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ORIGINAL PAPER

Identification of an inositol-3-phosphate synthase 1-B gene (*AccIPS1-B*) from *Apis cerana cerana* and its role in abiotic stress

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Abstract

Inositol phosphate synthase (IPS) is a rate-limiting enzyme in myo-inositol biosynthesis, which can regulate stress responses in plants and animals. However, there are few studies on the function of IPS in insects, especially in *Apis cerana cerana*. In this study, the inositol-3-phosphate synthase 1-B gene (*AccIPS1-B*) was isolated from *Apis cerana cerana*, and its connection to antioxidant defence was investigated. The open reading frame of *AccIPS1-B* was 1542 bp, encoding a 513 amino acid polypeptide. Quantitative real-time PCR analysis revealed that the expression level of *AccIPS1-B* was highest in pupae of *Apis cerana cerana*, and it was expressed at higher levels in the thorax than in other tissues tested. Moreover, the expression of *AccIPS1-B* was significantly upregulated by abiotic stresses. The recombinant AccIPS1-B also displayed significant tolerance to cumene hydroperoxide and $HgCl_2$. In addition, knockdown of *AccIPS1-B* significantly suppressed the expression of most of the antioxidant genes and decreased the antioxidant enzymatic activities of SOD, POD, and GST. Taken together, these findings indicate that *AccIPS1-B* may be involved in the response to antioxidant defence and development in *Apis cerana cerana*.

Keywords *Apis cerana cerana* · *AccIPS1-B* · Quantitative real-time PCR · Abiotic stress · Antioxidant defence

Introduction

Chinese honeybees, as pollinators, play important roles in ecological stability and agricultural development. However, the number of Chinese honeybees has decreased sharply because honeybees encounter environmental pollution and environmental changes in the process of honey gathering, flying, and drinking. Therefore, it is particularly important to the survival of Chinese honeybees to identify specific genes and proteins involved in the response to abiotic stress.

Inositol is the precursor for many inositol-containing compounds, such as signalling molecules, and plays important roles in many essential processes, including growth regulation, hormonal regulation, membrane trafficking, and signal transduction (Kaur et al. 2013; Tan et al. 2013). Therefore, regulation of inositol biosynthesis is crucial for the regulation of second messenger signalling. The de novo synthesis of inositol is catalysed by a series of enzymes in organisms. Among them, myo-inositol-1-phosphate synthase (MIPS or ino-1, EC 5.5.1.4) is the first and rate-limiting step enzyme that catalyses glucose-6-phosphate to myo-inositol-phosphate l (Frej et al. 2016; Smith and Major 2011). The first gene encoding MIPS to be identified was the yeast *INO1* (Culbertson et al. 1976; Donahue and Henry 1981). To date, many MIPS genes have been identified from many living organisms, including green algae, bacteria, higher plants, and animals. MIPS genes are highly conserved among eukaryotes (Majumder et al. 2003). The typical MIPS proteins are characterized by four highly conserved regions, including NAD^+-binding domains, putative catalytic domains, conserved region 1, and conserved region 2 (Frej et al. 2016).

It was reported that MIPS genes play a critical role in the responses to abiotic stress in organisms. For example, overexpression of the MIPS gene in plants could enhance various metabolites responsible for protecting plants from abiotic stress (Kusuda et al. 2015). In *Larimichthys crocea*, MIPS was involved in the response to cold stress (Li et al. 2018). In the gram-positive Actinobacteria *Corynebacterium glutamicum*, ino-1 played an important role in oxidative stress

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resistance (Chen et al. 2018). Moreover, deletion of the ino-1 gene led to a decrease in cell viability, an increase in ROS production, and the elevation of protein carbonylation levels under various stress conditions.

Given the important role of inositol in cellular functioning, surprisingly, little is known about MIPS in honeybees, particularly in *Apis cerana cerana*. In this study, *AccIPS1-B* was characterized from *Apis cerana cerana*, and the expression profiles of this gene were investigated. *AccIPS1-B* knockdown causes inositol depletion, which results in the downregulation of some ROS-related genes and the decrease in ROS-scavenging enzyme activities in response to oxidative stress. To our knowledge, this is the first study to examine the function of *AccIPS1-B* in response to abiotic stresses in honeybees.

Materials and methods

Insects and treatments

The Chinese honeybees, *Apis cerana cerana*, used in the following experiment were maintained at Shandong Agricultural University (Taian, China). Honeybees were generally divided into different developmental stages (larvae, pupae, and adults) according to age, eye colour, and shape (Michelette and Soares 1993). The whole bodies of the first to seventh larval instars (L1–L7), pupae (PP, prepupae; Pw, white eyes; Pp, pink eyes; Pb, brown eyes; Pd, dark eyes), and adults (A1, 1-day post-emergence workers; A15, 15-day post-emergence workers) were collected from the hive randomly and were kept in an incubator at a constant temperature (34 °C) with 70% relative humidity under a 24-h dark regimen. Then, the honeybees (A15) were divided into nine groups receiving different treatments, and each group consisted of 48 individuals. The honeybees in group 1 were injected with H_2O_2 (0.5 μL, final concentration of 2 mM) and treated for 0, 0.5, 1, 2, 3, and 4 h. Groups 2–4 were exposed to cold (4 °C), hot (44 °C), and UV radiation (30 mJ/cm^2 UV), respectively, for 0, 0.5, 1, 2, 3, and 4 h. The honeybees in groups 5 and 6 were fed $CdCl_2$ (3 mg/mL added to food) and $HgCl_2$ (0.5 mg/L added to food), respectively, for 0, 0.5, 1, 1.5, 2, and 3 h. Moreover, the honeybees in groups 7–9 were exposed to different pesticides (lambda cyhalothrin and emamectin benzoate) for 0, 1, 2, 3, 4, and 5 h. For the tissue-specific expression analysis, the honeybees (A15) were dissected into different tissue parts, including the epidermis (Ep), wing (Wi), honey sac (Hs), muscle (Ms), leg (Le), abdomen (Ab), thorax (Th), sting needle (Sn), and midgut (Mi). All samples were collected at a specified point in time and stored at − 80 °C for RNA and protein extraction. Each treatment was repeated at least three times.

RNA extraction and quantitative real-time PCR

Total RNA was extracted with RNAiso Plus (TaKaRa, Dalian, China), and cDNA was synthesized by purified RNA samples using the PrimeScript™ RT reagent kit with gDNA Eraser (Vazyme, Nanjing, China). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China). According to the supplier's instructions, the PCR mixture was as follows: 1.0 μL cDNA, 6.5 μL SYBR Premix Ex Taq, 0.3 μL of each primer (10 mM each), and 5.9 μL RNase-free water in a total volume of 15 μL. The qRT-PCR reaction conditions were as follows: initial denaturation at 95 °C for 30 s, 40 cycles (95 °C for 5 s, 55 °C for 15 s, and 72 °C for 15 s), and a single melt cycle. *β*-Actin (XM-640276) was used as the internal reference. The relative level of gene expression was automatically calculated by CFX Manager software (version 1.1) according to the $2^{-\Delta\Delta CT}$ method. All expression levels were tested with three biological replicates, and each reaction was repeated three times. All primers used in this study are listed in Table 1.

Protein expression

To obtain the AccIPS1-B recombinant protein, the open reading frame (ORF) of AccIPS1-B was amplified using the primers BD1/BD2 and then inserted into the expression vector pET-30a (+). Furthermore, pET-30a (+)-AccIPS1-B was transformed into *Escherichia coli* strain BL21 (DE3) for protein expression. The recombinant AccIPS1-B and pET-30a (+) *E. coli* cells were cultured at 28 °C with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). A 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of the AccIPS1-B recombinant protein, which was then stained using Coomassie brilliant blue.

