

AccPDIA6 from *Apis cerana cerana* plays important roles in antioxidation

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ABSTRACT

PDIA6 is a member of the protein disulfide isomerase (PDI) family, shows disulfide isomerase activity and oxidoreductase activity, and can act as a molecular chaperone. Its biological functions include modulating apoptosis, regulating the proliferation and invasion of cancer cells, supporting thrombosis and modulating insulin secretion. However, the roles of PDIA6 in *Apis cerana cerana* are poorly understood. Herein, we obtained the PDIA6 gene from *A. cerana cerana* (*AccPDIA6*). We investigated the expression patterns of *AccPDIA6* in response to oxidative stress induced by H₂O₂, UV, HgCl₂, extreme temperatures (4 °C, 42 °C) and pesticides (thiamethoxam and hexythiazox) and found that *AccPDIA6* was upregulated by these treatments. Western blot analysis indicated that *AccPDIA6* was also upregulated by oxidative stress at the protein level. In addition, a survival test demonstrated that the survival rate of *E. coli* cells expressing *AccPDIA6* increased under oxidative stress, suggesting a possible antioxidant function of *AccPDIA6*. In addition, we tested the transcripts of other antioxidant genes and found that some of them were downregulated in *AccPDIA6* knockdown samples. It was also discovered that the antioxidant enzymatic activity of superoxide dismutase (SOD) decreased in *AccPDIA6*-silenced bees. Moreover, the survival rate of *AccPDIA6* knockdown bees decreased under oxidative stress, implying that *AccPDIA6* may function in the oxidative stress response by enhancing the viability of honeybees. Taken together, these results indicated that *AccPDIA6* may play an essential role in counteracting oxidative stress.

1. Introduction

Protein disulfide isomerases (PDIs) are a family of multifunctional proteins that are well known to be oxidoreductases, isomerases and molecular chaperones (Lee and Lee, 2017; Puig et al., 1994; Schwaller et al., 2003; Soares Moretti and Martins Laurindo, 2017). To date, 21 members have been reported in the human PDI family, and except for ERp27, ERp29, CASQ1 and CASQ2, most PDIs contain at least one redox-active CXXC motif (Lee and Lee, 2017). The CXXC motif has been reported to be important for enzymatic activity (Eletto et al., 2014). PDIs have been confirmed to participate in regulating redox balance (Zeeshan et al., 2016; Lee and Lee, 2017). In the PDI family, some members have been extensively studied in humans and other mammals. PDI and PDIA3 have been reported to be related to neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and prion disease (Matsusaki et al., 2020). PDIA4 and PDIA6 have been reported to mediate cisplatin-induced death resistance in lung adenocarcinoma cells (Tufo et al., 2014). In insects, it seems that only one

paper has been published about PDI, which determined that the PDI of *Bombyx mori* (bPDI) may participate in exogenous bacterial infection of the fat body and that its expression level is regulated by hormones.

PDIA6 belongs to the PDI family, and it has two typical CGHC active site sequences like other PDIs. However, its length, domain arrangement and substrate specificity are different from those other members of the human PDI family (Lee and Lee, 2017). Some studies have shown that human PDIA6 functions as a chaperone, an oxidoreductase and an isomerase (Jordan et al., 2005; Alanen et al., 2006; Kikuchi et al., 2002; Dupuy and Passam, 2019). Human PDIA6 participates in several physiological processes, including modulating apoptosis (Bai et al., 2019), regulating the proliferation and invasion of cancer cells (Cheng et al., 2017), supporting thrombosis (Essex and Wu, 2018), limiting unfolded protein response (UPR) signaling and affecting insulin secretion (Eletto et al., 2016). In particular, rat PDIA6 has been implicated in oxidative stress and shown to participate in tert-butyl hydroperoxide (t-BHP, an inducer of oxidative stress)-induced apoptosis controlled by reactive oxygen species (ROS) and the IRE1α/ASK1/JNK1/2/p38 signaling

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pathway (Shen et al., 2018). Previous studies have demonstrated that human PDIA6 is also associated with several types of cancers (including lung cancer, ovarian cancer, bladder cancer, hepatocellular carcinoma and primary ductal breast cancer) and diabetes (Bai et al., 2019; Samanta et al., 2017; Cheng et al., 2017; Cho et al., 2019; Liu et al., 2019). Additionally, mouse PDIA6 is reported to be associated with myocardial infarction (Kiouptsis et al., 2019), and *Caenorhabditis elegans* PDIA6 affects the development of larvae (Eletto et al., 2014).

The Chinese honeybee (*Apis cerana cerana*) is an important pollinator that plays an significant role in maintaining the balance of regional ecologies. It has many advantages over Western honeybees (*Apis mellifera*), such as higher cold tolerance and disease resistance (Li et al., 2012). However, many adverse conditions, such as pesticide abuse, diseases, pollution and climate changes, which may result in oxidative stress, inevitably challenge their survival (Yang, 2005; Yao et al., 2013; Jia et al., 2017; Li et al., 2015; Park and Park, 2007; An and Choi, 2010; Lushchak, 2011). Oxidative stress has been reported to be associated with endoplasmic reticulum (ER) stress and to lead to multiple human pathologies (Cao and Kaufman, 2014). PDIs play an important role in ER-related redox cell signaling and homeostasis (Laurindo et al., 2012). Since PDIA6 plays a role in the redox signaling pathway, we suspected that AccPDIA6 may be important in defending against oxidative stress as well. Therefore, we isolated and characterized the PDIA6 gene from *A. cerana cerana* (*AccPDIA6*) and evaluated its expression patterns in different developmental stages and various tissues. We also determined its expression levels in honeybees treated with many kinds of stresses and found that *AccPDIA6* was induced to varying degrees at the transcriptional and protein levels. In addition, we discovered the function of recombinant *AccPDIA6* in resisting oxidative stress *in vitro*. CGHC motifs were shown to be crucial for the function of *AccPDIA6* as an antioxidant. Moreover, by testing the expression patterns of some antioxidant genes and the activity of some antioxidants, we found that *AccPDIA6* may function in resisting oxidative stress. Moreover, we found a sharp decline in the survival rate of *AccPDIA6* knockdown honeybees exposed to oxidative stress, which implies an antioxidative function of *AccPDIA6* *in vivo*. These results provide new insights into the molecular characterization and functions of PDIA6 in insects.

2. Materials and methods

2.1. Honeybee material

The honeybees (*A. cerana cerana*) used in this study were maintained in 5 beehives at the experimental apiary of the College of Animal Science and Technology, Shandong Agriculture University, Tai'an, Shandong, China. The honeybees were categorized as eggs, larvae, pupae and adults (Meng et al., 2014; Chen et al., 2015). Larvae and pupae were identified by counting the number of days after eggs were laid. We collected samples of day-1 (L1) and day-6 (L6) instar larvae, prepupae (Pr), white-eyed (Pw), brown-eyed (Pb), pink-eyed (Pp), and dark-eyed (Pd) pupae and day-1 adults from the hive. Newly emerged bees (day-1 adults) were marked with paint, and day-15 to day-30 adults were collected after 15 to 30 days. Then, we froze these samples in liquid nitrogen and stored them at -80°C to analyze temporal expression patterns. Day-15 to day-30 adult bees were randomly sampled from the hive and dissected to collect venom gland (VG), nectary (NE), midgut (MG), wing (WI), abdomen (AB), antenna (AN), thorax (TH), head (HE), thorax muscle (MS), foot (FO; including 3 pairs of feet) and abdomen epidermis (EP) specimens for the analysis of spatial expression patterns.

