Identification of an *Apis cerana cerana* MAP kinase phosphatase 3 gene (*AccMKP3*) in response to environmental stress

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Abstract

MAP kinase phosphatase 3 (MKP3), a member of the dual-specificity protein phosphatase (DUSP) superfamily, has been widely studied for its role in development, cancer, and environmental stress in many organisms. However, the functions of MKP3 in various insects have not been well studied, including honeybees. In this study, we isolated an MKP3 gene from *Apis cerana cerana* and explored the role of this gene in the resistance to oxidation. We found that *AccMKP3* is highly conserved in different species and shares the closest evolutionary relationship with *AmMKP3*. We determined the expression patterns of *AccMKP3* under various stresses. qRT-PCR results showed that *AccMKP3* was highly expressed during the pupal stages and in adult muscles. We further found that *AccMKP3* was induced in all the stress treatments. Moreover, we discovered that the enzymatic activities of peroxidase, superoxide dismutase, and catalase increased and that the expression levels of several antioxidant genes were affected after *AccMKP3* was knocked down. Collectively, these results suggest that *AccMKP3* may be associated with antioxidant processes involved in response to various environmental stresses.

Keywords MAP kinase phosphatase 3 · Oxidative stress · RNA interference · Environmental stresses · *Apis cerana cerana*

Introduction

Apis cerana cerana (*A. cerana cerana*), the unique local bee species found in China, plays a crucial part in the maintenance of ecological balance and in agricultural development as the main pollinator associated with traditional agriculture. Compared with *Apis mellifera*, *A. cerana cerana* has unparalleled advantages, such as a sensitive sense of smell, ability to use sparse pollen sources, strong collection ability, low food consumption, and strong resistance to mites and disease

(Ratnieks 2006). However, with the deterioration of the natural ecological environment and the threat of pests and diseases, the Chinese honeybee population is facing an increasingly severe situation. There are many environmental stresses that affect the survival and development of honeybees, such as extreme temperatures, ultraviolet (UV) light, hydrogen peroxide, heavy metals, and pesticides. These abiotic stresses can cause the accumulation of intracellular reactive oxygen species (ROS) and cause injury to organisms (Ali et al. 2017; Matsumura et al. 2017).

ROS production occurs through metabolic processes that generally maintain a dynamic balance under normal conditions in most aerobic organisms (Emanuele et al. 2018). However, this equilibrium can be disrupted by various environmental stresses, leading to ROS accumulation. Excessive ROS can promote oxidative stress in cells via lipid peroxidation, DNA damage, protein degradation, and enzyme inactivation (Graves et al. 2009). Therefore, removing ROS is necessary for organisms. To maintain cell homeostasis and avoid oxidative damage, insects have evolved intricate antioxidant defense systems, including enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD) and non-enzymatic antioxidants such as ascorbate (AsA) and glutathione (GSH)

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(Geng et al. 2016). Dual-specificity protein phosphatases (DUSPs) have been recognized as key regulators of dephosphorylation, and these enzymes are involved in regulating cellular responses to diverse stimuli (Karkali and Panayotou 2012; Patterson et al. 2009).

The DUSP superfamily is a conserved protein family that appears to be selective for dephosphorylating phosphotyrosine and phosphoserine/phosphothreonine residues in MAP kinases (Patterson et al. 2009). DUSPs participate in various signaling pathways and physiological processes, such as cancer development, organogenesis, and immune response (Jeong et al. 2014). For example, the low-molecular-mass DUSP gene LDP-4 is expressed exclusively in the rat brain and plays an important role in response to stress signals (Takagaki et al. 2007). There is also evidence that DUSPs participate in the heat shock response. Sharda et al. (2009) found that DUSP12 could protect cells from heat-shock-associated damage by interacting with Hsp70. Another phosphatase, encoded by a gene homologous to DUSP8 in mouse, has also been reported to respond to heat shock (Palacios et al. 2001). In addition, it has been reported that, in a neuroblastoma cell line, DUSP1 was induced under conditions of hypoxia/reoxygenation (Koga et al. 2012). In *Drosophila*, notably, M3/6 (DUSP8) can change its substrate preference during arsenite-induced oxidative stress (Oehrl et al. 2013). Another DUSP-coding gene in *Drosophila*, Puckered, was activated under oxidative stress and in turn dephosphorylated JNK (Karkali and Panayotou 2012). These reports indicate that DUSPs are involved in a wide range of responses to stress, especially oxidative stress. However, it is unclear whether DUSPs are related to resistance to environmental stresses in *A. cerana cerana*.

