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Isolation of carboxylesterase (esterase FE4) from *Apis cerana cerana* and its role in oxidative resistance during adverse environmental stress

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Abstract

Carboxylesterases (CarEs) play vital roles in metabolising different physiologically important endogenous compounds and in detoxifying various harmful exogenous compounds in insects. Multiple studies of CarEs have focused on pesticide metabolism in insects, while few studies have aimed to identify CarE functions in oxidative resistance, particularly in *Apis cerana cerana*. In this study, we isolated a carboxylesterase gene, esterase FE4, from *Apis cerana cerana* and designated it towards an exploration of its roles as an antioxidant and in detoxification. We investigated *AcceFE4* expression patterns in response to various stressors. A quantitative real-time PCR analysis revealed that *AcceFE4* was up-regulated by H₂O₂, imidacloprid, and paraquat, and was down-regulated by 4 °C, UV radiation, CdCl₂, and HgCl₂. Additionally, the protein expression of this gene was down-regulated at 4 °C and up-regulated by H₂O₂. Disc diffusion assays showed that the *AcceFE4* recombinant protein-expressing bacteria had a smaller killing zone than the control group with the paraquat, HgCl₂ and cumyl hydroperoxide treatments. Moreover, when the gene was knocked down by RNA interference, we observed that multiple oxidant genes (i.e., *AccSOD*, *AccGST*, *AccTrx*, *AccMsra*, and others) were down-regulated in the knockdown samples. Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activity levels were reduced in the knockdown samples relative to the control group. Finally, we measured the enzyme activity of carboxylesterase and found that the enzyme activity was also reduced in the silent samples. Together, these data suggest that *AcceFE4* may be involved in the oxidative resistance response during adverse stress.

1 **Isolation of carboxylesterase (esterase FE4) from *Apis***
2 ***cerana cerana* and its role in oxidative resistance during**
3 **adverse environmental stress**

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23 **Highlights**

24 **1 The first report about a carboxylesterase gene (*AcceFE4*) from *Apis cerana*
25 *cerana*.**

26 **2 We imitated the adverse environments that the bees may be encountered in
27 their life.**

28 **3 *AcceFE4* was knockdown successfully in the *Apis cerana cerana*.**

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42 **Abstract**

43 Carboxylesterases (CarEs) play vital roles in metabolising different physiologically
44 important endogenous compounds and in detoxifying various harmful exogenous
45 compounds in insects. Multiple studies of CarEs have focused on pesticide
46 metabolism in insects, while few studies have aimed to identify CarE functions in
47 oxidative resistance, particularly in *Apis cerana cerana*. In this study, we isolated a
48 carboxylesterase gene, esterase FE4, from *Apis cerana cerana* and designated it
49 towards an exploration of its roles as an antioxidant and in detoxification. We
50 investigated *AcceFE4* expression patterns in response to various stressors. A
51 quantitative real-time PCR analysis revealed that *AcceFE4* was up-regulated by H₂O₂,
52 imidacloprid, and paraquat, and was down-regulated by 4 °C, UV radiation, CdCl₂,
53 and HgCl₂. Additionally, the protein expression of this gene was down-regulated at
54 4 °C and up-regulated by H₂O₂. Disc diffusion assays showed that the AcceFE4
55 recombinant protein-expressing bacteria had a smaller killing zone than the control
56 group with the paraquat, HgCl₂ and cumyl hydroperoxide treatments. Moreover, when
57 the gene was knocked down by RNA interference, we observed that multiple oxidant
58 genes (i.e., *AccSOD*, *AccGST*, *AccTrx*, *AccMsrA*, and others) were down-regulated in
59 the knockdown samples. Superoxide dismutase (SOD), peroxidase (POD) and
60 catalase (CAT) activity levels were reduced in the knockdown samples relative to the
61 control group. Finally, we measured the enzyme activity of carboxylesterase and
62 found that the enzyme activity was also reduced in the silent samples. Together, these
63 data suggest that *AcceFE4* may be involved in the oxidative resistance response
64 during adverse stress.

65 **Keywords** *Apis cerana cerana*; *AcceFE4*; Oxidation resistance; RNA interference;
66 Enzyme activity; Adverse stresses.

67 **1. Introduction**

68 The increasing seriousness of environmental pressures, including global warming
69 and the excessive use of pesticides and heavy metals, has intensified threats to the

survival of numerous organisms. These environmental stresses can induce the production of reactive oxygen species (ROS) (e.g., superoxide radicals, hydrogen peroxide and hydroxyl radicals), which promote oxidative stress in cells and affect cellular signal transduction in ways that result in cell damage and apoptosis [1-2]. In aerobic cells, ROS result from metabolic processes that ordinarily maintain a dynamic equilibrium under normal conditions. However, this equilibrium can be disrupted by adverse environmental stresses, which are largely harmful to cellular macromolecules [3-5]. Therefore, ROS detoxification is vital for the organism's survival. Organisms have established complex antioxidant mechanisms to avoid oxidative damage. In the defence against harmful compounds, carboxylesterase functions as an important metabolic enzyme that plays essential roles in the detoxification of various harmful exogenous compounds [6-8].

The CarEs constitute a metabolic enzyme superfamily, the members of which have multiple functions in the metabolic detoxification of pesticides [9], drug resistance [10], juvenile hormone and pheromone degradation [11-12], and secondary metabolic processes in plants [13]. CarEs are widely distributed in plants, animals, microbes and insects [14]. Based on their sequence similarities and substrate specificities, CarEs can be divided into the following eight classes: α -esterases (ae), β -esterases (be), juvenile hormone esterases (jhe), acetylcholinesterases (AChE), neurotactins (nrt), neuroligins (nlg), gliotactins (gli), and glutactins (glu) [15]. In mammals, CarEs studies have focused on the biotransformation of multiple drugs and the transesterification of xenobiotics, such as insecticides [16-17]. Insect CarEs are of particular interest because of their contributions to insecticide resistance. Due to this important function, most insect studies have focused on CarEs roles in detoxifying dietary and environmental exogenous compounds, particularly insecticides [7, 9, 10, 6, 18].

Furthermore, the roles of insect CarEs in mediating metabolic detoxification processes and oxidative stress responses have been studied. For example, one study indicated that two carboxylesterases may play significant roles in detoxification of

99 carbaryl and deltamethrin and are most likely involved in the detoxification of
100 chlorpyrifos in *Locusta migratoria* [9]. One study indicated that infection by *H.*
101 *beicherriana* induced oxidative stress in *T. molitor* larvae, which resulted in
102 antioxidant responses: carboxylesterase activities as detoxification enzymes were
103 altered in accordance with SOD, CAT, and GST activity levels [19].

104 Insect carboxylesterases are rooted in the α -esterase gene cluster, which includes
105 α E7 (also known as E3) from *Lucilia cuprina* (*LcaE7*), they play important
106 physiological roles in lipid metabolism and are implicated in the detoxification of
107 organophosphorus (OP) insecticides [20]. Montella reported that the metabolic
108 mechanism of carboxylesterase is that the esterases are capable of hydrolysing ester
109 bonds to generate an acid and an alcohol as metabolites [21]. Carboxylesterase is an
110 important detoxifying enzyme that has been implicated in insecticide resistance by its
111 relevant metabolic functions (i.e., catalysis of ester, sulphate, and amid hydrolysis)
112 [22]. These reports have indicated that CarEs may play vital roles in protecting
113 organisms against oxidative damage and oxidative stress. However, the question of
114 whether *AcceFE4* plays effective roles in metabolic detoxification and oxygen free
115 radical scavenging when *A. cerana cerana* is confronted with adverse environmental
116 stress remains unanswered.

117 *A. cerana cerana* is the major honeybee species in China; it has specific
118 advantages over other species, including its longer collection period for honey, higher
119 disease resistance, and lower food cost [23]. The balance between regional ecologies
120 and agricultural development previously enabled strong performances by these
121 honeybees [24]. Today, the environmental quality in China is worsening, a condition
122 that is adverse to honeybee survival.

123 Based on the significant roles of antioxidant processes in insects, we isolated the
124 esterase FE4 gene from *A. cerana cerana* to study its role in oxidative stress
125 resistance. We assessed its expression patterns at different developmental stages and
126 in different tissues. Moreover, the honeybees were exposed to a cold temperature
127 (4 °C), ultraviolet (UV) radiation, H₂O₂, pesticides (acetamiprid, cyhalothrin and

128 phoxim), an herbicide (paraquat) and heavy metals (HgCl_2 and CdCl_2) to evaluate the
129 *AcceFE4* expression patterns in response to oxidative stress. Meanwhile, at the protein
130 levels, *AcceFE4* expression profiles were detected by western blot assays. Additional,
131 disc diffusion assays were performed to explore the role of the recombinant *AcceFE4*
132 protein in vitro. Finally, we used RNA inference technology to knock down *AcceFE4*
133 and to observe the expression levels of other antioxidant genes in the knockdown
134 samples, and we measured enzyme activity of antioxidant enzyme and
135 carboxylesterases. Based on these results, we speculate that *AcceFE4* might play an
136 important role in the oxidative stress response.

