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Cloning and Overexpression of *CYP6F1*, a Cytochrome P450 Gene, from Deltamethrin-resistant *Culex pipiens pallens*

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Abstract *CYP6F1* (GenBank/EMBL accession No. AY662654), a novel gene with a complete encoding sequence in the cytochrome P450 family 6, was cloned and sequenced from deltamethrin-resistant 4th instar larvae of *Culex pipiens pallens*. The cDNA sequence of *CYP6F1* has an open reading frame of 1527 bp, which encodes a putative protein of 508 amino acid residues. The deduced amino acid sequence of *CYP6F1* indicated that the encoded P450 has conserved domains of a putative membrane-anchoring signal, putative reductase-binding sites, a typical heme-binding site, an ETLR motif and substrate recognition sites. Semi-quantitative RT-PCR analysis indicated that the *CYP6F1* gene was expressed to a greater extent in the deltamethrin-resistant strain than in the susceptible strain of *Cx. pipiens pallens*. The expression levels of the *CYP6F1* gene in the deltamethrin-resistant 1st, 2nd, 3rd, 4th instar larvae and adult female mosquitoes differed, with highest expression levels in the 4th instar larvae. In addition, the *CYP6F1* gene was stably expressed in mosquito C6/36 cells, and the expected 61.2 kDa band was identified by Western blotting. The cells transfected with *CYP6F1* had an increased resistance to deltamethrin as compared with control cells. These results indicate that *CYP6F1* is expressed at higher levels in the deltamethrin-resistant strain, and may confer some insecticide resistance in *Cx. pipiens pallens*.

Key words *Culex pipiens pallens*; insecticide resistance; cytochrome P450; RT-PCR

Cytochrome P450 (CYP) monooxygenases constitute the largest gene superfamily found in nature, and possess a wide range of functions. In insects, more than 300 cytochrome P450s have been identified and are distributed throughout 27 CYP families, with more than 70 known CYP subfamilies. For example, 90 P450 genes from 25 families are present in the *Drosophila melanogaster* genome and 111 P450 genes are present in *Anopheles gambiae* [1, 2]. Cytochrome P450s are involved in the biosynthesis of several essential endogenous compounds, as well as in the detoxification of many xenobiotics [3]. In addition, the P450s constitute the enzyme systems that are essential for insecticide detoxification or activation. The insecticide-resistant attributes of P450 genes such as *CYP6A1*, *CYP6A2*, *CYP6D1*, *CYP6E1*, *CYP6Z1* and *CYP6G1*, which have been isolated from insecticide-resistant strains, have previously been analyzed [4–9]. Interestingly, these P450 isoforms are constitutively overexpressed in insecticide-resistant strains; for example, *CYP6A2* [10] and *CYP6G1* [11] are overexpressed in DDT-resistant *Drosophila melanogaster*, *CYP6A1* is overexpressed in the diazinon-resistant strain of *Musca domestica* [4], *CYP6B7* in the pyrethroid-resistant strain of *Helicoverpa armigera* [12], *CYP6E1* in pyrethroid-resistant *Culex pipiens quinquefasciatus* [7], and *CYP6Z1* in pyrethroid-resistant *Anopheles gambiae* [8].

Diversity in the developmentally-specific expression of insect P450s is apparent. Some P450s, such as *CYP4D1*

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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA end; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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and *CYP6A1*, are expressed throughout all developmental stages [13,14], whereas others, such as *CYP6B1*, *CYP6B2*, *CYP6B3*, *CYP6D1*, and *CYP6L1*, have age-dependent expression patterns [15–17].

Insecticide resistance in mosquito-borne diseases is a major public health concern. Pyrethroid insecticides are widely used both to protect bed nets and in indoor residual spray programs in efforts to control the transmission of mosquito-borne diseases. Involvement of the P450s in pyrethroid resistant mosquitoes has been demonstrated in synergistic studies using the monooxygenase inhibitor piperonyl butoxide (PBO), and increased heme levels [18, 19]. In some cases, the P450s involved, such as *CYP6A2*, have been functionally expressed and shown to be able to metabolize various insecticides [20].

In the present study, we utilized RT-PCR and RACE to clone a cDNA encoding a member of cytochrome P450 family 6, *CYP6F1*. The nucleotide sequence of the clone was subsequently determined. Semi-quantitative RT-PCR indicated that this gene is expressed to a greater extent in the deltamethrin-resistant strain than in the susceptible strain of *Cx. pipiens pallens*. We also established the expression profile of the gene in the mosquito life cycle. Increased deltamethrin resistance in C6/36 cells containing the stably-transfected *CYP6F1* gene provided further support for a role of *CYP6F1* in deltamethrin resistance in *Culex pipiens pallens*.