2.2. Stress treatment

Day-15 to day-30 adult bees were randomly collected from honeybees marked with paint when they emerged from hives and classified into 8 groups, each of which consisted of 30 bees. Groups 1 and 3 were fed 1 μL of 2 mM H₂O₂ and 1 μL of 3 mg/mL HgCl₂, respectively; Groups

2, 4–5 were exposed to UV (254 nm, 30 MJ/cm²), 4 °C or 42 °C treatment. Groups 6–10 were treated with 20 mg/mL pesticides (avermectin, kresoxim-methyl, thiamethoxam, hexythiazox and emamectin benzoate) via either injection or addition to the basic adult diet. The control group consisted of untreated adult bees. Finally, honeybees sampled at the specified times (Group 1, 4, 5, 7 and 9–10: 0 h, 0.5 h, 1 h, 1.5 h, 2 h and 2.5 h; Group 2: 0 h, 1 h, 2 h, 3 h, 4 h and 5 h; Group 3: 0 h, 3 h, 6 h 9 h, 12 h and 24 h; Group 6 and Group 8: 0 h, 0.5 h, 1 h, 1.5 h and 2 h) were flash frozen and stored at -80 °C.

2.3. RNA extraction and cDNA synthesis

RNA was extracted from the honeybees using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's protocol. cDNA synthesis was carried out with the HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) Kit (Vazyme, Nanjing, China) according to the steps specified by the kit. Then, we stored all the honeybee RNA and cDNA samples at -80 °C and -20 °C, respectively, until their use.

2.4. Primers

The primers used in this study are listed in Table 1, and all of the sequences were synthesized by Sangon Biotech Company (Shanghai, China).

2.5. Isolation of the *AccPDIA6* open reading frame and construction of the expression vector

The open reading frame (ORF) of *AccPDIA6* was isolated by PCR using the specific primers A6F and A6R. The PCR product was integrated into pEasy-T1 vectors (TransGen Biotech, Beijing, China) and introduced into *E. coli* Trans1-T1 (TransGen Biotech). To obtain the pET-30a (+)-*AccPDIA6* recombinant expression plasmid, the coding region of *AccPDIA6* flanked by *Bam*H I and *Sall* restriction sites was connected to the pET-30a(+) vector (Novagen, Darmstadt, Germany).

2.6. Bioinformatic and phylogenetic analysis

The molecular weight of *AccPDIA6* was predicted by using DNAMAN. Multiple protein sequence alignments of PDIA6 from various species were analyzed by using DNAMAN. Conserved domains were obtained using the BLAST search program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic tree analysis was performed via the neighbor-joining method using MEGA version 5.2 software.

2.7. qRT-PCR

qRT-PCR analysis was performed by using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Dalian, China) and the CFX96™ Real-Time System. Gene expression was normalized to the housekeeping gene β -actin (GenBank accession no. HM640276.1). All the primers used for qRT-PCR assays showed an efficiency within the recommended range (90–110%), and the corresponding correlation coefficients were higher than 0.99. The PCR amplification program was set as described by Yan et al. (2014). At least three biological replicates were carried out for each treatment. The relative gene expression levels were analyzed by using the 2^{-ΔΔCT} method and CFX Manager software (version 3.1).

2.8. Recombinant protein expression and antibody preparation

The pET-30a (+)-*AccPDIA6* recombinant plasmid was introduced into chemically competent Transetta (DE3) cells purchased from TransGen Biotech (Beijing, China). The bacterial solution containing recombinant *AccPDIA6* was grown in 10 mL of Luria-Bertani (LB) broth with 5 μL of kanamycin (100 mg/mL) at 37 °C overnight. Aliquots of 100–200 μL of the bacterial solution were subcultured in 10 mL of fresh

Table 1
PCR primers used in this study.

Primer	Primer sequence (5' – 3')	Description
For the cloning of the full-length cDNA		
A6F	ATGCAAGAATTAGGTATTTGCTATT	Full-length cDNA primer, forward
A6R	TTACAGTCGCTTTCATCAAGA	Full-length cDNA primer, reverse
For the constructing of the prokaryotic expression vector		
A6v-F	GGATCCATGCAAGAATTAGGTATTTGC	Protein expression primer, forward
A6v-R	GTCGACCAGTCGCTTTCATCAAG	Protein expression primer, reverse
For the cloning of the dsRNA		
A6d-F	TAATACGACTCACTATAGGGCAGGTGACACCTGAATATAACAAAGCTGC	RNAi primer, forward
A6d-R	TAATACGACTCACTATAGGGCACCTTAAGCTCTGTCAGATG	RNAi primer, reverse
GFP-F	TAATACGACTCACTATAGGGCAGGTGATGCAACATACGGAAAAC	Standard control primer of RNAi, forward
GFP-R	TAATACGACTCACTATAGGGCAGATAATGATCAGCGAGTTGC	Standard control primer of RNAi, reverse
For quantitative real-time PCR		
A6q-F	GTGCAAGAATATGATGGTGGTCG	RT-qPCR primer, forward
A6q-R	CGGTAAAACGGATATAACACATAACCG	RT-qPCR primer, reverse
β-s	TTATATGCCAACACTGTCCTT	Standard control primer, forward
β-x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
GSTO2-F	CCAGAAGTAAAGACAAGTTCGT	qPCR primer of <i>AccGSTO2</i> , forward
GSTO2-R	CCATTAACATCAACAAGTGTGGT	qPCR primer of <i>AccGSTO2</i> , reverse
Tpx5-F	GGGGTATTCTATTTCGCACTCCA	qRT-PCR primer of <i>AccTpx5</i> , forward
Tpx5-R	CCATTACGATGAGAACATCGACTGA	qRT-PCR primer of <i>AccTpx5</i> , reverse
CAT-F	GTCTTGGCCCGAACAAATTG	qPCR primer of <i>AccCAT</i> , forward
CAT-R	CATCTCTAGGCCACCAAA	qPCR primer of <i>AccCAT</i> , reverse
CYP-F	CGAAAGAGAAATGGGAAGG	qRT-PCR primer of <i>AccCYP4G11</i> , forward
CYP-R	CTTTTGTTACGGAGGTG	qRT-PCR primer of <i>AccCYP4G11</i> , reverse
GSTS4-F	CITCTTAGTTATGGAGGTGTTG	qRT-PCR primer of <i>AccGSTs4</i> , forward
GSTS4-R	GCCATCTGAAATCGTAAAGAG	qRT-PCR primer of <i>AccGSTs4</i> , reverse
Trx2-F	GGTTCGGTAGTACTTGTGAC	qPCR primer of <i>AccTrx2</i> , forward
Trx2-R	GGACCAACACACATAGCAAAG	qPCR primer of <i>AccTrx2</i> , reverse
GSTD-F	CGAAGGAGAAAATATGTGGCAG	qRT-PCR primer for <i>AccGSTD</i> , forward
GSTD-R	CGTAATCCACCAACCTCTATCG	qRT-PCR primer for <i>AccGSTD</i> , reverse
MsrA-F	GGTGATTGTTTATTGGCG	qPCR primer of <i>AccMsrA</i> , forward
MsrA-R	TTTGATTTGCTCTGTTCAAGC	qPCR primer of <i>AccMsrA</i> , reverse
For site-directed mutagenesis		
1F	CCATGGGCTGGACATTGCAAATG	C51A mutation primer of <i>AccPDIA6</i> , forward
1R	CATTGACAATGTCAGCCCCATGG	C51A mutation primer of <i>AccPDIA6</i> , reverse
2F	CCATGGTGTGGACATGCTCAAATG	C54A mutation primer of <i>AccPDIA6</i> , forward
2R	CATTGAGCATGTCACACCATGG	C54A mutation primer of <i>AccPDIA6</i> , reverse
12F	CCATGGGCTGGACATGCTCAAATG	C51A/C54A mutation primer of <i>AccPDIA6</i> , forward
12R	CATTGAGCATGTCACACCATGG	C51A/C54A mutation primer of <i>AccPDIA6</i> , reverse
3F	CATGGGCTGGTCAATTGAAAAATTAGC	C179A mutation primer of <i>AccPDIA6</i> , forward
3R	GCTAAATTTCACAATGACCAAGCCATG	C179A mutation primer of <i>AccPDIA6</i> , reverse
4F	CATGGTGTGGTCAATTGAAAAATTAGC	C182A mutation primer of <i>AccPDIA6</i> , forward
4R	GCTAAATTTCACAATGACCAAGCCATG	C182A mutation primer of <i>AccPDIA6</i> , reverse
34F	CATGGGCTGGTCAATTGAAAAATTAGC	C179A/C182A mutation primer of <i>AccPDIA6</i> , forward
34R	GCTAAATTTCACAATGACCAAGCCATG	C179A/C182A mutation primer of <i>AccPDIA6</i> , reverse

LB medium with 50 µg/mL kanamycin for 1–2 h until the OD₆₀₀ reached 0.4–0.6. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG, CWbiotech, Beijing, China) was added at a final concentration of 100 µg/mL to induce the expression of the recombinant *AccPDIA6* protein at 28 °C for 8 h.