DUSPs can be divided into six subgroups according to their sequence similarity. One of the best characterized subgroups is the MKP family, which has been widely studied. MAP kinase phosphatase 3 (MKP3, also known as DUSP6 or PYST1) is a novel tyrosine/threonine dual-specificity phosphatase belonging to the MKP subgroup (Muda et al. 1996; Zhang et al. 2011). Many studies have shown that MKP3 plays an important role in development and disease; for instance, MKP3 acts as a key inhibitor in tumorigenesis and invasiveness (Wong et al. 2012). Furukawa et al. have revealed that deletion of DUSP6 and mutations of KRAS2 can cause uncontrolled cell growth (Furukawa and Horii. 2004). Additionally, functional MKP3 is required during *Drosophila* wing vein formation (Molnar and de Celis 2013). Muda et al. (1996) have shown that MKP3 can specifically dephosphorylate activated ERK1/2. There is evidence that ERK1/2 is activated by some abiotic stresses, such as high temperature and oxidative stress. Therefore, we suspect that MKP3 may be also associated with the response to abiotic stresses. Although MKP3 has been extensively investigated with regard to many aspects, its role in *A. cerana cerana* has yet to be studied.

A. cerana cerana is the major native bee species in China, though the survival and persistence of its population is being affected by many environmental stresses. Accordingly, it is necessary to increase our understanding of gene expression and molecular mechanisms in the stress response of *A. cerana cerana*. In this study, we isolated the *AccMKP3* gene from *A. cerana cerana* and explored its role in growth, development, and response to stress. Using qRT-PCR, we examined the transcript levels of *AccMKP3* at different developmental stages and in different tissues. Additionally, we analyzed the expression patterns of *AccMKP3* under multiple environmental stresses. Moreover, we generated *AccMKP3*-silenced bees via RNAi technology. Then, we explored the expression levels of other antioxidant genes in these *AccMKP3*-silenced bees and evaluated the activities of some typical antioxidant enzymes. Overall, these results have indicated that *AccMKP3* may participate in the antioxidant processes of *A. cerana cerana*.

Materials and methods

Insects and treatments

Insects (*A. cerana cerana*) maintained at the experimental apiary of Shandong Agricultural University (Tai'an, Shandong, China) were used in this study. The developmental stages of honeybees, including eggs, larvae, pupae, and adults, were identified based on the shapes, ages, and eye colors of the bees (Zhu et al. 2016). To analyze the level of *AccMKP3* transcription during different development stages, fourth-day (L4), fifth-day (L5), and sixth-day (L6) instar larvae, prepupae (P0), white-eyed (Pw), pink-eyed (Pp), brown-eyed (Pb), and dark-eyed (Pd) pupae, and 1-day postemergence adults (A1) were obtained from directly from the beehive; 15-day postemergence adults (A15) and 30-day postemergence adults (A30) were collected at the entrance of the beehive. Newly emerged worker bees were marked with paint and regarded as 1-day worker bees. For specific expression analysis, the adult bees were randomly divided into 9 groups, 40 individuals each. Groups 1–3 were exposed to extreme heat (42 °C), extreme cold (4 °C), or UV light (30 mJ/cm²). Groups 4–6 were injected with CdCl₂ (3 mM, diluted with distilled water), HgCl₂ (3 mg/mL, diluted with distilled water), or H₂O₂ (1 mM, diluted with distilled water). In addition, one of three pesticides, namely, thiamethoxam, hexythiazox, and abamectin, was applied to groups 7–9 by fumigation, and the final concentrations of the pesticides were 125 mg/L, 125 mg/L, and 25 mg/L, respectively. All the honeybees used in this work were kept in an artificial incubator (34 °C and 70% relative humidity) and fed with a basic solution of pollen and sucrose (Alaux et al. 2010). Different bee tissues, such as the head (HA), muscle (MS), wing (WI), epidermis (EP),

midgut (MG), and sting shaft (SS), were anatomically separated from adult bees; the whole process was carried out on ice (Li et al. 2016). All the bee samples processed above were quick-frozen with liquid nitrogen and stored at -80°C .

RNA extraction and cDNA synthesis

RNA samples were extracted with RNAiso Plus (TaKaRa, Japan), and first-stand cDNA was synthesized using the HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) according to the Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) according to the manufacturer's protocol. The cDNA was used as a PCR template for the gene cloning and qRT-PCR processes.

Primer sequences

The primer sequences used in this study are listed in Table 1.