Materials and Methods

Mosquitoes

Deltamethrin-resistant and susceptible *Cx. pipiens pallens* mosquitoes were reared at 28 °C, with 70%–80% humidity and a constant light:dark cycle (14:10). The mosquitoes were fed with mouse blood. The deltamethrin-resistant colony was selected with deltamethrin at LC_{50}; the deltamethrin resistance in the resistant strain was 400-fold greater than that in the susceptible strain [21].

RNA extraction, RT-PCR and 3′ RACE PCR

Total RNA was extracted from approximately 20 mg of 1st, 2nd, 3rd and 4th instar larvae and female adult *Cx. pipiens pallens* mosquitoes, using Trizol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's protocol. RT-PCR was conducted to amplify the P450 cDNA fragments, and the RNA extracted from the 4th instar larvae was used as a template using an RT-PCR kit (TaKaRa, Dalian, China). The primers were designed based on the sequence reported by Kasai [22] (GenBank/EMBL accession No. AB001324): forward 5′-ATGTTTGCGTGGATAATCTGC-3′ and reverse 5′-ATTACGAGTCACTTCAATTTGTATTA-3′. PCR conditions were: initial denaturation at 94 °C for 2 min, followed by 27 cycles at 94 °C for 1 min, 58 °C for 40 s and 72 °C for 90 s with a final 10-min extension at 72 °C. cDNA fragments were successfully amplified and separated by electrophoresis on a 1% agarose gel. The cDNA fragment of interest was purified using a quick gel extraction kit (Qiagen, Hilden, Germany), and ligated into a pGEM-T easy vector (Promega, Toll Free, USA) overnight at 4 °C. *Escherichia coli* JM 109 competent cells were transformed with this ligation mixture and cultured on an LB plate containing ampicillin (100 μg/ml), IPTG (400 μg/ml), X-Gal (200 μg/ml). White colonies were selected and subjected to PCR amplification to confirm the presence of inserts with the expected sizes. Plasmid DNA was extracted using a plasmid mini kit (Qiagen) and sequenced using an automated DNA sequencer (Applied Biosystems model 373; Shanghai Sangon BioTech Company, Shanghai, China).

After part of the sequence was obtained, a forward primer was designed (5′-TGACCAAGTTCTCGCAAG-CC-3′) so that the amplified product would overlap with approximately 500 bp of the sequence, in order to confirm unequivocally that the sequence of the product obtained corresponded to the same cDNA. These products were subsequently used with oligo(dT) primer for PCR amplification starting at the 3′ end of the cDNA. Reverse transcription was performed, and the DNA fragment obtained was ligated into a pGEM-T easy vector and sequenced. The NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) was used to perform similarity searches and retrieve homologous sequences. The sequence analysis tools from the SWISS-PROT Internet server were used to process the data for the deduced protein sequences. Multiple sequence alignment was conducted using the Clustal Alignment Program [23].

Semi-quantitative RT-PCR analysis

RT-PCR was performed with the predetermined PCR cycle during the exponential phase. Results for samples isolated from deltamethrin-resistant and susceptible 4th instar larvae were compared using specific oligonucleotide primer pairs according to the *CYP6F1* sequence (forward primer 5′-GGAACAAGTCACAGCACACG-3′, and reverse primer 5′-GGCGTAAGTTTCATCAGCAA-3′). RT-PCR was also performed with samples isolated from deltamethrin-resistant 1st, 2nd, 3rd and 4th instar larvae

and female adult mosquitoes to confirm the expression levels at each developmental stage using the primers described above. Mosquito β-actin gene cDNA was amplified by PCR using the forward primer 5′-CGCTTCCTCGTCTACACTGG-3′, and the reverse primer 5′-GTGTTGGCGAACAGATCCTT-3′. The PCR conditions were: 94 °C for 5 min followed by 27 cycles of 94 °C for 1 min, 58 °C for 40 s, 72 °C for 40 s with a final 10-min extension at 72 °C. The *CYP6F1* and β-actin gene PCR products were resolved by electrophoresis on 1% agarose gels. Gels were photographed using Gel Doc 100 (Bio-Rad, Hercules, USA) and the images were analyzed by using Gel-Pro analyzer 3.1 software. The relative level of *CYP6F1* mRNA was indirectly quantified by calculating the ratio of the intensity of the *CYP6F1* band to that of the β-actin gene band.

