenetic tree analysis of the AK amino acid sequences from different species was carried out using the neighbor-joining method in Molecular Evolutionary Genetic Analysis (MEGA version 4.1).

Fluorescence real-time quantitative PCR

Quantitative PCR (Q-PCR) was performed with the SYBR Premix Ex Taq (TaKaRa) in the CFX96™ real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) to measure *AccAK* transcription at different developmental stages and under adverse abiotic and biotic stresses. The specific primers AKRTF and AKRTR were designed based on the *AccAK* cDNA sequence. The β -s and β -x primers were designed to amplify the housekeeping gene β -actin (GenBank accession no. HM_640276), which was used to normalize RNA levels. We have first validated the primers of β -actin and *AccAK*. The efficiency of β -actin and *AccAK* both approached 100 %. The melting curves had single peaks and the correlation coefficients (R^2) were 0.997 and 0.998, respectively. The amplification parameters were performed as described in Yao et al. (2013a). All the experiments were carried out in triplicate. The data were analyzed with the CFX Manager software program (version 1.1) using the $2^{-\Delta\Delta C_t}$ comparative CT method (Livak and Schmittgen 2001). Differences among the samples were determined by one-way ANOVA using the Statistical Analysis System (SAS) software, version 9.1. The significant differences were labelled with different letters. The same letter indicated that there was no significant difference between the two groups. The different letters indicated that there was significant difference. The overlapped letters indicated that there was difference between the two groups but the difference was not significant.

Expression and purification of recombinant AccAK

To obtain the AccAK protein, the *AccAK* ORF was amplified using a pair of primers containing BamHI and HindIII

restriction sites and was inserted into the expression vector PET-30(a+). Then the expression plasmid PET30a (+)-AccAK was transformed into *E. coli* strain Rosseta(DE3) which was reformed from BL21 for protein expression. The cells were cultured in Luria–Bertani (LB) broth with kanamycin at 37 °C until the cells density reached 0.4–0.6 OD₆₀₀. Then, the AccAK expression was induced with 1.0 mM isopropyl-1-thio- β -galactopyranoside (IPTG) at 28 °C for 8 h. The protein was purified on a HisTrap™ FF column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. The expression of the target protein was analyzed by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Anti-AccAK preparation and Western blot analysis

The anti-AccAK was obtained by injecting the purified AccAK protein subcutaneously into white mice as described by Yan et al. (2013). Total protein were extracted from *A. cerana cerana* according to Li et al. (2009) and quantified with the BCA Protein Assay Kit (Thermo Scientific Pierce, IL, USA). Equal amounts of protein from each sample were separated by 12 % (SDS-PAGE) and subsequently electrotransferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Western blot was performed according to Meng et al. (2010). The anti-AccAK serum was used as the primary antibody at a 1:400 (v/v) dilution. Peroxidase-conjugated goat anti-mouse immunoglobulin G (Dingguo, Beijing, China) was used as the secondary antibody at a 1:2,000 (v/v) dilution. The results were visualized using the SuperSignal® West Pico Trial Kit (Thermo Scientific Pierce, IL, USA).

AccAK activity assay

The AK activity assay was conducted according to the procedures described in “Enzymatic Assay of AK” available on the following website: http://www.sigmaaldrich.com/img/assets/18220/Arginine_Kinase.pdf. Briefly, in the reaction mix, the final concentrations are 178 mM glycine, 0.33 mM 2-mercaptoethanol, 13 mM magnesium sulfate, 133 mM

potassium chloride, 20 mM phospho(enol)pyruvate, 6.7 mM adenosine 5'-triphosphate, 0.13 mM β -nicotinamide adenine dinucleotide, 2 U pyruvate kinase, 3 U lactic dehydrogenase, 17 mM L-arginine. The reaction solution was preincubated for 2 min at 30 °C before reaction. The AK activity was measured at A340 nm immediately after adding 0.04 mL of AK enzyme solution into the 1 mL reaction mix. The progress was recorded as the decrease in the A340 nm for 5 min, and the activity was recorded as the Δ A340 nm/min using the maximum linear rate. One unit can convert 1.0 μ M of L-arginine and ATP to *N*-phospho-L-arginine and ADP per minute at pH 8.6 at 30 °C.

