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Identification and characterization of NADPH-dependent cytochrome P450 reductase gene and cytochrome b_5 gene from *Plutella xylostella*: Possible involvement in resistance to beta-cypermethrin

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ABSTRACT

NADPH–cytochrome P450 reductase (CPR) and cytochrome b_5 (b_5) are essential for cytochrome P450 mediated biological reactions. CPR and b_5 in several insects have been found to be associated with insecticide resistance. However, CPR and b_5 in the diamondback moth (DBM), *Plutella xylostella*, are not characterized and their roles remain undefined. A full-length cDNA of CPR encoding 678 amino acids and a full-length cDNA of b_5 encoding 127 amino acids were cloned from DBM. Their deduced amino acid sequences shared high identities with those of other insects and showed characteristics of classical CPRs and b_5s, respectively. The mRNAs of both genes were detectable in all developmental stages with the highest expression levels occurring in the 4th instar larvae. Tissue-specific expression analysis showed that their transcripts were most abundant in gut. Transcripts of CPR and b_5 in the beta-cypermethrin resistant DBM strain were 13.2- and 2.84-fold higher than those in the beta-cypermethrin susceptible strain, respectively. The expression levels of CPR and b_5 were enhanced by beta-cypermethrin at the concentration of 12 mg L^{-1} (~LC_{10}). The results indicate that CPR and b_5 may play essential roles in the P450 mediated resistance of DBM to beta-cypermethrin or even other insecticides.

1. Introduction

The diamondback moth (DBM), *Plutella xylostella*, is a worldwide distribution pest damaging crucifer vegetables which causes over 1 billion USD lost annually (Furlong et al., 2013; Talekar and Shelton, 1993). To date, its control remains mainly relying on the use of chemical insecticides. However, due to the frequent and unreasonable use of insecticides, as well as its polyvoltine characteristics and wide overlap of generations, DBM has developed resistance to >90 insecticides (APRD, 2014; Furlong et al., 2013). The enzymatic detoxification is considered as the major mechanism of DBM resistance to various insecticides. Cytochrome P450 monooxygenases (P450s), a superfamily of ancient enzymes that play dominant roles in the oxidative metabolism of endogenous compounds and xenobiotics (Feyereisen, 1999), have been suggested to be involved in DBM field resistance to organophosphates, pyrethroids, indoxacarb, avermectins, and benzoylureas (Eziah et al., 2009; Iqbal and Wright, 1997).

Generally, during the P450-dependent metabolic process, two electrons are required. The first one or both are considered to be transferred from NADPH by NADPH-dependent cytochrome P450 reductase (CPR) (Gan et al., 2009). The second one is thought to be transferred by cytochrome b_5 (b_5) in some cases (Porter, 2002; Zhang et al., 2005). Both two enzymes, playing crucial roles in supporting the P450 metabolic system, have been identified to be associated with insecticide resistance in several insects. In *Helicoverpa armigera*, the induction and over-expression patterns of b_5 were identical to those of CYP6B7 indicating its involvement in the CYP6B7-mediated pyrethroid resistance (Ranasinghe and Hobbs, 1999a). A recent study showed that the susceptibility of *H. armigera* to fenvalerate was significantly enhanced after knockdown of CYP6B7 together with CPR and b_5 (Tang et al., 2012). Knockdown of CPR also resulted in the reduced deltamethrin resistance in *Cimex lectularius* (Zhu et al., 2012), and the increased susceptibility to imidacloprid and beta-cypermethrin in *Nilaparvata lugens* (Liu et al., 2014), respectively.