137 **2. Materials and methods**

138 *2.1. Insects and treatments*

139 The experimental insects (*A. cerana cerana*) that were used in this study were reared
140 in the artificial honeycomb of Shandong Agricultural University (Taian, China). The
141 honeybees were classified into larvae, pupae, and adults based on age and shape [25].
142 The fourth (L4), fifth (L5) instar larvae and the white-eyed (Pw), brown-eyed (Pb),
143 pink-eyed (Pp) and dark-eyed (Pd) pupae were obtained from the hive; the 3- day-old,
144 the 15-day-old and 30-day-old adults were gathered from the honeycomb. The adult
145 honeybees were caged in bee boxes at a constant temperature of 34 °C and 80 %
146 humidity under a 24-h dark regimen [26].The adult bees were fed a basic adult diet of
147 water and powdered sugar [27]. The 15- to 30-day post-emergence adult workers were
148 divided into 10 groups, and each group contained 30 honeybees. Each group was
149 exposed to different harmful environmental conditions. Groups 1, 2 were exposed to a
150 cold temperature (4 °C) and UV light (30 mJ/cm^2) for 0, 0.5, 1.0, 1.5, 2.0, 2.5 h
151 respectively. In addition, the bees in group 3 were injected with 0.5 μL (2mM) H_2O_2
152 and treated for 0, 0.25, 0.5, 0.75, 1 h or 1.25 h, while the control group was injected
153 with 0.5 ul phosphate-buffered saline (PBS) (0.5 $\mu\text{L}/\text{adult}$). Groups 4-8 were treated
154 with pesticides (imidacloprid, cyhalothrin, or acetamiprid at a final concentration of
155 20 $\mu\text{g/mL}$), and herbicides (phoxim or paraquat at a final concentration of 20 $\mu\text{g/mL}$)

156 that were diluted in water and added to the food for the experimental groups (treat 0,
 157 0.5, 1.0, 1.5, 2.0, and 2.5 h). Group 9 and 10 were injected with HgCl₂ or CdCl₂ (0.5
 158 µL of a 2 mM dilution was applied to the thoracic nota of the adult bees), while the
 159 control group was injected with PBS (0.5 µL/adult), and the treated bees were
 160 collected after treatment at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 h. [28] The treated
 161 bees were flash-frozen in liquid nitrogen and stored at -80 °C until further use.

162 *2.2. Primer sequences in this study.*

163 The primers in this study are listed in Table 1.

164

165

166

167 **Table 1** PCR primers

Abbreviation	Primer sequence (5'-3')	Description
C1	CTTGGTTAACAAATGAAACAAAC	cDNA sequence primer, forward
C2	TTAAGATAATTGGATGATAAAAG	cDNA sequence primer, reverse
C3	CATGGTGGCGCATTTGTAGTAGGC	Real-time PCR primer, forward
C4	GGCCCAAGAACATCGTATCAATCC	Real-time PCR primer, reverse
β-s	TTATATGCCAACACTGTCCTTT	Standard control primer, forward
β-x	AGAATTGATCCACCAATCCA	Standard control primer, reverse
C5	GGTACCATGAACAAACAAATAGTAA	Protein expression primer, forward
C6	GTCGACAGATAATTGGATGATAAAAG	Protein expression primer, reverse
C7	TAATACGACTCACTATAAGGGCGAGTCAGCG	RNAi primer of <i>AccCarE</i> , forward
	CTGGAGGAGTT	
C8	TAATACGACTCACTATAAGGGCGAGATCTCC GCATCTTCTAA	RNAi primer of <i>AccCarE</i> , reverse
GSTDF	CGAAGGAGAAAACATATGTGGCAG	qPCR primer of <i>AccGSTD</i> , forward
GSTD R	CGTAATCCACCACCTCTATCG	qPCR primer of <i>AccGSTD</i> , reverse
GSTO1F	CCAGAAGTAAAGGACAAGTTCGT	qPCR primer of <i>AccGSTO1</i> , forward
GSTO1R	CCATTAACATCAACAAAGTGCTGGT	qPCR primer of <i>AccGSTO1</i> , reverse
GST4F	CTTCTTAGTTATGGAGGTGTTG	qPCR primer of <i>AccGSTS4</i> , forward
GST4R	GCCATCTGAAATCGTAAAGAG	qPCR primer of <i>AccGSTS4</i> , reverse
Trx1F	GGTTTGAGAATTATACGCACTGC	qPCR primer of <i>AccTrx1</i> , forward
Trx1R	GAGTAAGCATGCGACAAGGAT	qPCR primer of <i>AccTrx1</i> , reverse
Trx2F	GGTCGGTAGTACTTGTGGAC	qPCR primer of <i>AccTrx2</i> , forward
Trx2R	GGACCACACCACATAGCAAAG	qPCR primer of <i>AccTrx2</i> , reverse
MsrAF	GGTGATTGTTATTGGCG	qPCR primer of <i>MsrA</i> , forward

MsrAR	TTTGTATTGCTCTTGTCACG	qPCR primer of <i>MsrA</i> , reverse
P450F	CGCAAAGAGAAATGGGAAGG	qPCR primer of <i>AccCYP4GII</i> , forward
P450R	CTTTGTGTACGGAGGTGC	qPCR primer of <i>AccCYP4GII</i> , reverse
SOD1F	AAACTATTCAACTTCAAGGACC	qPCR primer of <i>AccSOD1</i> , forward
SOD1R	CACAAGCAAGACGAGCACC	qPCR primer of <i>AccSOD1</i> , reverse
SOD2F	TTGCCATTCAAGGTTCTGGTT	qPCR primer of <i>AccSOD2</i> , forward
SOD2R	GCATGTTCCCAAACATCAATACC	qPCR primer of <i>AccSOD2</i> , reverse
Tpx1F	GGTGGTCTTGGTGAATGAAC	qPCR primer of <i>AccTpx1</i> , forward
Tpx1R	CTAACACGCAAAGTCTCATCACAG	qPCR primer of <i>AccTpx1</i> , reverse
Tpx3F	CCTGCACCTGAATTTCGGG	qPCR primer of <i>AccTpx3</i> , forward
Tpx3R	CTCGGTGTATTAGTCCATGC	qPCR primer of <i>AccTpx3</i> , reverse
CATF	GTCTTGGCCCCAACAAATTG	qPCR primer of <i>AccCAT</i> , forward
CATR	CATTCTCTAGGCCACCAAA	qPCR primer of <i>AccCAT</i> , reverse

168

169 *2.3. RNA isolation and cDNA synthesis*

170 TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA.
 171 All RNA samples were treated with RNase-free DNaseI (Promega) to eliminate
 172 potential genomic DNA contamination. RNA samples were reverse-transcribed into
 173 first-strand cDNA using the EasyScript cDNA Synthetic SuperMix (TransGen Biotech,
 174 Beijing, China) according to the manufacturer's protocol, and the cDNA was used as
 175 the PCR template for gene cloning and qRT-PCR.

176 *2.4. Isolation the AcceFE4 ORF*

177 The open reading frame (ORF) of *AcceFE4* was isolated by PCR amplification with a
 178 primer pair (C1 and C2, Table 1) that was designed and synthesised by Biosune
 179 Biotechnological Company, Shanghai, China; all primers in this study were obtained
 180 from this company. The PCR products were purified and connected with pEASY-T1
 181 vectors (TransGen Biotech, Beijing, China) and were transformed into *Escherichia*
 182 *coli* cells (DH5α) for sequencing.

183 *2.5. Bioinformatics analysis and polygenetic tree construction*

184 The active sites and conserved domains of *AcceFE4* were obtained from
 185 SPDBV_4.10_PC. The *AcceFE4* protein sequence was compared with other

186 homologous carboxylesterases from various species that were obtained using the
187 Basic Local Alignment Search Tool from NCBI
188 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) [23]. We obtained the *AcceFE4* gene and
189 protein sequences to predict the isoelectric points and molecular weights of the
190 proteins using DNAMAN version 5.2.2. Phylogenetic trees were constructed based on
191 the homologous sequences of carboxylesterase from the other species with MEGA
192 version 4.1 software using the neighbour-joining method.

193 *2.6. Fluorescence real-time quantitative PCR*

194 qRT-PCR was performed using the SYBR Premix Ex Taq (TaKaRa Dalian, China)
195 and a server on the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules,
196 CA, USA) to detect the gene expression changes after various exposures to the
197 adverse stressors. The C3 and C4 specific primers were designed according to the
198 *AcceFE4* sequence. The β -x and β -s primers were designed to amplify the
199 housekeeping gene, β -*actin* (GenBank accession no. HM_640276), which was used as
200 a stable house-keeping gene to normalise the RNA levels [29]. The 20- μ L reaction
201 volumes contained 10 μ L of SYBR Taq, 0.5 μ L of C3 primer, 0.5 μ L of C4 primer, 1
202 μ L of cDNA template, and 8 μ L of ddH₂O. The amplification conditions were as
203 follows: initial denaturation at 95 °C for 30 s; 41 cycles at 95 °C for 5 s, 55 °C for 15
204 s and 72 °C for 15 s; with one melt cycle from 65 °C to 95 °C [30]. All experiments
205 were performed in triplicate. The data analyses were obtained from CFX Manager
206 Software version 1.1, and the significant differences between the samples were
207 determined by one-way ANOVA and Duncan's multiple range tests using SPSS
208 software version 17.0 [5].