Disc diffusion assay

Disc diffusion assay was performed according to Zhang et al. (2013). Approximately 5×10^8 bacterial cells overexpressing AccAK were plated on LB-kanamycin agar plates and incubated at 37 °C for 1 h. Cells with pET-30a (+) vector were used as the control. Filter discs (6-mm diameter) soaked in different concentrations of HgCl₂ (0, 10, 20, 40, or 80 mg/mL), paraquat (0, 10, 50, 100, or 200 mM), *t*-butylhydroperoxide (0, 100, 250, 500, or 1,000 mM), or cumene hydroperoxide (0, 10, 50, 100, or 200 mM) were placed on the surface of the top agar. The cells were incubated at 37 °C for 24 h before the killing zones around the discs were measured.

Results

Isolation of AccAK cDNA and bioinformatics analysis

The full-length *AccAK* cDNA was obtained from *A. cerana cerana* RNA using reverse-transcription PCR and rapid amplification of cDNA ends PCR. The full-length cDNA is 1,673 bp long and contains a 70-bp 5' untranslated region (UTR), a 539-bp 3' UTR, and a 1,065-bp ORF, which encodes a 355-amino-acid protein with a predicted molecular mass of 39 kDa and a theoretical isoelectric point of 5.34. Multiple sequence alignments of several AK protein sequences showed that the putative AccAK shared 96.90 %, 88.73 %, 87.92 %, and 87.32 % similarity with *A. mellifera*, *S. invicta*, *Periplaneta americana*, and *Helicoverpa armigera*, respectively (Fig. 1a). As shown in Fig. 1a, AccAK contained the typical conserved residues like other AKs, including seven conserved arginine (Arg) binding residues (Ser⁶², Gly⁶³, Val⁶⁴, Tyr⁶⁷, Glu²²⁴, Cys²⁷⁰, and Glu³¹³), five Arg residues (123, 125, 228, 279, and 308) that interact with ADP, and the active center sequence CPTNCGT (270–276), of which the first cysteine residue is the active site necessary for kinase activity.

Additionally, the Asp and Arg residues at positions 61 and 192, respectively, could form an ion pair structure (Fujimoto et al. 2005; Takeuchi et al. 2004; Zhang et al. 2011).

A neighbor-joining phylogenetic tree was built using 21 AK sequences from various insects to demonstrate the putative evolutionary relationships among the AKs (Fig. 1b). The result showed that AKs were highly conserved throughout evolution. The AK in *A. cerana cerana* shared the highest similarity with the AK found in *A. mellifera*. Additionally, this sequence was more closely related to the AKs in honeybees than to the homologues present in other species.

Analyses of partial potential *cis*-acting elements in the 5'-flanking region of AccAK

To predict the putative mechanism of AccAK regulation, a 1,207-bp 5'-flanking region of *AccAK* was isolated, and the putative transcription factor binding sites were predicted. Representative portions of these sequences are shown in Fig. 2. In this region, nearly 100 heat shock factor (HSF) binding sites were found. This transcription factor plays a crucial role in heat-induced transcriptional activation (Fernandes et al. 1994). In addition, the region contains a large number of binding sites for other transcription factors, including those for caudal-related homeobox (Cdx) protein, which participates in tissue-selective development (Ericsson et al. 2006; Hsu et al. 1992), fork head domain protein crocodile (Croc), which is a brain development-specific transcriptional activator (Jeffrey et al. 2000), and Broad-Complex, which is a key ecdysone-responsive regulator of metamorphosis (von Kalm et al. 1994). Additionally, some binding sites for transcription factors related to early stage tissue development and growth or involved in the response to adverse stress, such as Hunchback (Hb), Dfd, NIT2, cAMP-responsive element binding protein (CREB), and CCAAT/enhancer binding protein (C/EBP), were also found.

