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Gene expression analysis and enzyme assay reveal a potential role of the carboxylesterase gene *CpCE-1* from *Cydia pomonella* in detoxification of insecticides

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ABSTRACT

Carboxylesterases (CarEs) are responsible for metabolism of xenobiotics including insecticides in insects. Understanding the expression patterns of a such detoxifying gene and effect of insecticides on its enzyme activity are important to clarify the function of this gene relevant to insecticides-detoxifying process, but little information is available in the codling moth *Cydia pomonella* (L.). In this study, we investigated the expression profiles of CarE gene *CpCE-1* at different developmental stages and in different tissues of *C. pomonella*, as well as the larvae exposed to chlorpyrifos-ethyl and lambda-cyhalothrin by using absolute real-time quantitative PCR (absolute RT-qPCR). Results indicated that *CpCE-1* expression was significantly altered during *C. pomonella* development stages, and this expression differed between sexes, with a higher transcript in females than males. Meanwhile, *CpCE-1* is overexpressed in cuticle, midgut and head than silk gland, fat body and Malpighian tubules. Exposure of third instar larvae to a non-lethal dosage of chlorpyrifos-ethyl and lambda-cyhalothrin resulted in induction of *CpCE-1* transcript. The total carboxylesterase enzyme activity was inhibited by chlorpyrifos-ethyl *in vivo*; in contrast, the activity of *Escherichia coli* produced recombinant CpCE-1 was significantly inhibited by both lambda-cyhalothrin and chlorpyrifos-ethyl *in vitro*. These results suggested that *CpCE-1* in *C. pomonella* is potentially involved in the development and in detoxification of chlorpyrifos-ethyl and lambda-cyhalothrin.

1. Introduction

Carboxylesterases (CarEs, EC 3.1.1.1) are enzymes responsible for hydrolysis of chemicals which contain a functional group of carboxylic acid ester, amide, and thioester [1,2]. Most insect CarEs are associated with insecticide resistance or hormone and semiochemical metabolism [3,4]. They play a key role in the detoxification of many insecticides, including synthetic pyrethroids (SPs), carbamates (CBs) and organophosphates (OPs) and have been shown to be involved in development of resistance to insecticide groups SPs, CBs and OPs [5,6].

The codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is one of the most serious orchard pests of pome fruit production with huge economic losses suffered in cases where integrated control strategies are not implemented in the world [7–9]. This pest has been directly targeted by insecticides. As a result, resistance of codling moth to insecticides has been reported in many areas, including Europe [10], North America [11], South America [12], Australia [13] and northwestern China [14]. Previous studies observed CarEs activity in insecticide resistant populations increasing [8,9] or decreasing [10,12,15,16] compared with the laboratory strain, indicating that CarEs are likely associated with resistance to SPs, CBs and OPs insecticides. CarE gene overexpression contributing to insecticide resistance has also been reported in many insects such as in *Bombyx mori* [17], *Myzus persicae* [18], *Nilaparvata lugens* [19], *Aphis gossyppi* [20–21], and also in mite *Tetranychus cinnabarinus* [22]. Therefore, the study of CarEs promotes our understanding of the function of CarE involved in metabolism of xenobiotics and the development of resistance to insecticides in *C. pomonella*. However, the spatiotemporal and insecticide-induced expression profiles of specific CarE genes have not been described in *C. pomonella*.

Understanding the expression patterns of a detoxifying gene and its responses to insecticides are important to clarify the function of this gene relevant to insecticides-detoxification process. Currently, relative and absolute real-time quantitative PCR (RT-qPCR) methods are available. Relative quantification method requires normalization the mRNA levels of the gene of interest to housekeeping or reference genes through the number of PCR cycles. However, the relative quantification becomes inaccurate when target or reference genes are low constitutively expressed. In a preliminary experiment, a relative RT-qPCR was selected to construct a standard curve to determine the expression level of *CpCE-1* at different developmental stages and in different tissues; however, the *Ct* values in non-diluted cDNA were higher than 26.0 due to the low constitutive expression level of *CpCE-1*. Thus, it is difficult to determine the expression level of *CpCE-1* by relative RT-qPCR method. Alternatively, absolute quantification method relies on a standard plot constructed from series diluted concentrations of standards to measure the actual copy numbers of a specific target gene,

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and is therefore considered to be more informative and reliable for comparisons [23]. The only normalization done between samples is for the starting amount of total RNA [23].

We have reported the isolation of a CarE gene *CpCE-1* (GenBank number: KC832922) from *C. pomonella* in previous work [24]. To further understand the function of this gene relevant to the xenobiotics-detoxifying process, it is urgent to clarify the spatiotemporal and insecticide-induced expression patterns, as well as the effect of insecticides on the activity of recombinant protein. In the present study, the spatiotemporal, and chlorpyrifos-ethyl- and lambda-cyhalothrin-exposure (contact)-induced expression profiles of *CpCE-1* were determined using absolute RT-qPCR. The total carboxylesterase activity of *C. pomonella* exposure to chlorpyrifos-ethyl and lambda-cyhalothrin and the effect of these two insecticides on the activity of *E. coli* produced recombinant CpCE-1 *in vitro* were further determined.

