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Knockdown of NADPH-cytochrome P450 reductase increases the susceptibility to carbaryl in the migratory locust, *Locusta migratoria*

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HIGHLIGHTS

- The 2043 bp cDNA sequence of *CPR* gene from *Locusta migratoria* was cloned by RT-PCR.
- Higher mRNA expression was found in the antenna, ovary, integument, and hindgut.
- Silencing of *LmCPR* reduced the enzymatic activity of LmCPR.
- Silencing of *LmCPR* enhanced the susceptibility of *Locusta migratoria* to carbaryl.

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ABSTRACT

Background: NADPH-cytochrome P450 reductase (CPR) plays important roles in cytochrome P450-mediated metabolism of endogenous and exogenous compounds, and participates in cytochrome P450-related detoxification of insecticides. However, the CPR from *Locusta migratoria* has not been well characterized and its function is still undescribed.

Results: The full-length of *CPR* gene from *Locusta migratoria* (*LmCPR*) was cloned by RT-PCR based on transcriptome information. The membrane anchor region, and 3 conserved domains (FMN binding domain, connecting domain, FAD/NADPH binding domain) were analyzed by bioinformatics analysis. Phylogenetic analysis showed that *LmCPR* was grouped in the Orthoptera branch and was more closely related to the CPRs from hemimetabolous insects. The *LmCPR* gene was ubiquitously expressed at all developmental stages and was the most abundant in the fourth-instar nymphs and the lowest in the egg stage. Tissue-specific expression analysis showed that *LmCPR* was higher expressed in ovary, hindgut, and integument. The CPR activity was relatively higher in Malpighian tubules and integument. Silencing of *LmCPR* obviously reduced the enzymatic activity of LmCPR, and enhanced the susceptibility of *Locusta migratoria* to carbaryl.

Conclusion: These results suggest that LmCPR contributes to the susceptibility of *L. migratoria* to carbaryl and could be considered as a novel target for pest control.

1. Introduction

The oriental migratory locust (*Locusta migratoria*) is not only one of the most destructive agricultural pests in the world, but also a good research model for insect molecular biology and toxicology (Zhang et al., 2014). In past decades, the frequent application of insecticides has inevitably led to insecticide resistance in some natural populations of *L. migratoria* (Yang et al., 2009).

Cytochrome P450 monooxygenase (CYP) is a member of the hemoprotein family, and it catalyzes a series of crucial biological reactions in all living organisms from bacteria to humans (Feyereisen, 2011). The most common reaction catalyzed by CYP is the monooxygenase reaction, which introduces oxygen into various reducing substrates (Sezutsu et al., 2013). In insects, CYPs participate in the detoxification of insecticides, drugs, and plant secondary metabolites and play a key role in metabolizing ecdysteroids, juvenile hormones, and fatty acids (Feyereisen, 2006). Our previous studies suggested that *CYP409A1* and *CYP408B1* (Guo et al., 2012),

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CYP9A subfamily (Guo et al., 2015; Zhu et al., 2016) and *CYP6FF1* were involved in the detoxification of pyrethroid pesticides, and *CYP6FD2* and *CYP6FE1* were associated with the metabolism of carbaryl in *L. migratoria* (Guo et al., 2016).

The reaction of microsomal CYPs depends on electron transfer from NADPH through FAD and FMN to the ferrum atom of the prosthetic heme group in the CYP by its electron transfer partner, NADPH-Cytochrome P450 Reductase (Masters and Okita, 1980) (CPR; Fig. 2). Generally speaking, each insect genome possesses only 1 CPR gene. Since the first insect CPR was cloned from *Musca domestica* (Koener et al., 1993), more than 20 insect CPRs have been identified, and some upregulated CPRs were further proved to participate in insecticide resistance in several species, such as *Tetranychus cinnabarinus* (Shi et al., 2015), *Laodelphax striatellus* (Zhang et al., 2016), and *Bactrocera dorsalis* (Huang et al., 2015). Furthermore, RNAi-mediated silencing of insect CPR genes enhanced insecticide-induced mortality in *Cimex lectularius* (Zhu et al., 2012) and *Nilaparvata lugens* (Liu et al., 2015b). For its essential role in the CYPs/CPR electron cycle, insect CPRs were considered as a novel target for the development of synergists (Zhu et al., 2012). However, there is no report on the sequence and biological function of CPR from *L. migratoria* and whether it is related to insecticide-induced mortality.

In the present study, a full-length cDNA of the CPR from *L. migratoria* (*LmCPR*) was cloned and characterized. The developmental and spatial expression patterns of LmCPR were examined by RT-qPCR. Tissue distribution of CPR activity was determined by spectrophotometrical method at 550 nm. The transcription of *Lm*CP*R* was silenced by injection of *LmCPR*-specific dsRNA, which depressed CPR enzymatic activity. Then, a bioassay was performed to compare the carbaryl susceptibility of *LmCPR*-silenced and control *L. migratoria*. This study will help to elucidate the role of CPRs in CYP-mediated metabolic detoxification of carbaryl in *L. migratoria*.