209 *2.7. Expression of recombinant AcceFE4*

210 A bacterial prokaryotic expression vector, PET-30 (a+), was used together with
211 transient competent cells to produce the *AcceFE4* recombinant protein. The primers
212 (C5 and C6) design were based on the *AcceFE4* ORF and contained a Kpn I or Sal I
213 restriction site and were inserted into the PET-30 (a+) expression vector, which was

214 previously digested with these restriction sites. The PET-30 (a+)-*AcceFE4* expression
215 plasmid was transformed into transient *E. coli* cells. The bacterial solution (10 µL)
216 was added to 10 mL of Luria Bertani (LB) broth that contained 5 µL of kanamycin
217 and was incubated at 37 °C for approximately 1.5 h. When the cell density reached
218 0.4-0.6 (OD₆₀₀), the recombinant protein was induced with 0.5 mM
219 isopropyl-1-thio-β-galactopyranoside (IPTG) at 37 °C for 6-8 h. To collect the
220 bacterial cells, the bacterial liquid was centrifuged for 2 min at 13000 rpm. After
221 centrifugation, the supernatant was discarded, the loading buffer was added and the
222 sample was boiled for 10 min. Protein induction was detected by sodium dodecyl
223 sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a 12 %
224 polyacrylamide gel.

225 *2.8. Antibody preparation and Western blot analysis*

226 After protein induction was assessed by 12 % SDS-PAGE, the target protein was
227 excised from the gel and added to 1 mL of normal saline (NS) for grinding using a
228 mortar. White mice received four subcutaneous injections of the products once per
229 week. Blood was collected into a 1.5 mL centrifuge tube, which was placed in a 37 °C
230 water bath for 1 h then and at 4 °C for 6 h. After centrifugation at 3000 rpm for 15
231 min, the supernatant antibodies were stored at -80 °C.

232 The protein samples were obtained from the various honeybee treatment groups,
233 and the protein was extracted using the Tissue Protein Extraction Kit (CWBiotech,
234 Beijing, China). Each protein sample was added to the loading buffer, boiled for 10
235 min, separated on a 12 % SDS-PAGE and blotted onto a polyvinylidene difluoride
236 (PVDF) membrane. The membrane was blocked in 5 % (w/v) skim milk (diluted in
237 TBST) for 1 h and incubated overnight at 4 °C with primary antibodies at 1:500 (v/v)
238 dilutions. Tubulin (Beyotime, Shanghai, China) was used as the reference antibody for
239 the Western blot analysis. The membranes were washed three times in TBST,
240 incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2000 (v/v))
241 (Dingguo, Beijing, China) for 2 h at room temperature, and washed three times [5].
242 Finally, the results were observed using the SuperSignal® West Pico Trial Kit

243 (Thermo Scientific Pierce, IL, USA), based on the instructions provided in the
244 FDbio-Dura ECL Kit (FDbio Science Biotech, Hangzhou, China).

245 *2.9. Disc diffusion assay*

246 The disc diffusion assay was performed according to Liu et al. [5]. The recombinant
247 AcceFE4 was expressed in transient *E. coli* cells. The bacterial culture (5×10^8 cell/ml)
248 was seeded onto LB-kanamycin agar plates and incubated at 37 °C for 1 h. Cells with
249 the PET30 (a+) empty vector were used as the control. The filter discs (6-mm
250 diameter) were placed onto the surfaces of the agarose medium plates, and the filters
251 were soaked with different concentration of hazardous substances, including HgCl₂ (0,
252 10, 20, 40, and 80 mM), paraquat (0, 10, 50, 100, and 200 mM) and cumene
253 hydroperoxide (0, 10, 50, 100, and 200 mM). The cells were grown for 12 h at 37 °C.

254 *2.10. dsRNA (dseFE4-RNA and dsGFP-RNA) synthesis and injection*

255 We selected a 507-bp fragment that was part of the *AcceFE4* ORF and designed a
256 primer pair (C7 and C8, Table 1) according to the target fragment; T7 polymerase
257 promoter sequences were included at the start of the *AcceFE4* forward primer (C7)
258 and at the end of the *AcceFE4* reverse primer (C8). PCR products were purified and
259 transformed into *Escherichia coli* cells (DH5α) for sequencing. Briefly, the purified
260 products were used as templates for dsRNA synthesis with the T7 RiboMAX™
261 Express RNAi System (Promega, USA) according to the manufacturer's instructions.
262 The green fluorescent protein gene (GFP, GenBank accession number U87974) was
263 used as the control [31]. The genes in *Apis cerana cerana* doesn't share homology
264 with dsRNA-GFP, thus the RNAi response will not be triggered by dsRNA-GFP in the
265 body of *Apis cerana cerana*. The synthesised dsRNA samples were adjusted to final
266 concentrations of 6 µg/µL. The adult honeybees were injected with 0.5 µL of
267 dseFE4-RNA or 0.5 µL of dsGFP-RNA into the thoracic nota using microsyringes [9].
268 In addition, 0.5 ul water was injected with bees as the control group. The samples
269 were flash-frozen in liquid nitrogen at 12 and 36 h post-injection and were stored at
270 -80 °C until further use.

271 *2.11. mRNA level analysis of antioxidant genes after AcceFE4 knockdown*

272 To assess the transcriptional levels of antioxidant genes after the *AcceFE4* knockdown,
273 we designed primers (Table 1) for these genes, which included *AccSOD1* (GenBank
274 ID: JN700517), *AccSOD2* (GenBank ID: JN637476), *AccGSTD* (GenBank ID:
275 JF798573), *AccGSTS4* (GenBank ID: JN008721), *AccGSTO1* (GenBank ID:
276 KF496073), *AccTpx1* (GenBank ID: HM641254), *AccTpx3* (GenBank ID: JX456217),
277 *AccTrx1* (GenBank ID: JX844651), *AccTrx2* (GenBank ID: JX844649), *AccCAT*
278 (GenBank ID: KF765424), *AccMsrA* (GenBank ID: HQ219724), and *AccCYP4G11*
279 (GenBank ID: KC243984) [2]. qRT-PCR was performed to analyse these genes after
280 *AcceFE4* was knocked down.

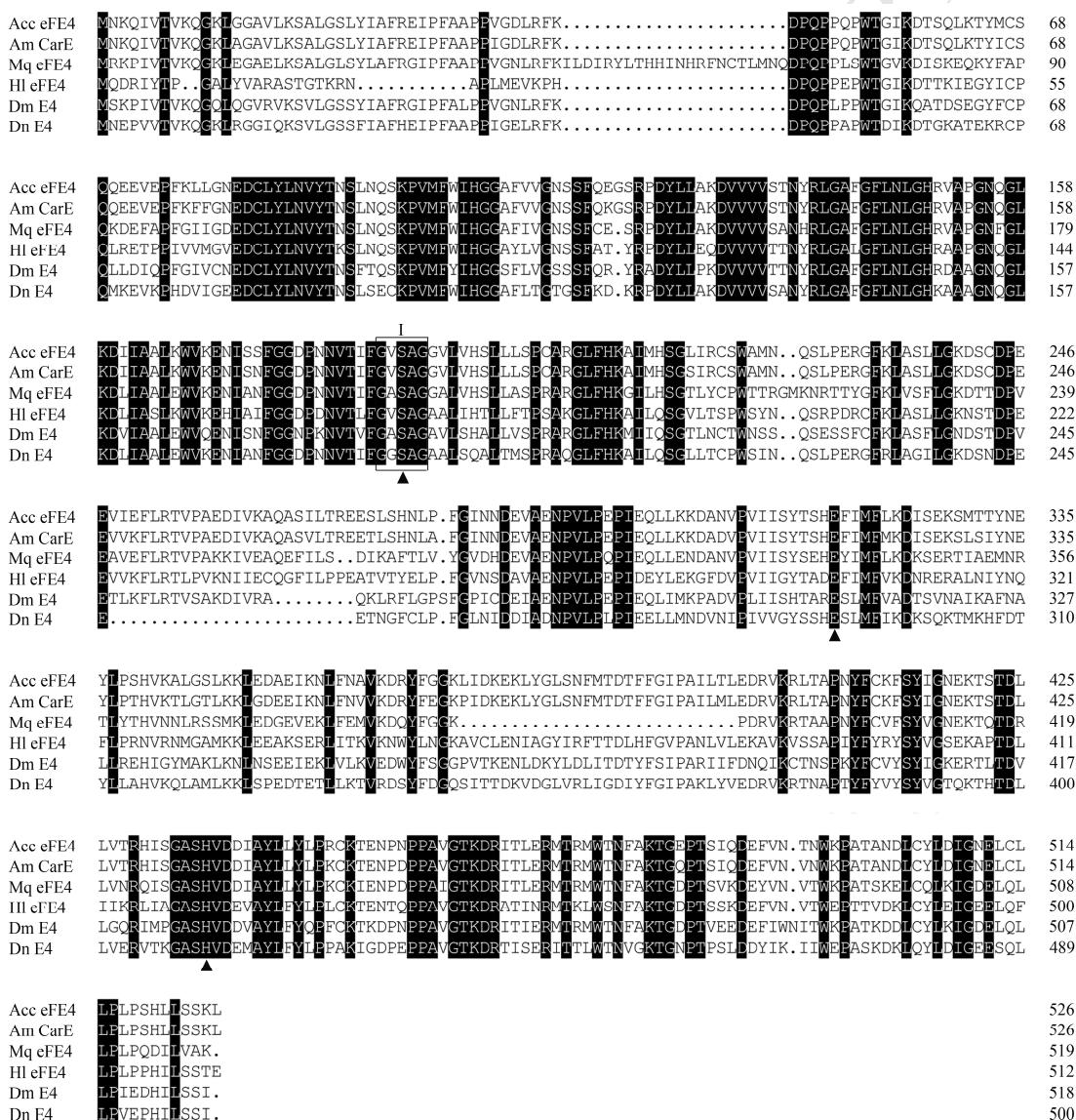
281 *2.12. Enzymatic activities in the AcceFE4 RNAi samples*