Developmental expression levels of AccAK

Q-PCR was performed to investigate the expression levels of *AccAK* at different developmental stages (Fig. 3). The results showed that the lowest expression level was during the egg stage. In the pupae, the expression levels in dark-pigmented phase pupae were somewhat higher than in the pupae of other phases. The highest level of expression appeared at the fourth larval instar, while the levels in the other larval instar stages and the prepupal phase pupae were not as high, although were still higher than the expression observed in pupae. The second highest expression level was observed in the 15- and the 30-day-old adult workers.

Fig. 1 Characterization and evolutionary relationship of arginine kinases (AKs) from various species. **a** Multiple amino acid sequence alignment of AccAK (GenBank accession no. KF772855) from *A. cerana cerana*, AmAK (NM_001011603.1) from *A. mellifera*, HaAK from *H. armigera* (EF600057.1), PaAK from *P. americana* (GU301882.1), and SiAK from *S. invicta* (EU817514.1). Conserved regions are shown in black. Conserved arginine binding residues are marked with black triangles. Arginine (Arg) residues that interact with ADP are marked with double lines. The active center sequence CPTNCGT is boxed. The Asp and Arg residues at positions 61 and 192, respectively, which can form an ion pair structure are marked with arrows. **b** Phylogenetic analysis of AK amino acid sequences from various species. The sequences were obtained

from the NCBI database. The homologous AKs are from the following species: *A. mellifera* (NM_001011603.1), *Bombus hypocrite* (JF751027.1), *Pteromalus puparum* (FJ882065.1), *Acromyrmex echinator* (GL888624.1), *S. invicta* (EU817514.1), *B. mori* (EU327675.1), *Danaus plexippus* (AGBW01011584.1), *Papilio xuthus* (AK401168.1), *H. armigera* (EF600057.1), *Spodoptera exigua* (GQ379235.1), *L. migratoria manilensis* (DQ513322.1), *P. americana* (GU301882.1), *Blattella germanica* (FJ855501.1), *Lucilia cuprina* (JQ088101.1), *Riptortus pedestris* (AK417004.1), *Anasa tristis* (JQ266746.1), *F. chinensis* (AY661542.1), *Litopenaeus vannamei* (EU346737.1), *Fenneropenaeus merguensis* (FJ895112.1), and *Metapenaeus ensis* (EU497674.1)

Fig. 2 The nucleotide sequence and the partial putative transcription factor binding sites in the 5'-flanking region. The transcription start site is marked with an *arrow*, and the putative transcription factor binding sites are *boxed*

Expression profiles of *AccAK* under adverse abiotic stress

The mRNA transcript levels under various environmental stresses were quantified using Q-PCR and were normalized to an untreated control group. Surprisingly, *AccAK* was induced by all of the tested treatments. *AccAK* robustly responded to CdCl_2 treatment, which caused an 86-fold increase in expression after treatment with 5 mg/L for 4 days

compared with the control group (Fig. 4b). Similarly, CdCl_2 treatment with different concentrations and for different times induced different patterns of *AccAK* expression (Fig. 4a, c, and d). Although *AccAK* expression changed in response to all tested pesticides and herbicides, it seemed that the induction varied by treatment type, as paraquat and pyriproxyfen caused a much more intense increase in expression than acaricide and phoxim (Fig. 5a, b, c, and d). Extreme temperatures that

Fig. 3 The relative expression of *AccAK* during different developmental stages. The data are the mean $\pm$ SE of three replicates. The letters above the bar indicate significant differences ($P<0.0001$) as determined by Duncan's multiple range tests using SAS software version 9.1

honeybees may experience (42 °C and 4 °C) were also examined. The results showed that *AccAK* expression was enhanced more drastically after exposure to the high temperature (Fig. 6a and b). We also analyzed the expression level after treatments with H₂O₂ and vitamin C, as H₂O₂ is a type of oxidant and vitamin C is an antioxidant. The two treatments both caused an increase in *AccAK* expression, but H₂O₂ caused a drastic increase, while vitamin C treatment resulted in only a slight increase (Fig. 6c and d).