Disc diffusion assay

To evaluate the tolerance of the recombinant AccIPS1-B protein under stress, disc diffusion assays were performed as described by Yan et al. (2013). pET-30a (+) was used as a control, and recombinant AccIPS1-B *E. coli* cells were plated on LB agar plates containing kanamycin and then incubated at 37 °C for 1 h. Next, filter discs (8-mm-diameter) soaked in different concentrations of cumene hydroperoxide (0, 10, 20, 50, or 100 mM) and $HgCl_2$ (0, 10, 20, 40, and 100 mM) were placed on the surface of the culture medium (Yan et al. 2013). Finally, the bacteria were cultivated at 37 °C for 24 h, and then the killing zones around the filter discs were measured three times from different angles. Every treatment was performed in triplicate.

Table 1 The primers used in this study

Primer name	Primer sequence (5′-3′)	Description
IZ1	ATGGCTTTGAAAATTCGCGTTC	cDNA primer of *AccIPS1-B*, forward
IZ2	TCATAATTGATTTTTGAAATTAACTTTG	cDNA primer of *AccIPS1-B*, reverse
β-s	GTTTTCCCATCTATCGTCGG	Standard control primer of qPCR, forward
β-x	TTTTCTCCATATCATCCCAG	Standard control primer of qPCR, reverse
BD1	GGATCCATGGCTTTGAAAATTCGCGTT BamHI	Primers of constructing expression vector, forward
BD2	GAGCTCTAATTGATTTTTGAAATTAACT SacI	Primers of constructing expression vector, reverse
IQ1	ACGGTGACACCTGTATCAAC	qPCR primer of *AccIPS1-B*, forward
IQ2	CTTTGTTTCCTTTGCCTAAT	qPCR primer of *AccIPS1-B*, reverse
IA1	TAATACGACTCACTATAGGGCGAGATTGTATTGAAGCACAGTACGA	RNAi primer of *AccIPS1-B*, forward
IA2	TAATACGACTCACTATAGGGCGATTGCACGTTGCATAGCATCAGCTA	RNAi primer of *AccIPS1-B*, reverse
GA1	TAATACGACTCACTATAGGGCGAGTCAGCGCTGGAGGGAGTT	RNAi primer of GFP, forward
GA2	TAATACGACTCACTATAGGGCGAGATCTCCGCATCTTCTAA	RNAi primer of GFP, reverse
AccSOD1-F	AAACTATTCAACTTCAAGGACC	qPCR primer of *AccSOD1*, forward
AccSOD1-R	CACAAGCAAGACGAGCACC	qPCR primer of *AccSOD1*, reverse
AccSOD2-F	TTGCCATTCAAGGTTCTGGTT	qPCR primer of *AccSOD2*, forward
AccSOD2-R	GCATGTTCCCAAACATCAATACC	qPCR primer of *AccSOD2*, reverse
AccGSTD-F	CGAAGGAGAAAACTATGTGGGCAG	qPCR primer of *AccGSTD*, forward
AccGSTD-R	CGTAATCCACCACCCACCTCTATCG	qPCR primer of *AccGSTD*, reverse
AccGSTO1-F	CCAGAAGTAAAAGGACAAGTTCGT	qPCR primer of *AccGSTO1*, forward
AccGSTO1-R	CCATTAACATCAACAAGTGCTGGT	qPCR primer of *AccGSTO1*, reverse
AccTpx3-F	CCTGCACCTGAATTTTCCGG	qPCR primer of *AccTpx3*, forward
AccTpx3-R	CTCGGTGTATTAGTCCATGC	qPCR primer of *AccTpx3*, reverse
AccP450-F	CGCAAAGAGAATGGGAAGG	qPCR primer of *AccP450*, forward
AccP450-R	CTTTTGTGTGACGGAGGTGC	qPCR primer of *AccP450*, reverse

Protein extraction and western blot

Total protein from different treatments was extracted from honeybees using a Tissue Protein Extraction Kit (Comwin Biotech, Beijing, China) and quantified using the BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). As described by Yan et al. (2013), AccIPS1-B antibody was obtained by injecting the AccIPS1-B recombinant protein into white mice at least four times, as described by Chen et al. (2015). Anti-α-tubulin antibody (Beyotime Biotechnology, Shanghai, China) was used as the control, and western blot was performed as previously described (Chen et al. 2015). All experiments were carried out three times.

RNA interference *AccIPS1-B*

Partial sequence of the coding region of AccIPS1-B was amplified by PCR with primers GA1/GA2, then was used for synthesized massively double-strand RNA *AccIPS1B* (dsRNA-AccIPS1-B) through RiboMAX T7 large-scale RNA production systems (Promega, Madison, WI, USA), and injected into honeybees. GFP (GenBank accession number: U87974) was used as a control because none of the genes in *Apis cerana cerana* share homology with *GFP*. The 15-day post-emergence adult workers were selected for RNA interference (RNAi) experiments and divided into three groups with 40 individuals in each group. Then, 8 μg dsRNA-AccIPS1-B or dsRNA-GFP was injected into each adult between the first and second abdominal segments using a microsyringe. The last group was not injected and was used as a control. qRT-PCR and western blotting were used to examine whether *AccIPS1-B* was silenced. Then, the silenced honeybees were collected at different time points (12, 24, 36, 48 h) and stored at − 80 °C until use.

Antioxidant enzyme activity analysis

Total protein of the silenced honeybees was extracted in 1 mL normal saline, and concentrations were quantified using a BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute). Next, the activities of the antioxidant enzymes superoxide dismutase (SOD), POD, and glutathione S-transferase (GST) were determined using SOD, POD, and GST kits (Nanjing Jiancheng Bioengineering Institute), respectively, following the instructions. All experiments were repeated three times.

Bioinformatic analysis

Many IPS genes and proteins were analysed using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The physical and chemical properties of *AccIPS1-B* were predicted by the ProtParam tool (http://www.expasy.ch/tools/protparam.html). An amino acid multiple alignment was conducted by DNAMAN (version 5.2.2). A phylogenetic tree was generated with the neighbour-joining method using MEGA 4.0 software. The protein tertiary structure was built and analysed with the SWISSMODEL software (https://swissmodel.expasy.org/). The putative *cis*-acting elements in the *AccIPS1-B* promoter region were predicted using TFBIND (http://tfbind.hgc.jp/). Statistical significance was

analysed using Duncan's multiple range tests with analysis of variance (ANOVA), and calculations were performed with SPSS Statistics. Significance was set at $P < 0.01$.

Results

Characterization of *AccIPS-1B*

The inositol-3-phosphate synthase 1-B gene was cloned by the primers IZ1/IZ2 from *Apis cerana cerana* and named *AccIPS1-B*. The full-length cDNA sequence of *AccIPS1-B* (GenBank Accession no: XM017065093.1) contains a 317-bp 5′UTR, a 228-bp 3′UTR, and a 1542-bp open reading frame (ORF). The ORF of *AccIPS1-B* encodes a 513 amino acid polypeptide with a predicted molecular mass of 57.14 kDa and a theoretical isoelectric point (p*I*) of 7.81. The AccIPS1-B protein contains four highly conserved regions, including NAD^+-binding domains (GWGGNNG), putative catalytic domains (SYNHLGNNDG), conserved region 1 (LWTANTERF), and conserved region 2 (NGSPQNTFVPG) (Fig. 1). The phylogenetic tree revealed that AccIPS1-B shares higher homology with AmIPS1-B and AfIPS1-B than with the other proteins (Fig. 2a). As shown in Fig. 3, the tertiary structure of the AccIPS1-B protein is similar to that of IPS in yeast and *Drosophila melanogaster*. To further analyse the gene structure of *AccIPS1-B*, a 3715-bp full-length sequence of DNA (GenBank accession number LOC108003032) was obtained from NCBI, which contained 5 introns and 6 exons. Mature mRNA of *AccIPS1-B* was spliced from six exons (Fig. 2b).