282 We extracted protein samples from whole-bee adults 12, 24 and 36 h after they had
283 been injected with dseFE4-RNA. The total protein levels were quantified with the
284 BCA Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).
285 We measured the activities of SOD, POD, CAT and CarEs enzyme using SOD,
286 POD, ,CAT and CarEs kits, respectively, according to the manufacturer's instructions
287 [32]; these kits were all obtained from the Nanjing Jiancheng Bioengineering
288 Institute.

289 **3. Results**290 *3.1. Isolation and characterisation of AcceFE4*

291 To determine whether *AcceFE4* played a role in oxidation resistance in honeybees, we
292 isolated the *eFE4* ORF in *A. cerana cerana*. The *AcceFE4* ORF consisted of 1,584 bp
293 and encoded a 527-amino-acid protein with a predicted molecular mass of 58 kDa and
294 a theoretical isoelectric point of 7.07. The amino acid sequence for *AcceFE4* was
295 compared with other homologous sequences, including *Apis mellifera*
296 carboxylesterase, *Melipona quadrifasciata* esterase FE4, *Habropoda laboriosa*
297 esterase FE4, and *Eufriesea mexicana* esterase E4, and is shown in Fig 1. The high

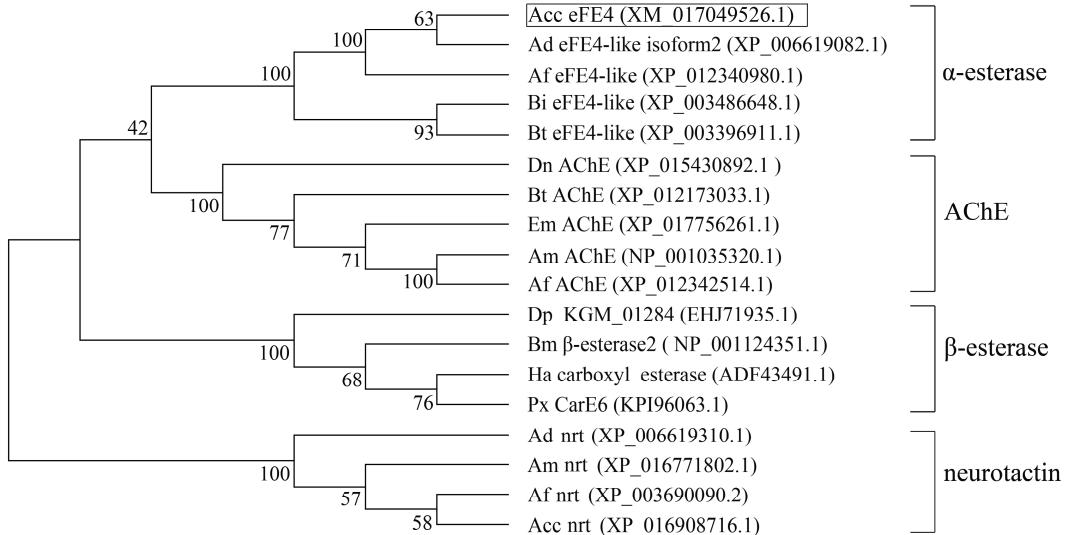
homology suggested that the CarEs family was highly conserved between these species and the common CarEs characteristics were associated with a catalytic triad consisting of Ser-Glu-His and a nucleophilic group that surrounded the active site serine residue (GXSXG) (Fig 1). To further explore the evolutionary relationships between the CarEs from the different insect species, we constructed a phylogenetic CarEs tree. As shown in Fig 2, we assembled four branches of CarEs, and *AcceFE4* was classified as α -esterase.



305

Fig. 1 Multiple amino acid sequence alignments for *AcceFE4* (GenBank accession no. XM_017049526.1) from *A. cerana cerana*, *AmCarE* (GenBank accession no. NP_001136081.1) from *A. mellifera*, *Mq E-FE4* (GenBank accession no. KOX71850.1) from *Melipona*

309 *quadrifasciata*, *Hl E-FE4* (GenBank accession no. KOC67535.1) from *Habropoda laboriosa*, *Em*
 310 *E4* (GenBank accession no. OAD58521.1) from *Eufriesea mexicana*, and *Dn E4* (GenBank
 311 accession no. KZC12051.1) from *Dufourea novaeangliae*. Conserved domains are shown in black.
 312 The Ser-Glu-His catalytic triads are indicated with black triangles. Domain I is the nucleophilic
 313 elbow that surrounds the serine residue in the active site (GXSXG).



314
 315 Fig. 2 Phylogenetic analysis of the CarEs homologous sequences from various species using the
 316 neighbour-joining (NJ) method with bootstrap values of 500 replicates. Four main groups of the
 317 CarE superfamily are shown, and *AcceFE4* is boxed. The sequences were obtained from the NCBI
 318 database.

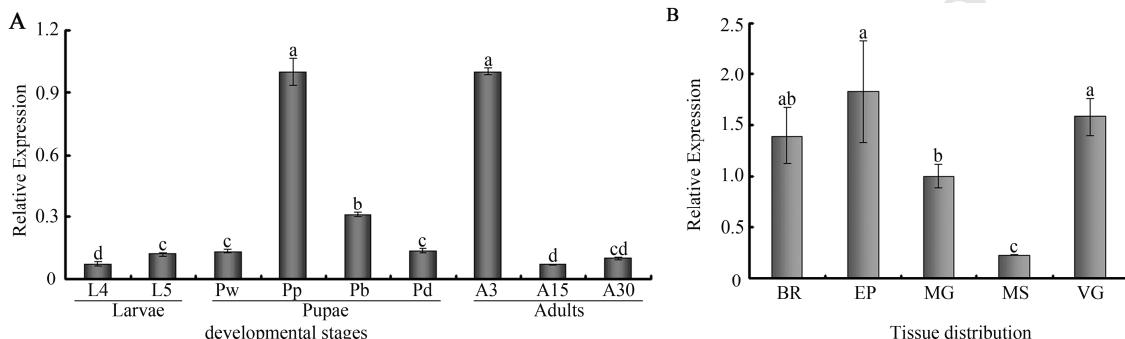
319

320 *3.2. Quantitative real-time PCR of AcceFE4 at different developmental stages and its*
 321 *tissue distribution*

322 The *AcceFE4* expression profile was assessed via qRT-PCR at the different honeybee
 323 developmental stages of L4, L5, Pw, Pb, Pp, Pd, A3, A15 and A30. As shown in Fig
 324 3a, *AcceFE4* expression was highest in the Pp phase. In the larvae, the expression
 325 levels in L5 were higher than in L4. In the pupae, the expression levels in pink-phase
 326 pupae were higher than in the other pupal phases, while expression levels were at their
 327 lowest in the Pd pupae developmental stage. The expression levels in 3-day-old adult

328 workers were higher than in other adult bees. Total RNA was collected from the head,
 329 epidermis, muscle, and venom gland. As shown in Fig 3b, their expression patterns
 330 indicated that the *AcceFE4* transcripts were most abundant in the epidermis, followed
 331 by the venom gland.

