Cell Stress and Chaperones
https://doi.org/10.1007/s12192-018-0920-3

ORIGINAL PAPER

Characteristics of *AccSTIP1* in *Apis cerana cerana* and its role during oxidative stress responses

Na Zhai[1] · Haihong Jia[1] · Manli Ma[1] · Yuzhen Chao[1] · Xingqi Guo[1] · Han Li[1]

Received: 4 September 2017 / Revised: 21 May 2018 / Accepted: 24 May 2018

Abstract

Various environmental stresses, such as heat shock, heavy metals, ultraviolet (UV) radiation and different pesticides, induce a cellular oxidative stress response. The cellular oxidative stress response is usually regulated by heat shock proteins (Hsps) acting as molecular chaperones. Stress-induced phosphoprotein 1 (STIP1), one of the most widely studied co-chaperones, functions as an adaptor that directs Hsp90 to Hsp70-client protein complexes. However, the biological functions of STIP1 remain poorly understood in honeybee (*Apis cerana cerana*). In this study, *AccSTIP1* was identified in *Apis cerana cerana*. *AccSTIP1* transcription was found to be induced by heat (42 °C), $HgCl_2$, H_2O_2 and different pesticides (emamectin benzoate, thiamethoxam, hexythiazox and paraquat) and inhibited by $CdCl_2$, UV and kresoxim-methyl. Moreover, western blot analysis indicated that the expression profiles of AccSTIP1 were consistent with its transcriptional expression levels. The disc diffusion assay showed that chemically competent transetta (DE3) bacteria expressing a recombinant AccSTIP1 protein displayed the smaller death zones than did control bacteria after exposure to paraquat and $HgCl_2$. The DNA nicking assay suggested that recombinant purified AccSTIP1 protected supercoiled pUC19 plasmid DNA from damage caused by a thiol-dependent mixed-function oxidation (MFO) system. After knocking down *AccSTIP1* gene expression via RNA interference (RNAi), the transcript levels of antioxidation-related genes were obviously lower in ds*AccSTIP1* honeybees compared with those in the uninjected honeybees. Collectively, these results demonstrated that *AccSTIP1* plays an important role in counteracting oxidative stress. This study lays a foundation for revealing the mechanism of *AccSTIP1* in the *Apis cerana cerana* antioxidant system.

Keywords *Apis cerana cerana* · Stress-induced phosphoprotein 1 · Oxidative stress · RNA interference · Abiotic stresses

Introduction

A significant question in biology is how organisms cope with various environmental stresses such as heat shock, oxidative stress, heavy metals and toxins and induce cellular stress responses (Song et al. 2009). It has become clear that organisms possess a common molecular response that includes a dramatic change in their gene expression profiles and elevated synthesis of a family of heat shock or stress-induced proteins (Morimoto 1993).

Various environmental stresses, such as heat shock, oxidative stress, heavy metals and toxins, lead to prior transcription and translation of heat shock proteins (Hsps); this heat shock response is one of the most highly conserved defence mechanisms for coping with stressful conditions (Song et al. 2009). Stress-inducible protein 1 (Stip1), a homologue of stress-induced phosphoprotein 1 (STIP1), is a co-chaperone protein that plays significant roles in response to stress and non-stress conditions (Wang et al. 2015). Stip1, one of the most widely studied co-chaperones, functions mainly as an adaptor protein that can synchronously bind to and transfer client proteins from Hsp70 and Hsp90 (Song et al. 2009). The stress-inducible protein 1 (STI1), a homologue of STIP1, was first identified in *Saccharomyces cerevisiae* in a search for transacting factors that induce heat shock gene expression (Nicolet and Craig 1989). Stip1 homologues have since been studied in various organisms, such as humans, mice, rats,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12192-018-0920-3) contains supplementary material, which is available to authorized users.

✉ Han Li
lihan@sdau.edu.cn

[1] State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong 271018, People's Republic of China

Published online: 20 August 2018

insects, yeast, plants, parasites and even viruses, indicating the broad biological importance of this protein (Wang et al. 2015).

Organisms constantly exhibit oxidative stress responses when they are exposed to various environmental stresses (such as heat shock, heavy metals, ultraviolet (UV) radiation or pesticides), which can lead to increased reactive oxygen species (ROS) level (Lushchak 2011; Kottuparambil et al. 2012). ROS are a component part of aerobic life with both favourable and detrimental effects, potentially causing damage to DNA, proteins and lipid membranes (Jia et al. 2014). In *Caenorhabditis elegans*, *CeSTI-1*, a homologue of STIP1, is a co-chaperone protein that maintains homeostatic functions during episodes of stress and can regulate longevity in nematodes (Song et al. 2009). In mice, STI1, a homologue of STIP1, is a multifunctional protein that is required during development; in the absence of STI1, cells show decreased resilience to stress (Beraldo et al. 2013). Thus, *AccSTIP1* may play roles in the oxidative stress response to ROS-induced damage.

In this study, we first identified *AccSTIP1* in *Apis cerana cerana* and investigated its function via gene expression analyses, disc diffusion assay, DNA nicking assay and RNA interference (RNAi) assays. Moreover, the relationship between the role of *AccSTIP1* and oxidative stresses was determined. Collectively, we speculate that *AccSTIP1* plays a significant role in the oxidative stress response.

Materials and methods

Animals and treatments

The Chinese honeybees (*Apis cerana cerana*) used in this work were routinely maintained in the experimental apiary at the College of Animal Science and Veterinary Medicine of Shandong Agricultural University (Taian, Shandong, China). Adult worker honeybees were randomly collected at the hive entrance, fed adult diet water, 30% honey and 70% powdered sugar and reared in an incubator at 34 °C, with constant darkness and 60% relative humidity (Zhang et al. 2014). The worker honeybees were randomly divided into 10 groups of 30 individuals each and subjected to the various treatments described below. Groups 1 and 2 were placed at a high temperature (42 °C) and under ultraviolet radiation (254 nm, 30 mJ/cm^2 UV) for 0, 1, 2, 3, 4 and 5 h, respectively. Groups 3, 4 and 5 were injected in the thoracic nota using a sterile microscale needle containing with 1 μL H_2O_2 (1 mM), 1 μL $CdCl_2$ (3 mg/mL) or 1 μL $HgCl_2$ (3 mg/mL) for 0, 0.5, 1, 1.5, 2 and 2.5 h, respectively. Groups 6–10 were exposed to five different pesticides (30 μg/mL emamectin benzoate, 125 μg/mL thiamethoxam, 125 μg/mL hexythiazox, 700 μg/mL kresoxim-methyl or 1670 μg/mL paraquat) for 0, 0.5, 1, 1.5, 2 and 2.5 h, respectively. The untreated adult worker honeybees used as the control group were fed a normal diet. All of the adult worker honeybee specimens collected at appropriate time points (four honeybees were collected at every time point) were quick-frozen in liquid nitrogen and then stored at − 80 °C for subsequent analysis.