Isolation of the *AccMKP3* ORF sequence

The open reading frame (ORF) of *AccMKP3* was cloned by PCR amplification with the specific primers MF1 and MR1 (as shown in Table 1). Then, the PCR product was ligated into the pEASY-T1 simple vector (TransGen Biotech, Beijing, China) after purification and transformed into *Escherichia coli* cells (Trans1-T1 phage-resistant chemically competent cells) (TransGen Biotech, Beijing, China) for sequencing. The sequencing analysis and primer synthesis were conducted by Biosune Biotechnological Company (Shanghai, China).

Bioinformatics analysis of *AccMKP3*

The ORF sequence and conserved domains of *AccMKP3* were identified by the bioinformatics tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) available on the National Center for Biotechnology Information (NCBI) website. Multiple sequence alignment analysis was conducted by DNAMAN software (version 5.2.2), and the molecular mass and isoelectric

Table 1 PCR primers

Abbreviation	Primer sequence (5'–3')	Description
MF	ATGCCAGGTGGAAGTGTATAG	cDNA sequencing primer, forward
MR	GCTTCTCTTTGCTACTTTCTG	cDNA sequencing primer, reverse
MQF	CGAGTGGTGTGAAGGTAGTC	Real-time PCR primer, forward
MQR	GGCAAATCAGGGGTACAT	Real-time PCR primer, reverse
β -s	TTATATGCCAACACTGTCTTT	Standard control primer, forward
β -x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
RNAiF	TAATACGACTCACTATAGGGCGACGAG	RNAi primer for <i>AccMKP3</i> , forward
RNAiR	TGGTGTGAAGGTAGTC TAATACGACTCACTATAGGGCGACCA GC	RNAi primer for <i>AccMKP3</i> , reverse
CATF	ACTCCCTTATCTGAG GTCTTGCCCCGAACAATTTG	qPCR primer for <i>AccCAT</i> , forward
CATR	CATTCTCTAGGCCACCAAA	qPCR primer for <i>AccCAT</i> , reverse
GSTs4F	CTTCTTAGTTATGGAGGTGTTG	qPCR primer for <i>AccGSTs4</i> , forward
GSTs4R	GCCATCTGAAATCGTAAAGAG	qPCR primer for <i>AccGSTs4</i> , reverse
SOD2F	TTGCCATTCAAGGTTCTGGTT	qPCR primer for <i>AccSOD2</i> , forward
SOD2R	GCATGTTCCCAAACATCAATACC	qPCR primer for <i>AccSOD2</i> , reverse
Tpx3F	CCTGCACCTGAATTTTCCGG	qPCR primer for <i>AccTpx3</i> , forward
Tpx3R	CTCGGTGTATTAGTCCATGC	qPCR primer for <i>AccTpx3</i> , reverse
P450F	CGCAAAGAGAATGGGAAGG	qPCR primer for <i>AccCYP4G11</i> , forward
P450R	CTTTTGTGTACGGAGGTGC	qPCR primer for <i>AccCYP4G11</i> , reverse
MsrAF	GGTGATTGTTTATTTGGCG	qPCR primer for <i>AccMsrA</i> , forward
MsrAR	TTTGTATTGCTCTGTTCACG	qPCR primer for <i>AccMsrA</i> , reverse
Trx1F	GGTTTGAGAATTATACGCACTGC	qPCR primer for <i>AccTrx1</i> , forward
Trx1R	GAGTAAGCATGCGACAAGGAT	qPCR primer for <i>AccTrx1</i> , reverse
Trx2F	GGTTCGGTAGTACTTGTGGAC	qPCR primer for <i>AccTrx2</i> , forward
Trx2R	GGACCACACCACATAGCAAAG	qPCR primer for <i>AccTrx2</i> , reverse
STIP1F	CATGGTGGCGCATTTGTAGTAGGC	qPCR primer for <i>AccSTIP1</i> , forward
STIP1R	GGCCCAAGAACATCGTATCAATCC	qPCR primer for <i>AccSTIP1</i> , reverse

point were also predicted. The phylogenetic tree for analysis of amino acid sequences of MKP3 from different species was constructed using the neighbor-joining method in Molecular Evolutionary Genetic Analysis (MEGA version 4.1). The on-line software TFBIND (<http://tfbind.hgc.jp/>) was used to predict binding sites in the *AccMKP3* promoter sequence.