Construction of the expression vector

The entire coding region of *CYP6F1* was amplified by PCR using the specific primers designed. The forward primer contained a Kozak translation sequence [24] and an ATG start codon for proper initiation of translation. The reverse primer lacked the native stop codon and maintained the frame through the DNA encoding the C-terminal peptide. The forward primer used was 5′-GAG**ATG**GAAATGTTTGCGTGGATAATC-3′, and the reverse primer was 5′-CGAAGCCCTCCGTTCCGAAAT-3′. The PCR conditions were: 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 90 s, then 72 °C for 20 min. The PCR product was purified from the gel following electrophoresis using a quick Gel extraction kit (Qiagen). The purified PCR product was ligated with T4 DNA ligase to the pIB/V5-His-TOPO vector (Invitrogen, Carlsbad, USA), and the ligation reaction solution was transformed into TOP *Escherichia coli* competent cells (Invitrogen). Positive clones were identified by restriction analysis of recombinants with *Not*I and *Bam*HI, and by PCR with specific primers and vector primers. The accuracy of the expression plasmid pIB/V5-His-TOPO-*CYP6F1* was further verified by sequencing.

Cell culture and stable transfection

Mosquito C6/36 cells were obtained from the China Center for Type Culture Collection (Wuhan, China). Cells were maintained in RPMI 1640 medium supplemented with 10% (*V*/*V*) fetal bovine serum (FBS, Sigma), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The cells were grown in a 5% CO_2 humidified incubator at 28 °C and were plated in a six-well culture plate prior to transfection. When the cells were at 50%–60% confluence, they were transfected using the Cellfectin reagent (Invitrogen) according to the manufacturer's instructions. Briefly, for each well, 2 μg DNA was added to 100 μl serum-free RPMI 1640 medium, then separately, 5 μl Cellfectin reagent was added to 100 μl serum-free medium, then the two solutions were combined, mixed gently, and incubated at room temperature for 30 min. Subsequently, 0.8 ml of serum-free medium was added to this mixture and the entire mixture was layered on to cells after washing the cells with antibiotic-free medium. The cells were incubated with the transfection mixture for 24 h in a 5% CO_2 humidified incubator at 28 °C, and the transfection medium was then replaced with 2 ml normal growth medium-containing serum. The cells were then incubated for an additional 48 h before being harvested for RT-PCR and Western blotting.

Once it had been confirmed that the cells were expressing the expected protein, stable expression cell lines were created according to the manufacturer's instructions. Briefly, a kill curve was performed to test the cell line for sensitivity to 20 μg/ml blasticidin, which can kill cells within one week. Forty-eight hours post-transfection, the transfection solution was removed and fresh medium without blasticidin was added. The cells were split in a 1:5 ratio and allowed to attach for 20 min before the selective medium was added. The medium was removed and replaced with medium containing 20 μg/ml blasticidin, and the cells were incubated at 28 °C. The selecting medium was replaced every 3 to 4 days until clones were observed. The resistant cell lines were isolated using a dilution method until only one colony was found in each well of a 96-well microtiter plate, after which the plate was incubated until the colony filled a majority of the well. The cells were harvested and transferred to a 24-well plate with 0.5 ml fresh medium containing 20 μg/ml blasticidin, then the clone was expanded in 12- and 6-well plates, and finally a T-25 flask. The cells were analyzed for expression using RT-PCR and Western blot assays.

Isolation of total RNA and RT-PCR analysis of the specific *CYP6F1* transcript

Total RNA was isolated from transfected cells by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Five micrograms of isolated total RNA from each sample was used as a template for first-strand cDNA synthesis. The cDNA was synthesized at 30 °C for 10 min, then 42 °C for 30 min with a random primer using Avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa). The reverse transcriptase was then inactivated at 99 °C for 5 min. PCR amplification of the

CYP6F1 gene was performed with the forward gene-specific primer and the reverse vector primer for confirmation of transcriptional expression. One microliter of the RT reaction product was used as the template for routine PCR. The following cycling parameters were used: 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 90 s, followed by a final extension step of 72 °C for 20 min.