Expression profiles of *AccAK* under biotic stress

To further understand the role *AccAK* plays in honeybees, we treated fourth instar larvae with different concentrations of ecdysone. As described above, Q-PCR was performed to examine the accumulation of *AccAK* transcripts. As shown

in Fig. 7a, transcription was similarly induced under all ecdysone concentrations. Additionally, we diluted *A. apis* in the larval food. The expression of *AccAK* initially decreased after treatment with *A. apis* for 1 day, but increased immediately on the second day. Then, on the third day, its expression decreased again but remained higher than that of the control group (Fig. 7b).

Western blot analyses

To further detect *AccAK* in honeybees under adverse stress, Western blot was performed. Anti-*AccAK* was used to detect *AccAK*. Following 42 °C treatments for 0.5 and 1.5 h, the expressions of *AccAK* were induced; especially after 1.5 h, the protein level was several times induced (Fig. 8a). After exposure to phoxim for 0.5 and 1.0 h, the *AccAK* expressions

Fig. 4 Expression profiles of *AccAK* under heavy metal stress. The treatments are as follows: CdCl₂ (24 h) (a), CdCl₂ (4 days) (b), CdCl₂ (5 days) (c), and CdCl₂ (6 days) (d). The data are the mean $\pm$ SE of three replicates. The letters above the bar indicate significant differences ($P<0.0001$) as determined by Duncan's multiple range tests using SAS software version 9.1

Fig. 5 Expression profiles of *AccAK* under pesticide and herbicide stress. These stresses are as follows: acaricide (**a**), pyriproxyfen (**b**), phoxim (**c**), and paraquat (**d**). The data are the mean $\pm$ SE of three replicates. The letters above the bar indicate significant differences ($P<0.0001$) as determined by Duncan's multiple range tests using SAS software version 9.1

were also induced (Fig. 8b), although not that high as they were after 42 °C treatment. The results at protein level were consistent with the expressions of *AccAK* at mRNA level.

AccAK enzyme activity analyses under adverse stress

AccAK enzyme activities were examined under various stresses. After 42 °C treatments for 0.5, 1.0, and 2.5 h, the *AccAK* enzyme activity increased several times compared with the untreated group and reached the peak at 1.0 h (Fig. 9a). After phoxim treatment for 0.5, 1.0, and 1.5 h, the enzyme activity increased constantly though only slightly (Fig. 9b). Following H₂O₂ treatment for 10, 20, and 30 min

and CdCl₂ (1 mg/mL) treatment for 2, 4, and 6 h respectively, the enzyme activity drastically increased (Fig. 9c and d). The enzyme activities all increased after the four adverse stresses, though not strictly consistent with the results at mRNA level which might result from the accumulation and degradation of protein.

Disc fusion assay under various stresses

The target protein contained the *AccAK* protein and the His tag. SDS-PAGE showed that the target protein could be induced by IPTG, and only the amount of the target protein increased constantly as time increased (Fig. 10). The protein

Fig. 6 Expression profiles of *AccAK* under extreme temperature, oxidant, and antioxidant stresses. These stresses are 42 °C (**a**), 4 °C (**b**), H₂O₂ (**c**), and V_C (**d**). The data are the mean $\pm$ SE of three replicates. The letters above the bar indicate significant differences ($P<0.0001$) as determined by Duncan's multiple range tests using SAS software version 9.1