Quantitative real-time PCR

To quantify the expression patterns of *AccMKP3* after exposure to various environmental stresses, quantitative real-time PCR (qRT-PCR) experiments were performed using the Bestar SybrGreen qPCR master mix (DBI, Germany) and the CFX96™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The primers β -s and β -x were designed to amplify the housekeeping gene β -actin (GenBank accession no. HM_640276), which was used as an internal control to normalize RNA levels. The specific primers MQF and MQR (as shown in Table 1) for amplification of the *AccMKP3* gene were designed according to the cDNA sequence of the gene. PCR was performed with a 20- μ L reaction volume (10 μ L of SYBR Premix Ex Taq, 1.0 μ L of cDNA, 0.5 μ L of each primer (10 mM), and 8 μ L of ddH₂O) by using the following program: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 15 s, and 72 °C for 15 s (Zhao et al. 2018). All the samples were prepared with three individuals, and each sample was analyzed in triplicate. The relative mRNA levels of the *AccMKP3* gene were analyzed with CFX Manager software (version 1.1) and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Statistical Analysis System software (version 9.1) was used to analyze the significant differences among samples by one-way ANOVA and Duncan's multiple range tests.

RNAi of *AccMKP3*

To silence the expression of *AccMKP3*, an RNAi experiment was performed as described by Jia et al. (2017). First, we selected a 431-bp non-conserved domain present in the *AccMKP3* ORF and designed two primers, RNAiF and RNAiR, which contained T7 polymerase promoter sequences at their termini. The PCR products were purified and cloned into *E. coli* cells (Trans-T1) for sequencing. Then, the double-stranded RNA (dsRNA) was synthesized using RiboMAX T7 large-scale RNA production systems (Promega, Madison, WI, USA) with the purified PCR products as templates. The GFP gene (GenBank accession no. U87974) was used as a control because this gene is not homologous to any *A. cerana cerana* gene. The final concentrations of the newly synthesized dsRNA-*AccMKP3* and dsRNA-*GFP* were adjusted to 7 μ g/ μ L. The 15-day postemergence adult honeybees were collected randomly and used in this study. Next, we used a microsyringe to inject adult honeybees in the first and second

abdominal segments with 0.5 μ L of dsRNA-*AccMKP3* or dsRNA-*GFP*. All the above samples were flash-frozen in liquid nitrogen and stored at -80 °C until further analysis.

The expression levels of antioxidant genes after knockdown of *AccMKP3*

To detect the expression levels of some antioxidant genes after *AccMKP3* silenced, we designed specific primers for these genes, which included *AccCAT* (no. KF765424), *AccGSTs4* (no. JF798572), *AccSOD2* (no. JN637476), *AccTpx3* (no. JX456217), *AccCYP4G11* (no. KC243984), *AccMsrA* (no. HQ219724), *AccTrx1* (no. JX844651), *AccTrx2* (no. JX844649), and *AccSTIP1* (no. XP016918784). The efficiency of the silencing of the *AccMKP3* gene and the transcript levels of these antioxidant genes were identified by qRT-PCR.

Determination of antioxidant enzymatic activities

For enzyme activity analysis, we selected *AccMKP3*-silenced bees at 36 and 48 h to prepare the 10% tissue homogenate with normal saline solution, and then, the protein concentrations in the supernatants were measured by using the BCA Protein Assay Kit. The enzymatic activities of the POD, SOD, and CAT enzymes were measured using POD, SOD, and CAT kits, respectively, according to the manufacturer's protocols. These kits were all produced by the Nanjing Jiancheng Institute (Nanjing, China).

Statistical analysis

All experiments were conducted in triplicate. The results are presented as the mean $\pm$ standard error (SE) of replicates. Significant differences were determined by Duncan's multiple range tests with Statistical Analysis System software (version 9.1). Significance was set at $p < 0.05$.

Results

Isolation and characterization of *AccMKP3*

The coding sequence (CDS) of *AccMKP3* contains 1203 bp, encoding a 400-amino acid protein with a predicted theoretical isoelectric point (pI) of 4.90 and a molecular mass of 43.922 kDa. The amino acid sequence of *AccMKP3* (XP_016918316.1) was aligned with homologous sequences, including AmMKP3 (*A. mellifera*, XP_006564577.1), ArMKP3 (*Athalia rosae*, XP_012257927.1), BtMKP3 (*Bombus terrestris*, XP_012173008.1), N1MKP3 (*Neodiprion lecontei*, XP_015521709.1), and PdMKP3 (*Polistes dominula*, XP_015180223.1). As shown in Fig. 1, the sequence analysis indicated that MKP3 proteins