To our knowledge, although sequences of CPR and b_5 cDNAs of many insects are available on NCBI database, only a few CPRs from several species of insects, including *Musca domestica* (Koener et al., 1993), *Drosophila melanogaster* (Hovemann et al., 1997), *Bombyx mori* (Horike et al., 2000), *Mamestra brassicae* (Maibeche-Coisne et al., 2005), *Anopheles gambiae* (Lycett et al., 2006), *Anopheles minimus* (Kaewpa et al., 2007), *C. lectularius* (Zhu et al., 2012), *Chilo suppressalis* (Liu et al., 2013), *H. armigera* (Zhao et al., 2014), and *N. lugens* (Liu et al., 2014), have

Abbreviations: DBM, diamondback moth; *CPR*, NADPH–cytochrome P450 reductase; b_5, cytochrome b_5; P450s, cytochrome P450 monooxygenases; RT-qPCR, real-time quantitative polymerase chain reaction; RACE, rapid amplification of cDNA ends; NCBI, National Center for Biotechnology Information; ANOVA, analysis of variance.

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been characterized. And reports about insect b_5 were even fewer. There is no report concerning the sequences and functions of CPR and b_5, as well as their interactions with individual P450 in DBM. In this work, the full-length cDNAs of CPR and b_5 from DBM were cloned and characterized. Their transcriptional levels at different developmental stages and in various tissues were investigated. The transcriptional responses of CPR and b_5 to beta-cypermethrin, as well as their expression profiles in beta-cypermethrin population were examined. The present study may provide a foundation for further investigation to uncover the functions of CPR and b_5 in the P450-mediated beta-cypermethrin or other insecticide resistance in DBM.

2. Materials and methods

2.1. Insects

An insecticide-susceptible strain of DBM (L-SS) with an LC_{50} value of 48.9 mg L^{-1} for beta-cypermethrin was initially obtained from a culture at the Biorational Pesticides Research and Development Center, Northwest A&F University, Shaanxi, China, has been maintained in the laboratory for >5 years without exposure to insecticides. A field strain of DBM (TB-RS) was originally collected from cabbage field in Tai Bai county, Shaanxi, China, in August 2013, and showed an LC_{50} value of 7.92 g L^{-1} for beta-cypermethrin with the resistance ratio about 162-fold.

The two strains were reared on Pak choi cabbage seedlings at 25 ± 2 °C, 50% relative humidity and with a photoperiod of 16:8 (light:dark) in the laboratory. Newly molted moths were collected and supplied with 5% honey solution as nutrients and permitted to oviposition on moist gauze sterilized with a 1% sodium hypochlorite solution.

2.2. Beta-cypermethrin treatment

Beta-cypermethrin (purity ≥ 95%, Jingchun Co. Ltd., Shanghai, China) was diluted in acetone to produce a stock solution and was further diluted to 12 mg L^{-1} (~LC_{10}) and 50 mg L^{-1} (~LC_{50}) by 0.5‰ Triton X-100 solution, respectively. Newly molted 4th instar larvae of L-SS were selected and starved for 4 h. The leaf-dipping method was employed (Baek et al., 2010). Fresh radish leaves were dipped into the beta-cypermethrin solutions for 10–15 s and air dried at room temperature, then fed to the starved larvae. Larvae that fed with radish leaves treated with solution in the absence of beta-cypermethrin were taken as the controls. All larvae collected after 2 h, 6 h, 12 h, and 24 h post-treatment were snap frozen in liquid nitrogen and then stored at −80 °C.

2.3. Total RNA isolation and cDNA synthesis

Total RNA was extracted from different developmental stages, (egg, 1st to 4th instar larvae, pupae and adult) and various tissues (head, gut, epidermis, and hemolymph) of the 4th instar larvae using RNAiso Plus (TaKaRa, Dalian, China) following the manual instructions.

For real-time quantitative polymerase chain reaction (RT-qPCR) analysis, cDNAs were synthesized from 1.0 μg RNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). For amplification of CPR and b_5, cDNAs were synthesized from the 4th instar larvae RNA using 5′-Full RACE Kit and 3′-Full RACE Core Set (TaKaRa, Dalian, China), respectively. All cDNAs were stored at −20 °C until use.