Fig. 7 Expression profiles of *AccAK* under various biotic stresses. The stresses included ecdysone (a) and *A. apis* treatments (b). The data are the mean $\pm$ SE of three replicates. The letters above the bar indicate significant differences ($P < 0.0001$) as determined by Duncan's multiple range tests using SAS software version 9.1

was detected soluble (data not shown). It took longer time for the bacteria containing *AccAK* to produce the same number of cells as the bacteria without *AccAK*. After overnight exposure to various stressors, including HgCl_2 , paraquat, *t*-butylhydroperoxide, and cumene hydroperoxide, the bacteria overexpressing *AccAK* were more susceptible to the adverse stress, as the killing zones of the bacteria were bigger on the plates compared with the control bacteria. The sensitivity of the bacteria overexpressing *AccAK* varied from the different stressors, as the killing zones around HgCl_2 , paraquat, and *t*-butylhydroperoxide-soaked filters were much bigger than the control group compared with those around cumene hydroperoxide-soaked filters (Fig. 11). To explore the mechanism of the inhibiting effect of the *AccAK* to the growth of bacteria, the putative antimicrobial motifs were predicted on the website: <http://aps.unmc.edu/AP/>, and the result showed that there is at least one antimicrobial motif existing in the *AccAK* (Fig. 12). The motif has neutral charge, and may have antimicrobial activity.

Discussion

Previous studies demonstrated that AK plays a major role in energy metabolism and contributes to growth and development in invertebrates. An increasing number of studies have been conducted on the roles of AK in response to adverse stress. However, no studies had systematically imitated the various types of stresses that animals may experience during their life.

In this study, we imitated several stresses that *A. cerana cerana*, the main species used in Chinese farming, may encounter during their lifespan, and we explored the resulting changes in AK expression at the mRNA and protein level as well as the AK enzyme activity. Also, we explored the resistance of the bacteria overexpressing *AccAK* to determine the role that AK may play. The honeybees used in this study were obtained from artificial beehives, and they were raised for collecting honey. So, they were always subjected to wild environment.

To achieve this goal, we first isolated a predicted AK gene from *A. cerana cerana* and named it *AccAK*. Similar to other typical AKs found in the NCBI database, *AccAK* contained conserved AK residues. Through sequence alignment, it was shown that *AccAK* showed a high degree of similarity with several typical AK amino acid sequences. Furthermore, its phylogenetic tree indicated that AK sequences have been conserved throughout evolution, and *AccAK* is in the typical AK cluster, sharing the highest degree of homology with the AK of *A. mellifera*. Taken together, these results indicate that the gene we isolated is a typical AK.

A promoter determines the timing and level of gene expression, both of which depends on the role the gene plays. To predict the putative roles of *AccAK*, a 1,207-bp 5'-flanking region was cloned. The region contained a large number of putative binding sites for transcription factors involved in development and response to environmental stress. This result drove us to study the putative roles that *AccAK* potentially played. To achieve this goal, we analyzed *AccAK* expression at different growth stages and under various abiotic and biotic stresses. Stage-specific expression analysis indicated that the

Fig. 8 Western blot analysis of *AccAK* changes after 42 °C (a) and phoxim (b) treatments. The total protein was immunoblotted with anti-*AccAK*. The signal of the binding reactions was visualized with HRP substrates

Fig. 9 Relative enzyme activities of AccAK under various stresses. These stresses are 42 °C (a), phoxim (b), H₂O₂ (c), and CdCl₂ (d). The data are the mean±SE of three replicates. The letters above the bar indicate significant differences ($P<0.001$) as determined by Duncan's multiple range tests using SAS software version 9.1

highest accumulation of *AccAK* was detected in the fourth larval instars. The fourth larval instars are at the stage when protein synthesis is the highest (Li et al. 2007), and these larvae are the most vulnerable to *A. apis* infection and the easiest to die of chalkbrood disease (Bailey 1967). Although lower than in the fourth larval instars, *AccAK* expression is still very high in adults, as the adult workers' main duty is to work outside where they consume more energy and are exposed to worse environmental stresses. The prepupal phase also showed a high level of *AccAK* expression. In *Drosophila*, the prepupal phase is the stage at which AK mostly accumulated and when the most ecdysone was secreted (Jiang et al. 2000). Ecdysone plays a crucial role in coordinating moulting, metamorphosis, and reproduction in insects (Liu et al. 2007). It is reasonable to infer that AKs play an ecdysone-related role in the insects' growth and developmental progress (James and

Collier 1992). Similarly, at the egg and pupae stages, when the bees stay in the hives and have little exposure to adverse environments, *AK* expression was the lowest. The stage-specific expressions of *AccAK* provided clues that *AccAK* might play roles when the honeybees were exposed to adverse stress.