RNA extraction and cDNA synthesis

Total RNA was obtained from the honeybee specimens using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then employed for first-strand cDNA synthesis with EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Next, the cDNA was used as the PCR template for *AccSTIP1* gene cloning and transcriptional analysis.

Primers

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

AccSTIP1 gene isolation

To obtain the open reading frame (ORF) of the *AccSTIP1* gene, SF and SR primers were designed based on the sequence of the *AccSTIP1* gene and then synthesised by the Biosune Biotechnological Company, Shanghai, China. The PCR product of the *AccSTIP1* gene was purified and cloned into the *pEASY*-T1 vector (TransGen Biotech, Beijing, China), which was then transformed into *Escherichia coli* cells for sequencing.

Bioinformatics analysis

Multiple protein sequence alignments among different species were carried out with DNAMAN version 6.0.3. Conserved domains were obtained using the BLAST search program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The molecular weight and theoretical isoelectric point were predicted with DNAMAN.

Fluorescent quantitative real-time PCR (qRT-PCR)

To explore expression profile of the *AccSTIP1* gene under various environmental stresses, qRT-PCR was performed using a CFX96TM Real-Time system (Bio-Rad, Hercules, CA, USA). Specific primers (DLF/DLR) based on the *AccSTIP1* gene ORF were used. *β-actin* (GenBank accession no. HM640276) was used as the control gene for transcriptional analysis. The PCR mixture was composed of 8 μL of ddH_2O, 0.5 μL of forward and reverse primers, 1 μL of cDNA template and 10 μL of SYBR Premix Ex Taq, and the PCR amplification programme was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 15 s and

Table 1 Primers used in this work

Abbreviation	Primer sequence (5′–3′)	Description
SF	ATGGATCAGGTATATTTAC	cDNA sequence primer, forward
SR	TCAATGAATAGCTATTAGACC	cDNA sequence primer, reverse
DLF	CATGGTGGCGCATTTGTAGTAGGC	Real-time PCR primer, forward
DLR	GGCCCAAGAACATCGTATCAATCC	Real-time PCR primer, reverse
β-s	TTATATGCCAACACTGTCCTTT	Standard control primer, forward
β-x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
YHF	GGTACCATGGATCAGGTATATTTA	Protein expression primer, forward
YHR	GTCGACATGAATAGCTATTAGACC	Protein expression primer, reverse
STiF	TAATACGACTCACTATAGGGCGA GGACAAACGAATACTTAC	RNAi primer for *AccSTIP1*, forward
STiR	TAATACGACTCACTATAGGGCGA CCAATTCTAGTAAATGCC	RNAi primer for *AccSTIP1*, reverse
SOD1F	AAACTATTCAACTTCAAGGACC	qRT-PCR primer for *AccSOD1*, forward
SOD1R	CACAAGCAAGACGAGCACC	qRT-PCR primer for *AccSOD1*, reverse
SOD2F	TTGCCATTCAAGGTTCTGGTT	qRT-PCR primer for *AccSOD2*, forward
SOD2R	GCATGTTCCCAAACATCAATACC	qRT-PCR primer for *AccSOD2*, reverse
GSTDF	CGAAGGAGAAAACTATGTGGCAG	qRT-PCR primer for *AccGSTD*, forward
GSTDR	CGTAATCCACCACCTCTATCG	qRT-PCR primer for *AccGSTD*, reverse
GSTO1F	CCAGAAGTAAAAGGACAAGT TCGT	qRT-PCR primer for *AccGSTO1*, forward
GSTO1R	CCATTAACATCAACAAGTGCTGGT	qRT-PCR primer for *AccGSTO1*, reverse
Tpx1F	GGTGGTCTTGGTGAAATGAAC	qRT-PCR primer for *AccTpx1*, forward
Tpx1R	CTAAACGCAAAGTCTCATCAACAG	qRT-PCR primer for *AccTpx1*, reverse
Tpx3F	CCTGCACCTGAATTTTCCGG	qRT-PCR primer for *AccTpx3*, forward
Tpx3R	CTCGGTGTATTAGTCCATGC	qRT-PCR primer for *AccTpx3*, reverse
Trx1F	GGTTTGAGAATTATACGCACTGC	qRT-PCR primer for *AccTrx1*, forward
Trx1R	GAGTAAGCATGCGACAAGGAT	qRT-PCR primer for *AccTrx1*, reverse
Trx2F	GGTTCGGTAGTACTTGTGGAC	qRT-PCR primer for *AccTrx2*, forward
Trx2R	GGACCACACCACATAGCAAAG	qRT-PCR primer for *AccTrx2*, reverse

72 °C for 15 s. The experiments were repeated at least three times. The experimental data were analysed with CFX Manager Software version 1.1, and significant differences were determined via one-way ANOVA and Duncan's multiple range tests using SPSS software version 17.0 (Liu et al. 2016).

Expression of recombinant AccSTIP1

To express recombinant AccSTIP1, the *AccSTIP1* ORF was amplified with the YHF and YHR primers and subcloned into the pET-30a (+) expression vector with a His-tag, after which the recombinant plasmid was transformed into chemically competent transetta (DE3) cells. The bacterial solution containing recombinant AccSTIP1 was cultured in 10 mL of Luria-Bertani (LB) broth with 5 μL of kanamycin (100 mg/mL) at 37 °C until the optical density of the solution reached 0.2–0.4 (OD_{600}). Next, the recombinant AccSTIP1 protein was induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 28 °C for 6–8 h. To obtain bacterial cells harbouring recombinant AccSTIP1, the bacterial liquid was harvested via centrifugation at 13000 rpm for 2 min at room temperature. After centrifugation, the supernatant was discarded, sample loading buffer was added and the sample was boiled for 10 min at 100 °C. The expression of recombinant AccSTIP1 was confirmed through 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining.

Anti-AccSTIP1 preparation and western blotting

After the expression of recombinant AccSTIP1 was assessed by 12% SDS-PAGE, the target protein were excised from two SDS gel and added to 1 mL sodium chloride (0.9%) for grinding using the mortar. Then, the grinding (200 μL) was intraperitoneally injected into every white mouse to produce the anti-AccSTIP1. The white mice were injected a total of four times at 1-week intervals. Blood was collected on the third day after the last injection through the eyeball, which was placed in the 37 °C water bath for 1 h then and at 4 °C for 6 h. The blood was centrifuged at 3000 rpm for 15 min, and the

supernatant antibodies were stored at − 80 °C. Total protein was extracted from honeybee specimens using a Tissue Protein Extraction Kit (CWBiotech, Beijing, China). Each protein specimen was added to sample loading buffer, which was then boiled for 10 min at 100 °C. Western blot analysis was conducted according to the procedure of Liu et al. (2016). Anti-tubulin (Beyotime, Shanghai, China) was employed as the control antibody for western blot analysis. The western blot results were obtained using the SuperSignal® West Pico Trial Kit (Thermo Scientific Pierce, IL, USA).