To obtain anti-*AccPDIA6*, we separated the recombinant *AccPDIA6* protein using 12% SDS-PAGE. The target protein band was excised from the SDS-PAGE gel, homogenized with stroke-physiological saline solution and injected into white mice. The mice were injected once per week for 4 weeks to produce antibodies. Next, we collected and stored the antibodies following the method described by Zhai et al. (2018).

2.9. Cell growth in stress medium

A survival test on LB agar was performed to identify the function of *AccPDIA6* in *E. coli*. Tranetta cells transformed with the pET-30a (+)-*AccPDIA6* recombinant plasmid were incubated until reaching an optical density of 0.6 at 600 nm (OD₆₀₀) and induced with IPTG diluted to a final concentration of 100 µg/mL for 2 h at 28 °C, after which the bacterial liquid was diluted to an OD₆₀₀ of 0.6 and then diluted to 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵. For the H₂O₂ treatment, different concentrations of solutions were spotted on LB agar supplemented with 0.3 mM H₂O₂

and then incubated at 37 °C for 12 h. For the 42 °C treatment, solutions at different dilutions were spotted on LB agar and then incubated at 42 °C.

2.10. Protein extraction and Western blot analysis

Total protein extraction was carried out by using a tissue protein extraction kit purchased from CWBiotech (Beijing, China). Then, protein samples were mixed with 2× sample loading buffer and boiled at 100 °C for 10 min for denaturation. Next, to analyze the expression pattern of *AccPDIA6* at the protein level, Western blot analysis was performed. Total proteins were separated using 12% SDS-PAGE, and the target band was excised and transferred to a PVDF membrane (ComWin Biotech, Beijing, China) via semidry electroblotting. Anti-*AccPDIA6* (1:500 (v/v) dilution) was used as the primary antibody, POD-conjugated goat anti-mouse immunoglobulin G (1:2000 (v/v) dilution, Dingguo, Beijing, China) was used as the secondary antibody, and antitubulin (1:2000 (v/v) dilution, Beyotime Biotechnology, Beijing, China) served as the control. Finally, the Western blotting results were analyzed by using Image-Pro Plus 6.0 (Media Cybernetic, Rockville, MD, USA).

2.11. RNA interference

To study the contribution of the *AccPDIA6* gene to the oxidative stress response, RNAi technology was employed to silence this gene. A pair of specific primers (A6d-F and A6d-R) with a T7 promoter sequence added at the 5' end were constructed based on a partial *AccPDIA6* coding region sequence. We performed numerous rounds of PCR amplification of the partial *AccPDIA6* coding region sequence and purified the PCR products. A large set of dsRNAs for *AccPDIA6* was produced using the T7 RiboMAX™ Express RNAi System (Promega, Madison, United States)

according to the manufacturer's instructions. Additionally, dsRNA for the green fluorescent protein gene (*GFP*, GenBank: U87974) was produced to serve as a control (Wang et al., 2019). Day-15 to day-30 adult honeybees were sampled at 15 to 30 days after marking the newly emerged honeybees and divided into three groups ($n = 30$). Group 1 was subjected to sham injection, group 2 was injected with 7 μg dsRNA-*GFP*, and group 3 was injected with 7 μg dsRNA-*AccPDIA6*. Then, all groups were raised in an incubator set at 34 °C and 70% relative humidity (RH) with a 0 h:24 h light:dark cycle for two days and were supplied with fresh pollen dough and a 30% sucrose solution. Healthy honeybees were