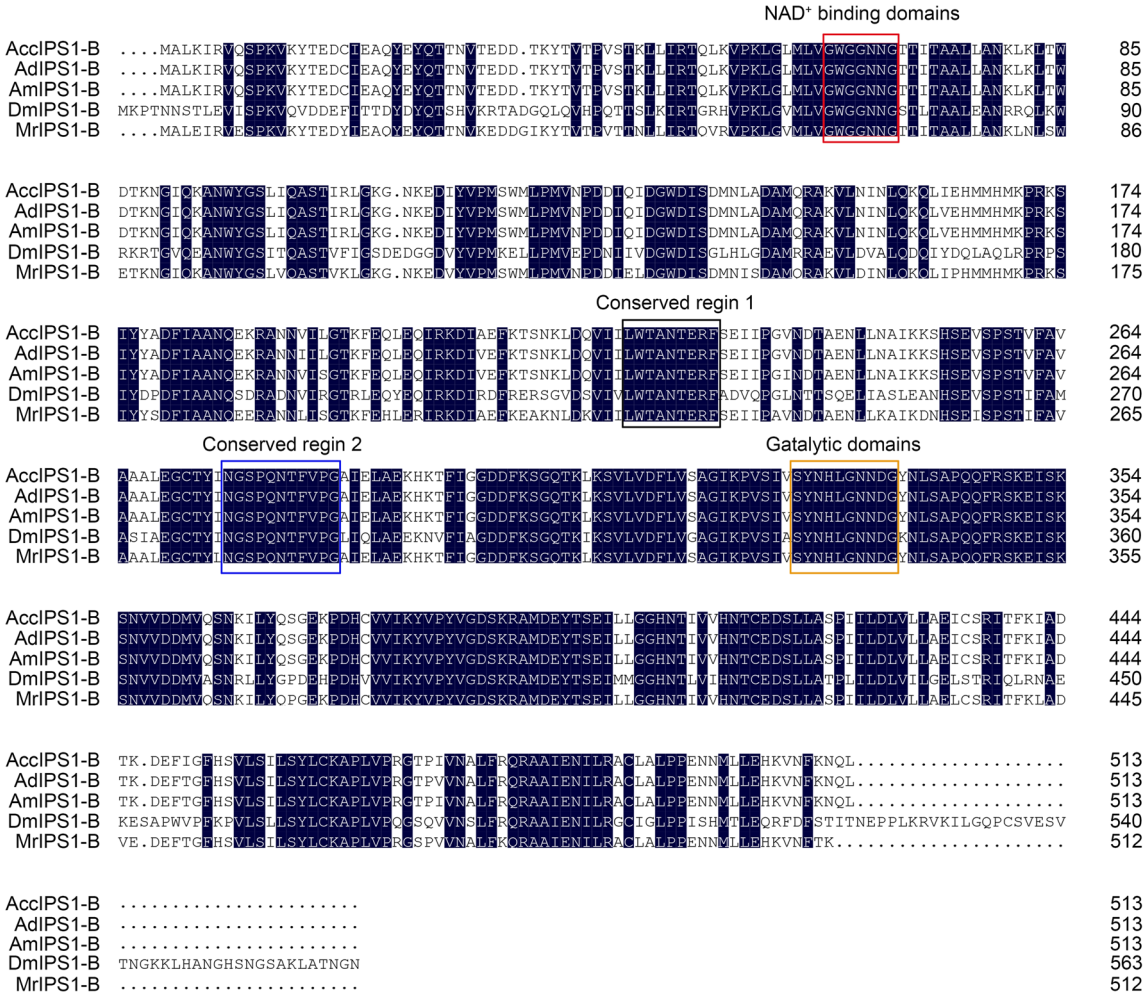

Fig. 1 Multiple alignment of AccIPS1-B with other known IPS1 genes. All species abbreviations and sequence IDs are as follows: AccIPS1-B (*Apis cerana cerana*, XP_016920582.1), *AdIPS1-B (Apis dorsata*, XP_006623674.1), AmIPS1-B (*Apis mellifera*, XP_623377.1), DmIPS1 (*Drosophila melanogaster*, NP_477405.1), MrIPS1-B (*Megachile rotundata*, XP_003706882.1). Identical regions are shaded in black markers, and the four conserved regions are marked with boxes, including NAD^+-binding domains, catalytic domains, conserved region 1, and conserved region 2

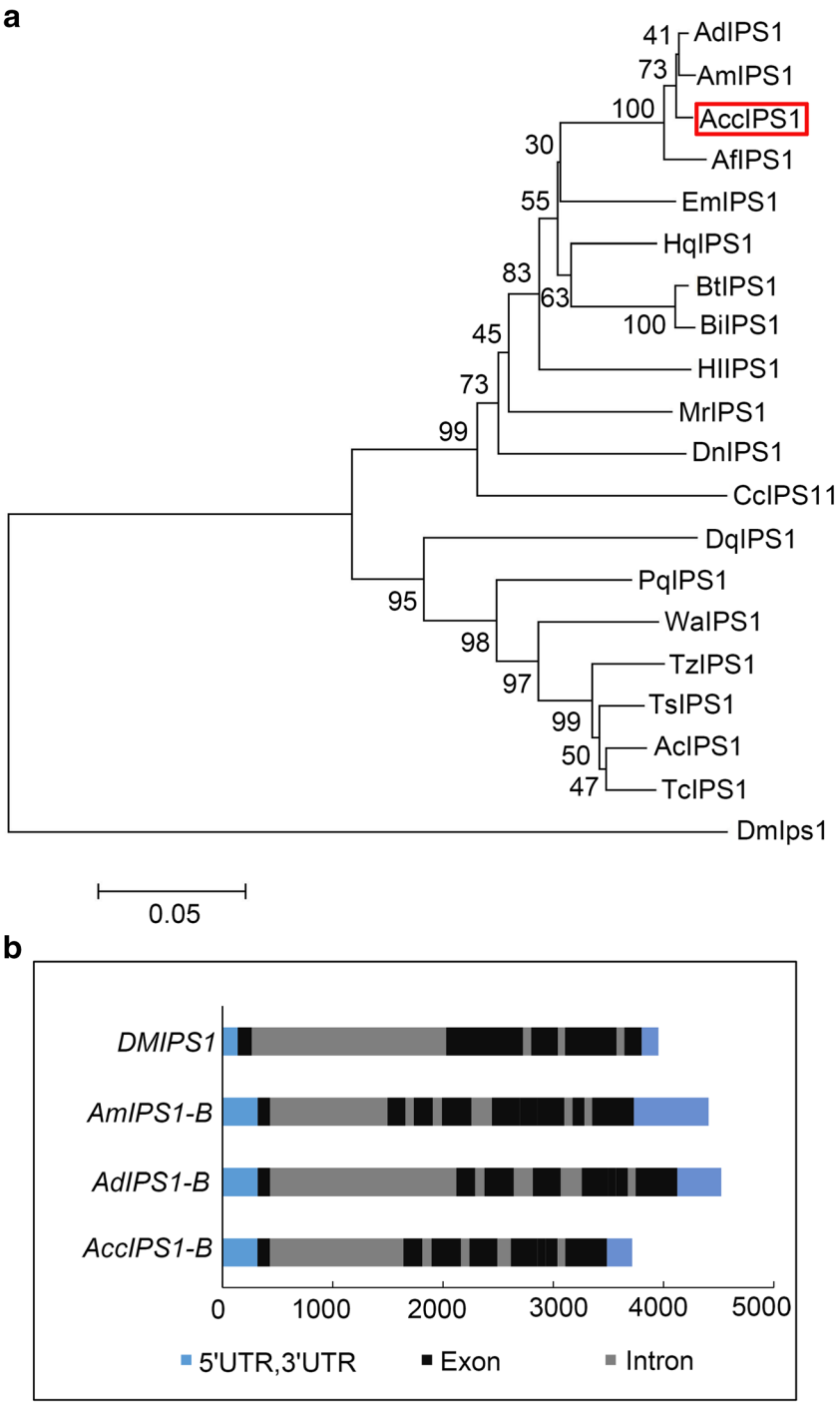

Fig. 2 Analysis of the biological information of IPS1. **a** A phylogenetic tree of IPS1 from different species. All species abbreviations and sequence IDs are as follows: AccIPS1-B (*Apis cerana cerana*, XP_016920582.1), *AdIPS1-B (Apis dorsata*, XP_006623674.1), AmIPS1-B (*Apis mellifera*, XP_623377.1), AfIPS1-B (*Apis florea*, XP_003692777.1), HqIPS1-B (*Melipona quadrifasciata*, KOX68055.1), BtIPS1-B (*Bombus terrestris*, XP_003403362.1), BiIPS1-B (*Bombus impatiens*, XP_003484397.1), DmIPS1 (*Drosophila melanogaster*, NP_477405.1), HlIPS1-A (*Habropoda laboriosa*, XP_017791627.1), MrIPS1-B (*Megachile rotundata*, XP_003706882.1), DnIPS1-B (*Dufourea novaeangliae*, XP_015431343.1), CcIPS1-A (*Ceratina calcarata*, XP_017885676.1), EmIPS1-A (*Eufriesea mexicana*, XP_017756426.1), PgIPS1-A (*Pseudomyrmex gracilis*, XP_020281034.1), WaIPS1-B (*Wasmannia auropunctata*, XP_011702940.1), TsIPS1-A (*Trachymyrmex septentrionalis*, XP_018347046.1), AcIPS1-A (*Atta cephalotes*, XP_012062538.1), TcIPS1-A (*Trachymyrmex cornetzi*, XP_018374519.1), TzIPS1-A (*Trachymyrmex zeteki*, XP_018314715.1), DqIPS1-B (*Dinoponera quadriceps*, XP_014474412.1). **b** Genomic structure of some IPS1 genes