Western blot analysis

Cells transfected with the *CYP6F1* construct were washed with phosphate buffered saline (PBS) and lysed in extraction buffer containing 50 mM Tris, pH 7.8, 150 mM NaCl, 1% Nonidet P-40 (Sigma, St. Louis, USA). The cell lysate was centrifuged at 12,000 *g* for 2 min, and the supernatant was collected and stored at –80 °C until use. Protein concentration was confirmed by using the Bradford assay. Twenty micrograms of protein per lane was used for SDS-PAGE electrophoresis. Sample treatment buffer (0.125 M Tris-HCl, pH 6.8, 10% Glycerol, 2% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue) was added to the sample and heated at 70 °C for 5 min. The gels were run for 120 min at 120 V, and then transferred to a PVDF membrane at 300 mA for 90 min. The membrane was washed and blocked in PBS containing 0.05% Tween-20 and 5% non-fat dry milk, and then incubated at 4 °C with mouse anti-V5 antibody (Invitrogen; 1:2000 dilution). The membrane was washed twice for 5 min with PBS-T, incubated for 1 h with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000; Santa Cruz, USA) at room temperature, and then washed five times as described above. Detection was done with the Amersham ECL reagents according to the manufacturer's instructions. The membrane was exposed to radiographic film (Kodak, Rochester, USA) for 3 min. Molecular weight was determined by comparison with molecular weight markers (Bio-Rad).

MTT assay

Cell viability was assessed by quantifying 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction by using a colorimetric reaction [25, 26] at indicated time points after exposure to deltamethrin. The assay is based on the ability of living cells to convert dissolved MTT to insoluble formazan. Briefly, the cultures were incubated with 0.5 mg/ml MTT in medium at 28 °C with 5% CO_2 for 3 h. The formazan salt was solubilized in 200 μl DMSO. Absorption was measured at a wavelength of 570 nm using a microtiter plate reader. Relative viability (%) was calculated as the ratio of MTT reduction in treated cells to that of control cells.

Results

Cloning of cytochrome P450 cDNA

A cDNA fragment was amplified using primers based on the nucleotide sequence of previously isolated cytochrome P450 *CYP6F1* from *Cx. quinquefasciatus* Say [22]. A forward primer was designed from the start codon to downstream 21 nucleotides; the reverse primer was generated from the stop codon to upstream 26 nucleotides. A fragment of the expected size (1500 bp) was cloned and sequenced. However, the cDNA sequence did not contain the 3′ untranslated region (3′ UTR), which was obtained instead by 3′ RACE PCR. The resulting total cDNA sequence consisted of 1639 bp (**Fig. 1**). The longest open reading frame encoded 508 amino acids with a predicted molecular mass of 58.2 kDa. A poly-addition signal, AATAAA, was presented at the 3′ untranslated region. Only two nucleotides and one amino acid from the nucleotide sequence of the cloned P450 differed from *CYP6F1* of *Cx. quinquefasciatus* [22]: the clone contained a T and a C at positions 223 and 1584, respectively, whereas in *CYP6F*, the same positions were C and T. Position 223 was located in the coding region, and the other was present in the 3′ UTR. The amino acid residue at position 74 in the sequence was F, whereas it was L in *CYP6F* (99% identity), suggesting the presence of multiple isoforms of *CYP6F1*. Sequence analysis indicated that the protein encoded by the *CYP6F1* contains important domains that are conserved in the P450s: a putative membrane-anchoring signal, putative reductase binding sites, a typical heme-binding site, an ETLR motif and substrate recognition sites. The determined amino acid sequence of the fragment showed 38% identity to *CYP6E1* from *Cx. quinquefasciatus*, 40% identity to *CYP6Z1* and 34% identity to *CYP6Y1* from *Anopheles gambiae*, and 32% identity to *CYP6A1* from *Musca domestica* [4,7,8, 27] (**Fig. 2**).

Expression of the P450 gene at various developmental stages in deltamethrin-susceptible and resistant 4th instar larvae

Cytochrome P450 tissue expression specificity was determined by PCR amplification of RT-PCR products prepared from different tissue RNAs, including those from 1st, 2nd, 3rd and 4th instar larvae, and adult females from the resistant mosquito group. A 547-bp cDNA fragment

