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Multiple P450 genes: Identification, tissue-specific expression and their responses to insecticide treatments in the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidea)

Yong Huang, Guang-Mao Shen, Hong-Bo Jiang, Xuan-Zhao Jiang, Wei Dou, Jin-Jun Wang *

Key Laboratory of Entomology and Pest Control Engineering, College of Plant Protection, Southwest University, Chongqing 400716, PR China

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ABSTRACT

The oriental fruit fly *Bactrocera dorsalis* is an economically important pest, yet little research has focused on the mechanisms underlying the increasing insecticide resistance of this species. In this study, 12 cytochrome P450 genes were isolated from *B. dorsalis*, and alignments of the deduced amino acid sequences confirmed that they contained the conserved motifs of the P450 superfamily. Phylogenetic analyses indicated relationships with CYP3, CYP4, and mitochondrial cytochrome P450 gene families. Expression profiles of the P450 genes were compared between control and insecticide-treated adult flies using real-time quantitative PCR (RT-qPCR) methods. Results showed that five, four, and seven P450s were up-regulated following exposure to LD_{50} doses of malathion, abamectin, and beta-cypermethrin, respectively, and that transcriptional levels reached a maximum at 36 h post-exposure. In addition, several genes did not show significant changes in expression, and several down-regulated genes were also noted. Most of the P450s (except *CYP28F1*) in untreated adults showed higher levels of transcription in the midgut, fat body, and/or Malpighian tubule than in the head. Taken together, these findings showed that multiple P450s are up-regulated in response to insecticide exposure and have various levels in different tissues. Further studies are needed to determine their possible role in the insecticide metabolism.

1. Introduction

Cytochrome P450 monooxygenases (P450s) are hemoproteins that act as the terminal oxidase in monooxygenase systems [1], and comprise a large superfamily (CYP, cytochrome P450 superfamily) of proteins that are found in virtually all living organisms. They play a key role in the detoxification and activation of xenobiotics such as drugs, pesticides, plant toxins, and mutagens [2]. Most eukaryotic P450s are dependent microsomal enzymes that, coupled with NADPH dependent P450 reductases, constitute one of the primaries for phase I detoxification, often the first step/s of detoxification of xenobiotics encountered by organisms [3,4]. Nearly all insects possess some capacity to detoxify insecticides and other xenobiotics. It was reported that transcriptional induction in response to the substrate is a means of activating gene expression only when required [5]. Increased tolerance to insecticides by various detoxification processes has frequently been associated with P450 genes [6]. Though the importance of P450s in insect toxicology is widely recognized, no P450 gene has so far been associated with insecticide detoxification in the oriental fruit fly, *Bactrocera dorsalis*.

B. dorsalis (Hendel), is one of the most economically important fruit fly pests in East Asia and the Pacific, where it is a serious pest of a wide range of tropical, subtropical, and temperate fruit crops [7,8]. It was reported that the damage caused by the oriental fruit fly consists both of punctures of the host tissue during oviposition and feeding on the fruit pulp by the developing larvae [9]. Insecticides have been a principal tool in many control practices worldwide [10], however, resistance in *B. dorsalis* has evolved in some areas [11,12]. In this study, we isolated P450s from *B. dorsalis*, and investigated their responses to various insecticides at doses similar to those (lethal doses such as LD_{50}) used in the field. Gene expression profiles in different tissues were also investigated. The current study provides the first insights into the *B. dorsalis* P450 genes at the molecular level and is the first step to understand the relationship between the P450 genes and insecticide metabolism in this insect.

2. Materials and methods

2.1. Insects

The laboratory stock of *B. dorsalis*, originally collected in 2008 from fields in Dongguan, Guangdong Province, China, was cultured

Abbreviations: CYP, cytochrome P450; FB, fat body; GSPs, gene specific primers; M, midgut; MT, Malpighian tubule; RT-qPCR, real-time quantitative PCR; UTR, untranslated region.

* Corresponding author. Fax: +86 23 68251269.
E-mail addresses: jjwang7008@yahoo.com, wangjinjun@swu.edu.cn (J.-J. Wang).

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in an insectary under the following conditions: 27 ± 0.5 °C, 14 h light:10 h dark photoperiod, 75% relative humidity. The larvae were reared on an artificial larval diet and adults on an artificial diet of 2.5% yeast extract, 7.5% sugar, 2.5% honey, 0.5% agar, and 87% H_2O.

2.2. Insecticide treatment

Three insecticides, malathion, abamectin, and beta-cypermethrin, belonging to different chemical classes, were used as xenobiotics in this study. LD_{50} concentrations (the dose at which 50% of the test animals are killed) for each insecticide were determined empirically. Insecticide solutions (dissolved with acetone, 0.5 μL per fly) were spotted onto the thoracic terga of adults after they had been anesthetized by freezing at −20 °C for 1 min. A great number of adults in both sexes were treated to remaining enough survivors. The surviving flies were randomly collected for RNA extraction at 24, 36, and 48 h post-treatment. The control flies were treated with acetone only. Eight survivors were used for each RNA sample, with three biological replicates for each treatment.