Disc diffusion assay

Disc diffusion assays were adopted to characterise recombinant AccSTIP1. The recombinant AccSTIP1 was expressed in chemically competent transetta (DE3) cells. Chemically competent transetta (DE3) bacterial cells (5×10^8 cell/mL) carrying the recombinant pET-30a (+)-AccSTIP1 and empty pET-30a (+) vectors were evenly coated on LB-kanamycin agar plates, which were then incubated for 1 h at 37 °C. Subsequently, filter discs (6-mm diameter) soaked with different concentrations of paraquat (0, 50, 100, 200 and 400 mM) and $HgCl_2$ (0, 20, 40, 70 and 100 mM) were placed on the surface of plates containing agarose medium, and growth on the plates was allowed to occur for 12 h at 37 °C. Transetta (DE3) cells transfected with the empty pET-30a (+) vector were employed as a negative control.

Purification of recombinant AccSTIP1 and antioxidant activity assay

The recombinant AccSTIP1 protein was induced with 0.4 mM IPTG for 6–8 h at 28 °C. To obtain the bacterial cells harbouring recombinant AccSTIP1, the bacteria were harvested via centrifugation at 6000 rpm for 5 min at 4 °C and resuspended in binding buffer (Tris-HCl, pH 7.4). After suspension, the liquid mixture was sonicated for 30 min on ice and then centrifuged at 11,000 rpm for 15 min at 4 °C. Next, the supernatant was employed to purify the recombinant AccSTIP1 protein under native conditions using a HisTrap™ FF column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Subsequently, the recombinant AccSTIP1 protein was collected using elution buffer (pH 7.4). After elution, recombinant AccSTIP1 expression was assessed via 12% SDS-PAGE and Coomassie Brilliant Blue staining. The protein concentration was quantified with the BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). To detect the ability of AccSTIP1 to protect against oxidative damage to DNA, a thiol-dependent mixed-function oxidation (MFO) system was employed, as described previously by Yan et al. (2014). The MFO reaction was performed in a total volume of 50 μL, containing ddH_2O, 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 3 μM $FeCl_3$, 10 mM dithiothreitol (DTT) and the purified recombinant AccSTIP1 protein (0.32, 0.64, 0.96, 1.28, 1.6, 1.92, 2.24, 2.56, 2.88 and 3.2 μg), which was cultured for 30 min at 37 °C. Next, 3 μL of supercoiled pUC19 plasmid DNA (100 ng/μL) was added to the reaction mixture, followed by incubation for another 2.5 h at 37 °C. Oxidative damage to DNA caused by the MFO system was evaluated through electrophoresis in a 1.5% agarose gel with ethidium bromide.

Knockdown of *AccSTIP1* gene expression in *Apis cerana cerana*

An RNA interference (RNAi) experiment was performed to knock down the *AccSTIP1* transcript. The non-conserved region of the *AccSTIP1* ORF sequence was determined to design specific primers. The specific primers, containing a T7 polymerase promoter sequence at their 5′-end, were then employed to amplify a linear DNA template via RT-PCR. *AccSTIP1* double-stranded RNA (dsRNA) was synthesised using the RiboMAX T7 large-scale RNA production system (Promega, Madison, WI, USA). The *Apis cerana cerana* gene does not share homology with *GFP* derived dsRNA-*GFP*, and an RNAi response should not be triggered by dsRNA-*GFP* in *Apis cerana cerana*. Therefore, the green fluorescent protein gene (*GFP*, GenBank accession number U87974) was used as a control (Elias-Neto et al. 2010). Adult worker honeybees were selected for the RNAi experiments and divided into three groups ($n = 30$ individuals/group). Four micrograms of dsRNA-*AccSTIP1* or dsRNA-*GFP* was then injected into the thoracic nota of *Apis cerana cerana* individuals using a microsyringe (1 μL). The last group was left untreated, receiving no injection, and was used as a control. The above three groups were reared in an incubator under conditions at 34 °C under constant darkness and 60% relative humidity. The living adult worker honeybee specimens collected at appropriate time points (four honeybees were collected at each time point) were quick-frozen in liquid nitrogen and then stored at − 80 °C for subsequent analysis.

Results

Isolation and sequence analysis of *AccSTIP1*

The *AccSTIP1* ORF (GenBank accession no. XM_017063295.1) was isolated based on specific primers (SF and SR). The *AccSTIP1* ORF contained 1623 bp and encoded a 540 amino acid residue protein with a predicted molecular mass and theoretical isoelectric point of 61 kDa and 6.80, respectively. As shown in Fig. 1, multiple