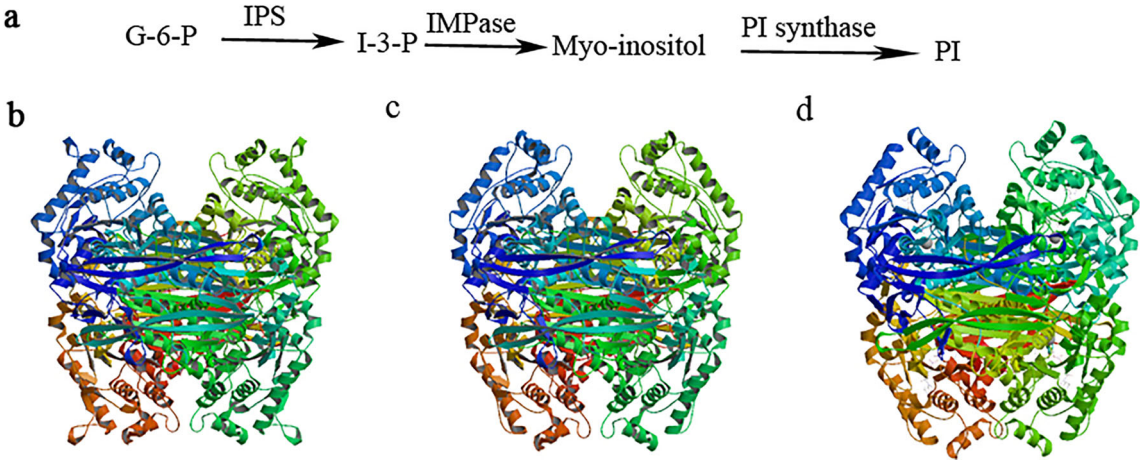

Fig. 3 **a** Three-dimensional structure and catalysis of IPS. De novo synthesis of inositol and PI. The abbreviations and full names of the substances and enzymes in the inositol synthesis are as follows: G-6-P (glucose-6-phosphate), IPS (inositol-3-phosphate synthase), IMPase (inositol monophosphatase), PI (inositol monophosphate). **b**–**d** The tertiary structure of the IPS1 protein in yeast, honeybee, and *Drosophila melanogaster*, respectively. The amino acid sequences of IPS in *Saccharomyces cerevisiae*, *Apis cerana cerana*, and *Drosophila melanogaster* were downloaded from the NCBI database, and their GenBank accession numbers are NP_012382.2, XM017065093.1, and NP_477405.1, respectively

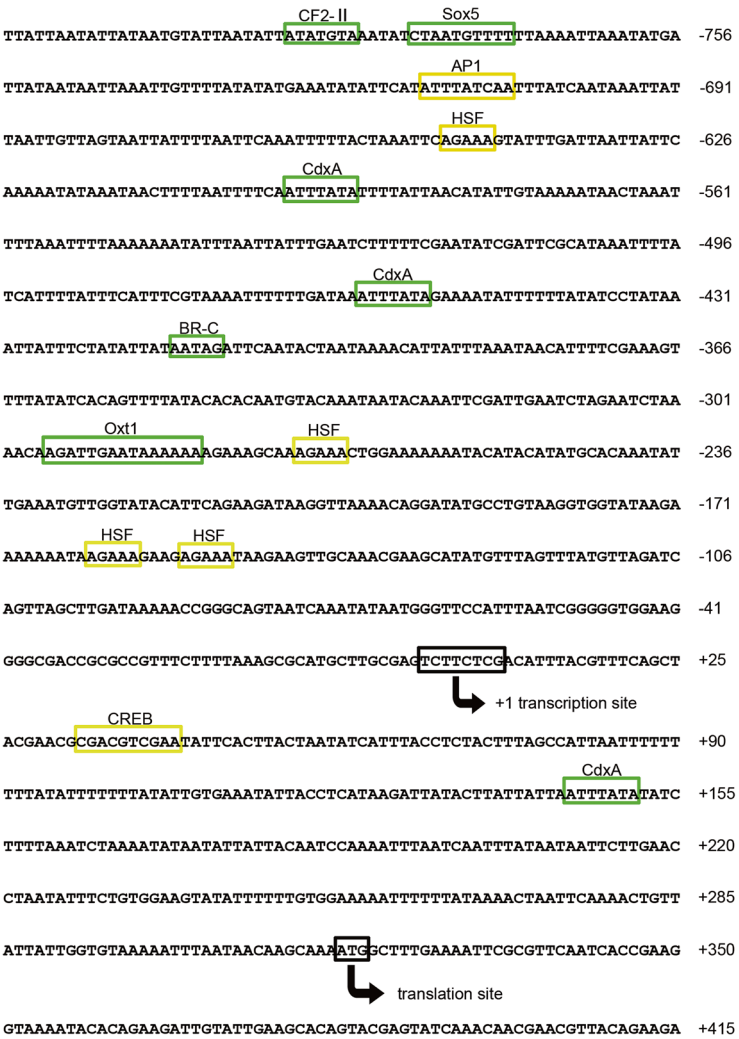

Fig. 4 Nucleotide sequence of the 5′-flanking region of *AccIPS1-B*. The translation and transcription start sites are marked with arrows, and the *cis*-acting elements are boxed. The *cis*-acting elements of environmental stress and immune response are marked with yellow boxes, and the *cis*-acting elements involved in tissue development and early growth stages are marked with green boxes

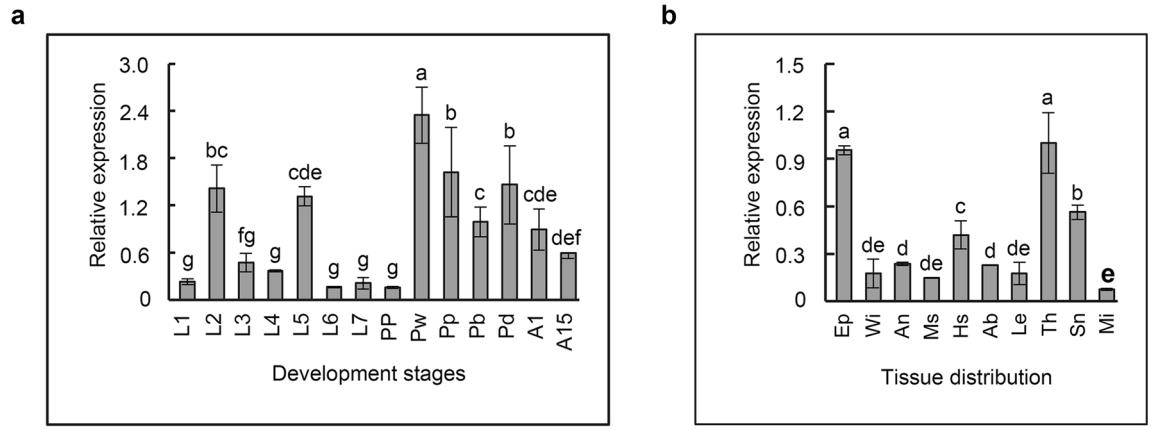

Fig. 5 The expression profile of *AccIPS1-B* at different developmental stages and in different tissues. **a** The expression levels of *AccIPS1-B* at different developmental stages. The honeybees were larvae (L1–L7, the first to seventh instars), pupae (PP, prepupae; Pw, white eyes; Pp, pink eyes; Pb, brown eyes; and Pd, dark eyes), and adults (A1, 1 day post-emergence; A15, 15 days post-emergence). **b** The expression levels of *AccIPS1-B* in different tissues, including epidermis (Ep), wing (Wi), honey sac (Hs), muscle (Ms), leg (Le), abdomen (Ab), thorax (Th), sting needle (Sn), and midgut (Mi). Vertical bars represent the means ± SEM ($n = 3$). Letters above the bars designate significant differences ($P < 0.01$)