Fig. 1 Multiple sequence alignment of *AccMKP3*. Conserved regions are shaded in black. The conserved catalytic domain is boxed, and the corresponding amino acid residues are marked with black dots. The

critical KIM motif, which specifically recognizes MAPKs in MKPs, is indicated by a horizontal line

from different species share high homology. In addition, MKP3 has a highly conserved sequence- $DX_{26}(V/L)X(V/I)HCXAG(I/V)SRSXT(I/V)XXAY(L/I)M$ (where X is any amino acid) within its C-terminal region and a KIM motif within its N-terminal region. The C-terminal catalytic domain is made up of a tyrosine/threonine specific phosphatase designated sequence $HCXXXXXR$ at the active site, where cysteine acts as the enzymatic nucleophile and arginine interacts directly with the phosphate group on phosphotyrosine or phosphothreonine. A highly conserved aspartic acid also participates in the enzymatic catalysis (Farooq et al. 2001; Stewart et al. 1999). In addition, the conserved KIM sequence in the noncatalytic N-terminal domains has been reported to be important for substrate specificity (Muda et al. 1998). It mediates the binding of MKPs/DUSPs with the conserved MAPK common docking domain without

requiring the phosphorylation of MAPK for activation, thus mediating the enzyme-substrate interaction (Camps et al. 1998).

Furthermore, we constructed a phylogenetic tree to explore the evolutionary relationship of *AccMKP3* among different species. As shown in Fig. 2, we assembled six clades of DUSPs, and *AccMKP3* was classified into the MKP subgroup. *A. cerana cerana* MKP3 was most closely related to *A. mellifera* MKP3 in terms of evolution, and both these proteins belong to the MKP subgroup.

Putative transcription binding sites in the *AccMKP3* promoter

A 1094-bp promoter sequence of *AccMKP3* (XM_017062827) located upstream of the transcription start

Fig. 2 Phylogenetic analysis of *AccMKP3* from different species. The phylogenetic tree was constructed by MEGA version 4.0 with the neighbor-joining (NJ) method and the bootstrap values of 1000

replicates. All amino acid sequences were obtained from the NCBI database. Six main groups of the DUSP superfamily are shown, and *AccMKP3* is boxed

site was obtained from the NCBI database. As shown in Fig. 3, many putative transcription factor binding sites were predicted by TFBIND. The promoter region shows nearly 37 sites for the caudal-related homeobox (*CdxA*) protein, which is associated with embryo or tissue development (Ericsson et al. 2006). Sequences involved in environmental stress and immune response, including 17 heat shock factor (HSFs), 38 cAMP response element-binding protein (CREB), 21 activating protein-1 (AP-1), 12 p53, 14 Nkx-II, and 3 nuclear factor kappa B (NFκB) sites, were also identified in the region (Lee et al. 2018; Li et al. 2018; Mazaira et al. 2018). The promoter region also harbors a site for nuclear factor-erythroid 2-related factor 2 (Nrf2), which plays a crucial role in controlling cellular adaptation to oxidants and electrophiles (Hayes and McMahon 2009). Additionally, some GATA motifs related to gene expression in terminally differentiated endodermal tissue and development of the endoderm were also found (Burmeister et al. 2008). *Apis cerana cerana* MKP3 was most closely related to *Apis mellifera* MKP3 in terms of evolution, then we also explored the promoter of *AmMKP3* (Supplementary Fig. 1) and found that there are 36 *CdxA*,

23 CEBP, 16 AP-1, 12 HSF, 5 P53, 11 Nkx-II, 5 CREB, and 1 NFκB sites and several GATA motifs. The sites for *CdxA*, CREB, AP-1, P53, Nkx-II, NFκB, and GATA were repeated between the two species. Therefore, we speculate that these repetitive binding sites related to growth and stress response are of great significance.

Developmental and tissue-specific expression patterns of *AccMKP3*

The expression profile of *AccMKP3* at different developmental stages and in different tissues was determined by qRT-PCR. As shown in Fig. 4(A), we found that the relative expression of *AccMKP3* was significantly higher in the Pw and P0 phases than in other phases. In the pupae, the transcript level was highest in the Pp phase, followed by the P0 phase, and in the remaining phases, the transcript levels were almost the same. In the larval stage, the relative expression of *AccMKP3* in the L6 phase was almost 3-fold that in the L4 or L5 phase. In the adult stage, the level in the A15 phase was somewhat higher than that in the other phases. Then, we

Fig. 3 Partial nucleotide sequence and putative transcription factor binding sites in the *AccMKP3* promoter. The transcription factor binding sites are boxed, and the transcription start site is marked with an arrow

examined the transcript levels in different tissues, including the brain, wing, muscle, epidermis, midgut, and sting shaft tissues, and found that the MS exhibited the highest expression level of *AccMKP3*, whereas MG exhibited a low level of expression (Fig. 4(B)).