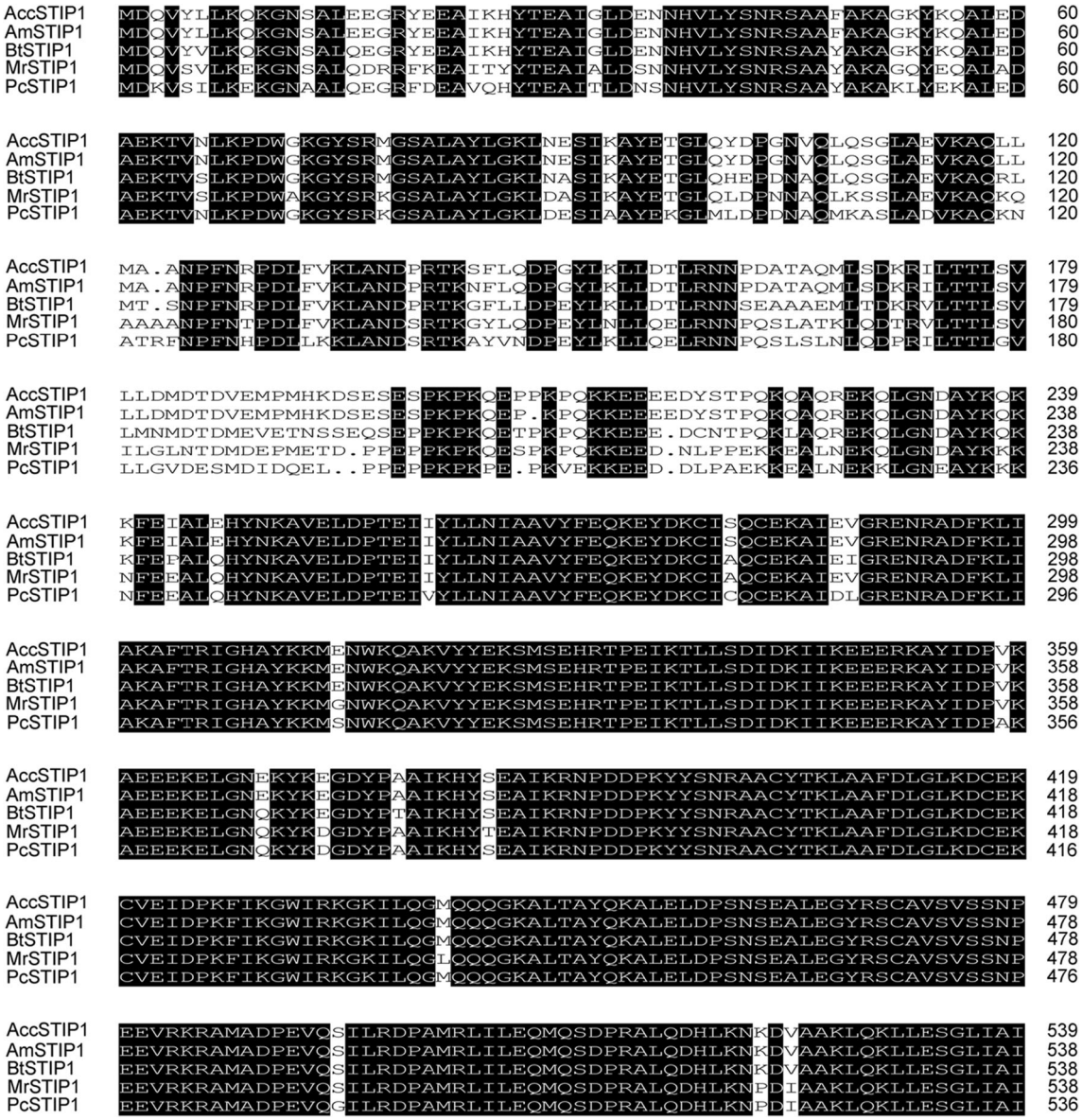

Fig. 1 AccSTIP1 sequence analysis. Alignment of the AccSTIP1 amino acid sequence with AmSTIP1 (XP_006567267), BtSTIP1 (XP_012174112), MrSTIP1 (XP_012152804) and PcSTIP1 (XP_014604812). Identical amino acids are highlighted in black

sequence alignment demonstrated that the amino acid sequence of AccSTIP1 was similar to that of other typical insect STIP1s, revealing high homology to AmSTIP1 from *Apis mellifera* (99.63%), BtSTIP1 from *Bombus terrestris* (91.85%), MrSTIP1 from *Megachile rotundata* (84.47%) and PcSTIP1 from *Polistes canadensis* (80.96%). The result suggests that STIP1 is highly conserved across hymenopteran species.

AccSTIP1 transcriptional expression profiles under stress treatments

To explore the putative role of *AccSTIP1* in the responses to various environmental stresses, its expression profile was studied via qRT-PCR after exposure to different abiotic stresses. As shown in Fig. 2a, *AccSTIP1* expression was obviously induced by heat (42 °C). Among the heavy metal treatments, $CdCl_2$ mildly inhibited *AccSTIP1* expression, while $HgCl_2$ increased *AccSTIP1* expression, especially at 1 h (Fig. 2b, c). In the UV and H_2O_2 treatments, *AccSTIP1* expression was notably decreased by UV and increased to different degrees by H_2O_2 (Fig. 2d, e). *AccSTIP1* expression levels were also increased by the applied pesticides (emamectin benzoate, thiamethoxam, hexythiazox and paraquat) except for kresoxim-methyl (Fig. 2f–j). These results suggest that *AccSTIP1* may be involved in regulating defence responses to various environmental stresses.