2.4. Cloning of full-length cDNA of CPR and b_5

Using the full-length cDNA of *H. armigera* CPR (accession: HM347785) and b_5 (accession: AF061105) as probes, we blasted the genomic and transcriptomic database for DBM (KONAGAbase) (Jouraku et al., 2013). The fragments of DBM CPR and b_5 were obtained. Full-length sequences were cloned by rapid amplification of cDNA ends PCR (RACE-PCR) with universal primers supplied in the RACE kits and specific primers listed in Table 1 according to the manual instructions. The open reading frames (ORFs) of DBM CPR and b_5 were amplified and confirmed by PCR using corresponding specific primers (Table 1).

All PCR products were cloned into pMD19-T vectors (TaKaRa, Dalian, China) and transformed into *Escherichia coli* DH5α cells and then sequenced (AuGCT, Inc., Beijing, China).

2.5. Sequence analysis of CPR and b_5

The full-length cDNAs of DBM CPR and b_5 were assembled using the Contig Express program suite Vector NTI6.0 (InforMax, Frederick, MD, USA), and the ORFs were obtained by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The ExPASy Compute pI/Mw tool, (http://web.expasy.org/compute_pi/), was used to predict the molecular weight and isoelectric point of the deduced amino acid sequence. Sequence similarities were analyzed by using BLAST programs (http://www.ncbi.nlm.gov/blast/). The N-terminal signal peptide and transmembrane domain were predicted by using SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively. For the analysis of protein domains, the InterProScan 4 from EMBL-EBI (http://www.ebi.ac.uk/Tools/pfa/iprscan/) was used to search the InterPro collection of protein signature databases. The phylogenetic trees were conducted by the neighbor-joining method with bootstrap test of 1000 replicates using MEGA 5.0 software (Tamura et al., 2011).

2.6. RT-qPCR analysis

All primers used in RT-qPCR reactions were designed by Primer3 (http://www.simgene.com/Primer3) and listed in Table 1. Using 100-fold diluted cDNAs as templates, RT-qPCR was performed on an iCycler iQ5 real-time PCR detection system (Bio-Rad, Philadelphia, PA, USA) using the UltraSYBR mixture (CWBIO, Beijing, China) according to the manual's instructions. Thermal cycling conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. This was followed by a dissociation analysis to confirm the homogeneity of the PCR product. The actin gene (accession: JN410820) was used as a reference. The RT-qPCR was repeated three times for each gene. Each replicate was performed with an independent RNA sample preparation and consisted of three technical replicates. The comparative Ct method was used to assess the different expression levels of DBM CPR and b_5 (Livak and Schmittgen, 2001).

Table 1
Primers used in this work.

Gene	Primer name	Sequence (5′–3′)	Function
CPR	CPR-5R	CGGCGGGCAACATCCAGGTGAC	5′-RACE
	CPR-3R	ACCTGCTCGGCAACCAGAACGC	3′-RACE
	CPR-F	CATATGCACCATCACCATTCGGAGGACCTCGCGCA	ORF amplification
	CPR-R	AAGCTTCTAGCTCCACACGTCGGCGGAG	–
	CPR-qF	CTCTCGACGACGTGTTTTCA	RT-qPCR analysis
	CPR-qR	TGGTATAACGCCTTGCCTTC	–
b_5	b_5-5R	GGCGTTCCTGGACGACCACCCG	5′-RACE
	b_5-3R	CCTCCGACTGCTTCCACGACAT	3′-RACE
	b_5-F	CATATGCACCATCACCATACCA CGGAGTTCACGCG	ORF amplification
	b_5-R	AAGCTTTCACCCGAACAGATAGAAGTAG	–
	b_5-qF	TCATCATCATCGACAACGTG	RT-qPCR analysis
	b_5-qR	GAACTGCAGCATCCAGTCG	–
β-Actin	qF	GCGACTTGACCGACTACC	RT-qPCR analysis
	qR	GGAATGAGGGCTGGAACA	–