Analysis of transcript levels of *AccMKP3* in response to various stresses

Honeybees are exposed to various environmental stresses during their lifecycle, such as heat, cold, heavy metals, UV rays (UV), and pesticides, and all these abiotic stresses are

considered to lead to increased ROS levels (Lushchak 2011). To determine the role of *AccMKP3* in oxidation-related processes, we examined the transcriptional patterns under environmental stress with qRT-PCR. As shown in Fig. 5, the *AccMKP3* transcript level was clearly upregulated in response to all these treatments. First, we tested the extreme temperatures (42 °C and 4 °C) that honeybees experience. The results showed that the expression of *AccMKP3* gradually increased and peaked after 5 h (3.0-fold increase) during the 42 °C treatment (Fig. 5(A)), and during the 4 °C treatment, expression induced markedly after 2.5 h (Fig. 5(B)). After the UV and H₂O₂ treatments, *AccMKP3* expression was significantly

Fig. 4 Expression patterns of *AccMKP3* during different developmental stages and in various tissues. The β -actin gene was used as an internal control. The data represent the means $\pm$ SEs of three independent

experiments. Letters above the error bars showed significant differences ($p < 0.05$) based on Duncan's multiple range tests with the SAS software (version 9.1)

elevated, with peak expression observed at 3 h (Fig. 5(C)) and 1.5 h (Fig. 5(D)), respectively. Heavy metals can harm organisms when absorbed; therefore, we analyzed the expression of

AccMKP3 after treatment with heavy-metal-containing compounds, including HgCl_2 and CdCl_2 (Fig. 5(E, F)). Both treatments led to slightly increased transcript levels. In addition,

Fig. 5 Expression patterns of *AccMKP3* under various abiotic stresses. The transcript levels of *AccMKP3* were detected by qRT-PCR in total RNA extracted from adult worker bees. The adult honeybees were exposed to the following stresses: 42 °C, 4 °C, UV, H_2O_2 , CdCl_2 ,

HgCl_2 , thiamethoxam, hexythiazox, and abamectin. The data represent the means $\pm$ SEs of three independent experiments. The letters above the error bars indicate significant differences ($p < 0.05$) based on Duncan's multiple range tests with SAS software (version 9.1)

we analyzed the relative expression level of *AccMKP3* after pesticide treatment. As shown in Fig. 5(G–I), all three pesticide treatments, namely, thiamethoxam, hexythiazox, and abamectin, resulted in substantially elevated levels of *AccMKP3* expression.

***AccMKP3* knockdown**

Based on the expression pattern analysis, we speculated that the *AccMKP3* gene may be involved in the process of antioxidant defense in *A. cerana cerana*. To verify the function of *AccMKP3*, the RNAi method was used to knock down the gene. Then, dsRNA-*AccMKP3* and dsRNA-*GFP* were injected into 15-day postemergence adult bees for silencing. As shown in Fig. 6, the relative expression level of *AccMKP3* was decreased by approximately 50% and 80% compared with that of the dsRNA-GFP-injected groups at 36 and 48 h, respectively. Based on the qRT-PCR results, we concluded that *AccMKP3* had been successfully knocked down.

Effects of the silenced *AccMKP3* on transcript profiles of antioxidant genes

To further determine the role played by *AccMKP3* in defense against oxidative damage, we chose *AccMKP3*-silenced bee samples at 36 and 48 h after injection to examine the relative expression of some other antioxidant genes. The qRT-PCR results showed that the transcript levels of *AccCAT*, *AccGSTs4*, *AccSOD2*, and *AccTpx3* were upregulated when *AccMKP3* was knocked down, while the transcript levels of *AccCYP4G11*, *AccMsrA*, *AccTrx2*, and *AccSTIP1* were inhibited (Fig. 6). The results indicated that the transcript levels of some antioxidant genes were influenced by the knockdown of *AccMKP3*.

Determination of antioxidant enzymatic activities after *AccMKP3* silenced

To further verify the antioxidant function of *AccMKP3* when *A. cerana cerana* were exposed to environmental stresses, we examined the activities of some antioxidant enzymes at 36 and 48 h after injection. As shown in Fig. 7, the POD, SOD, and CAT enzyme activities were obviously increased following the knockdown of *AccMKP3*. This result is consistent with the expected result. Therefore, we hypothesized that *AccMKP3* may play a role in the resistance of *A. cerana cerana* to oxidative stress.