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CTTATGTTTGCGTGGATAATCTGCGCTGCGGCAGCAGTTCCGCTGGTGTACTTCCTGATC      −60
    M  F  A  W  I  I  C  A  A  A  A  V  P  L  V  Y  F  L  I
GTGTACCAGTTCAGCTACTGGAAACGTCGTGGGATCACACAACTCACTCCATCATTCCCA     −120
 V  Y  Q  F  S  Y  W  K  R  R  G  I  T  Q  L  T  P  S  F  P
TTTGGAGATCTTGGACCGTTCTTTCGGCAACGGTCCAGCCTCGGAGTGGTCTACGCCGAT     −180
 F  G  D  L  G  P  F  F  R  Q  R  S  S  L  G  V  V  Y  A  D
GTGTACCGGCTGTGCAAGCGCCTACCCTTTGTGGGGATCTACTTTTCCTTGCGGCCAATG     −240
 V  Y  R  L  C  K  R  L  P  F  V  G  I  Y  F  S  L  R  P  M
CTGGTGGTCAACGACCCCGAGTTGATTAAAAATGTGCTTGTGCGTGATTTTGACCACTTT     −300
 L  V  V  N  D  P  E  L  I  K  N  V  L  V  R  D  F  D  H  F
CACGATCGTGGACTGTACGTGAACGAGGAGAAGGACCCACTCAGTGGGCATTTGTTTGCA     −360
 H  D  R  G  L  Y  V  N  E  E  K  D  P  L  S  G  H  L  F  A
CTCGGTGGCGAACAGTGGCGCCATCATCGGTCCAAGCTAACGCCAACGTTCACCTCGGGA     −420
 L  G  G  E  Q  W  R  H  H  R  S  K  L  T  P  T  F  T  S  G
AGGTTGAAGGAGATGTTCACGAACTTGGTCCAAATTGGGCGTGTTCTCCAAGATCACGTG     −480
 R  L  K  E  M  F  T  N  L  V  Q  I  G  R  V  L  Q  D  H  V
GCGAAACGTGCTGGGGAGGACATCGAAATTCGGGACGTGATGGCGCGGTACACTACCGAT     −540
 A  K  R  A  G  E  D  I  E  I  R  D  V  M  A  R  Y  T  T  D
ATCATTGCATCGGTTGGATTCGGAATCGAAAATGACTCCATCAACGAAAAGGGCAACATT     −600
 I  I  A  S  V  G  F  G  I  E  N  D  S  I  N  E  K  G  N  I
TTCAGGGAAATGGGAACGAAGGTGTTCTCTCCTGATCTTAAGACGATACTTCGATTGACG     −660
 F  R  E  M  G  T  K  V  F  S  P  D  L  K  T  I  L  R  L  T
AGCACATTTTTCACTCCAAAGCTGAACGCACTGTTTGGATTCAAATTTATCGCACAGGAG     −720
 S  T  F  F  T  P  K  L  N  A  L  F  G  F  K  F  I  A  Q  E
ATTGAAGACTTCATCATGAACGTTGTACGTGAAACCCTGGAGTACAGAGAAAGCAACAAA     −780
 I  E  D  F  I  M  N  V  V  R  E  T  L  E  Y  R  E  S  N  K
GTCGTCCGGAAGGATATGATGCAGCTGCTCATGCAGCTACGTAACTCCGGAACGGTTTCG     −840
 V  V  R  K  D  M  M  Q  L  L  M  Q  L  R  N  S  G  T  V  S
ATCGACGATCGATGGGACATCGAAGTGTCAACCAACAAGAAAAAGCTGTCCCTGGAACAA     −900
 I  D  D  R  W  D  I  E  V  S  T  N  K  K  K  L  S  L  E  Q
GTCACAGCACACGCGTTCGTATTCTTCATAGCAGCATACGAAACATCATCGACCACCATT     −960
 V  T  A  H  A  F  V  F  F  I  A  A  Y  E  T  S  S  T  T  I
TCGTTCTGCTTGTTCGAACTGGCACGCAATCCGGAGATTCAAAAGAAAGTGCAACAAGAA    −1020
 S  F  C  L  F  E  L  A  R  N  P  E  I  Q  K  K  V  Q  Q  E
ATTGACCAAGTTCTCGCAAGCCACAACGGCGAAATCACCTACGACAACATCAACGAAATG    −1080
 I  D  Q  V  L  A  S  H  N  G  E  I  T  Y  D  N  I  N  E  M
AAATACCTCGAAAACTGCATCGACGAAACGCTCCGAAAGTATCCGGCAGTTCCGTTCCTG    −1140
 K  Y  L  E  N  C  I  D  E  T  L  R  K  Y  P  A  V  P  F  L
AACCGTGAGTGTTCTAAGGATTACAAAATCCCCGGAACAGACACCACCATCGAGAAAGGA    −1200
 N  R  E  C  S  K  D  Y  K  I  P  G  T  D  T  T  I  E  K  G
ACATCGTTAGTCATTCCAGTCCTCGGACTACACCGCGATCCCGATCACTACCCGGAACCG    −1260
 T  S  L  V  I  P  V  L  G  L  H  R  D  P  D  H  Y  P  E  P
GACAGGTTCATTCCGGAACGGTTCAGCAACTTTGAAGATATTTCCACCAAACCGTATCTT    −1320
 D  R  F  I  P  E  R  F  S  N  F  E  D  I  S  T  K  P  Y  L
CCGTTTGGGGCAGGACCTCGCAACTGTATTGGACTGAGATTGGGCAAGCTGCAAACAAAG    −1380
 P  F  G  A  G  P  R  N  C  I  G  L  R  L  G  K  L  Q  T  K
GCGGGACTGGTGATGATGCTGTCCAAGTTTAACGTGCGGCTTGCTGATGAAACTTACGCC    −1440
 A  G  L  V  M  M  L  S  K  F  N  V  R  L  A  D  E  T  Y  A
AGCAAAGAGCTAGCGCTCGATGCGCGAAGTGTGGTTCTAATGCCGGTTGGAGGTATTAAG    −1500
 S  K  E  L  A  L  D  A  R  S  V  V  L  M  P  V  G  G  I  K
GTGTCGATTTCGGAACGGAGGGCTTCGTAAtacaaattgaagtgactcgtaatataatac    −1560
 V  S  I  S  E  R  R  A  S  *
ttaaatatcacaataaatgataccaaataaactatttaatgaacaaaaaaaaaaaggt    −1620
ccggtacctctagatcaga                                           −1639
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Fig. 1 cDNA sequence and deduced amino acid sequence of *CYP6F1*