332



333

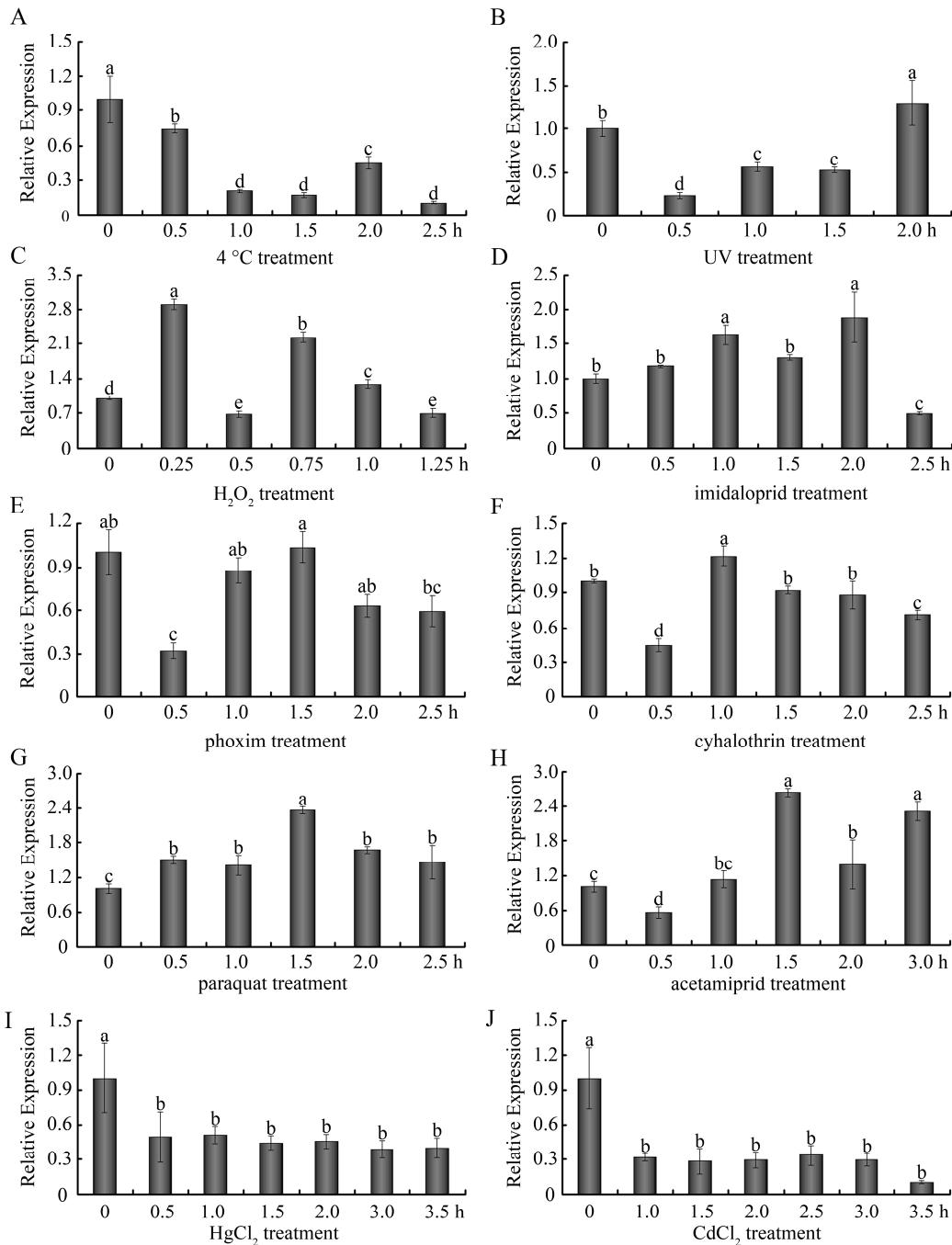
334 Fig. 3A *AcceFE4* transcription expression levels during the different developmental stages.
 335 *AcceFE4* expression was measured at multiple developmental stages, including the larvae (L4 and
 336 Pw, Pp, Pb, Pd) and pupae (Pp, Pb, Pd) stages, and in adult workers (A3, A15 and A30). The data are
 337 presented as the means \pm SE of three replicates. The letters above the bar indicate significant
 338 differences ($P < 0.005$) as determined by Duncan's multiple range tests using SPSS software
 339 version 17.0. Fig. 3B *AcceFE4* expression profiles in different tissues from *A. cerana cerana*.
 340 *AcceFE4* expression was detected in epidermis (EP), venom gland (VG), muscle (MS), brain (BR),
 341 and midgut (MG). The data are presented as the means \pm SE of three independent experiments.
 342 The letters above the columns represent significant differences ($P < 0.05$) based on one-way
 343 ANOVA and Duncan's multiple range tests using SPSS software version 17.0.

344

345 3.3. Transcriptional expression profiles of *AcceFE4* in response to various stressors

346 We assessed the *AcceFE4* transcriptional levels in *A. cerana cerana* in response to
 347 various stressor treatments by qRT-PCR; the untreated honeybees were used as the
 348 control group for qRT-PCR normalisation. As shown in Fig 4, the transcription levels
 349 were clearly down-regulated at 4 °C and with the HgCl₂, CdCl₂ and phoxim
 350 treatments. Fig 4A shows that the transcription level continued to decline and reached

351 its minimum at 2.5 h of the 4 °C treatment. Fig 4I and 4J show that with the HgCl₂
352 and CdCl₂ treatments, the *AcceFE4* expression levels continued to noticeably decline,
353 particularly at 3.5 h. Fig 4C shows that the expression levels at 15, 45 and 60 min
354 were higher relative to the control group during the H₂O₂ treatment. We also analysed
355 the expression levels after treatments with UV radiation (Fig 4B), the expression
356 profile of *AcceFE4* was reduced at 0.5, 1.0 and 1.5 h than the control group. After
357 bees treated for imidacloprid and acetamiprid, *AcceFE4* expression levels were
358 induced as showed in Fig 4D and Fig 4H. As shown in Fig 4F the gene's expression
359 was up-regulated at 1.0, 1.5 and 2.0 h than the control group. after treatment with
360 cyhalothrin condition. In Fig 4G, the expression of *AcceFE4* was up-regulated by
361 paraquat treatment.



362

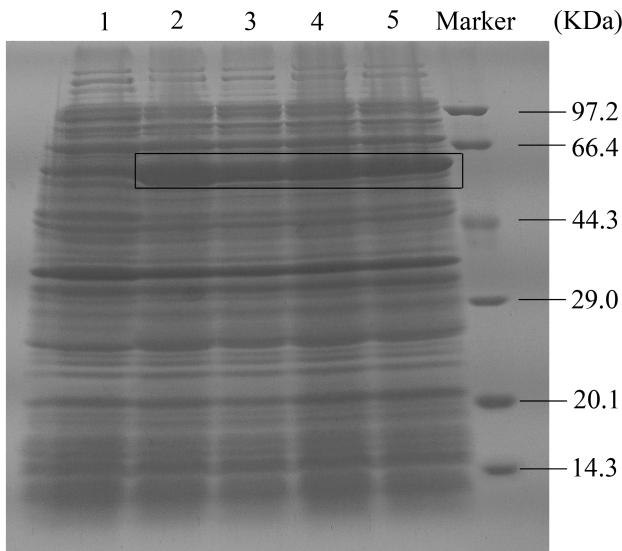
363 Fig. 4 *AcceFE4* expression patterns in response to abiotic stress. Total RNA was extracted from *A.*
 364 *cerana cerana* after treatments with various stressors. The adult worker bees were exposed to the
 365 following conditions: 4 °C, UV, H₂O₂, imidacloprid, phoxim, cyhalothrin, paraquat, acetamiprid,
 366 HgCl₂ or CdCl₂. The data are presented as the means ± SE of three replicates. The letters above
 367 the bar indicate significant differences ($P < 0.005$) as determined by Duncan's multiple range tests
 368 using SPSS software version 17.0.

369

370

371 *3.4. Expression of the recombinant AcceFE4 protein*

372 As shown in Fig 5, the induced protein was clearly apparent on the albumen glue. The
 373 second to fifth samples were the induced protein; the first sample was the control
 374 group, which did not receive IPTG. The target protein was approximately 58 kDa.