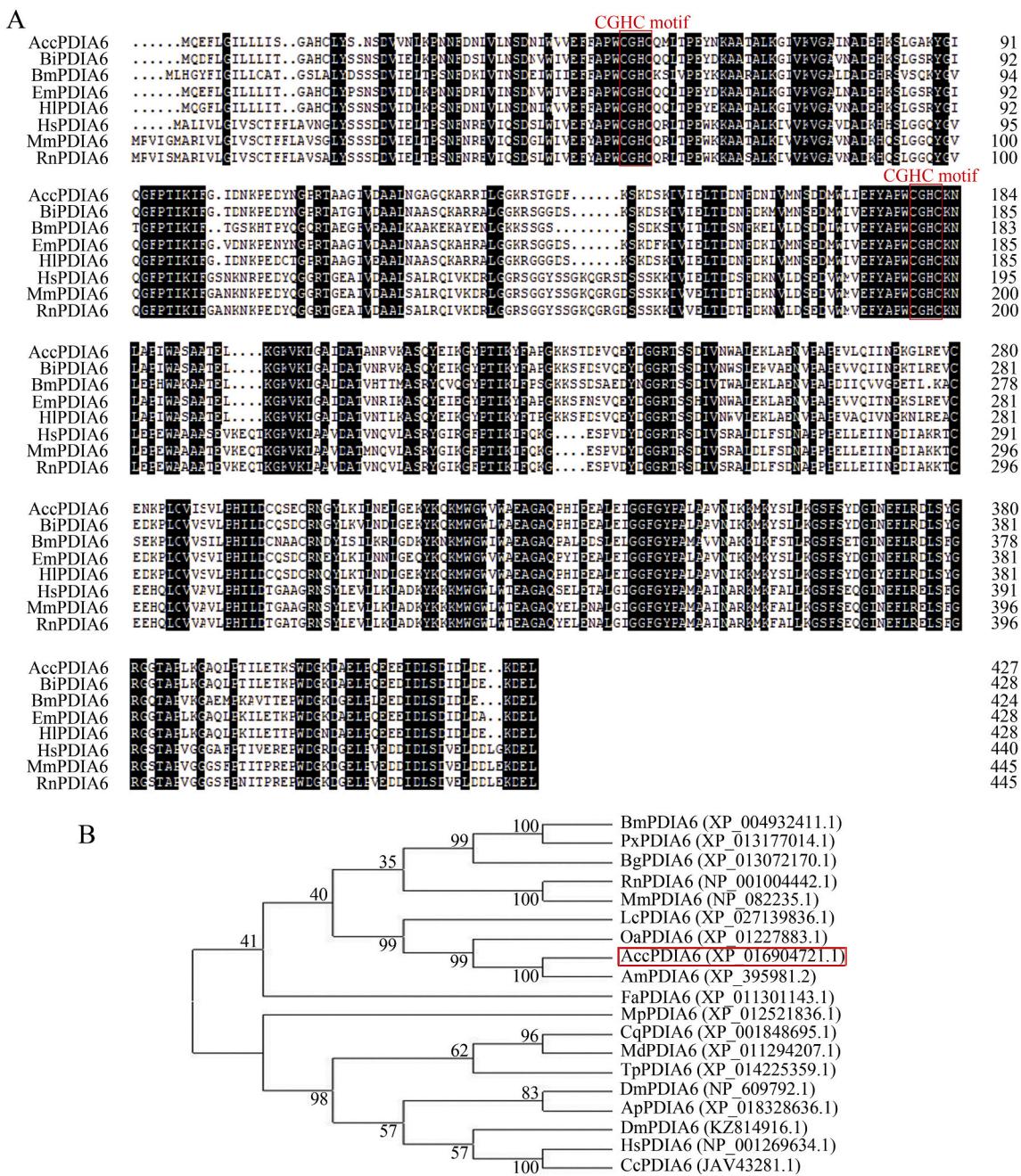


Fig. 1. Alignment and phylogenetic analysis of multiple PDIA6 sequences. (A) Alignment analysis of multiple PDIA6 sequences: PDIA6 from *A. cerana cerana* (AccPDIA6), *B. impatiens* (BiPDIA6), *B. mori* (BmPDIA6), *E. mexicana* (EmPDIA6), *H. laboriosa* (HIPDIAG), *M. musculus* (MmPDIA6), and *R. norvegicus* (RnPDIA6). The two CXXC motifs are marked with red boxes. (B) Phylogenetic tree of AccPDIA6 and PDIA6 from other species, namely, *B. mori* (BmPDIA6), *Papilio xuthus* (PxPDIA6), *Biomphalaria glabrata* (BgPDIA6), *R. norvegicus* (RnPDIA6), *M. musculus* (MmPDIA6), *Larimichthys crocea* (LcPDIA6), *Orius abietinus* (OaPDIA6), *A. mellifera* (AmPDIA6), *Fopius arisanus* (FaPDIA6), *Monomorium pharaonis* (MpPDIA6), *Culex quinquefasciatus* (CqPDIA6), *Musca domestica* (MdPDIA6), *Trichogramma pretiosum* (TpPDIA6), *Daphnia magna* (DmPDIA6), *Agrilus planipennis* (ApPDIA6), *H. saltator* (HsPDIA6) and *Castor canadensis* (CcPDIA6). AccPDIA6 is marked with a red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sampled for the verification of RNAi efficiency after 12, 24, 36 and 48 h respectively.

2.12. Oxidative tolerance evaluation

Day-15 to day-30 adult honeybees were sampled at 15 to 30 days after marking the newly emerged honeybees and divided into nine groups ($n = 30$). Groups 1–3 were subjected to sham injection, groups 4–6 were injected with 7 μg dsRNA-GFP, and groups 7–9 were injected with 7 μg dsRNA-AccPDIA6. Groups 1–2, 4–5 and 7–8 were raised in an incubator at 34 °C with 70% relative humidity under constant darkness; groups 3, 5 and 9 were raised in an incubator at 42 °C with 70% relative humidity under constant darkness. The survival rates of groups 2, 5, and 8 were calculated and recorded every 6 h, and those of the other groups were recorded every 3 h.

2.13. Site-directed mutagenesis and construction of mutant expression vectors

First, the pET-30 (+)-AccPDIA6 recombinant plasmid was used as a template, and specific primers containing *Bam*H I and *Sall* restriction sites (A6v-F and A6v-R) at both ends of the gene were paired with the mutant primers (1F and 1R, 2F and 2R, 3F and 3R, 4F and 4R, 12F and 12R, 34F and 34R) to amplify the upstream and downstream fragments. Then, the purified PCR products and the primer pair A6v-F and A6v-R were employed to produce the C51A, C54A, C179A, C181A, C51A/C54A, C179A/C181A sequences by using overlap PCR. Next, the PCR products were purified and introduced into the pET-30a(+) vector to generate the pET-30 (+)-C51A, pET-30 (+)-C54A, pET-30 (+)-C79A, pET-30 (+)-C81A, pET-30 (+)-C51A/C54A and pET-30 (+)-C179A/C181A recombinant plasmids. The pET-30 (+)-C51A/C54A recombinant plasmid and two primer pairs (A6v-F/A6v-R, C179A/C181A) were used to generate the pET-30 (+)-C51A/C54A/C179A/C181A recombinant plasmid according the method described above.

3. Results

3.1. Identification and sequence analysis of AccPDIA6

To explore the function of AccPDIA6 in *A. cerana cerana*, we isolated the open reading frame (ORF) of the AccPDIA6 gene, which consists of 1284 bp and encodes a protein of 427 amino acids with a predicted average mass of 47.093 kDa, by using specific primers (A6-F and A6-R). As shown in Fig. 1, multiple sequence alignments indicated that AccPDIA6 has a high similarity ranging from 57.12% to 90.89% to PDIA6 from *Bombus impatiens* (BiPDIA6), *B. mori* (BmPDIA6), *Eufriesea mexicana* (EmPDIA6), *Habropoda laboriosa* (HPDIA6), *Harpegnathos saltator* (HsPDIA6), *Mus musculus* (MmPDIA6) and *Rattus norvegicus* (RnPDIA6). In addition, AccPDIA6 has two redox-active thioredoxin (TRX)-like domains, and each domain consists of CGHC motifs; therefore, AccPDIA6 is similar to human PDIA6 of the TRX-like superfamily PDI (Lee and Lee, 2017) (Fig. 1A).

To investigate the evolutionary relationships among PDIA6 from different species, a phylogenetic tree of PDIA6 was constructed based on the amino acid sequences, which showed that AccPDIA6 was more closely related to PDIA6 from *A. mellifera* (AmPDIA6) than to that from other species (Fig. 1B). In summary, these results suggested that PDIA6 is highly conserved throughout evolution and that AccPDIA6 has a closer relationship with AmPDIA6 than with PDIA6 from other species.

3.2. Temporal and spatial expression profiles of AccPDIA6

To determine the expression patterns of AccPDIA6 in different developmental stages and in different tissues, we used quantitative real-time polymerase chain reaction (qRT-PCR). As shown in Fig. 2A, the highest expression level of AccPDIA6 was detected in brown-eyed pupae,

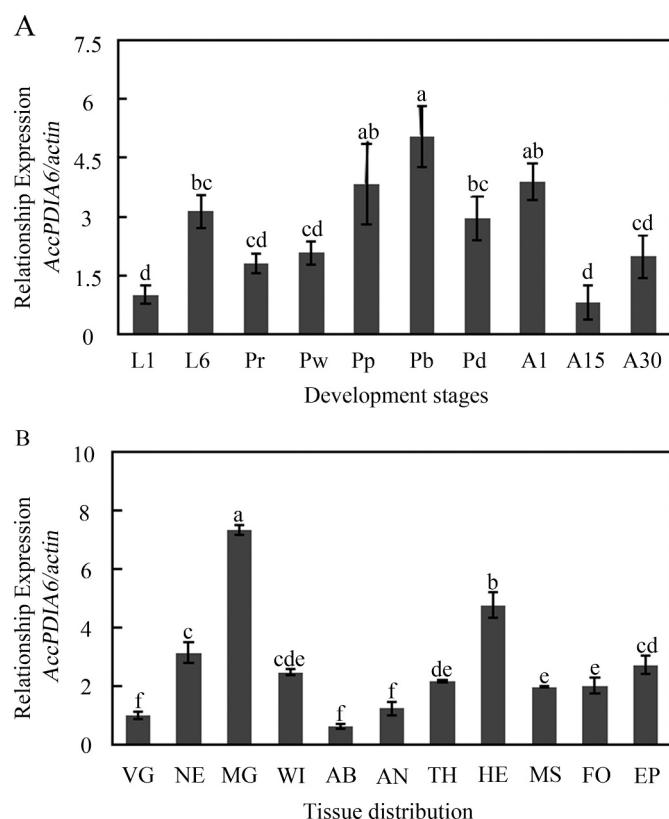


Fig. 2. Temporal and spatial gene expression patterns of AccPDIA6. (A) Expression patterns of AccPDIA6 during different developmental stages. L1: the day-1 larval instars; L6: the day-6 larval instars; Pr: the prepupae; Pw: the white-eyed pupae; Pp: the pink-eyed pupae; Pd: the brown-eyed pupae; A1: the day-1 adults; A15: the day-15 adults; A30: the day-30 adults. (B) Expression levels of AccPDIA6 in different tissues. VG: venom gland, NE: nectary, MG: midgut, WI: wing, AB: abdomen, AN: antenna, TH: thorax, HE: head, MS: thorax muscle, FO: foot, EP: thorax epidermis. The data are expressed as the mean \pm SEM of three independent experiments. The different letters above the bar indicate significant differences based on Tukey tests ($P < 0.05$). SEM, standard error of the mean. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

followed by pink-eyed pupae and day-1 adults. As shown in Fig. 2B, the mRNA levels of AccPDIA6 in the midgut (MG) and head (HE), where AccPDIA6 may play crucial roles, were remarkably higher than those in other tissues. Concisely, we found that AccPDIA6 was expressed at various levels in different developmental stages and tissues.