The two putative polyadenylation signals are in bold letters. Primers designed to confirm developmental expression are underlined. Stars denote the stop codon TAA. The GenBank accession number for this sequence is AY662654.

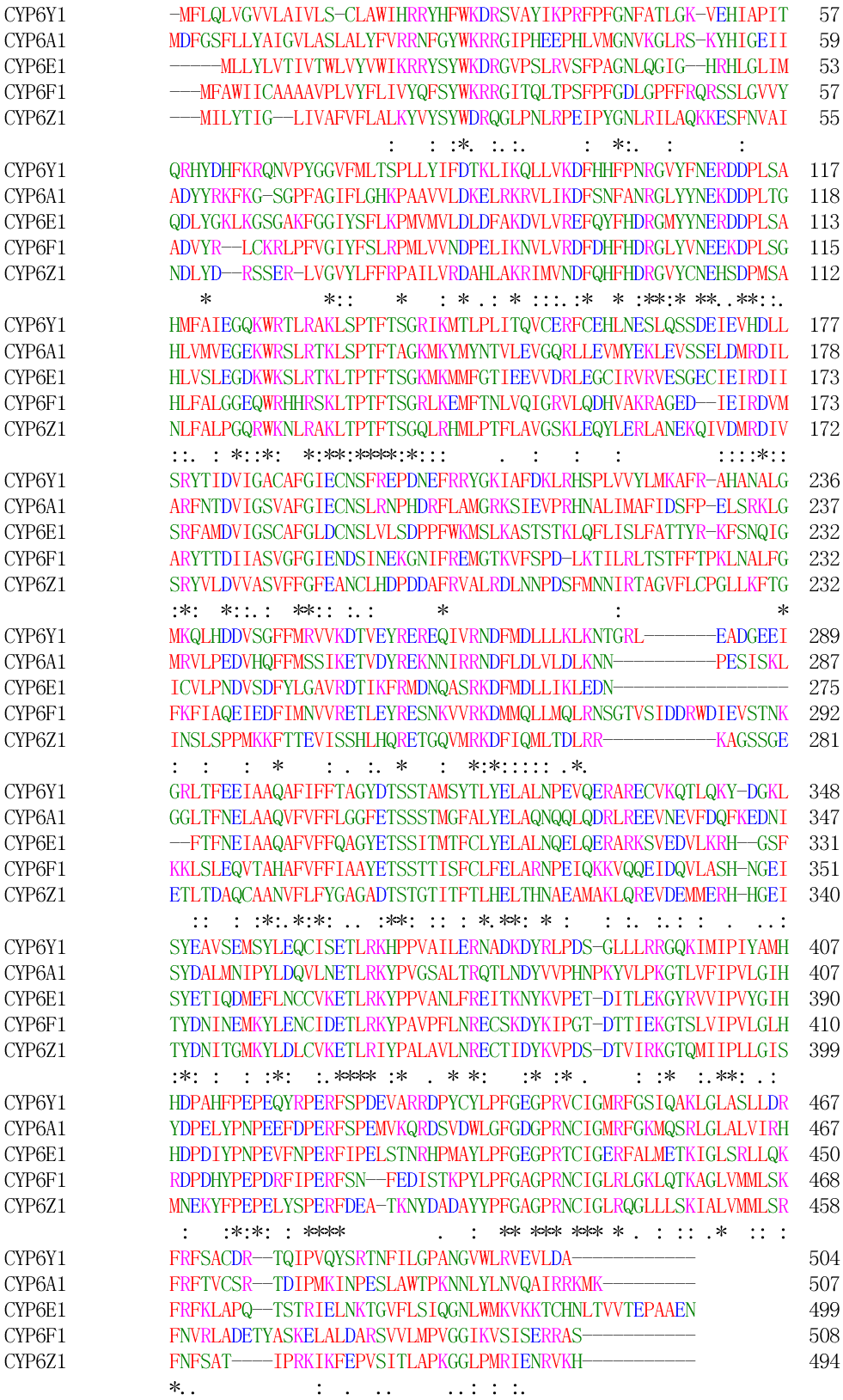

Fig. 2 Amino acid sequence alignment of selected members of insect *CYP6*

Culex quinquefasciatus CYP6E1 (GenBank accession No. AB001323), *Anopheles gambiae CYP6Z1*, *CYP6Y1* (AF487535, AF487536), *Musca domestica CYP6A1* (L27241). Identical amino sequences are denoted with a star.

was selectively amplified by using the specific primers (**Fig. 3**). The amount of amplified cDNA product was normalized by comparison with the amplification product of the β-actin gene from *Cx. pipiens pallens* (194 bp, **Fig. 3**). The maximal amplification of the *CYP6F1* PCR product was in the 4th instar larvae, followed by the 3rd instar larvae and the female adult mosquito, whereas the 1st and 2nd instar larvae had similarly low expression levels (**Fig. 3**). To assess the transcription expression level of the *CYP6F1* gene in deltamethrin-resistant and susceptible strains, semi-quantitative RT-PCR was used to measure the expression level of mRNA in *Cx. pipiens pallens*. The band intensities were significantly stronger in the resistant strain than in the susceptible strain (**Fig. 4**).

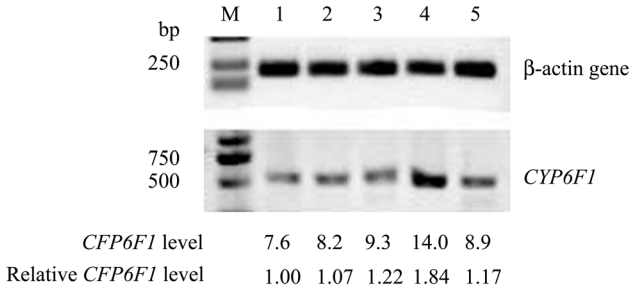