Discussion

DUSPs are a heterogeneous group of the protein tyrosine phosphatase superfamily and are key regulators of MAPK

activity in conditions of growth, illness, environmental stress, and so on (Camps et al. 2000; Patterson et al. 2009). As a member of the DUSP family, MKP3 not only play important roles in morphology development and cancer signal regulation but also involved in various cellular stresses (Kim et al. 2002; Molnar and de Celis 2013). Although functions of MKP3 have been widely studied in many species, there is limited information on these in insects. In this study, we isolated a dual-specificity MKP gene from *A. cerana cerana* and named it *AccMKP3*. Sequence analysis of *AccMKP3* indicated that this protein shared high homology with MKP3s from different species, and *AccMKP3* possessed the conserved characteristics of the DUSP family. The KIM motif, which is well conserved in all dual-specificity MKPs, was found in the noncatalytic N-terminal region of *AccMKP3* and may account for the specificity of MKP3 toward ERKs and for the subcellular localization of MKP3 (Liu et al. 2006). The phylogenetic analysis suggested that *AccMKP3* belongs to the MKP subgroup and shares the highest homology with *AmMKP3*. Taken together, these results proved that the *AccMKP3* gene is a typical dual-specificity MKP gene.

Promoters are a class of *cis*-acting elements involved in the initiation of gene expression that control the initiation of transcription through the binding of specific transcription factors. To elucidate the possible roles of *AccMKP3*, we analyzed its promoter sequence, which contains many putative binding sites for transcription factors related to growth and development as well as stress responses. We then explored the relative expression of *AccMKP3* in different development stages and tissues. Analysis of different developmental stages revealed that the transcript levels of *AccMKP3* were significantly higher in the pupal stages than in the other stages. The pupal stage is mainly associated with internal organ differentiation and formation. At this stage, bees are metabolically exuberant and grow fast; as a result, the insects are highly susceptible to ROS-induced oxidative damage (Corona and Robinson 2006). However, the prepupal stage is a transient transition period between the larval stage and the pupal stage and is often accompanied by morphological changes and differences in physiological function. Therefore, we suspect that *AccMKP3* may be essential for the morphological development of bees and may help bees resist the damage caused by ROS. Tissue-specific expression analysis revealed that *AccMKP3* was expressed in all tissues examined, indicating that *AccMKP3* is widely expressed in bees and may play an important role. It has been reported that in *Drosophila*, DMKP3 is expressed at low levels throughout the developmental process, but functional DMKP3 is required by the various compartments of the adult body (Kim et al. 2002). Taken together, these data suggest that *AccMKP3* may play a vital role in the development of bees and may protect bees from adversities in at different developmental stages and in different tissues.

Fig. 6 Assessment of the efficiency of *AccMKP3* silencing. (A) The effects of RNA interference on the transcript levels in 15-day postemergence adult honeybees were determined by qRT-PCR. (B–J) Expression profiles of other antioxidant genes after *AccMKP3* knockdown. The relative expression levels of several antioxidant genes (*AccCAT*, *AccGSTs4*, *AccSOD2*, *AccTpx3*, *AccCYP4G11*, *AccMsrA*,

AccTrx1, *AccTrx2*, and *AccSTIP1*) were examined on the *AccMKP3*-silenced bee samples at 36 and 48 h after injection. The β -actin gene was used as an internal control. The data represent the means $\pm$ SEs of three independent experiments. The letters above the error bars indicate significant differences ($p < 0.05$) based on Duncan's multiple range tests with SAS software (version 9.1)

In addition to developmental and tissue-specific analysis, we also investigated the transcriptional levels of *AccMKP3* under different environmental stresses that *A. cerana cerana* may suffer during its lifecycle. Temperature is a common

stress factor in the external environment and can affect insect growth, lifecycle, and fitness traits; therefore, many insects are sensitive to temperature, both physiologically and metabolically (Gray 2013). It has been reported that heat stress can

Fig. 7 Measurement of antioxidant enzyme activities after *AccMKP3* knockdown. The activities of antioxidant enzymes, including (A) POD, (B) SOD, and (C) CAT, were examined in the *AccMKP3*-silenced bee samples at 36 and 48 h after injection. The data represent the means $\pm$ SEs

of three independent experiments. The letters above the error bars indicate significant differences ($p < 0.05$) based on Duncan's multiple range tests with SAS software (version 9.1)

harm organisms by promoting the production of ROS (Geng et al. 2016), and low temperatures can reduce the rate of metabolism and accelerate the formation of oxidative radicals during bee flight (Harrison and Fewell 2002). We found that the relative expression of *AccMKP3* was increased to different extents during heat or cold treatment, which indicated that *AccMKP3* may help *A. cerana cerana* resist extreme temperatures. UV is a formidable and detrimental environmental stress factor that can significantly affect insect life by increasing the production and accumulation of endogenous ROS (Ali et al. 2017). Hydrogen peroxide can also cause oxidative damage as a typical oxidant. In our study, *AccMKP3* expression was upregulated after UV and H_2O_2 treatment, suggesting that *AccMKP3* may play essential roles in preventing cellular oxidative stress.