3.3. AccPDIA6 was regulated by oxidative stress at the transcriptional and protein levels

We tested the expression patterns of AccPDIA6 after honeybees were exposed to a variety of abiotic stresses. As shown in Fig. 3, AccPDIA6 was induced by these stresses to varying degrees. H₂O₂ treatment resulted in extreme increases in AccPDIA6 that peaked at 2 h (Fig. 3A). Following UV treatment, we found that AccPDIA6 was induced slightly and peaked at 1 h (Fig. 3B). After HgCl₂ treatment, the expression level of AccPDIA6 increased slightly and was higher at 6 h and 9 h than that at other time points (Fig. 3C). Following treatment at 4 °C, we discovered that the expression level of AccPDIA6 increased significantly and reached its highest level at 1.5 h (Fig. 3D). The expression level of AccPDIA6 increased sharply and peaked at 1 h after 42 °C treatment (Fig. 3E). AccPDIA6 was determined to be downregulated by avermectin (Fig. 3F). We also observed a substantial increase in AccPDIA6 expression after thiamethoxam and hexythiazox treatment, and the expression levels

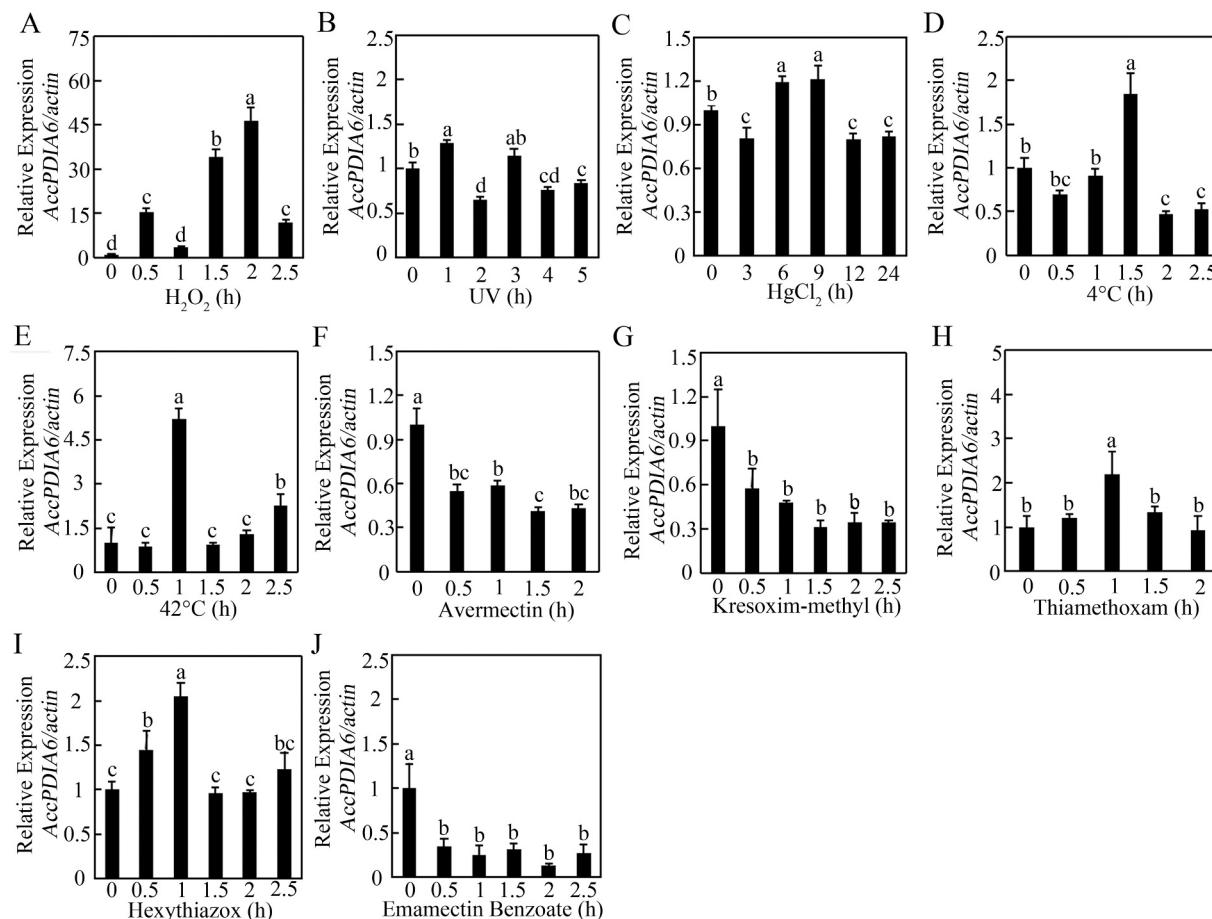


Fig. 3. Expression patterns at the mRNA level of *AccPDIA6* in *A. cerana cerana* under different abiotic conditions measured by qRT-PCR. Total RNA was extracted from adult worker honeybees sampled at the indicated times that were treated with conditions including H₂O₂ (A), UV (B), HgCl₂ (C), 4 °C (D), 42 °C (E), avermectin (F), kresoxim-methyl (G), thiamethoxam (H), hexythiazox (I) and emamectin benzoate (J). The data are presented as the mean ± SEM ($n = 3$). Different letters above the bar present significant differences ($P < 0.05$) based on Tukey tests.

peaked at 1 h for both of these pesticides (Fig. 3H–I). Following kresoxim-methyl and emamectin benzoate treatment, *AccPDIA6* expression levels were decreased (Fig. 3G and Fig. 3J).

To further confirm *AccPDIA6* expression at the protein level in response to oxidative stress, Western blot analysis was performed. Similar to the transcriptional expression level, the protein level of *AccPDIA6* was also upregulated (Fig. 4). These results indicate that *AccPDIA6* expression was upregulated by oxidative stress not only at the transcriptional level but also at the protein level, suggesting that *AccPDIA6* may have important functions in responding to oxidative stress.

3.4. Heterogeneous expression of *AccPDIA6* improved the antioxidant capacity of *E. coli* cells

To further explore whether *AccPDIA6* is involved in the response to oxidative stress, we tested the viability of *E. coli* under oxidative stress induced by H₂O₂ and high temperatures. As shown in Fig. 5A, no significant differences in cell growth were observed among cells expressing an empty vector or expressing *AccPDIA6* on LB agar without treatment. However, on the medium containing H₂O₂, there were substantially more colonies of *E. coli* expressing *AccPDIA6* than of *E. coli* expressing an empty vector. In addition, the number of *E. coli* cells transformed with the plasmid containing the *AccPDIA6* gene obviously increased in the culture medium at 42 °C. These results suggest that *AccPDIA6* confers powerful oxidative stress tolerance to cells.