2. Materials and methods

2.1. Insects and insecticide treatments

The *C. pomonella* larvae were originally collected from abandoned apple orchards in Wuwei City (Gansu Province, China), and fed on flesh of apple debris in the laboratory. The codling moth strain reared for 6 generations, and has never been exposed to insecticides and any chemicals, was used in this study. One microliter (μl) of 12.5 mg/L chlorpyrifos-ethyl and 0.19 mg/L lambda-cyhalothrin (a LC_{10} dosage) was applied on the dorsum of each third instar larva. Detailed description of the rearing condition, insecticide treatments and sample preparation were given in [25].

2.2. Sampling at different developmental stages

Three replicates of samples (twenty first to second instar larvae, ten third instar larvae, five fourth to fifth instars larvae, two-day old pupae and two-day old adults per replicate) were independently prepared in this experiment. Pupae and adults were divided to males and females, respectively. Samples were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.3. Larval dissections

Head, cuticle, silk gland, midgut, fat body and Malpighian tubules were dissected from 3 groups of 30 third instar larvae. Samples were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

2.4. RNA extraction and cDNA synthesis for molecular cloning

The total RNA was extracted from five third instar larvae using the RNAiso Plus Kit (Takara, Dalian, China) based on the manufacturer's instructions. The quality and concentration of extracted RNA was examined by electrophoresis and spectrophotometer (Infinite M200 PRO, Switzerland). The RNA samples were then digested with DNase I (MBI, Fermentas) to remove the genomic DNA. cDNA was synthesized (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas) using Oligo $(dT)_{18}$ primer (100 μM) in a 20 μl reaction mixture containing 1.0 μg total RNA. The product was stored at −20 °C before used in PCR.

2.5. Construction of the standard plasmids

The primers QCpCE-1-F and QCpCE-1-R for amplification of a 217 bp length of *CpCE-1* sequence, and the QActin-F and QActin-R (Table 1) for amplification of a 155 bp length of β-actin sequence [26], were designed using the Primer3 web-tool (http://primer3.sourceforge.net). The PCR was performed using high-fidelity Ex *Taq* polymerase (Takara) under the thermocycler conditions as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and with a final extension at 72 °C for 7 min. The PCR products was separated on a 1% agarose gel electrophoresis and the band of expected size was purified using the Biospin Gel Extraction Kit (Bioer Technology Co., Ltd) as described by the manufacturer. The purified fragments were inserted into the TA cloning vector pMD-19 T (Takara) and three clones were sequenced by Shanghai Sunny Biotech Co., Ltd., China. The positive clones were subjected to extract their plasmids DNA for later use. The quality of extracted plasmid DNA was detected by electrophoresis and spectrophotometer (Infinite M200 PRO, Switzerland) to determine the $A_{260/A280nm}$ value for potential bacterial DNA or protein contaminations. The final concentration of plasmid DNA stock was 645 ng/μl for *CpCE-1-pMD-19 T*, and 100 ng/μl for *β-actin-pMD-19 T*.

Plasmids from independent plasmid extractions were used as template DNA at concentrations ranging from 1 ng/μl to 0.01 pg/μl to produce standard curves. The plasmid whose constructed standard curve appears amplification efficiencies ~100% and correlation coefficients (R^2) ~1.0 was selected as for construction of standard curve.

2.6. Standard curve development and absolute quantitative real-time PCR (RT-qPCR)

Standard curves of *CpCE-1* and β-actin were obtained using linear gradient dilution of standard plasmid of *CpCE-1-pMD-19 T* (20-fold: 675, 33.75, 1.69, 0.084, 0.0042, 0.00021 and 0.0000105 ng/μl, corresponding to 2.02×10^{11}, 1.01×10^{10}, 5.06×10^{8}, 2.53×10^{7}, 1.26×10^{6}, 6.32×10^{4} and 3.16×10^{3} *CpCE-1* copy numbers) and *β-actin-pMD-19 T* (10-fold: 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/μl, corresponding to 3.20×10^{10}, 3.20×10^{9}, 3.20×10^{8}, 3.20×10^{7}, 3.20×10^{6}, 3.20×10^{5} and 3.20×10^{4} β-actin copy numbers) as templates, respectively. The β-actin gene was reported to be an excellent reference gene, which with a constitutive expression among tissues, life stages and insecticide treatments [26]. Thus, the reliability of the quantification method was determined according the expression stability of β-actin. The RT-qPCR reactions were carried out on a BioRad iCycler iQ5 (BioRad, USA). The total RNA of each replicate from tissues, the whole body of various developmental stages, insecticide- and acetone-exposed larvae of *C. pomonella* was independently extracted using the RNAiso Plus Kit (Takara, Dalian, China). For the tissue-specific expression assay, each tissue dissected from 30 third instar larvae was regarded as an independent specimen to isolate the total RNA. For the developmental expression assay, the qPCR was repeated three times with the total RNA and cDNA of extracted from each replicate. For the insecticide-induced gene expression, the qPCR was repeated three times with independently extracted total RNA. One ml of RNAiso Plus was used for each independently extraction. Subsequently, the RNA samples were then digested with DNase I (MBI) to remove the genomic DNA. The quality and concentration of extracted RNA was

Table 1
The information of primers used in this study.

Primer name	Sequence(5′-3′)	Length (bp)	Tm (°C)	Reference
QCpCE-1-F[a]	GTTTCAAGCACCTGGACCGC			
QCpCE-1-R[a]	GAACTTTCCTCCGCTGCCTAA	217	55	–
QActin-F	CGGCATCCACGAAACCACCT			
QActin-R	TGGAAGGAGCCAGTGCGG	155	55	Yang and Zhang (2014)

[a] The prime pair was designed using the Primer3 web-tool (http://primer3.sourceforge.net).