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Overexpression of cytochrome P450 genes in pyrethroid-resistant *Culex quinquefasciatus*

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ABSTRACT

JPal-per strain of *Culex quinquefasciatus* exhibits extremely high resistance against pyrethroids in larvae, though the resistance is greatly lower in adults. Increased microsome monooxygenase metabolism is one of the major factors of the larval resistance in this strain. We cloned 46 novel cytochrome P450 cDNAs from JPal-per strain. An oligonucleotide microarray was designed for the novel 46 genes plus 16 previously reported P450 genes along with other non-P450 gene probes. Of these, five P450 genes were upregulated (>2.5-fold) in JPal-per larvae as compared with a susceptible strain. The expression ratios for the highest three among the five P450 genes screened in the microarray analysis, *CYP9M10*, *CYP4H34* and *CYP6Z10*, were further validated by qPCR as 264-, 8.3-, and 3.9-fold, respectively. In JPal-per, the transcription levels of *CYP9M10* and *CYP4H34* showed a similar stage-dependent pattern as a high expression level during the larvfrom Ogasawara Islands in Japanal stage dramatically decreases in the adult stage. This larval specific overexpression manner of the two genes was consistent with the characteristic of stage-dependent resistance of JPal-per strain previously reported, suggesting that the two P450s, CYP9M10 and CYP4H34, are involved in pyrethroid detoxification in JPal-per strain.

1. Introduction

Mosquitoes transmit various human pathogens. Controlling of vector mosquitoes is an important measure in reducing these mosquito-borne diseases and such strategy often relies on the use of insecticides such as pyrethroids (WHO, 2006). Historically, the widespread use of insecticides has resulted in the development of insecticide resistance in both agriculturally and medically important insect pests. Elucidation of resistance mechanisms is important for developing the tools of monitoring resistance in populations, thereby contributing to mosquito control programs.

In most cases reported so far, pyrethroid resistances are conferred by decreased sensitivity of the voltage-gated sodium channel (Hemingway and Ranson, 2000), which is the target of pyrethroids and DDT (dichloro-diphenyl-trichloroethane) (Lund and Narahashi, 1983). Additionally, increased metabolisms mediated by cytochrome P450 monooxygenases (P450s), carboxylesterases (CEs), and glutathione transferases (GSTs) are confirmed to be involved in pyrethroid resistance. In some arthropod pests (the cattle tick *Boophilus microplus* and the peach-potato aphid *Myzus persicae*), the overproduction of CE contributes to both pyrethroid and organophosphorous insecticides (OPs) (Devonshire and Moores, 1982; Hernandez et al., 2002). GST confers pyrethroid resistance without catalyzing pyrethroid metabolism, but may reduce pyrethroid toxicity by sequesteration (Kostaropoulos et al., 2001; Vontas et al., 2001). In mosquitoes, CE is well known to be involved in OP resistance (Mouches et al., 1986), but there is no evidence for connection between CE and pyrethroid-resistance so far (Hemingway et al., 2004).

P450s are important metabolic enzymes, catalyzing a number of lipophilic compounds. Although insect endocrinological roles of entire P450s are not established, some P450s are responsible for synthesis and degradation of insect hormones including ecdysteroids (Gilbert, 2004) and juvenoids (Helvig et al., 2004). P450s are also involved in detoxification or activation of exogenous chemical substances, such as plant toxins and insecticides. Recently the role of P450s for various insecticide resistance have been accumulated in many pest species (Feyereisen, 2005).