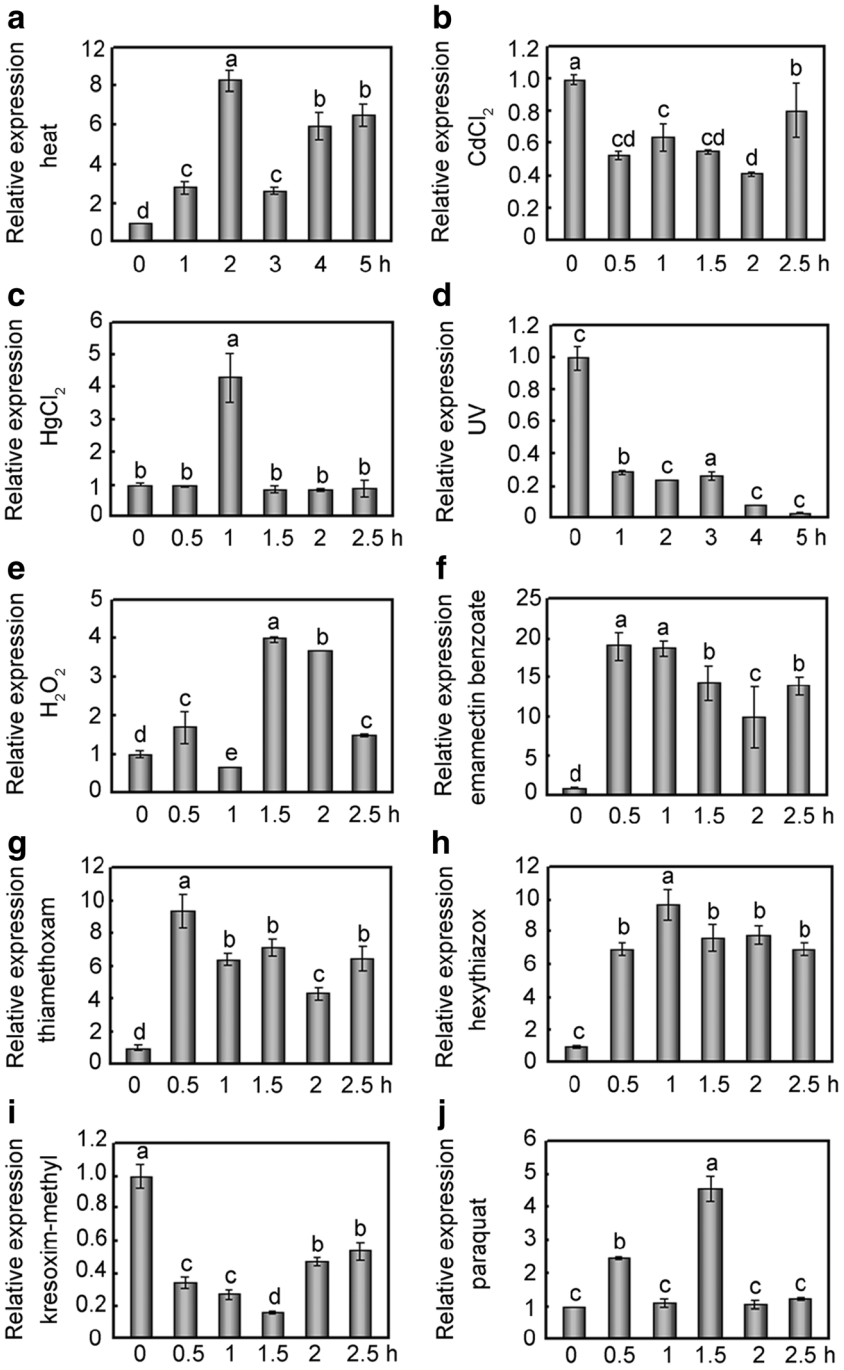

Fig. 2 Transcriptional expression profiles of *AccSTIP1* in *Apis cerana cerana*. Fifteen- to 30-day-old adult worker honeybees were examined after the treatments involving **a** 42 °C: 0, 1, 2, 3, 4 and 5 h; **b** $CdCl_2$: 0, 0.5 1, 1.5, 2 and 2.5 h; **c** $HgCl_2$: 0, 0.5 1, 1.5, 2 and 2.5 h; **d** UV: 0, 1, 2, 3, 4 and 5 h; **e** H_2O_2: 0, 0.5 1, 1.5, 2 and 2.5 h; **f** emamectin benzoate: 0, 0.5 1, 1.5, 2 and 2.5 h; **g** thiamethoxam: 0, 0.5 1, 1.5, 2 and 2.5 h; **h** hexythiazox: 0, 0.5 1, 1.5, 2 and 2.5 h; **i** kresoxim-methyl: 0, 0.5 1, 1.5, 2 and 2.5 h; and **j** paraquat: 0, 0.5 1, 1.5, 2 and 2.5 h. *AccSTIP1* transcriptional expression profile of was determined via qRT-PCR. Total RNA was extracted from adult worker honeybees at the indicated time points. *β-actin* (GenBank accession number: HM640276) was used as an internal control, and the experiments were repeated at least three times. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. This significance in the groups is established in relation to the each other

Expression of recombinant AccSTIP1 protein

To characterise the AccSTIP1 protein, recombinant AccSTIP1-pET-30a (+) was successfully expressed in chemically competent transetta (DE3) cells as a histidine fusion protein. After the recombinant AccSTIP1 protein was induced by IPTG overnight at 28 °C, the bacterial cells were collected from the LB medium. The results of SDS-PAGE demonstrated that the recombinant AccSTIP1 protein was overexpressed and had a molecular mass of approximately 68 kDa (Fig. 3). To further confirm the molecular mass of recombinant AccSTIP1 protein, western blot analyses demonstrated that the molecular mass of recombinant AccSTIP1 protein is correct (Fig. S1).

Western blot analysis

To further confirm AccSTIP1 expression profile in response to various environmental stresses, western blot analysis was employed. Tissue protein extracts were obtained from the above honeybee specimens. As shown in Fig. 4, western blot

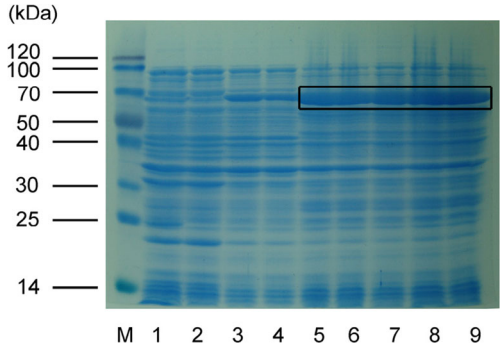

Fig. 3 Expression of the recombinant AccSTIP1 protein. Expression of recombinant AccSTIP1 protein was analysed via SDS-PAGE and Coomassie Brilliant Blue staining (M, protein molecular weight marker; lane 1, chemically competent transetta (DE3) cells; lane 2, chemically competent transetta (DE3) cells containing an empty pET-30 (+) vector; lanes 3 and 4, recombinant AccSTIP1 protein without IPTG; lanes 5–9, recombinant AccSTIP1 protein with IPTG)

analyses of AccSTIP1 were performed after exposure to abiotic stresses (heat, $CdCl_2$, UV, emamectin benzoate, kresoxim-methyl, thiamethoxam and hexythiazox). The results indicated that this trend of the expression profiles of AccSTIP1 and its transcriptional expression levels is consistent.

Characterisation of the recombinant AccSTIP1 protein

To elucidate the function of the recombinant AccSTIP1 protein in response to oxidative stress, disc diffusion assays were employed. As shown in Fig. 5a, b, AccSTIP1-overexpressing transetta (DE3) cells displayed smaller death zones than control bacteria after exposure to paraquat and $HgCl_2$. The results from the disc diffusion assays demonstrated that AccSTIP1 may play a significant role in protecting Chinese honeybees from oxidative stresses.

To further verify the role of AccSTIP1, a DNA nicking assay was performed to assess the protection of DNA from damage by a thiol-dependent MFO system. After induction of the recombinant AccSTIP1 protein with IPTG, the AccSTIP1 fusion protein was further purified using HisTrap™ FF columns and assessed via SDS-PAGE and Coomassie Brilliant Blue staining; the concentration of the recombinant AccSTIP1 protein was 80 μg/mL (Fig. 6a). In the thiol-dependent MFO reaction system, hydroxyl radicals that are generated can disrupt the supercoiled form of pUC19 plasmid DNA in the presence of DTT and $FeCl_3$. The hydroxyl radicals can cause conversion of supercoiled pUC19 plasmid DNA into the nicked form (NF) in the absence of the recombinant AccSTIP1 protein, while the presence of the purified recombinant AccSTIP1 protein prevents the nicking of the supercoiled form by hydroxyl radicals. The amount of supercoiled pUC19 plasmid DNA nicking declined gradually with increasing concentrations of the purified recombinant AccSTIP1 protein (Fig. 6b). Thus, the recombinant AccSTIP1 protein may play a significant role in counteracting oxidative stress.