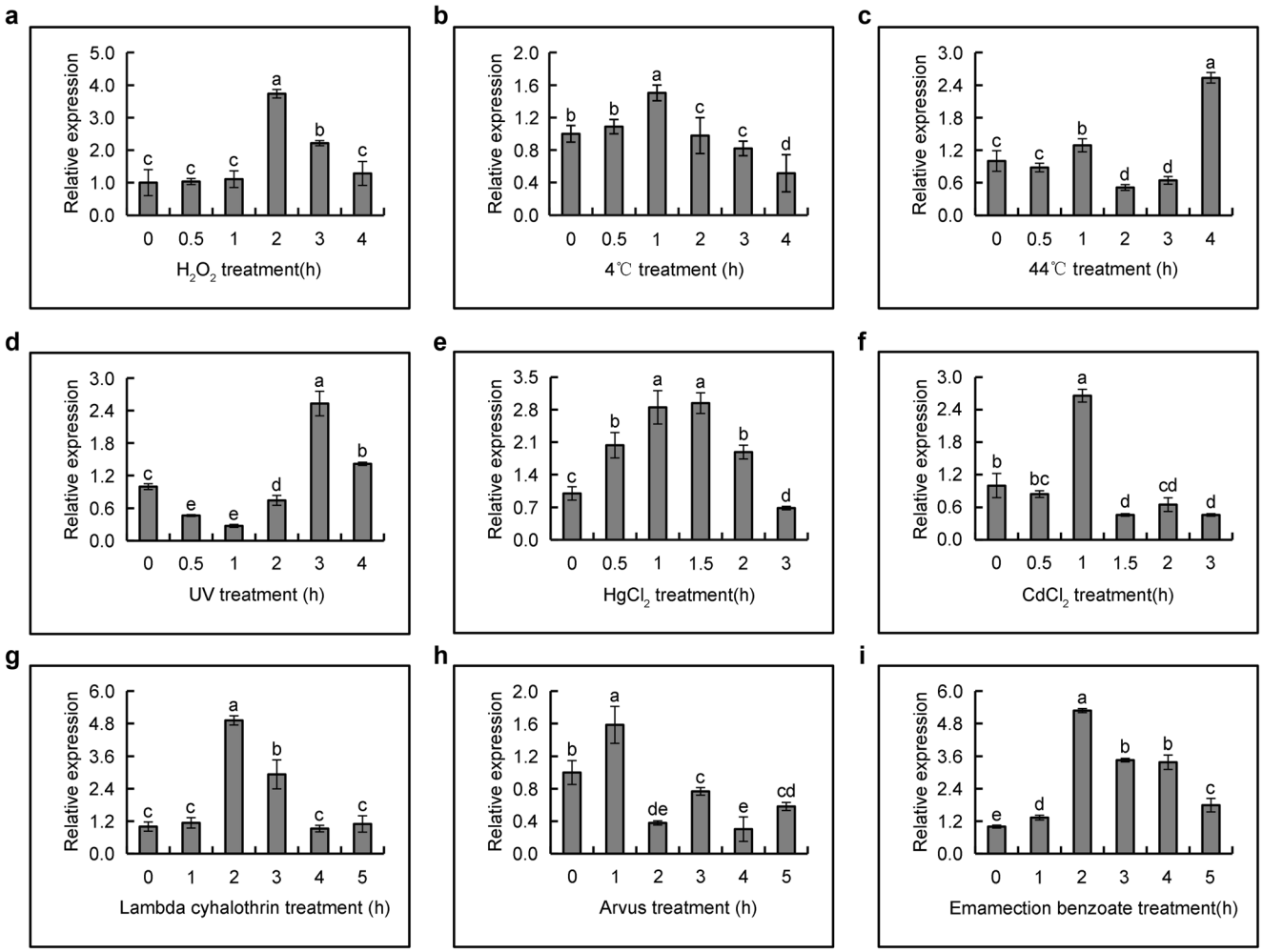

Fig. 6 The transcriptional expression profile of *AccIPS1-B* under different abiotic stresses. Total RNA was extracted from adult bees (15 days) at the specific time points and treated with H_2O_2 (**a**), 4 °C (**b**), 44 °C (**c**), UV (**d**), $HgCl_2$ (**e**), $CdCl_2$ (**f**), and different pesticides (**g**–**i**). β-Actin (GenBank accession number: HM640276) was used as an internal control. Vertical bars represent the means ± SEM ($n = 3$). Letters above the bars designate significant differences ($P < 0.01$)