375

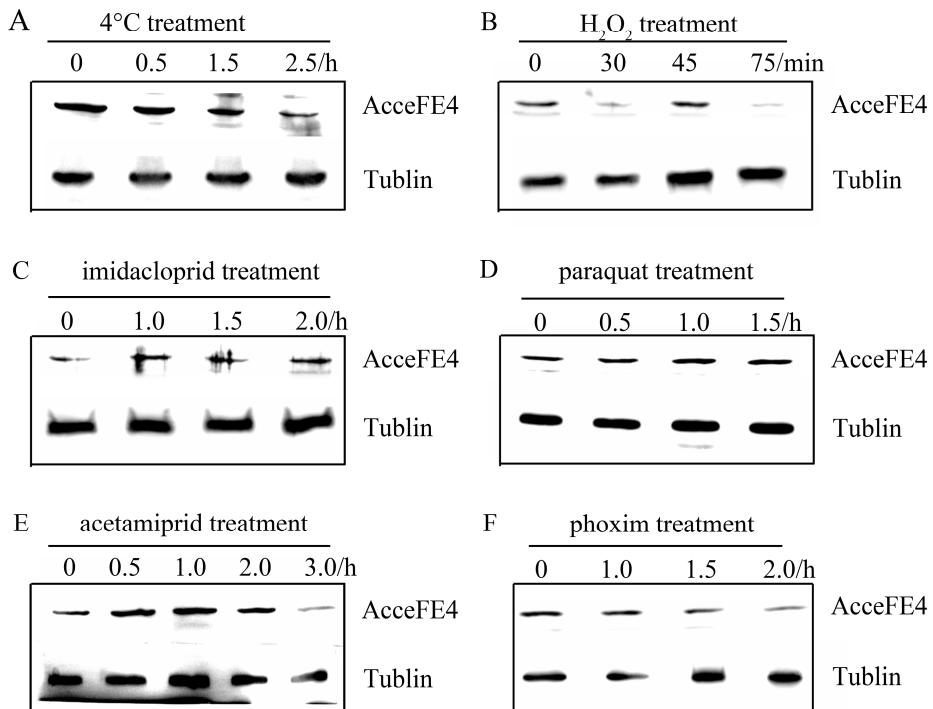
376 Fig. 5 Recombinant AcceFE4 protein expression. The SDS-PAGE gel lanes are as follows: lane 1 -
 377 recombinant AcceFE4 expression without IPTG; lanes 2-5 – recombinant AcceFE4 expression
 378 with IPTG.

379

380 *3.5. Western blot analyses*

381 To further assess the role of eEF4 in the oxidative response in *A. cerana cerana* and to
 382 verify the results of the transcription expression profiles for *AcceFE4*, we analysed
 383 protein expression in response to adverse stress by Western blot. The anti-AcceFE4
 384 antibody was used to detect the *AcceFE4* protein, and tubulin was used as the
 385 normalisation reference. As shown in Fig 6A, protein expression was consistent with
 386 the transcriptional level at 4 °C, and *AcceFE4* expression was inhibited; its lowest
 387 expression level occurred at 2.5 h after treatment. The expression level was induced at

388 45 min after the treatment with H₂O₂, as shown in Fig 6B. The AcceFE4 protein was
 389 reduced with the phoxim treatment, particularly at 2 h. In summary, *AcceFE4*
 390 expression at the protein level was consistent with its expression at the mRNA level.

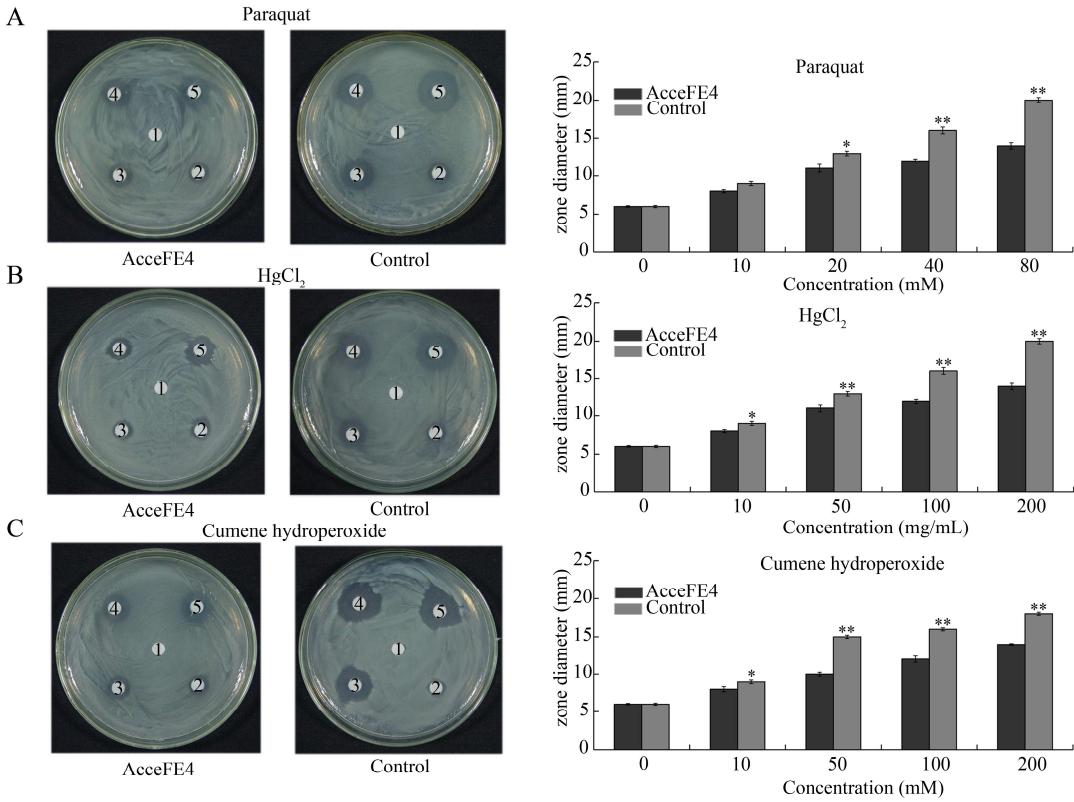


391
 392 Fig. 6 Western blot analysis of AcceFE4 expression levels after treatments with various abiotic
 393 stressors. Total protein was extracted from adult bees that were treated with various stressors,
 394 including 4 °C, H₂O₂, imidacloprid, paraquat, acetamiprid and phoxim. Tubulin was used as a
 395 reference protein for the Western blot analysis.

396

397 *3.6. AcceFE4 assessment by disc diffusion under various conditions*

398 The disc diffusion method was used to explore the role of the recombinant AcceFE4
 399 protein. The overexpressed AcceFE4 recombinant protein was exposed to various
 400 stressors, including cumyl hydroperoxide, paraquat and HgCl₂. As shown in Fig 7, the
 401 bacteria that expressed the recombinant protein had a smaller killing zone than the
 402 PET-30 (a+) empty vector control, and the results indicated that the recombinant
 403 protein contained antioxidant activity.



404

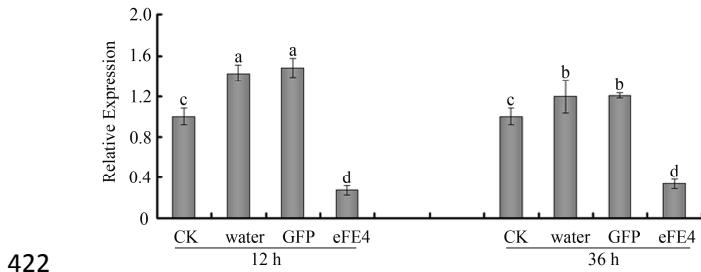
405 Fig. 7 Disc diffusion assays to detect the resistance of AcceFE4-overexpressing bacterial cells to
 406 paraquat, cumyl hydroperoxide and HgCl_2 . The halo diameters of the killing zones were compared
 407 using histograms. A. The paraquat concentrations are 0, 10, 20, 40, and 80 mM. B. The HgCl_2
 408 concentrations are 0, 10, 50, 100, and 200 mg/mL. C. The cumyl hydroperoxide concentrations are
 409 0, 10, 50, 100, and 200 mM. Independent-sample *t* tests were used for comparisons of
 410 baseline characteristics between the treatment and control groups. Significant
 411 differences are indicated with two asterisks at $P < 0.05$.

412

413 3.7. Knockdown of AcceFE4 in *A. cerana cerana*

414 The RNAi method was used to confirm the antioxidant function of *AcceFE4* in *A.*
 415 *cerana cerana*. dseFE4-RNA, dsGFP-RNA and water were injected into adult bees.
 416 As shown in Fig 8, lower *AcceFE4* transcript expression quantities were observed in
 417 the dseFE4-RNA-injected adults at 12 and 36 h post-injection, and the silencing was
 418 efficient. Additionally, the expression assessment of the control adult group, which

419 was injected with dsGFP-RNA and injected with water, showed minimal expression
 420 changes, indicating that the dsGFP-RNA and water injections did not influence
 421 *AcceFE4* expression [33].