Knockdown of *AccSTIP1* gene expression

The various expression profile analyses (Figs. 2 and 4), disc diffusion assays (Fig. 5) and DNA nicking assay (Fig. 6) indicated that the *AccSTIP1* gene may play a significant role in multiple stress defence responses, particularly with regard to oxidative stresses. To confirm the role of *AccSTIP1* in stress responses, RNAi experiments were employed to knock down *AccSTIP1* expression in *Apis cerana cerana*. RNAi has proven to be a very promising tool in several research fields, including genomics, for the determination of gene functions and

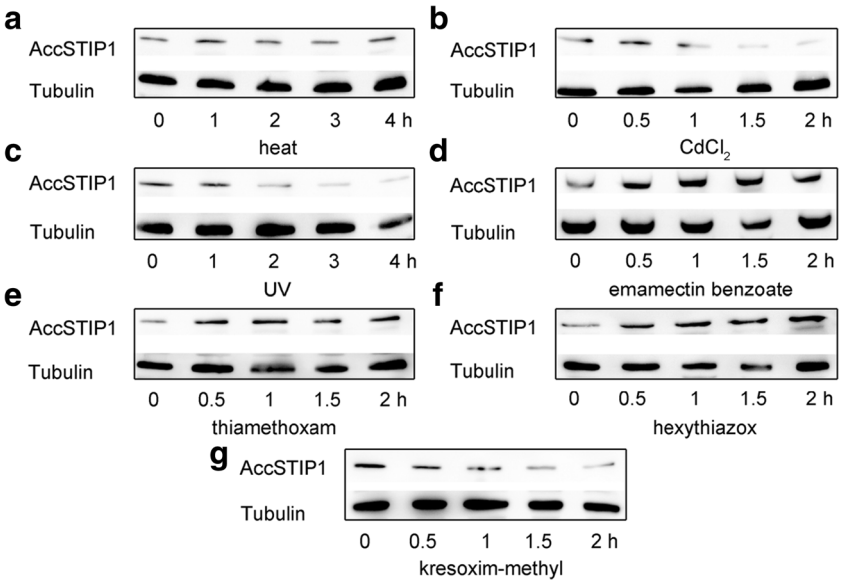

Fig. 4 Western blot analysis of AccSTIP1. Fifteen- to 30-day-old adult worker honeybees were examined after the treatments involving heat **a** (42 °C), **b** $CdCl_2$, **c** UV, **d** emamectin benzoate, **e** thiamethoxam, **f** hexythiazox and **g** kresoxim-methyl. The AccSTIP1 expression profiles were determined via western blot analysis. Total protein was extracted from adult worker honeybees at the indicated time points. Tubulin was used as an internal control. Three honeybees were used to western blot analysis at the indicated time points

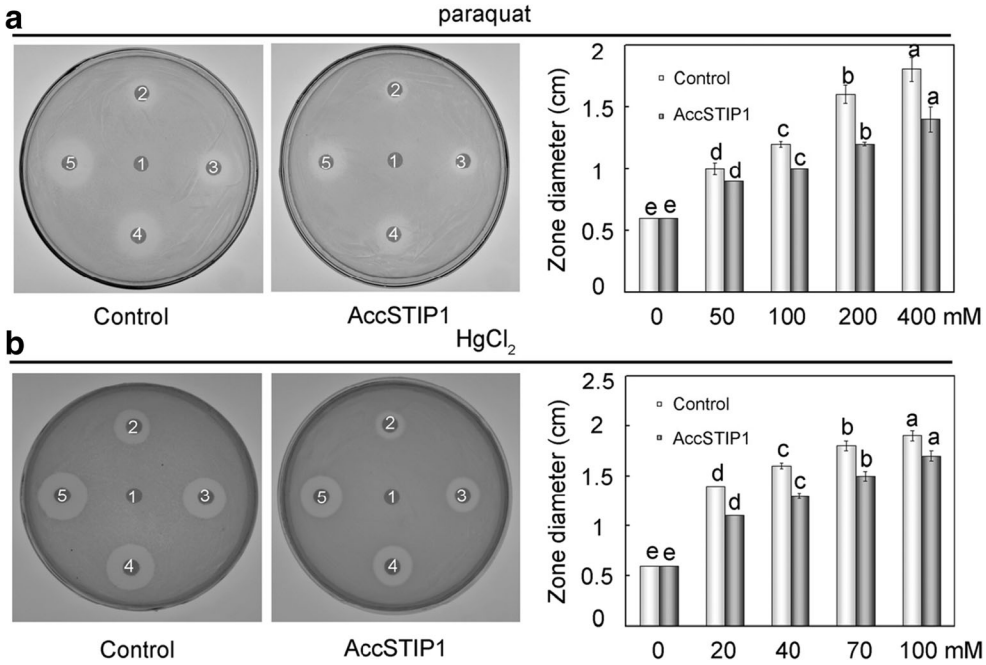

Fig. 5 Disc diffusion assay using AccSTIP1-overexpressing chemically competent transetta (DE3) bacterial cells. After exposure to **a** paraquat and **b** $HgCl_2$, the diameter of the death zone halo was compared between the control and AccSTIP1-overexpressing cells using histograms. Transetta (DE3) cells transfected with pET-30 (+) (only the empty vector) were used as the control. The paraquat and $HgCl_2$ concentrations for filter discs 1–5 were 0, 50, 100, 200 and 400 mM and 0, 20, 40, 70 and 100 mM, respectively. The experiments were repeated at least three times. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. This significance in the groups is established in relation to the control

gene knockdown in eukaryotes, and medicine, to control cancers and viral disease (Huvenne and Smagghe 2010). *AccSTIP1* transcript levels were examined via qRT-PCR in the uninjected, ds*GFP* and ds*AccSTIP1* honeybees. The qRT-PCR results suggested that *AccSTIP1* had been successfully knocked down in *Apis cerana cerana* (Fig. 7).

Effects of silencing *AccSTIP1* on the expression profiles of antioxidant genes

To further study the effects of *AccSTIP1* knockdown, the expression levels of antioxidation-related genes were examined after *AccSTIP1* silencing. We examined the transcription levels of *AccSOD1*, *AccSOD2*, *AccGSTD*, *AccGSTO1*, *AccTpx1*, *AccTpx3*, *AccTrx1* and *AccTrx2*. As shown in Fig. 8, after *AccSTIP1* knockdown, the transcript levels of eight evaluated antioxidation-related genes were markedly lower in the ds*AccSTIP1* honeybees compared with those in the uninjected honeybees. These results demonstrated that *AccSTIP1* may play a significant role by enhancing the ability of cells to evade oxidative stress.

Discussion

It is well established that heat shock protein (Hsp) 70 and Hsp90 complexes play roles in the regulation and folding of diverse signalling proteins and that stress-induced phosphoprotein 1 (STIP1), an Hsp70-Hsp90 organising protein, promotes the association of this multichaperone machinery (Wang et al. 2015). The honeybee (*Apis cerana cerana*) should be a more highly sensitive bioindicator of environmental pollution caused by toxic substances, be these heavy metals, radioactive elements or persistent organic pollutants such as pesticides (Bargańska et al. 2016; Li et al. 2017). The population of *Apis cerana cerana* has severely declined, which can be attributed to abiotic stresses such as extreme temperature, heavy metals, ultraviolet radiation and pesticides (Li et al. 2016). In this study, *AccSTIP1* was first isolated from *Apis cerana cerana*. We further characterised *AccSTIP1* as well as its potential roles during stress responses.