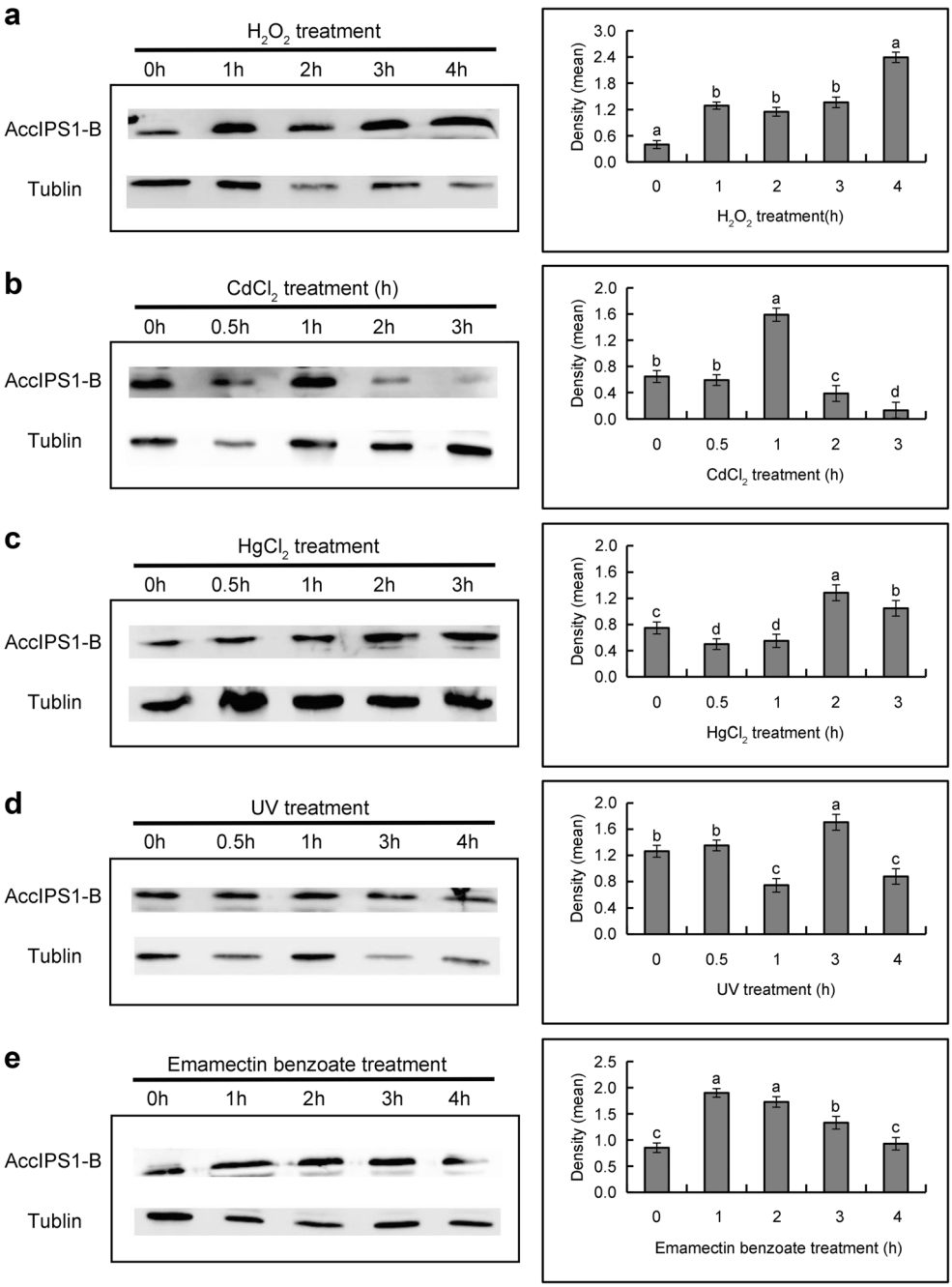

Fig. 7 The protein profile of AccIPS1-B under different abiotic stresses was obtained using western blots. Honeybees were exposed to the following treatments: H_2O_2 (**a**), $CdCl_2$ (**b**), $HgCl_2$ (**c**), UV (**d**), and emamectin benzoate (**e**). Anti-α-tubulin antibody was used as an internal control

Identification and analysis of the promoter region

The 821-bp promoter sequence was obtained from NCBI (gene symbol LOC108003032) to examine the transcription regulatory region of *AccIPS1-B* using the online software program TFBIND (http://tfbind.hgc.jp/). Many important transcription factor binding sites (TFBS) were identified (Fig. 4). Some transcription factors play important roles in environmental stress and the immune response, including heat-shock factors (HSFs), activating protein-1 (AP1), and cAMP response element binding protein (CREB). In addition, several transcription factors involved in tissue development, such as the caudal-related homeobox (CdxA) protein, cell factor 2-II (CF2-II), Oct-1, broad complex (BR-C), and Sox-5, were also detected in the promoter region of *AccIPS1-B*.

Temporal and spatial expression of *AccIPS1-B*

qRT-PCR was used to determine the expression profiles of *AccIPS1-B* during different developmental stages and in different tissues. As shown in Fig. 5a, *AccIPS1-B* was expressed at all developmental stages of *Apis cerana cerana*, and the highest expression level was found at the white eyes stage (Pw). Among the selected tissues, the highest expression of *AccIPS1-*

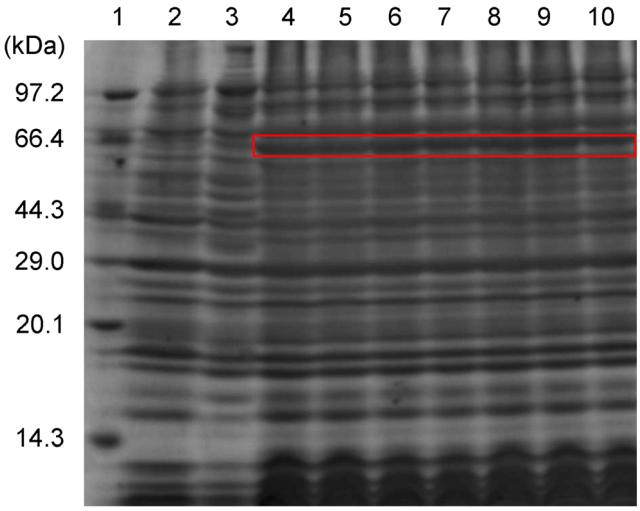

Fig. 8 The expression of the recombinant AccIPS1-B protein in *E. coli*. Recombinant AccIPS1-B was separated by SDS-PAGE. Lane 1, protein molecular weight marker; lane 2, induced expression of pET30a(+) in *E. coli* BL21; lane 3, uninduced expression of recombinant AccIPS1-B protein; lanes 4–6, expression of recombinant AccIPS1-B protein induced at 37 °C; lanes 7–10, expression of recombinant AccIPS1-B protein induced at 28 °C. The site of recombinant AccIPS1-B is marked with a red box

B was displayed in the thorax (Th), followed by the epidermis (Ep) (Fig. 5b). The above results indicated that *AccIPS1-B* might play an important role in the development of *Apis cerana cerana*.

Expression of *AccIPS1-B* under abiotic stresses

To elucidate the impact of *AccIPS1-B* on different abiotic stress responses, its expression profile was evaluated by qRT-PCR. In the present study, the test honeybees were exposed to a variety of adverse environmental stresses, such as cold (4 °C), heat (44 °C), heavy metals ($HgCl_2$ and $CdCl_2$), UV radiation, and pesticides, all of which may cause the formation of ROS. As shown in Fig. 6a, the expression of *AccIPS1-B* was significantly upregulated under H_2O_2 treatment and peaked after 2 h. The *AccIPS1-B* transcription dramatically increased after cold and hot treatment and reached a peak after 1 and 4 h, respectively (Fig. 6b, c). In addition, *AccIPS1-B* transcription increased significantly under UV treatment, and the expression peak was observed at 3 h (Fig. 6d). Following the $HgCl_2$ treatments, *AccIPS1-B* expression was significantly upregulated, with the highest levels observed at 1.5 h (Fig. 6e). As shown in Fig. 6f, *AccIPS1-B* expression under $CdCl_2$ treatment reached a peak after 1 h. Moreover, different pesticides also significantly increased *AccIPS1-B* expression levels (Fig. 6g–i). All of the above results suggest that *AccIPS1-B* might be involved in responses to various abiotic stresses.

To further examine *AccIPS1-B* expression under different types of abiotic stresses, western blot analysis was performed as previously described (Chen et al. 2015). Following exposure of bees to UV, H_2O_2, $CdCl_2$, $HgCl_2$, emamectin benzoate, and lambda cyhalothrin, samples were collected to detect *AccIPS1-B* expression using anti-*AccIPS1-B*. The results showed that *AccIPS1-B* expression was significantly induced under all of the abovementioned treatments (Fig. 7a–e), which indicates that the expression levels of the *AccIPS1-B* protein and mRNA were consistent.

Characterization of recombinant AccIPS1-B protein

AccIPS1-B was overexpressed in *E. coli* with several histidine tags after IPTG induction. The recombinant AccIPS1-B protein was separated by SDS-PAGE and showed an apparent molecular mass of 64.17 kDa (containing His-tag) (Fig. 8), which is consistent with the predicted result from DNAMAN.

To assess the ability of AccIPS1-B to resist oxidative stress, a disc diffusion test was performed with methods similar to those in a previous report (Jia et al. 2017). Compared with plates containing control bacteria, the halo diameters of the death zones on the plates containing *E. coli* overexpressing *AccIPS1-B* were significantly smaller after exposure to different concentrations of cumene hydroperoxide and $HgCl_2$ (Fig. 9). This result indicated that overexpression of *AccIPS1-B* can improve the tolerance of *E. coli* to oxidative stress.

Knockdown of *AccIPS1-B*

To further confirm the function of *AccIPS1-B*, *AccIPS1-B* was knocked down using the RNAi technique. Fifteen-day post-emergence adult workers were microinjected with dsRNA of GFP or *AccIPS1-B*. The qRT-PCR results showed that the transcript levels of *AccIPS1-B* after dsAccIPS1-B injection significantly decreased compared to those after dsGFP injection or those with no injection, specifically at 12 h and 36 h (Fig. 10a). Then, honeybees microinjected with *dsAccIPS1-B* at 12 h and 36 h were selected for protein extraction and western blot analysis. As shown in Fig. 10b, the protein expression levels of AccIPS1-B at 12 and 36 h after injection were clearly lower than those of the control groups. Thus, the results suggest that *AccIPS1-B* was successfully silenced.