In addition to stage-specific expression analysis, the expression patterns of *AccAK* were also characterized after various treatments imitating adverse environments. When the honeybees were treated with a low concentration of CdCl₂ for a short time, *AccAK* expression was either lower or only slightly higher than that of the control group. Increasing the concentration or extending the treatment time resulted in increased expression. A high concentration of CdCl₂ for a short time could induce an obvious increase of *AccAK* enzyme activity. A lot of heavy metals including CdCl₂ (Liu et al. 2009a) can induce the formation of endogenous reactive oxygen species (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). H₂O₂ and vitamin C, a typical oxidant and antioxidant, respectively, both cause oxidative damage. H₂O₂-induced ROS elevation and resulted in cell death caused by oxidative stress (Goldshmit et al. 2001; Casini et al. 1986). On the other hand, it was reported that vitamin C, a well known antioxidant, could induce decomposition of lipid hydroperoxides to endogenous genotoxins and resulted in DNA oxidative damage (Lee et al. 2001). *AccAK* expression was upregulated after these two treatments, and the enzyme activity also obviously increased after H₂O₂ treatment. It is noticeable that the *AccAK* expressions and enzyme activities all

Fig. 10 The expression of the recombinant *AccAK* protein. Lane 1, protein molecular weight marker; lane 2, expression of *AccAK* without IPTG induction; lanes 3–9, expression of *AccAK* after IPTG induction for 2, 3, 4, 5, 6, 7, and 8 h

Fig. 11 The resistance of bacteria cells overexpressing AccAK to HgCl₂, paraquat, *t*-butylhydroperoxide, and cumene hydroperoxide. The halo diameters of the killing zones were compared in the histograms. **a** The HgCl₂ concentrations of discs 1–5 are 0, 10, 20, 40, and 80 mg/mL respectively. **b** The paraquat concentrations of discs 1–5 are 0, 10, 50,

100, and 200 mM, respectively. **c** The *t*-butylhydroperoxide concentrations are 0, 100, 250, 500, and 1,000 mM, respectively. **d** The cumene hydroperoxide concentrations are 0, 10, 50, 100, and 200 mM, respectively. The data are the mean±SE of three replicates

Fig. 12 The putative antimicrobial peptide motif. The amino acids sequence is boxed

MVDQAVLDKLETGFTKLANSDSKSLKKYLTKEVFDQLKTKKTSFSDSTL
 LDCIQSGVENLDSGVGIYAPDAEAYTLFADLFDPPIEDYHGGFKKTDEHP
 PKDFGDDVDSLGNLDPANE **FIVSTRVRCGRSLEGYPFNPCLTEAQYKEME**
EKVSSTLSGLEDELKGTFFYPLTGMSKEIQQLIDDHFLFKEGDRFLQAA
 ACRFWPTGRGIYHNDDKTFLVWCNEEDHLRIISMQMGDDLQGVYRRLV
 HAVNEIERRLPFSSHNDRLGFLTFCPTNLGTTVRASVHIKLPKLAANRAKL
 EEIAGKFNLQVRGTRGEHTEAEGGIYDISNKRRLGLTEYQAVKEMHDGI
 AELIKLEKEL