2. Material and methods

2.1. Insect cultures and treatments

L. migratoria eggs were purchased from Insect Protein Co., Ltd. (Cangzhou, China). The eggs were hatched in sandy soil, and instar nymphs were reared on fresh leaves of *Setaria viridis* (L.) at 30 ± 1 °C under a 14 h:10 h light: dark cycle. Adults were bred under the same light and temperature conditions, and were supplied with fresh leaves of *Setaria viridis* (L.) and wheat bran.

2.2. Reagents and assay kits

The pEASY-Blunt Zero cloning kit and Fastpfu DNA polymerase were obtained from Transgen Biotech Co. Ltd. (Beijing, China). RevertAid H minus reverse transcriptase was purchased from Fermentas (MA, USA). SYBR Green Real-time PCR Master Mix was obtained from Toyobo (Osaka, Japan). dNTPs, TRIzol, and the gel extraction kit were obtained from TaKaRa Bio Group (Dalian, China). The T7 RiboMAx Express RNAi System was from Promega (WI, USA). The Cytochrome *c* reductase (NADPH) assay kit was obtained from Sigma-Aldrich (MO, USA). All other reagents were of the highest grade commercially available.

2.3. Synthesis of cDNA and cloning of LmCPR

Total RNA, which used to clone full-length *LmCPR*, was extracted from nymphs and adults of *L. migratoria* by the TRIzol method, according to the manufacturer's protocol. The quality and concentration of the extracted RNA was determined with a Nanodrop 2000. The first-strand cDNA was synthesized from 4 μg of total RNA by RevertAid H minus reverse transcriptase in a reaction containing dNTPs, RNase inhibitor, and oligo$(dT)_{18}$. Based on the *L. migratoria* transcriptome data, a pair of full-length primers for RT-PCR was designed and is shown in Table 1. The RT-PCR was performed with FastPfu DNA polymerase. The PCR product was recovered with the gel extraction kit and ligated into the pEASY Blunt Zero vector, which was then transformed into *Trans*-T1 competent cells and sequenced in both directions by Sangon Biotech (Shanghai, China).

2.4. Bioinformatics analysis of LmCPR gene

The predicted open reading frame (ORF) and protein sequence of LmCPR was deduced from the *LmCPR* gene by DNA translate (http://web.expasy.org/translate/). The theoretical isoelectric point and molecular weight of LmCPR were predicted by ProtParam (http://web.expasy.org/protparam/). The amino acid sequences of various insect CPRs were aligned using Muscle software. Then, a phylogenetic tree was constructed by the neighbor-joining method using MEGA 6.0 software (Tamura et al., 2013), with 1000 bootstrap replicates.

2.5. Reverse transcription quantitative PCR analysis

The primers used for the RT-qPCR analysis are shown in Table 1. The RT-qPCR assay was performed on an ABI 7300 real-time PCR system in a final volume of 20 μL, containing 4 μL of cDNA,10 μL of 2 × SYBR Green Real-time PCR Master Mix (with ROX), 1.5 μL of each RT-qPCR primer (10 pmol/μL), and 3 μL of DNase-free water. The PCR started with a denaturation step at 94 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 61 °C for 31 s. For the tissue-specific expression profiles, total RNA samples were prepared from the brain, foregut, midgut, gastric caecum, hindgut, fat bodies, Malpighian tubules, ovary, spermary, hemolymph, integument and muscles from the fifth-instar nymphs of *L. migratoria*. For the stage-specific expression profiles, the eggs, the first-, second-, third-, fourth- and fifth-instar nymphs and adults were collected for RNA extraction. *EF1α*, *RpL32* and *Hsp70* were selected as the reference genes. All data were analyzed by one-way ANOVA, Tukey's HSD test. Relative transcript levels were determined by using the double standard curve method. The amplification efficiency of the primer used for RT-qPCR was higher than 0.95. The experiment was repeated with five biological replicates.

Table 1
Primer used in this study.

Application	Name	5′-3′ sequence
Full-length	*CPR*-Full FW	ATGGAGGCAGAGGCAGGCAACG
RT-PCR	*CPR*-Full RV	TCAGCTCCATACATCTGAAGAAT
RT-qPCR	*CPR*-FW	TTACCCACCTGCTACAAAAGAA
	CPR-RV	CACAACAATGTCATGCACATCA
	Hsp70-FW	CTGGTGTGCTCATTCAGGTAT
	Hsp70-RV	TCGTGGGGCAGGTGGTATT
	EF1α-FW	AGCCCAGGAGATGGGTAAAG
	EF1α-RV	CTCTGTGGCCTGGAGCATC
	RPL32-FW	ACTGGAAGTCTTGATGATGCAG
	RPL32-RV	CTGAGCCCGTTCTACAATAGC
	β-actin-FW	CGAAGCACAGTCAAAGAGAGGTA
	β-actin-RV	GCTTCAGTCAAGAGAACAGGATG
RNAi template	CPR-RNAi FW	taatacgactcactatagggTATGAAATGGGTCTTGGGGA
PCR	CPR-RNAi RV	taatacgactcactatagggGGGTGCTTCTTCGTTGACTC