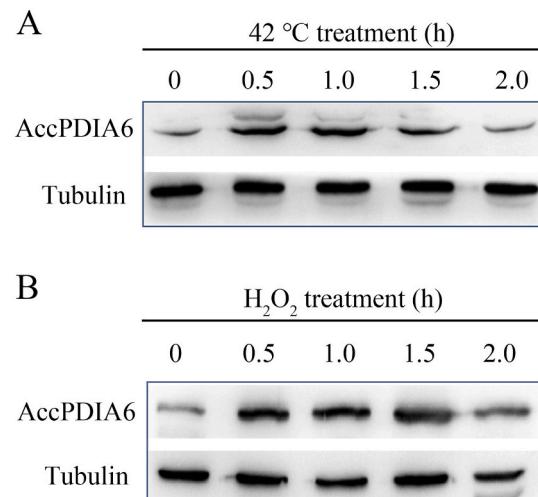


Fig. 4. Western blot analysis of *AccPDIA6* under H₂O₂ (A) and 42 °C (B) treatment conditions. Total protein was extracted from adult worker honeybees at the indicated time points. Tubulin was used as an internal control.

3.5. Defective mutations of cysteines of CGHC motifs of *AccPDIA6* almost lose the anti-stress activities for catalyzing function

Previous analysis showed that *AccPDIA6* has two redox-active TRX-

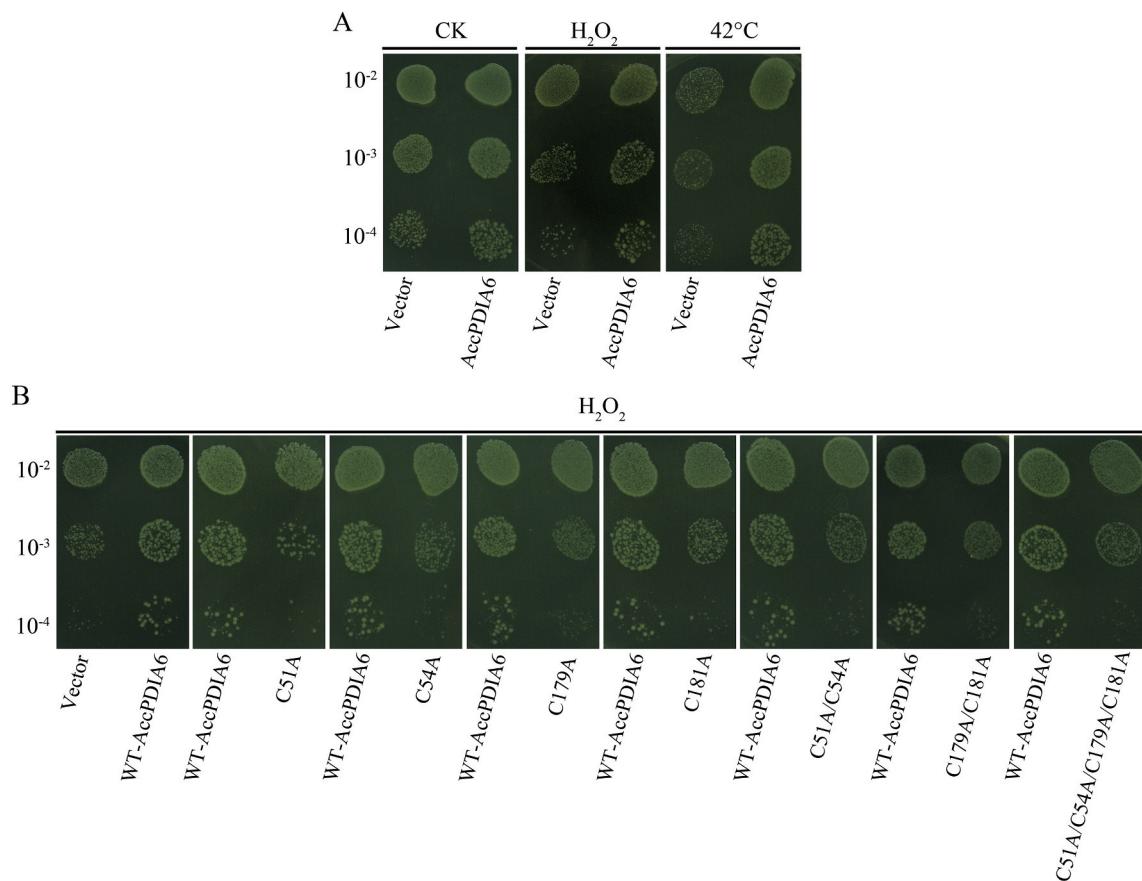


Fig. 5. Survival test of *E. coli* cells carrying AccPDIA6, defective AccPDIA6 or empty vector under H_2O_2 and $42^\circ C$ stress conditions. (A) Survival test of *E. coli* cells carrying AccPDIA6 or empty vector under H_2O_2 and $42^\circ C$ stress. Ten microliters of cultures from 10^{-2} to 10^{-4} dilutions were spotted on LB agar with or without H_2O_2 or placed in an incubator at either $37^\circ C$ or $42^\circ C$. (B) Survival test of *E. coli* cells carrying AccPDIA6, defective AccPDIA6 or empty vector under H_2O_2 stress conditions. Ten microliters of cultures from 10^{-2} to 10^{-4} dilutions were spotted on LB agar supplemented with or without H_2O_2 .

like domains and that each domain contains a typical CGHC motif. This motif has enzymatic activity (Bowley et al., 2017). In addition, Zhang (2018) reported that the two cysteine residues in CXXC motifs were pivotal for the catalytic function of PDI1 in *Arabidopsis thaliana* (AtPDI1). We assumed that the stress tolerance of AccPDIA6 against oxidative stress was related to the cysteines of the CXXC motifs. Therefore, we constructed cysteine-deficient mutants of AccPDIA6, including C51A, C54A, C179A, C181A, C51A/C54A, C179A/C181A and C51A/C54/C179A/C181A, and tested the viability of these mutants in *E. coli* under the condition of H_2O_2 . As shown in Fig. 5B, the colonies of *E. coli* carrying these defective cysteine mutations of AccPDIA6 were similar to those carrying the empty vector, whose numbers were fewer than those carrying wild-type AccPDIA6. In other words, defective mutations of cysteines of AccPDIA6 practically lose the anti-stress activity against H_2O_2 . These results confirmed our assumption that cysteines of CXXC motifs are indispensable for AccPDIA6 in response to oxidative stress.

3.6. Knockdown of AccPDIA6 influenced the transcription levels of antioxidant genes and enzymatic activities of antioxidants

To further investigate the effect of AccPDIA6 in *A. cerana cerana* on oxidative damage, we performed double-stranded RNA (dsRNA)-mediated RNAi to silence AccPDIA6. According to the qRT-PCR results, we found that the AccPDIA6 transcription level of the dsRNA-AccPDIA6-injected group was significantly lower than that of the control groups at 24 and 36 h after injection (Fig. 6A). These results proved that AccPDIA6 was silenced.

Then, we measured the transcriptional levels of several antioxidant genes at 36 h after injection. As shown in Fig. 6B–I, the expression levels of *AccGSTO2*, *AccTpx5*, *AccGSTs4* and *AccCYP4G11* were notably reduced, while the expression levels of *AccGSTD*, *AccCAT*, *AccTrx2* and *AccMsra* were significantly upregulated.

In addition, the enzymatic activities of antioxidants (catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD)) in the AccPDIA6 knockdown bees were analyzed. The activities of POD (Fig. 7A) and CAT (Fig. 7C) in dsRNA-AccPDIA6-injected bees were higher than those in control bees. However, for SOD (Fig. 7B), the activity was lower in dsRNA-AccPDIA6-injected bees than in control bees. In summary, the above evidence certified that AccPDIA6 may be of importance for *A. cerana cerana* in response to oxidative stress.