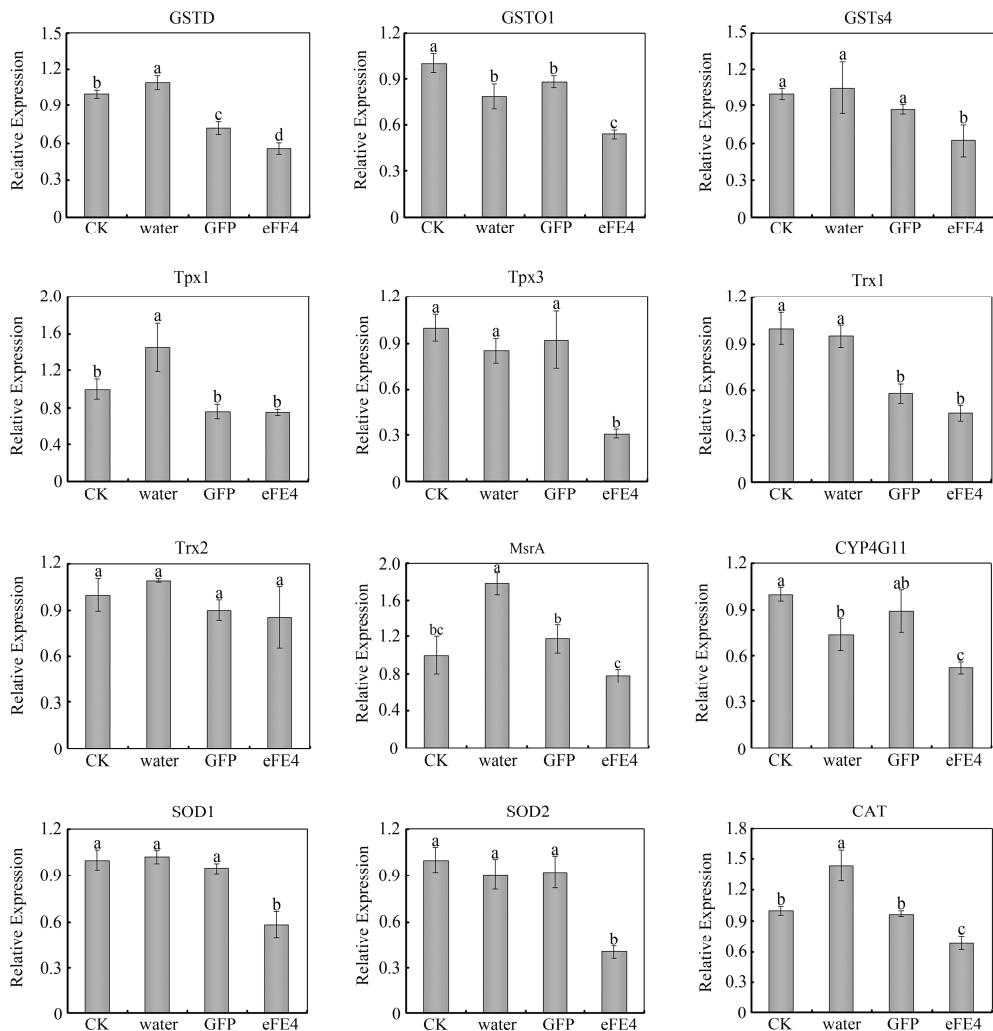


422
 423 Fig. 8 Silencing efficiency of *AcceFE4* after the adult bees were injected with 3 µg of dsRNA.
 424 Groups ck (no injection), water and dsGFP were used as the controls. Total RNA was extracted
 425 and detected by qRT-PCR at 12 and 36 h after injection. β-actin was used as an internal reference
 426 gene. The data are presented as the means ± SE of three replicates. The letters above the bar
 427 indicate significant differences ($P < 0.005$) as determined by Duncan's multiple range tests using
 428 SPSS software version 17.0.

429

430 3.8. Transcription levels of antioxidant genes after *AcceFE4* knockdown

431 To further investigate the role of *AcceFE4* against oxidative damage, we analysed the
 432 transcript levels of other antioxidant genes. The qRT-PCR results in Fig 9 show that
 433 *AccSOD1*, *AccGSTD*, *AccGSTS4*, *AccGSTO1*, *AccTpx1*, *AccTpx3*, *AccTrx1*, *AccTrx2*,
 434 *AccCAT*, *AccMsra*, and *AccCYP4G11* were reduced when *AcceFE4* was knocked
 435 down at 36 h.



436

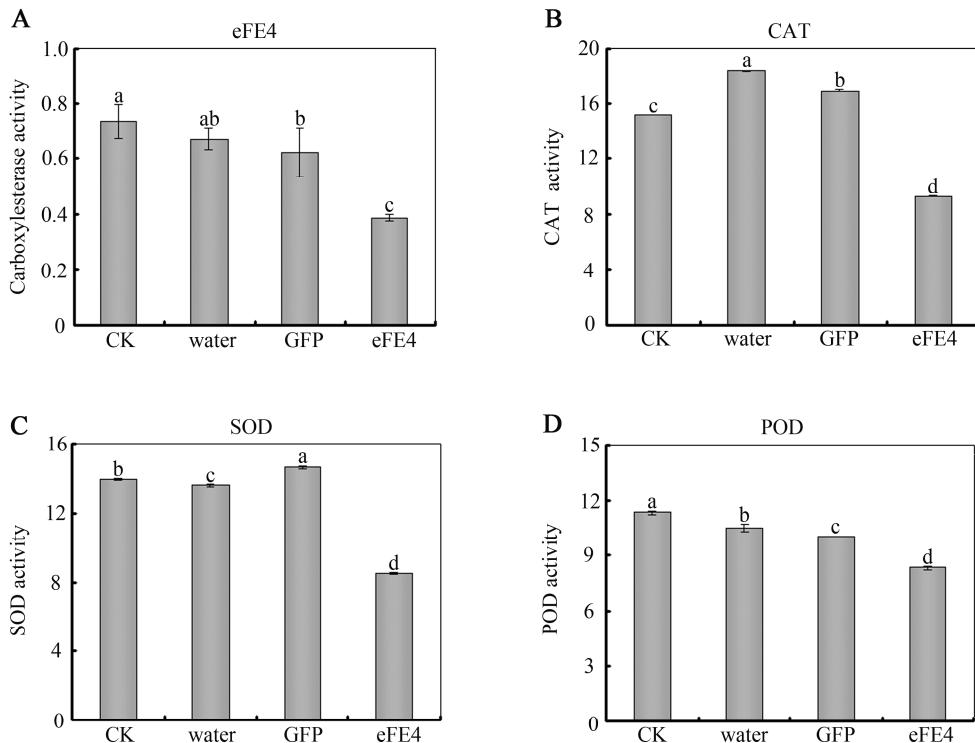
437 Fig. 9 Expression levels of other antioxidant genes (GSTD, GSTO, Trx1, Trx2, MsrA, CYP4G11,
 438 SOD1, SOD2, Tpx1, Tpx3, CAT and GST4) following *AcceFE4* knockdown at 36 h were
 439 analysed by qRT-PCR. The β -actin gene was used as the internal control. The data are presented as
 440 the means \pm SE of three replicates. The letters above the bar indicate significant differences ($P <$
 441 0.005) as determined by Duncan's multiple range tests using SPSS software version 17.0.

442

443 3.9. Determination of enzymatic activities after *AcceFE4* knockdown

444 To confirm the oxidation resistance of *AcceFE4* in *A. cerana cerana* at the protein
 445 level, we measured the enzymatic activities of the knockdown samples (at 36 h).
 446 Fig10 B, C, D indicated that the CAT, SOD, POD activities in the honeybees in which

447 *AcceFE4* was knocked down were reduced relative to the control honeybees, while
 448 the change in the control group was minimal. As shown in Fig 10 A the enzyme
 449 activity of carboxylesterases was reduced in the silent samples than in the control
 450 group.



451

452 Fig. 10 The antioxidant enzymatic activity changes for (A) Carboxylesterase, (B) CAT, (C) SOD,
 453 (D) POD when *AcceFE4* was knocked down at 36 h. Total protein was extracted from adult bees
 454 36 h after the injections. Groups ck (no injection), water and dsGFP were used as the controls. The
 455 data are presented as the means \pm SE of three replicates. The letters above the bar indicate
 456 significant differences ($P < 0.005$) as determined by Duncan's multiple range tests using SPSS
 457 software version 17.0.

458

459 4 Discussion

460 Many previous studies indicated that carboxylesterases (CarEs), cytochrome
 461 P450 monooxygenases (P450) and glutathione S-transferases (GSTs) are the major

462 metabolic detoxification enzymes that are involved in detoxification of insecticides
463 and in resistance development in insect populations [34-37]. And these enzymes may
464 play significant roles in protecting organisms from adverse environmental stress.
465 However, many studies have reported that P450 and GSTs play important roles in
466 oxidative stress [27, 38-40]. Thus, we predict that CarE may protect against oxidative
467 stress. And we have conducted a series of experiments to prove our prediction. Thus,
468 we guessed that *AcceFE4* may participate in oxidative stress and metabolic
469 detoxification process in *A. cerana cerana*.

470 In this work, we imitated several types of ROS damage that *A. cerana cerana*
471 may encounter during their lifespan and explored the resulting changes in eFE4
472 expression at the mRNA and protein levels to look for evidence of their antioxidant
473 functionality environmental stresses. We first identified an esterase FE4 from *A.*
474 *cerana cerana* and named it *AcceFE4*. Sequence analysis indicated that *AcceFE4*
475 shared a characteristic α/β hydrolase structure, including a catalytic triad consisting of
476 Ser-Glu-His and a GQSAG consensus sequence, suggesting that *AcceFE4* was
477 biologically active [9]. Additionally, phylogenetic analysis revealed an *AcceFE4* clade
478 that was implicated in dietary detoxification. Taken together, these results indicated
479 that *AcceFE4* was a member of the carboxylesterase family.