Fig. 3 Semi-quantitative RT-PCR of each life stage showed the relative amount of amplified transcripts of *CYP6F1* (Panel 2) in comparison with amplified mosquito β-actin gene transcripts (Panel 1)

M, maker; 1, 1st larvae; 2, 2nd larvae; Lane 3, 3rd larvae; 4, 4th larvae; 5, adult mosquito.

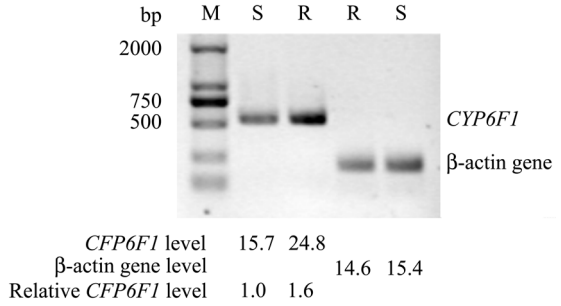

Fig. 4 Semi-quantitative RT-PCR of deltamethrin-resistant and -susceptible 4th larvae demonstrates the relative amount of amplified transcripts of *CYP6F1* compared to amplified mosquito β-actin gene transcripts

M, marker; S, susceptible strain; R, resistant strain.

Transcription and expression of CYP6F1 in C6/36 cells

To effect expression of the P450 gene in C6/36 cells, *CYP6F1* cDNA was inserted into the expression vector pIB/V5-His-TOPO. To improve the expression levels of the gene in the mosquito cell system, a Kozak sequence was introduced and a codon (GAA) was added between ATG and the sequence. Total RNA was isolated from control cells and from the cultured C6/36 cells transfected with the constructed recombinant vector, and RT-PCR was performed using forward specific primers and the reverse vector primer. A PCR product of the expected size, about 1800 bp, was observed only in cells that were transfected with the *CYP6F1* gene [**Fig. 5(A)**], confirming that *CYP6F1* had been transcribed in the transfected cells. Western blot analysis using anti-V5 antibodies was used to identify a protein with a molecular mass of 61 kDa in cells transfected with the *CYP6F1* gene [**Fig. 5(B)**].