3.7. AccPDIA6 gene silencing reduced the survivability of *A. cerana cerana*

To investigate whether AccPDIA6 participated in the response to oxidative stress *in vivo*, we analyzed the survival of AccPDIA6-silenced bees exposed to oxidative stress at different time points. The control groups, the dsRNA-GFP-injected group and the uninjected group, had no noticeable differences in the survival when exposed to oxidative stress. The viability of the AccPDIA6-silenced group was distinctly lower than that of the control groups (Fig. 8). These results showed that AccPDIA6 may enhance the viability of *A. cerana cerana* in response to oxidative stress.

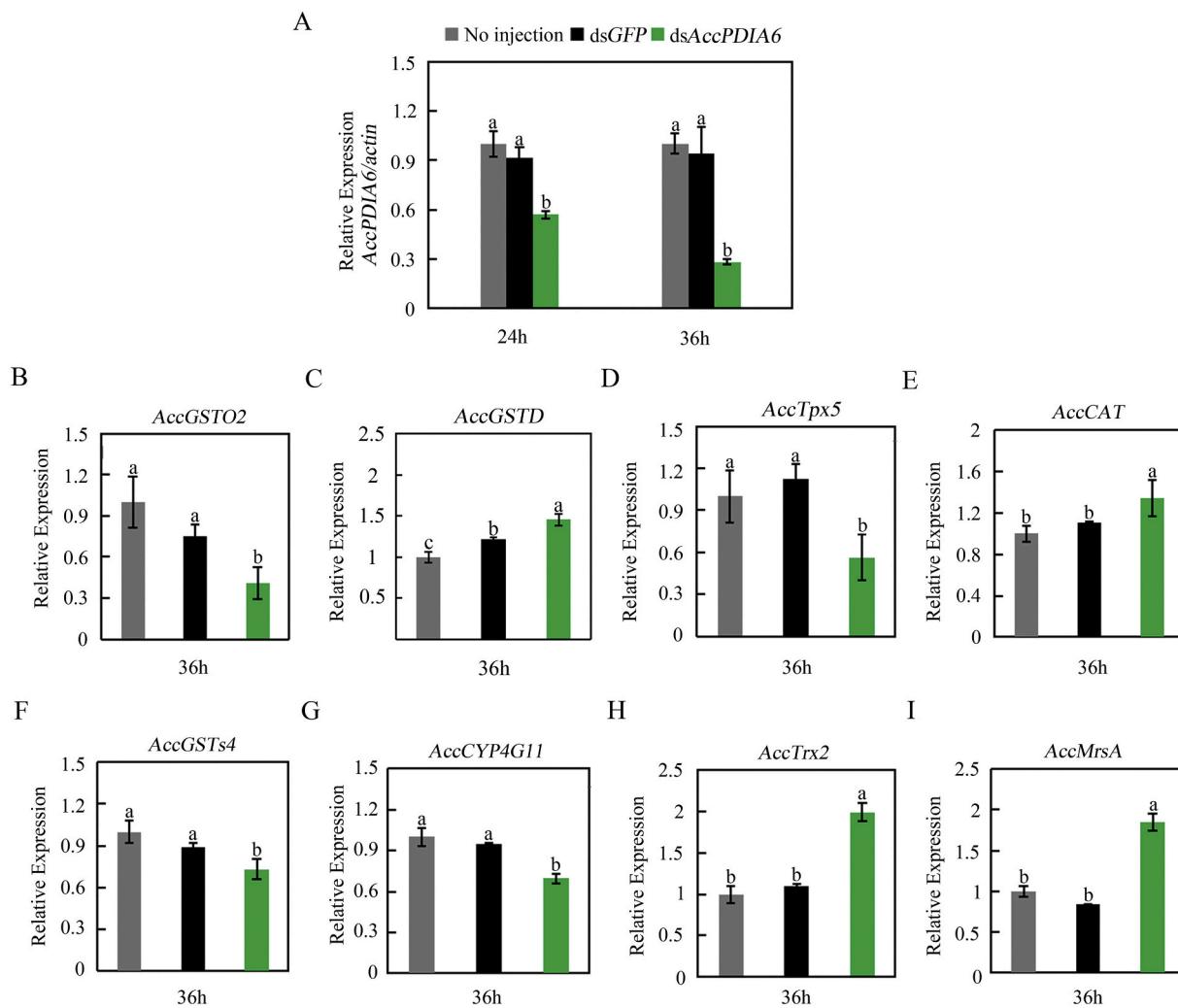


Fig. 6. Effects of *AccPDIA6* knockdown on other antioxidant genes. (A) Efficiency of RNAi on mRNA levels of *AccPDIA6* in *A. cerana cerana* at 24 and 36 h. Expression levels of other antioxidant genes (*AccGSTO2* (B), *AccGSTD* (C), *AccTpx5* (D), *AccCAT* (E), *AccGSTs4* (F), *AccCYP4G11* (G), *AccTrx2* (H), and *AccMsrA* (I)) following *AccPDIA6* knockdown in adult honeybees at 36 h. No injection: uninjected group; dsGFP: dsRNA-GFP-injected group; dsAccPDIA6: dsRNA-AccPDIA6-injected group. The values are expressed as the mean \pm SEM of three independent experiments. The different letters above the bar indicate significant differences based on Tukey tests ($P < 0.05$).

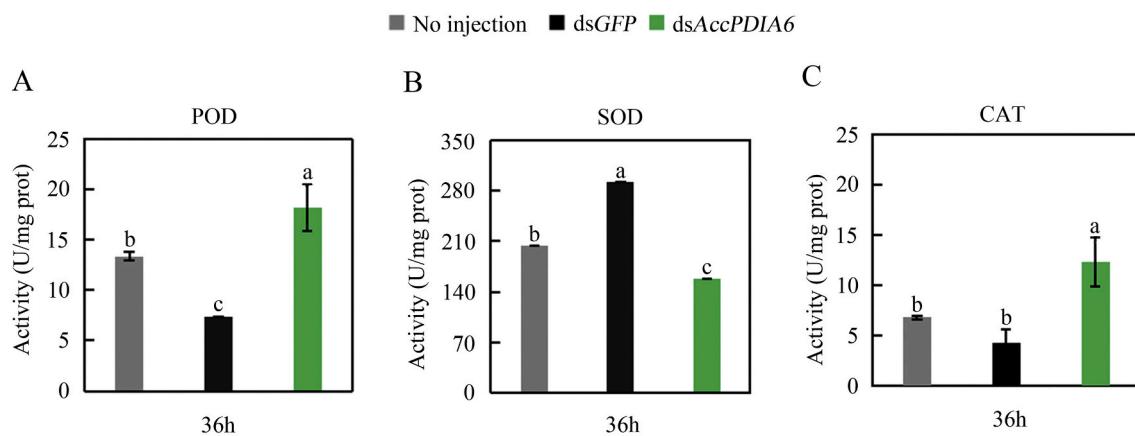


Fig. 7. Effects of *AccPDIA6* knockdown on other antioxidants. Enzymatic activity of the POD (A), SOD (B), and CAT (C) antioxidants following *AccPDIA6* knockdown in adult honeybees at 36 h.

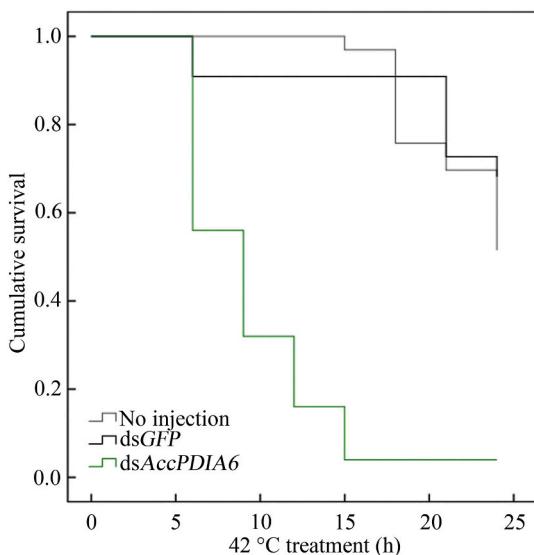


Fig. 8. Survival function of honeybees exposed to a temperature of 42 °C after the knockdown of AccPDIA6. No injection: uninjected group; dsGFP: dsRNA-GFP-injected group; dsAccPDIA6: dsRNA-AccPDIA6-injected group. The experiment was repeated three times with essentially identical results, and survival curves were compared using the log-rank (Mantel-Cox) test.