increased drastically after CdCl_2 and H_2O_2 treatments compared with other treatments. These findings indicated that AccAK might play a prior role in the response to oxidative damage. Bad weather is a common adverse situation experienced by *A. cerana cerana*. There is a large number of putative HSF binding sites in the *AccAK* 5'-flanking region that participate in heat-induced transcriptional activation. Previous studies showed that heat and cold could both upregulate *AK* expression in *Helicoverpa assulta* (Zhang et al. 2011). Similarly, our results demonstrated that *AccAK* expression increased under both high and low temperatures. Western blot and enzyme activity assay of AccAK after 42 °C treatment both confirmed the result, suggesting that AccAK might play a role in response to extreme temperature. Pesticides and herbicides are the main threat to a honeybee's life. The two treatments are thought to influence the insect development process. Although different patterns of AK expression were observed, both pesticide and herbicide treatments resulted in an increase in the expression of *AccAK*. In addition, phoxim treatment resulted in increase in both protein expression and enzyme activity of AccAK, which may play roles in *A. cerana cerana* developmental processes. All the results indicated that the AccAK would be induced and activated after abiotic stress which indicated that AccAK quite possibly plays a significant role in the response of *A. cerana cerana* to adverse environmental situations. Quite a lot of genes that involved in response to adverse stress have been well studied in *A. cerana cerana*. The suite of stressors would result in changes of these genes expression in different patterns. For example, high temperature (42 °C) treatment inhibited the expression of *AccTrx2* (Yao et al. 2013a). HgCl_2 treatment inhibited the expression of *Acctpx-3* (Yao et al. 2013b). Pyriproxyfen treatment inhibited the expression of *AccSOD2* (Jia et al. 2014). Though some of these stressors also cause increases of gene expressions in *A. cerana cerana*, the levels and patterns are quite different from that of *AccAK*. These findings showed that the suit of stressors would not result in induction of all other genes in *A. cerana cerana*, and the *AccAK* mRNA is specifically upregulated during stress.

In addition to the abiotic treatments, we also imitated biotic situations. In *B. mori* (L.), the expression of *AK* is consistent with the accumulation of ecdysone (Wang and Xu 2006). As mentioned above, *AccAK* was highly expressed during the prepupal phase when ecdysone levels were also the highest. BRCZ is an ecdysone-regulated transcription factor and can regulate gene expression at a low ecdysone concentration (Karim et al. 1993). In the *AccAK* 5'-flanking region, there are plenty of potential binding sites for the BRCZ, which suggests that AccAK might be regulated by ecdysone. The Q-PCR results confirmed this prediction. It has been shown that AKs participate in immune responses. After injection of *Vibrio alginolyticus*, *AK* expression in *Portunus trituberculatus* peaked after 3 h with a 5.01-fold increase

(Song et al. 2012). Using the mRNA differential display technique, the *AK* level in *Penaeus stylirostris* was found to be upregulated following *white spot virus* injection (Astrofsky et al. 2002). Similarly, treatment with *A. apis*, which is a main fungal parasite of *A. cerana cerana*, resulted in an increase in *AccAK* expression on the second and third days. This result confirmed the role AK played in immunization.

To perform the disc diffusion assay, we cultured the bacteria with AccAK and the control bacteria to achieve the same cell density. It is worth noting that it took longer time for the bacteria with AccAK to reach the same OD_{600} which provides a clue that AccAK may be an inhibitor for the growth of bacteria. After exposure to four adverse treatments, overexpression of AccAK reduced the resistance of the bacteria cells to the adverse stress. Then the amino acid sequence was input on website for prediction of antimicrobial activity, and there is a putative anti-microbial peptide in the AccAK protein. The result suggested that AccAK possibly acts as an inhibitor of the bacterial growth and protects honeybees from bacterial damage.

In conclusion, we identified the *AK* gene in *A. cerana cerana*. Then, to predict its role, we cloned the 5'-flanking region and predicted putative binding sites for transcription factors. Next, the *AK* expression patterns during different developmental stages and under different adverse stresses were examined at the transcriptional level. Then the expression patterns at protein level and the enzyme activities were examined to confirm the results. At last, a disc fusion assay indicated the inhibiting effect of AccAK to the growth of bacteria. Taken together, our research provides evidence that AccAK is potentially involved in response to adverse stress. However, these findings just raised the possibility that AccAK plays a role in response to adverse stress. To further understand the characteristics of AccAK, more studies that directly address the functional significance of the gene and its products should be carried out.

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