Stress-inducible protein 1 (Stip1), a homologue of STIP1, is a well-studied co-chaperone that is produced in response to stress and mediates the formation of a complex between the stress proteins Hsp70 and Hsp90 (Wang et al. 2015; Schmidt et al. 2011). Heat shock and other stresses have been demonstrated to enhance the expression level of STI-1 (a homologue of STIP1) in different organisms (Zhang et al. 2003; Song et al. 2009; Schmidt et al. 2011).

The dynamic equilibrium of reactive oxygen species (ROS) can be disturbed, leading to increased ROS levels and damage to cellular constituents, which is referred to as "oxidative stress." It has been clearly determined that

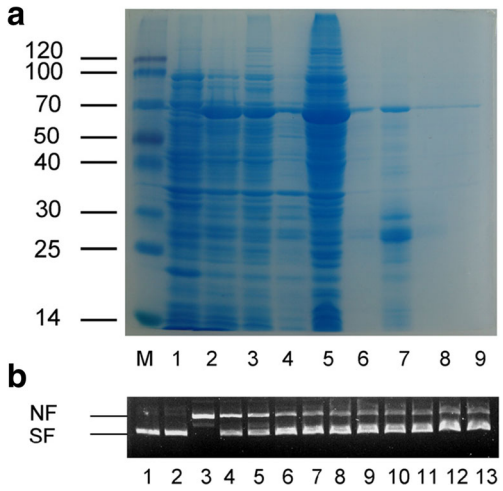

Fig. 6 Purification of the recombinant AccSTIP1 protein and DNA cleavage assay. **a** The recombinant AccSTIP1 protein was expressed and analysed by SDS-PAGE and Coomassie Brilliant Blue staining. Lane M, marker. Lane 1, expression of the recombinant AccSTIP1 protein without IPTG induction. Lane 2, expression of the recombinant AccSTIP1 protein after IPTG induction. Lane 3, mixture of the recombinant AccSTIP1 protein after sonication. Lane 4, supernatant of the recombinant AccSTIP1 protein after centrifugation. Lane 5, sedimentation of the recombinant AccSTIP1 protein after centrifugation. Lanes 6–9, purified recombinant AccSTIP1 protein. **b** A DNA nicking assay was performed using an MFO system. Lane 1, pUC19 plasmid DNA only. Lane 2, pUC19 plasmid DNA and $FeCl_3$. Lane 3, pUC19 plasmid DNA, $FeCl_3$ and DTT. Lanes 4–13, pUC19 plasmid DNA, $FeCl_3$, DTT and varying concentrations of the recombinant AccSTIP1 protein (6.4, 12.8, 19.2, 25.6, 32, 38.4, 44.8, 51.2, 57.6 and 64 μg/mL). NF, nicked form; SF, supercoiled form

practically any strong stress is generally accompanied by oxidative stress (Lushchak 2011). Chinese honeybees constantly experience oxidative stress responses when they are exposed to various environmental stresses. Stress induced by changes in water temperature has also been shown to be accompanied by increased ROS generation and oxidative stress (An and Choi 2010). Heavy metal ions are well-known inducers of oxidative stress (Lushchak 2011). Ultraviolet (UV) radiation, an abiotic stress factor, significantly affects

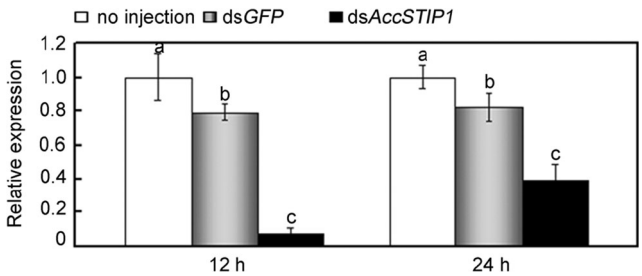

Fig. 7 Effect of RNA interference on *AccSTIP1* transcript levels in *Apis cerana cerana*. Relative *AccSTIP1* transcript levels in the uninjected, ds*GFP* and ds*AccSTIP1* honeybees were examined via qRT-PCR. The experiments were repeated at least three times. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. This significance in the groups is established in relation to each other

insect life because it enhances ROS production and causes oxidative damage in the cell (Ali et al. 2017). Additionally, pesticides, which are physical, chemical or biological agents intended to kill undesirable plant and animal pests, may induce oxidative stress via several mechanisms (Lushchak 2011).

In this study, *AccSTIP1* transcription levels were found to be significantly induced by heat (42 °C), $HgCl_2$, H_2O_2 and pesticides (emamectin benzoate, thiamethoxam, hexythiazox and paraquat). These results suggest that *AccSTIP1* expression is increased by abiotic stress and that *AccSTIP1* may play a significant role in survival in response to various environmental conditions. $CdCl_2$, UV and kresoxim-methyl mildly decreased *AccSTIP1* transcriptional levels. These results indicate that *AccSTIP1* may play various roles in coping with adverse environmental conditions. The mRNA expression level of *AccSTIP1* is significantly increased by some stresses and decreased by other stresses, and these results demonstrate that *AccSTIP1* is a mediator of the stressors. The possible explanation for the phenomenon was that other stress-related genes might play crucial roles in response to these stresses. Moreover, the role of *AccSTIP1* might be weakened in response to stresses. The various treatment concentration, intensity and mechanism of stress may lead to different response. The western blot analyses of *AccSTIP1* were consistent with its transcriptional expression profiles under the different oxidative stresses. It is well established that the expression profile of a gene often implies its biological function. In *Caenorhabditis elegans*, the expression of *Sti-1*, a homologue of STIP1, is increased by heat stress, and a *sti-1* (jh125) null mutant shows decreased fertility under heat stress conditions (Song et al. 2009). The epidermis and midgut showed the highest *AccRBM11* expression in tissue-specific analyses, indicating that *AccRBM11* may protect Chinese honeybees from environmental stresses (Li et al. 2016). *AccERR* expression levels were induced by extreme temperature (4 or 42 °C), UV, heavy metals and different pesticides, which suggested that *AccERR* may play a significant role in stress responses (Zhang et al. 2016). Nevertheless, these results should be further confirmed in *Apis cerana cerana*.