Response of *AccIPS1-B*-silenced honeybees to oxidative stress

To further explore the impact of *AccIPS1-B* silencing, the transcript levels of various antioxidant genes, including *AccSOD1*, *AccSOD2*, *AccGSTD*, *AccGSTO1*, *AccTpx3*, and *AccP450*, were determined using qRT-PCR at 12

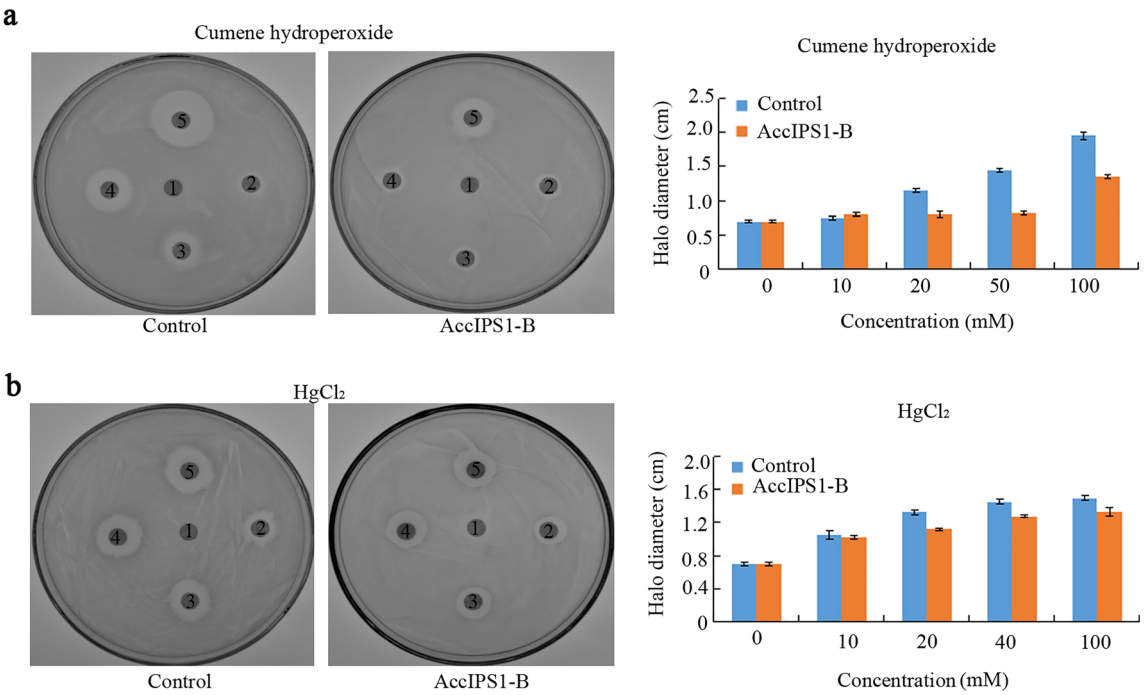

Fig. 9 Resistance of *E. coli* cells overexpressing *AccIPS1-B* to cumene hydroperoxide and $HgCl_2$. LB agar plates were inoculated with 5×10^8 cells. *E. coli* cells were transfected with a plasmid to overexpress *AccIPS1*-B; bacteria transfected with empty pET-30a (+) vector were used as the negative controls. **a** The diameter (1, 2, 3, 4, 5) after soaking in different concentrations of cumene hydroperoxide (0, 10, 20, 50, or 100 mM). **b** The concentrations of $HgCl_2$ were 0, 10, 20, 40, and 100 mM, respectively. After overnight exposure, the diameters of the death zones surrounding the drug-soaked filters were measured. The data were presented in three independent experiments

and 36 h after *dsAccIPS1-B* injection. In addition, the activities of antioxidant enzymes (including SOD, POD, and GST) were measured. As shown in Fig. 11, the transcript levels of various antioxidant genes were significantly lower than those of the control groups. Moreover, the SOD, POD, and GST activities were significantly decreased in *AccIPS1-B*-silenced honeybees (Fig. 12). The results clearly suggested that AccIPS1-B may help to prevent oxidative damage to proteins and membranes that is commonly caused by abiotic stresses.

Discussion

IPS catalyses the rate-limiting step in the de novo synthesis of inositol, which plays a critical role in the growth and survival of organisms (Frej et al. 2016; Seelan et al. 2011; Li et al. 2016). However, few studies of IPS have been systematically conducted in insects, especially in *Apis cerana cerana*. In the present study, *AccIPS1-B* was isolated from *Apis cerana cerana*, and its response to different oxidative stresses was investigated. In addition, we also examined the expression

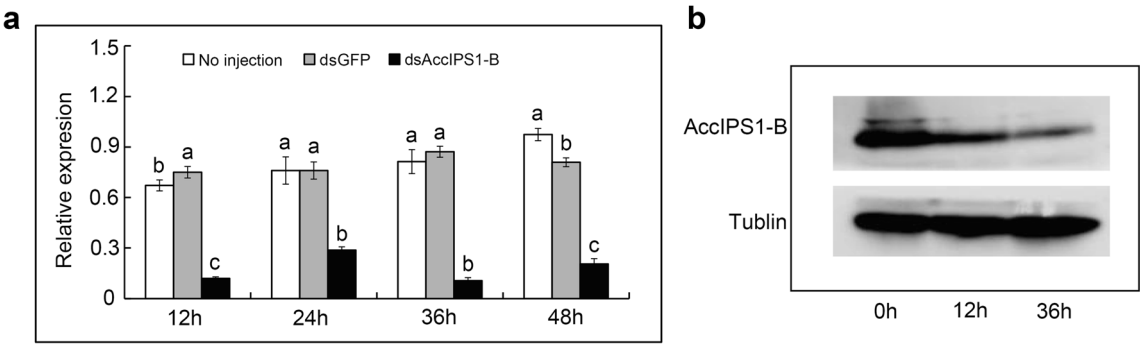

Fig. 10 Effects of *AccIPS1-B* RNAi in *A. cerana cerana* adults. **a** The transcription levels of *AccIPS1-B* after RNA interference in adult honeybees (15-day post-emergence workers). **b** Western blot analysis of the AccIPS1-B protein in control and *AccIPS1-B*-silenced honeybees. The data presented are from three independent experiments. Vertical bars represent the means ± SEM ($n = 3$). Letters above the bars designate significant differences ($P < 0.01$)