Identification of the P450 gene that is responsible for the insecticide resistance often becomes a major problem, since each insect species encodes a substantial number of P450 genes in its genome. For instance, *Anopheles gambiae* and *Aedes aegypti* have 105 and 160 P450 genes, respectively (Strode et al., 2008). In addition, each P450 isozyme has either narrow or broad substrate specificity, and their catalytic spectra may overlap. As a consequence, despite numerous P450 genes in various insect species are now annotated, the associations of individual P450 genes with insecticide metabolism or resistance remain poorly understood

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(Berge et al., 1998; Feyereisen et al., 1989; Nikou et al., 2003). As a few exception, *CYP6A1* of the housefly *Musca domestica* which is overexpressed in diazinon-resistant strains (Carino et al., 1994) was exemplified as metabolizing diazinon (Sabourault et al., 2001). *Cyp6g1* of *Drosophila melanogaster* is overexpressed in DDT-resistant strains and confirmed to metabolize DDT (Joussen et al., 2008). *CYP9A12* and *CYP9A14* of *Helicoverpa armigera* are overexpressed in pyrethroid-resistant strains and confirmed to metabolize pyrethroids (Yang et al., 2008). Since antiserum of CYP6D1 inhibit deltamethrin metabolism *in vitro*, CYP6D1 is suspected to be involved in pyrethroid resistance in the housefly (Wheekock and Scott, 1992).

Microarray analysis that allows genome-wide screening of overexpressed P450 genes associating with insecticide resistance is becoming a powerful tool in post-genomic era of insecticide resistance research, especially for *An. gambiae* (David et al., 2005) and *Ae. aegypti* (Strode et al., 2008). Moreover, Wondji et al. (2007) applied QTL (quantitative trait loci) analysis followed by performing the positional cloning of resistant-associated genes (Wondji et al., 2009) in *Anopheles funestus*, showed another promising strategy. With these methods, several P450 genes as candidates for the factors of pyrethroid resistance have been proposed, such as *CYP6Z1*, *CYP6Z2*, and *CYP6P3* along with GST genes (*GSTe2* and *GSTd1-6*) in *An. gambiae* (David et al., 2005; Djouaka et al., 2008; Muller et al., 2007), *CYP9J32* along with *GSTe2* in *Ae. aegypti* (Strode et al., 2008), and *CYP6P9* and *CYP6P4* in *An. funestus* (Wondji et al., 2009).

Culex quinquefasciatus is an important vector mosquito inhabiting tropical and subtropical regions worldwide. This species is involved in transmission of bancroftian filariasis, West Nile encephalitis, St. Louis encephalitis, and Western equine encephalitis. The strain of *C. quinquefasciatus*, JPal-per, showed a marked resistance to permethrin (2500-fold compared to an insecticide susceptible strain) (Kasai et al., 1998a) and to other pyrethroids such as phenothrin (2460-fold) and etofenprox (4160-fold) (Weerasinghe et al., 2001) during larval stage. All these pyrethroids possess a 3-phenoxybenzyl moiety. On the other hand, JPal-per showed moderate resistance to five other pyrethroids that posses an α-cyano-3-phenoxybenzyl moiety, for which the resistance ratios were 39- to 59-fold (Kasai et al., 1998b). Synergism studies with several detoxification enzyme inhibitors have revealed that the high level of permethrin resistance was mainly due to increased activity of P450s (Kasai et al., 1998b). *In vitro* studies also showed that the accelerated metabolism of pyrethroids by P450s and hydroxylation of the 3-phenoxybenzyl moiety is the main metabolic pathway of permethrin in JPal-per (Kasai et al., 1998b).

In order to identify the P450 that is responsible for the pyrethroid metabolism and resistance in JPal-per, we cloned 46 novel putative-P450 genes by degenerative PCR. Then microarray analysis was conducted for these 46 genes and 16 already-known P450 genes in *C. quinquefasciatus* to detect P450s overexpressed specifically in JPal-per, comparing to a susceptible strain. As a result, we obtained two P450s, designated *CYP9M10* and *CYP4H34*, being extraordinarily overexpressed in JPal-per larvae but not in adult. The larval specific overexpression of these genes correlated with the characteristic of the pyrethroid resistance of JPal-per (Hardstone et al., 2007), thus indicates *CYP9M10* and *CYP4H34* are strong candidates of the P450 gene that is responsible for the pyrethroid resistance of JPal-per.