2.3. RNA isolation and first-strand cDNA synthesis

Total RNA was extracted from whole bodies of control/treated adult flies using TRzol (Biomed, Beijing, China), and was also extracted from various tissues (head, midgut, fat body, and Malpighian tubule) from untreated adults using an RNAeasy Micro Kit (Qiagen, Hilden, Germany). These tissues were dissected using a needle in physiological saline solution under a stereomicroscope (Olympus SZX12, Tokyo, Japan). Thirty individuals were dissected for each sample, with three biological replicates. RNA quantity was ascertained by measuring the absorbance at 260 nm using a Nanovue UV–Vis spectrophotometer (GE Healthcare, Fairfield, CT), and the quality was assessed at the absorbance ratio of $OD_{260/280}$. The RNA integrity was further confirmed with 28S/18S rRNA ration by 1% formaldehyde agarose gel electrophoresis. RNA was digested using DNase I (Takara, Shiga, Japan) to eliminate genomic DNA contamination. First strand cDNA was synthesized using a SMARTer RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) for cloning and a Perfect Real Time Kit (Takara) for RT-qPCR. cDNA quantity was checked as described above.

2.4. Cloning of full-length P450 cDNA

P450 gene fragments were selected from previous transcriptome analyses of *B. dorsalis* [13]. To obtain the full-length cDNA sequences of the P450s, 5′ and 3′ rapid amplification of cDNA ends (RACE) analyses were performed by nested PCR with two gene specific primers (GSPs) and two universal primers, UPM and NUP (UPM: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCA ACGCAGAGT-3′, NUP: 5′-AAGCAGTGGTATCAACGCAGAGT-3′). GSPs (Table S1) used in RACE analyses were designed based on the cDNA fragments derived from the transcriptome analyses. A pair of primers were designed to amplify the complete open reading frame (ORF) of each gene, based on the 5′ untranslated region (UTR) and 3′ UTR of the cloned cDNA fragments obtained from the 5′ and 3′ RACE analyses (Table S1). Amplifications were performed with the following conditions: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 49–68 °C for 30 s, and 72 °C for 30–90 s, with a final extension of 72 °C for 10 min. Following purification by agarose gel electrophoresis and gel extraction, the amplified products were cloned into a pGEM-T easy vector (Promega, Madison, WI) and sequenced (Invitrogen, Shanghai, China).

2.5. Sequence analysis and phylogenetic tree construction

Sequences were edited and aligned using ClustalW software (www.ebi.ac.uk/clustalw/) [14]. Similarity searches were performed using BLASTP in the non-redundant protein sequences (nr) database of the NCBI website (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed with MEGA 5 [15], using the neighbor-joining (NJ) method. CYP18A1 from *Drosophila melanogaster* was used as an outgroup. The branch support of the NJ phylogenetic tree was estimated by the bootstrap analysis with 1000 replicates.

2.6. RT-qPCR of P450s

RT-qPCR was conducted on a Stratagene Mx3000P System using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Each 20 μL reaction contained 400 ng cDNA templates, 10 μL SYBR Green Supermix, 10 pmol each primer (Table 1), and double distilled water. All reactions were performed under the following conditions: 95 °C for 30 s, 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. To verify the specificity of the amplicon for each primer pair, a dissociation curve was included from 60–95 °C at the end of each RT-qPCR run.

A cDNA dilution series (1, 1/3, 1/9, 1/27, and 1/81) of sample cDNA was used to construct the standard curve and calculate the efficiency of amplification. Relative expression levels were calculated by the comparative C_T method [16]. The relative quantities were normalized to the stable reference gene α-tublin, selected with the specific tools, *geNorm* and *Normfinder*, as described previously [17], among different tissues and also in insecticide treatments. Relative quantity was calibrated against the control samples in insecticide treatments, and against head samples in different tissues.

2.7. Statistical analysis

The PCR efficiencies of each P450 gene and the reference were calculated using Mxpro-Mx3000P version 3.20 (Agilent-Stratagene, Santa Clara, CA). Fold changes in gene expression between control and treated flies, and differences among the tissues, were subjected to a Mann–Whitney test at a significance level of 0.05 (SPSS 19.0 for Windows).

3. Results

3.1. cDNA cloning and characterization

Twelve P450 genes were isolated from *B. dorsalis*. Of these, six belonged to the CYP4 family, two to the CYP12 family, and one to each of the CYP6, CYP28, CYP302, and CYP314 families. Protein sequence alignments revealed the typical conserved P450 motifs, including the conserved residues WXXXR located in Helix-C, GXE/DTT in Helix-I, the absolutely conserved EXXR motif found in Helix-K, the PERF region (PXXFXPE/DRF), and the heme-binding domain (PFXXGXRXCXG/A) (Fig. S1). The substrate recognition domains, SRS1-6, were proposed in the CYP2A5 sequence (Fig. S1) [18,19]. Pairwise percentage identities of predicted amino acid sequences among the 12 P450 genes were also compared (Table 2). The pairwise percentage of their encoded proteins was 14.8–64.4%.

3.2. Phylogenetic analysis

To analyze the sequence homology and phylogenetic relationships, 60 other insect P450s and three published P450s from *B. dorsalis* [20,21] were downloaded from GenBank and aligned with the