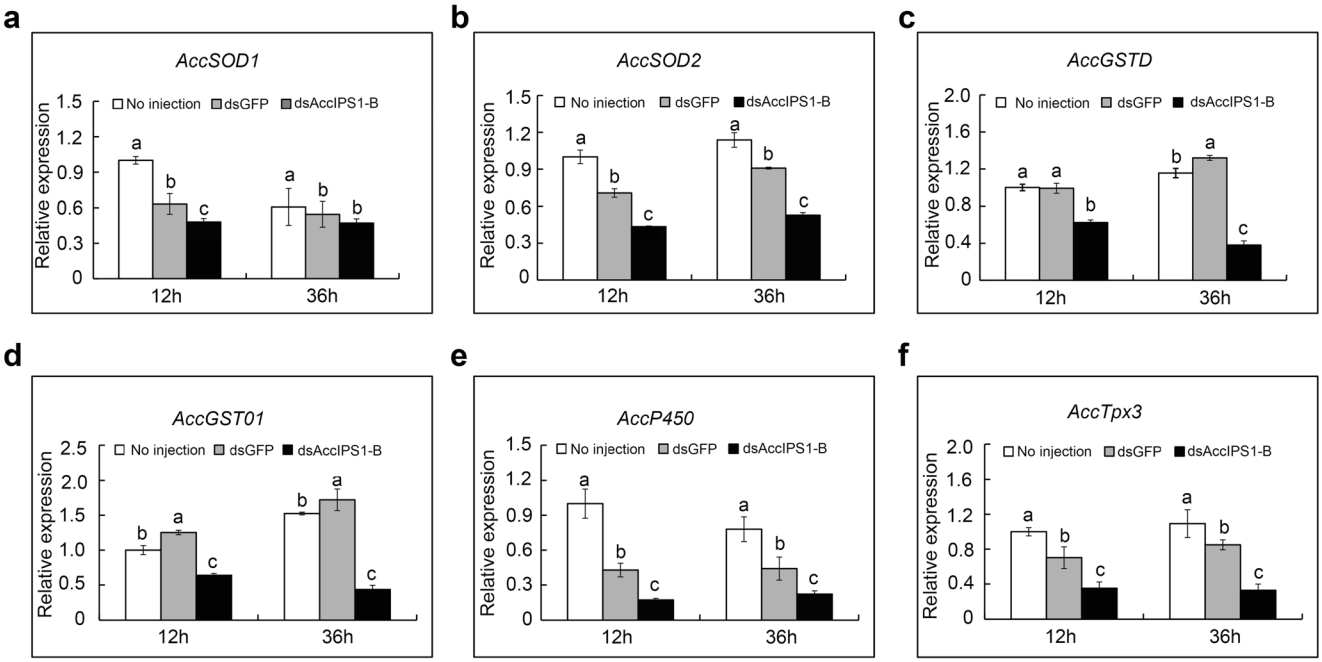

Fig. 11 Expression patterns of antioxidant genes after *AccIPS1-B* knockdown. **a** *AccSOD1* (GenBank ID: JN700517). **b** *AccSOD2* (GenBank ID: JN637476). **c** *AccGSTD* (GenBank ID: JF798572). **d** *AccGSTO1* (GenBank ID: KF496073). **e** *AccP450* (GenBank ID: KC243984). **f** *AccTPX3* (GenBank ID: JX456217). Vertical bars represent the means ± SEM ($n = 3$). Letters above the bars designate significant differences ($P < 0.01$)

levels of some antioxidant genes and the activities of several antioxidant enzymes in *AccIPS1-B*-silenced honeybees. According to the results, we speculate that *AccIPS1-B* might play important roles in the oxidative stress response in Chinese honeybees.

IPS1 proteins of different species contain four highly conserved regions, including NAD^+-binding domains (GWGGNNG), putative catalytic domains (SYNHLGNNDG), conserved region 1 (LWTANTERF), and conserved region 2 (NGSPQNTFVPG) (Frej et al. 2016). In this study, sequence alignment analysis revealed that the AccIPS1-B protein also harboured the four typically conserved domains (Fig. 1a), which suggested that they may have the same functions. In addition, our data showed that AccIPS1-B has the same Ser residue as IPS of other species, including S277 and S355 in NGSPQN and SKSNV, which may relate to the regulation of inositol synthesis (Parthasarathy et al. 2013; Zhai et al. 2016; Deranieh et al. 2013). The phylogenetic tree revealed that AccIPS1-B shared higher homology with AmIPS1-B and AfIPS1-B than with the other proteins (Fig. 2a).

It has previously been reported that IPS is involved in growth and has tissue-specific expression (Kuwano et al. 2010; Luo et al. 2011). In the present study, some predicted transcription factor binding sites related to growth and development, such as CdxA, CF2-II, Oct-1, BR-C, and Sox-5, were found in the 5′ flanking region of AccIPS1-B. Tissue-specific expression analysis indicated that *AccIPS1-B* was comparatively highly expressed in the epidermis and thorax (Fig. 5b). Previous studies suggested that the epidermis is the first place to resist the adverse external environment and that the thorax is the major movement centre and is required for collecting pollen and clearing combs (Meng et al. 2014; Von

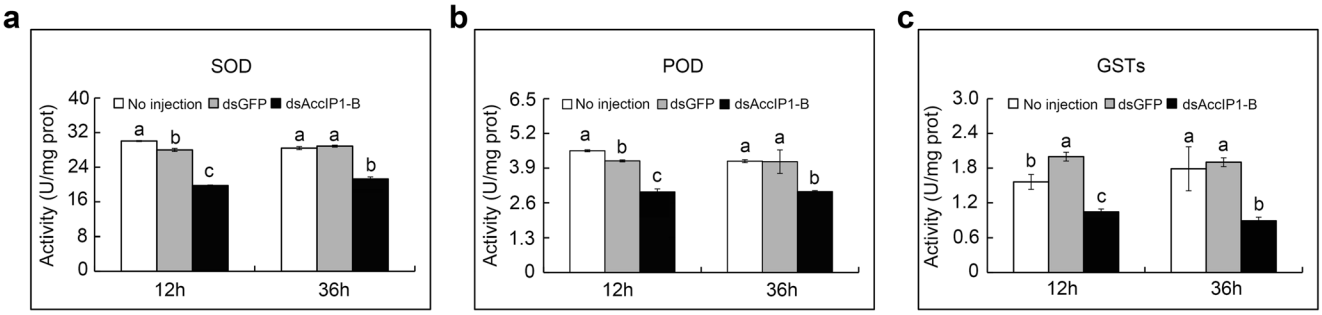

Fig. 12 The antioxidant enzymatic activities of SOD (**a**), POD (**b**), and GST (**c**) in *AccIPS1-B*-silenced honeybees compared to the GFP control. The data presented are from three independent experiments. Letters above the bars designate significant differences ($P < 0.01$)

et al. 1994). Thus, the specific expression pattern of *AccIPS1-B* in different tissues may reflect the unique demands of the specific tissue. In addition, the expression of *AccIPS1-B* was higher in the pupae than in larvae and adults during all development stages, which indicates that AccIPS1-B might play an important role in the early developmental stages.

Honeybees usually suffer from different environmental stresses in their lifetime, including temperature, ultraviolet radiation, heavy metals, and pesticides, all of which may induce the formation of reactive oxygen species (Jia et al. 2014; Lushchak 2010). A previous study indicated that MIPS1 plays important roles in resistance to different environmental stresses (Butzin et al. 2013; Huang and Hernick 2015; Ju et al. 2004). However, the function of *AccIPS1-B* in response to environmental stresses remains unclear in *Apis cerana cerana*. In this study, some transcription factors involved in environmental stress and the immune response, including HSFs, AP1, and CREB, were identified from the promoter region of *AccIPS1-B*, which suggested that *AccIPS1-B* may have the potential to respond to oxidative stress. Furthermore, qRT-PCR and western blot results showed that the expression of *AccIPS1-B* could be induced by cold, heat, heavy metals, ultraviolet radiation, and pesticides (Fig. 6). In addition, analysis of the bacteriostatic experiment showed that overexpression of *AccIPS1-B* in *E. coli* protected these cells from oxidative stressors, such as $HgCl_2$ and H_2O_2 (Fig. 9). These results suggest that *AccIPS1-B* may be involved in resisting oxidative stress.

High ROS concentrations in cells can lead to oxidative damage to nucleic acids, proteins, and lipids (Cadet et al. 2005; Kottuparambil et al. 2012; Ravanat et al. 2001). Cells possess a variety of antioxidant enzymes, including superoxide dismutases (SODs), glutathione S-transferases (GSTs), catalase (CAT), and thioredoxin peroxidase (Tpx), to scavenge ROS (Jia et al. 2014; Tang et al. 2012; Yu et al. 2011). A previous study reported that deletion of the ino-1 gene in the gram-positive Actinobacteria *Corynebacterium glutamicum* resulted in a decrease in cell viability and an increase in ROS production under various abiotic stresses (Chen et al. 2018), which indicates that IPS may take part in the resistance to ROS damage. In this study, we found that the transcript levels of various antioxidant genes and the enzyme activities of SOD, POD, and GST were significantly decreased when *AccIPS1-B* was knocked down (Fig. 12). Thus, we speculate that *AccIPS1-B* may play a critical role in scavenging ROS when *Apis cerana cerana* encounters different environmental stresses.

In conclusion, we have identified *AccIPS1-B* from *Apis cerana cerana*, which possesses the conserved domains of IPS1. The expression pattern of *AccIPS1-B* under different environmental stresses indicated that *AccIPS1-B* might participate in the response to antioxidant defence in honeybees. The recombinant AccIPS1-B also enhanced the tolerance to environmental stresses. Finally, the knockdown of *AccIPS1-B* significantly suppressed the expression of most of the antioxidant genes and decreased the antioxidant enzymatic activities. These findings unequivocally indicated that *AccIPS1-B* may be involved in the antioxidant defence response.

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