Disc diffusion assays using *Escherichia coli* overexpressing *GSTO-1* provide a test for determining resistance to long-term exposure under oxidative stress (Burmeister et al. 2008). The recombinant AccSTIP1 protein may play a significant role in counteracting oxidative stresses (paraquat or $HgCl_2$) according to disc diffusion assays. Various environmental stresses enhance ROS levels, which exerts a destructive effect on DNA (Boesch et al. 2011; Augustyniak et al. 2014). The purified BmPrx enzyme protects supercoiled plasmid DNA from damage in a metal-catalysed oxidation (MCO) system (Wang et al. 2008). The recombinant AccSTIP1 protein was able to prevent DNA strand breaks caused by hydroxyl radicals in supercoiled plasmid DNA in the MFO system

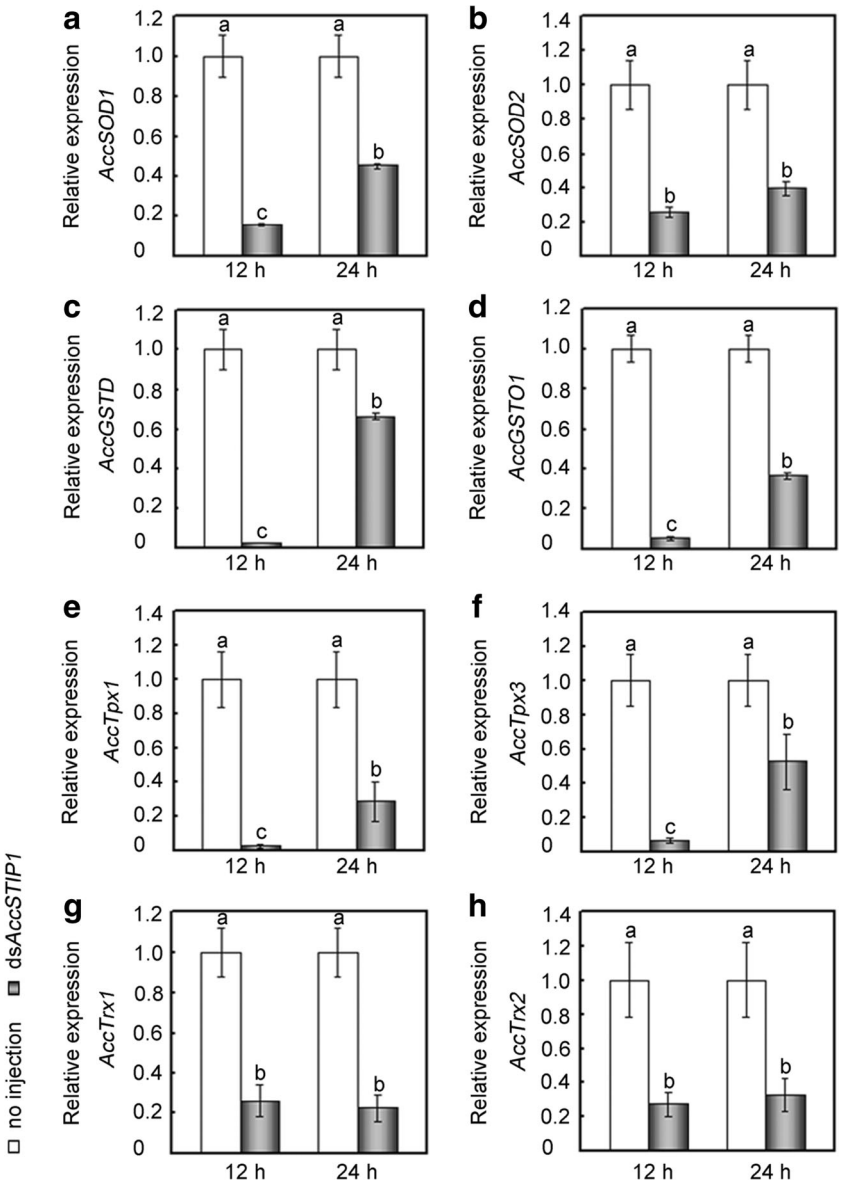

Fig. 8 Relative transcript levels of antioxidation-related genes in the uninjected and ds*AccSTIP1* honeybees after *AccSTIP1* knockdown. Relative expression levels of **a** *AccSOD1* (GenBank ID: JN700517), **b** *AccSOD2* (GenBank ID: JN637476), **c** *AccGSTD* (GenBank ID: JF798572), **d** *AccGSTO1* (GenBank ID: KF496073), **e** *AccTpx1* (GenBank ID: HM641254), **f** *AccTpx3* (GenBank ID: JX456217), **g** *AccTrx1* (GenBank ID: JX844651) and **h** *AccTrx2* (GenBank ID: JX844649). The experiments were repeated at least three times. Different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test. This significance in the groups is established in relation to each other

employed in the present study, which demonstrated that AccSTIP1 may be involved in a mechanism to protect DNA from ROS damage. Collectively, these results indicated that AccSTIP1 may play important roles in counteracting oxidative stress.

Organisms living in aerobic environments require defence mechanisms that prevent oxidative damage caused by ROS (Lee et al. 2005). ROS can cause damage to DNA, proteins and lipid membranes (Jia et al. 2014). To protect against ROS toxicity, aerobic organisms have evolved protective enzymatic systems, including the superoxide dismutase (SOD), glutathione S-transferases (GSTs), thioredoxin peroxidase (Tpx) and thioredoxin (Trx) systems. In *Apis cerana cerana*, *AccSOD2* plays an important role in cellular stress responses and antioxidative processes and could be of critical importance to honey bee survival (Jia et al. 2014). Similarly, it has been reported that *AccGSTT1* may have an important function in antioxidant processes under adverse stress conditions (Liu et al. 2016), and *AccTpx5* most likely plays an essential role in antioxidant defence (Yan et al. 2014). Additionally, thioredoxin 1 may be critical for controlling redox status in *Paracoccidioides lutzii*, which could contribute to this organism's virulence (Cintra et al. 2017). The transcriptional levels of antioxidation-related marker genes can indirectly demonstrate that a gene might play a significant role in oxidative stress. As we all know, the function of a gene is presented by its expression products. Hence, to present the function of a gene, we may examine the transcriptional levels of antioxidation-related marker genes. After knockdown of *AccSTIP1* gene expression, the transcriptional levels of antioxidation-related genes (*AccSOD1*, *AccSOD2*, *AccGSTD*, *AccGSTO1*, *AccTpx1*, *AccTpx3*, *AccTrx1* and *AccTrx2*) were found to be markedly lower in

the ds*AccSTIP1* honeybees compared with those in the uninjected honeybees. Based on previous results and those of the present study, we infer that *AccSTIP1* may play significant role in oxidative stress.

In conclusion, we examined the characteristics of *AccSTIP1* and its potential roles during oxidative stress responses. The results of this study provide significant information for further exploring the mechanisms involved in the responses to various environmental stresses in Chinese honeybees.

Funding information This work was financially supported by the earmarked funds for the China Agriculture Research System (No. CARS-45), the National Natural Science Foundation of China (No. 31572470), the Shandong Province Modern Agricultural Technology System Innovation Team Special Fund (No. SDAIT-24-04) and Shandong Province Agriculture Fine Varieties Breeding Projects (2014–2016).

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