2. Materials and methods

2.1. Insects

Two strains of *C. quinquefasciatus* were used. Ogasawara (OGS) is an insecticide susceptible standard strain. This strain was originally collected from Ogasawara Islands in Japan in 1968, maintained without insecticidal selection at laboratory of National Institute of Infectious Diseases (NIID) of Japan. An original colony of the pyrethroid-resistant strain, JPal-per, was collected in Saudi Arabia in 1981, and JPal-per was established by larval selection of the original colony with permethrin for 20 consecutive generations at a mortality level of 60–75% in Liverpool School of Tropical Medicine (Amin and Hemingway, 1989). JPal-per shows 2500-fold larval resistance to OGS for permethrin (Kasai et al., 1998a). Since 1998 JPal-per has been maintained in NIID with confirmative bioassay and selection at 10 ppm permethrin every two year in order to secure the constancy of the resistance level. The resistance mechanism of JPal-per involves both decreased nerve sensitivity and increased microsome monooxygenase activity (Kasai et al., 1998b). An authentic *kdr* (knock down resistance) mutation equivalent to *M. domestica* L1014F in voltage-gated sodium channel gene is fixed in JPal-per strain (Komagata et al., 2008). The insects were reared at 26 ± 1 °C under a 16L:8D photoperiod. Larvae were fed with a diet of ground insect pellets (Oriental Yeast, Tokyo, Japan). Adults were maintained on 2% sucrose, and females were fed mouse blood meal for oviposition.

2.2. Degenerative PCR-based cDNA screening

Total RNA was extracted from ca. 500 embryos, 10 larvae, and 10 adults of each sex with Isogen (Nippon Gene, Tokyo) and then purified with RNeasy Mini Kit to which the optional DNase digestion kit was applied (Qiagen). First-strand cDNA was synthesized from 5 μg of total RNA with QT' primer (CCAGTGAGCAGAGTGACGAGGACTCGTGCTCAAGC$(T)_{15}$) and a reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan) in 50 μl of reaction solution.

Partial fragments of P450 cDNAs were amplified by degenerative PCR with Ex Taq DNA polymerase and its accessory reagents (Takara, Otsu, Japan) according to the manufacturer's instructions. The final concentration of each degenerate primer was 10 μM. Primer pairs used for degenerative PCR (Table 1) were designed for several consensus protein sequences among the P450s of *D. melanogaster* and *An. gambiae* (http://p450.sophia.inra.fr/index.html). The P450 domains utilized for primer design are shown in Fig. S1. PCR was performed under the following conditions: initial denaturing at 94 °C for 2 min; 35 thermal cycles of 94 °C for 30 s, 45 °C for 30 s, and 68 °C for 1 min; and final elongation at 72 °C for 5 min. The amplified products were separated by gel electrophoresis, purified with QIAquick Gel Extraction Kit (Qiagen), and then inserted into cloning vectors by using TOPO TA Cloning Kit for Sequencing (Invitrogen). One Shot TOP10 competent cells (Invitrogen) were chemically transformed with the inserted vectors. Transformed cells were selected on LB plates containing 50 μg/ml ampicillin. Positive clones were cultured overnight in LB liquid medium containing 50 μg/ml ampicillin. Plasmids were extracted and purified by using FastPlasmid Mini Kit (Eppendorf). The inserts were sequenced from their both ends with BigDye Terminator Cycle Sequencing Kit on ABI PRISM 3130 Genetic Analyzer system (Applied BioSystems). The inserts' sequences of 1200 clones obtained were searched for National Center for Biotechnology Information (NCBI) by BLASTX program.

2.3. Microarray analysis

Microarray experiments were performed by following the instructions of Agilent Technologies (Santa Clara, CA, USA). Oligonucleotide probes were designed from 62 unique P450 sequences of *C. quinquefasciatus* and its sibling species. Of these, 46 were newly obtained sequences by degenerative PCR in this study and