4. Discussion

PDIs have been widely studied in some mammals and are involved in regulating redox balance (Zeeshan et al., 2016; Lee and Lee, 2017) and proteostasis (Matsusaki et al., 2020). Recently, a report on PDIA6 implies that it has a possible function in redox signaling (Shen et al., 2018). However, there is no report about PDIA6 in insects. Here, we obtained and characterized *AccPDIA6* from *A. cerana cerana* for the first time, and our results imply its important antioxidant function.

We found that *AccPDIA6* was highly homologous to known PDIA6 proteins through sequence alignment (Fig. 1A), and using phylogenetic analysis, *AccPDIA6* was closer to AmPDIA6 than PDIA6 from other species (Fig. 1B). Importantly, like many other PDIs, *AccPDIA6* has two redox-active CGHC motifs, which are important for enzymatic activity. This result demonstrated that *AccPDIA6* is a member of the PDI family and may have similar functions to other family members.

Previous studies showed that PDIA6 of *Caenorhabditis elegans* is necessary during larval development (Eletto et al., 2014). To study whether *AccPDIA6* is related to development, the transcription levels of *AccPDIA6* were tested. The expression levels were higher in brown-eyed pupae, day-6 larvae, pink-eyed pupae and day-1 adults, suggesting that *AccPDIA6* may be involved in the development of honeybees. In addition, *AccPDIA6* was found to be expressed mostly in the MG and HE. Enayati et al. (2005) confirmed that the MG is the main metabolic center and participates in protecting the organism from oxidative damage. The HE has been reported to be highly sensitive to oxidative damage (Ament et al., 2008; Rival et al., 2004). Hence, *AccPDIA6* may be necessary and sufficient to protect the MG and HE from injury caused by oxidative stress.

Honeybees inevitably suffer from various adverse environmental conditions as described above. Recent studies have shown that PDIs are important in regulating redox balance and that PDIA6 is associated with the response to oxidative stress (Zeeshan et al., 2016; Lee and Lee, 2017; Laurindo et al., 2012). To explore whether *AccPDIA6* plays a part in resisting oxidative stress induced by these conditions, we tested its expression patterns at the transcriptional level in honeybees exposed to various adverse conditions. The results show that *AccPDIA6* was induced by some stresses, including H₂O₂, UV, HgCl₂, extreme temperatures (4 °C, 42 °C) and pesticides (thiamethoxam and hexythiazox), at

different levels. In contrast, *AccPDIA6* was reduced by some stresses, including avermectin, kresoxim-methyl and emamectin benzoate. These results determined that *AccPDIA6* may be a mediator of stressors and that other stress-related genes instead of *AccPDIA6* may play decisive roles in responding to these stresses (Zhai et al., 2018). Eletto et al. (2014) confirmed that PDIA6 controls the decay of IRE1α by interacting directly with it. In addition, the existence of IRE1α is not related to the duration and intensity of stress (Lin et al., 2007). These reports imply that *AccPDIA6*'s different responses to those stresses may be due to different treatment intensities and durations.

At the protein level, *AccPDIA6* was also induced by oxidative stress caused by high temperatures and H₂O₂. Previous research has shown that extreme temperatures can result in oxidative stress in organisms (An and Choi, 2010) and that the stress caused by temperature changes is related to the production of ROS and oxidative stress (Yan et al., 2012). PDIA6 has been reported to form a multimeric complex with heat shock protein member A2 (HSP2) and angiotensin-converting enzyme (ACE) in human spermatozoa, and its surface expression has been confirmed to be vulnerable to oxidative stress (Bromfield et al., 2016). t-BHP treatment of liver cells has been found to increase cell cytotoxicity as well as generate ROS (Shen et al., 2018). Shen (2018) also certified that t-BHP induced an apoptosis cascade and ER stress in hepatocytes by upregulating the expression of PDIA6. Therefore, *AccPDIA6* may be related to antioxidation. Furthermore, *AccPDIA6* was reported to inhibit oxidative stress-induced cell death (Shitara et al., 2012). To determine whether *AccPDIA6* affects the survival of cells exposed to oxidative stress, the viability of *E. coli* cells carrying *AccPDIA6* under oxidative stress conditions was tested. The results showed that *AccPDIA6* significantly enhanced the viability of *E. coli* exposed to H₂O₂ and high temperature. These results implied that *AccPDIA6* confers a powerful stress tolerance to cells against H₂O₂-induced oxidative stress and high temperature-induced oxidative stress.

In addition, it is well known that the CXXC motif is crucial for the catalytic activities of PDIs (Edman et al., 1985; Hawkins and Freedman, 1991). Eletto et al. (2014) reported that the activity of the C58A-C193A mutant of PDIA6 showed decreased activity and that a mutant of PDIA6 that lacked four cysteines of the two CXXC motifs showed no activity. In addition, Lee et al. (2014) reported that neither the C36S mutant nor the C171S mutants of PDIA6 bound to thioredoxin-interacting protein (TXNIP), and the mutation of the C-terminal cysteine residue of the CXXC motifs does not lead to abrogation of the interaction. These reports imply that cysteines of the N-terminus of the CXXC motifs are more important for the activity of PDIA6. However, in Zhang's (2018) study, cysteines of the C-terminal seemed to have no difference in function from those of the N-terminal. To study whether cysteines in the CGHC motifs are necessary for the enzymatic activity of *AccPDIA6* and whether cysteines of the N-terminus are more important than those of the C-terminus, we tested the viability of *E. coli* expressing mutants of *AccPDIA6*, including C51A, C54A, C179A, C181A, C51A/C54A, C179A/C181A and C51A/C54/C179A/C181A. Our results showed that defective mutations in cysteines of *AccPDIA6* almost lose the anti-stress activity, which suggests that CXXC motifs are also important for the activity of *AccPDIA6*. However, there were no significant differences among these mutants.

To prevent the oxidative damage, organisms have evolved various antioxidant systems, including the SOD, POD and CAT systems, and many antioxidation-related genes, including *AccGSTO2*, *AccGSTD*, *AccTpx5*, *AccCAT*, *AccGSTs4*, *AccCYP4G11*, *AccTrx2* and *AccMsrA* (Ma et al., 2018). Knockdown of *AccPDIA6* downregulated or upregulated the expression levels of some antioxidant genes. These results suggest that *AccPDIA6* may play an important role in oxidative stress. Our results also showed that the knockdown of *AccPDIA6* enhanced the enzymatic activity of SOD while inhibiting the enzymatic activity of POD and CAT. These results provided further evidence of the involvement of *AccPDIA6* in antioxidant processes. In addition, knockdown of *AccPDIA6* reduced the survival rate of honeybees exposed to oxidative

stress. This result further indicated that AccPDIA6 may protect honeybees from oxidative damage induced by high temperature *in vivo*.

In summary, we isolated the PDIA6 gene from *A. cerana cerana* (*AccPDIA6*) for the first time. We found that *AccPDIA6* was induced by oxidative stress at the mRNA and protein levels. *AccPDIA6* enhanced the viability of *E. coli* exposed to oxidative stress and the viability of honeybees. In addition, the cysteines in CGHC motifs were important for *AccPDIA6* to protect cells from oxidative damage.

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