480 To better understand the role of *AcceFE4* might play, we analysed *AcceFE4*
481 mRNA expression at multiple developmental stages and its tissue distribution. The
482 expression of developmental stages revealed that the *AcceFE4* mRNA expression was
483 higher in the pupal stages than in the adult stages. Because ROS cause oxidant
484 damage (particularly in fast-growing organisms) due to high oxygen levels [39], we
485 speculated that *AcceFE4* may be played a vital role in preventing ROS-mediated
486 damage during the pupae growth stages and would be essential for the honeybee's
487 development. Indeed, several studies have demonstrated that midgut is the first barrier
488 of xenobiotics in peroral toxicity [34-37]. It has been reported that metabolic process
489 occurred in midgut tissue [41]. Similarly, the expression level of *AcceFE4* in tissues
490 showed that highly expressed in midgut. Taken together, these data indicate that

491 *AcceFE4* may play important roles in preventing adverse damage during different
492 developmental stages and in different tissues.

493 In order to explore the function of *AcceFE4*, we imitated adverse life-threatening
494 environmental conditions that could induce ROS-mediated damage and pose threats to
495 the honeybee over the course of its life; these conditions included cold temperature,
496 H₂O₂, UV radiation [42, 43]. Temperature is an abiotic environmental factor that
497 causes physiological changes in organisms [44, 45], and ROS are key mediators of
498 cold-induced apoptosis [46]. In this study, we found that when the honeybees were
499 exposed to 4 °C, the *AcceFE4* mRNA levels were down-regulated. Cold stress can
500 induce hepatocytes and liver endothelial cells to undergo apoptosis due to
501 ROS-mediated damage in mammals. [47]. Low temperatures decrease metabolic rates
502 and increase oxygen radical formation during flight in honeybees [48]. However, after
503 H₂O₂ treatment, the gene was highly induced. H₂O₂ is a typical oxidant that causes
504 oxidative damage and can induce ROS elevation, which results in cell death by
505 oxidative stress [49-50]. Furthermore, there is also evidence that UV is a harmful
506 environmental stressor that can produce oxidative stress in honeybees and can cause
507 oxidative stress through ROS production, which damages membrane lipids and
508 proteins [51-52]. The qRT-PCR results showed that *AcceFE4* was repressed during the
509 UV exposure, which indicated that *AcceFE4* may play different roles in UV resistance
510 and may be involved in different signal transduction processes [45]. Moreover, heavy
511 metals, such as HgCl₂ and CdCl₂, can enhance intracellular ROS formation, which
512 promoting cellular oxidative stress [53-54]. We found that the gene was repressed
513 after the HgCl₂ and CdCl₂ treatments. These findings indicated that *AcceFE4* was
514 responsive to heavy metals and played a significant role in the honeybee's physiology.
515 Taken together, these results showed that *AcceFE4* was responsive to adverse and
516 oxidative stressors, indicating that *AcceFE4* was important in the life of *A. cerana*
517 *cerana*.

518 Nowadays, the mechanism of carboxylesterases insecticides metabolic
519 detoxification has been studied very clear however, few studies demonstrated that

520 antioxidative mechanism about carboxylesterase in insects [55]. For instance, as an
521 important detoxifying enzyme, carboxylesterase has been implicated in insecticide
522 resistance with its relevant metabolic functions, such as catalyzing hydrolysis of ester,
523 sulfate, and amid [56]. Studies have shown that CarE is important in the hydrolysis of
524 a broad range of endogenous and xenobiotic ester-containing compounds, such as OPs,
525 carbamates, and pyrethroids [21]. Insecticides are significant threats to a honeybee's
526 life and can induce the oxidation of lipid biomembranes, leading to impaired
527 biochemical and physiological functions [57]. In this work, we imitated adverse
528 conditions that bees may encounter in their life, such as, phoxim, paraquat, acaricide,
529 cyhalothrin and so on. Nearly all treatments caused a change of *AcceFE4* expression
530 profiles. For example, imidacloprid treatment promoted increased *AcceFE4*
531 expression. Ge reported that imidacloprid can induce oxidative stress and DNA
532 damage in zebrafish [58]. We suggest that *AcceFE4* may play different roles in the
533 honeybee. Additionally, *AcceFE4* transcription levels were consistent with its protein
534 expression levels. These data confirmed our speculation that eFE4 may be involved in
535 metabolic detoxification and adverse stress responses in *A. cerana cerana*.

536 Additionally, in the disc diffusion assay, we imitated three types of
537 environmental stress to explore the function of *AcceFE4*. When the paraquat, HgCl₂
538 and cumene hydroperoxide treatments were used, we observed that the killing zones
539 of the *E. coli* that overexpressed AcceFE4 were smaller than those of the control
540 bacteria. All together with previous results indicate that the bacterial growth may be
541 medicated by the overexpression of AcceFE4. And we speculate that AcceFE4
542 overexpression may increase ROS resistance in bacterial cells and protect *A. cerana*
543 *cerana* from bacterial damage.

544 RNAi technology has become a promising investigative strategy for
545 understanding gene roles, particularly for insects where transgenic technology is
546 insufficient [59]. We silenced the gene by microinjecting dseFE4-RNA into *A. cerana*
547 *cerana*. The controllable dose and minimal invasiveness make the microinjection
548 method widely useful for diverse insect types [31,60,61]. Here, we knocked down

549 *AcceFE4* using RNAi technology to further explore *AcceFE4* functions. In our
550 research, we found that the expression levels of *AccSOD*, *AccTrx*, *AccMsra*, *AccTpx*,
551 *AccGST* and *AccCAT* and other genes were reduced when *AcceFE4* was silenced.
552 However, these genes has been reported that they play important roles in the oxidative
553 stress [30, 33, 62, 63, 45], which indicating that *AcceFE4* may participate in the
554 oxidative process. Organism possess a variety of antioxidant systems for resisting
555 oxidative damage, including the glutathione S-transferases (GSTs), superoxide
556 dismutases (SODs), catalase (CAT), thioredoxin peroxidase (Tpx), methionine
557 sulfoxide reductase A (Msra), and thioredoxin (Trx) systems, which consist of
558 multifunctional proteins and enzymes. The omega-class glutathione S-transferase
559 (GSTO2) is required for reducing oxidative damage in *A. cerana cerana* [2]. Tsuda
560 has reported that the loss of Trx-2 enhances oxidative stress-dependent phenotypes in
561 Drosophila, which are hypersusceptible to paraquat and exhibit high levels of
562 expression of antioxidant genes, such as those encoding SOD, CAT, and glutathione
563 synthetase [64]. Moreover, it has been reported that the Msra system may mediate
564 methionine sulfoxide modifications and that absence of the Msra enzyme may result
565 in elevated levels of protein carbonyls in yeast and mice [65]. Thus, the antioxidant
566 genes expression changed after *AcceFE4* knockdown, indicating that *AcceFE4* may be
567 response to the oxidative stress. Antioxidant enzymes (CAT, POD and SOD) play
568 important roles in scavenging H₂O₂ [39, 45]. When their activity levels were
569 down-regulated by the *AcceFE4* knockdown, which may be result in the change of
570 H₂O₂ levels. This finding provided further confirmation of *AcceFE4*'s involvement in
571 the antioxidant process. In our study, the enzyme activities decreased in the
572 knockdown protein samples compared to the control groups. Moreover, the declining
573 activity of CarE indicated that *AcceFE4* may play important roles in response to
574 oxidative damage. Based on these findings, we speculate that *AcceFE4* may play
575 important roles in oxidative stress resistance when the bees encountered the adverse
576 environmental stresses in their life.

577 In conclusion, we have isolated a metabolism and detoxification enzyme, eFE4,

578 from *A. cerana cerana*, and we have studied the transcript and protein expression
579 levels of *AcceFE4* in response to various environmental stressors. *AcceFE4* was
580 involved in the oxidative process and protected the organism against ROS-mediated
581 damage. Additionally, after successful knockdown of *AcceFE4*, the declines in the
582 levels of multiple antioxidant genes and the reduced enzymatic activities of CAT,
583 SOD, and POD confirmed our results. This article provides a better understanding of
584 the detailed mechanisms that underlie the *AcceFE4* response to environmental stress
585 and will be useful for studying CarE in the organism. However, these data address
586 only the possibility that *AcceFE4* plays roles in adverse stress responses. To better
587 understand the characteristics of *AcceFE4*, further studies that validate the gene's
588 function will be necessary.

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595

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Highlights

- 1 The first report about a carboxylesterase gene (*AcceFE4*) from *Apis cerana cerana*.**
- 2 We imitated the adverse environments that the bees may be encountered in their life.**
- 3 *AcceFE4* was knockdown successfully in the *Apis cerana cerana*.**