Heavy metal ions are usually highly toxic because of their redox properties and chemical coordination ability, especially the ability to bind with cellular components (Bafana et al. 2017). There is evidence that heavy metals can accelerate intracellular ROS generation and increase cellular oxidative stress (Tang et al. 2012). *Toxoplasma gondii* organelles are damaged by low concentrations of $CdCl_2$ and $HgCl_2$, resulting in loss of viability and organelle death and elimination (de Carvalho and de Melo 2018). Our qRT-PCR results showed that *AccMKP3* transcription was slightly induced after $CdCl_2$ and $HgCl_2$ injection, which suggested that *AccMKP3* may respond to heavy metals stresses and protect honeybees from oxidative damage.

Previous studies have shown that pesticides can pose threats to the survival of honeybees (Chen et al. 2015). Thiamethoxam, hexythiazox, and abamectin are contact insecticides; i.e., these insecticides are absorbed by insects via direct contact with the body surface to have a toxic effect. The relative expression of *AccMKP3* was significantly induced after exposure to these pesticide treatments. Asghari et al. (2017) have reported that pesticide exposure causes mitochondrial dysfunction, endocrine disruption, and oxidative stress. Therefore, we suspect that *AccMKP3* may participate in the

physiological activities of honeybees and protect honeybees from adverse stress.

Antioxidant genes and antioxidant enzymes are integral parts of intracellular defense systems and can inhibit the production of ROS and maintain cellular homeostasis (Emanuele et al. 2018). It has been reported that changes in the expression of one antioxidant enzyme may affect the expression of other antioxidant enzymes, thus maintaining the balance of intracellular oxidation (Mates et al. 2012). In our study, the relative expression levels of *AccCAT*, *AccGSTs4*, *AccSOD2*, and *AccTpx3* were upregulated when *AccMKP3* was knocked down, suggesting that the intracellular oxidative balance was destroyed and the induction of these antioxidant genes may compensate for the lack of *AccMKP3* function in response to oxidative stress. In addition, the expression levels of some other antioxidant genes, namely, *AccCYP4G11*, *AccMsrA*, *AccTpx3*, and *AccSTIP1*, were downregulated when *AccMKP3* was knocked down. CYP4G11 is associated with detoxification in insects and exhibits peroxidase activity; loss function of *AccCYP4G11* in honeybees may lead to the accumulation of hydrogen peroxide (Shi et al. 2013). Similarly, the MsrA system has been reported to mediate methionine sulfide modification and downregulation of *MsrA* in yeast and mice may lead to increased protein carbonyl levels (Moskovitz and Oien 2010). Therefore, the reduction of these antioxidant genes further proved that *AccMKP3* participates in the antioxidant process and that they may respond to oxidative stress through a different way or regulated by *AccMKP3*. In addition, POD, SOD, and CAT are important antioxidant enzymes that can effectively scavenge free radicals and peroxides in organisms and prevent cells from oxidative damage (Corona and Robinson 2006; Schatzman and Culotta 2018). We found that the enzyme activities of POD, SOD, and CAT were increased following the silence of *AccMKP3*, which provided further evidence for the resistance of *AccMKP3* to oxidative stress. However, the effect of RNAi on the regulation of other stress response genes is complex; the specific mechanism remains to be studied.

In conclusion, we isolated an *MKP3* gene from *A. cerana cerana* and explored the possible functions of this gene. We studied the expression patterns of *AccMKP3* at different developmental stages and in different tissues and then measured the relative expression of *AccMKP3* under various environmental stresses. To further confirm the results, we measured the expression levels of several antioxidant genes and examined the enzyme activities of POD, SOD, and CAT. Taken together, our data provide evidence that *AccMKP3* may play indispensable roles in the response to oxidative stress. This work is of great significance for the study of the resistance of Chinese honeybees. However, to better understand the roles of *AccMKP3*, further in-depth research is required.

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