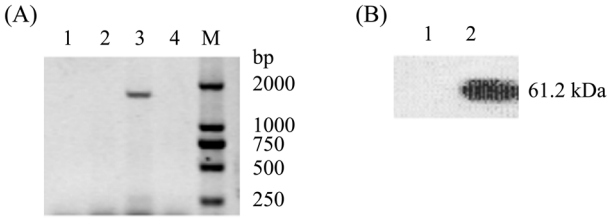

Fig. 5 Effects of expression and transcripts of *CYP6F1* in cells

(A) RT-PCR analysis of *CYP6F1* mRNA in mosquito cells using primers specific for the vector transcripts. The production of the transcripts was detected in cells transfected with the *CYP6F1* gene (lane 3); No signal was obtained in normal cells (lane 4) either in control reactions in which the reverse transcriptase was omitted (lane 2) or in reaction in which RNA cell extracts were replaced by water (lane 1). (B) Western blot analysis. No signal was obtained in the control cells (lane 1); expression was detected in cells transfected with the *CYP6F1* gene (lane 2).

Viability assay

To investigate the role of the *CYP6F1* gene, we transfected a vector harboring the *CYP6F1* gene and a vector containing a cat gene as a control into the mosquito C6/36 cell line and cells expressing the genes were selected to obtain a stable transfection cell line. After the cells were treated with various concentrations of deltamethrin, cell survival was analyzed using an MTT assay. The response and time course of cell viability (MTT reduction) were first measured over a wide range of concentrations of deltamethrin (data not shown). To avoid excessive toxicity,

in the subsequent experiments, we selected concentrations of deltamethrin that induced an approximately 30%–50% reduction in cell viability. We found that cell survival was reduced by at least 30% in the cells treated with 160 μM deltamethrin for 96 h. These parameters (160 μM deltamethrin, 96 h incubation) were used for all subsequent experiments. Expression of the *CYP6F1* gene improved the viability of deltamethrin-treated C6/36 cells by 12.7% and 76.5%, respectively ($P<0.05$, $P<0.01$), compared with cells that were not transfected with the *CYP6F1* gene or were transfected with the control vector (**Fig. 6**).

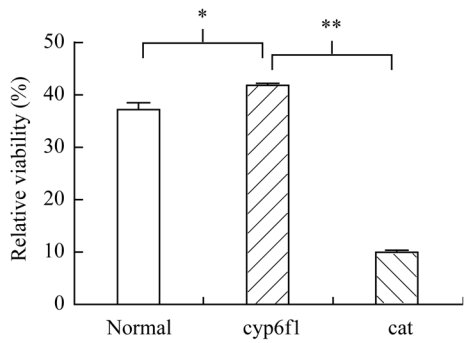

Fig. 6 Effects of deltamethrin on the viability of cultured C6/36 cells

The cultured cells were treated with 160 μM of deltamethrin and incubated for 96 h. Transfected *CYP6F1* provided statistically significant protection from deltamethrin toxicity (**$P<0.01$, *$P<0.05$). Mean viability and SD error bars are presented for three independent studies. Student's *t*-test was used for statistical comparisons.

Discussion

In the present study, we isolated cytochrome P450 cDNA from deltamethrin-resistant *Cx. pipiens pallens*. The predicted *CYP6F1* protein has many characteristics common to the *CYP6* family, such as a putative membrane-anchoring signal, putative reductase binding sites, a typical heme-binding site, an ETLR motif and substrate recognition sites. These results strongly suggest that *CYP6F1* is a member of the cytochrome P450 *CYP6* family. The *CYP6F1* nucleotide sequence shared 99% identity with *CYP6F* from *Cx. quinquefasciatus*. Only one amino acid and two nucleotides of the cloned P450 differed from that of *CYP6F*. *Cx. pipiens pallens* and *Cx. quinquefasciatus* are two subspecies of *Cx. pipiens*; the genes isolated from mosquitoes of these two families have high homology, suggesting that the *CYP6F* gene has been well conserved throughout evolution.

Diversity in the development, expression and regulation of insect P450s is well established. *CYP4D1* is expressed in a very similar pattern throughout life stages [13]. In contrast, *CYP6A1* is expressed at low levels in embryos, rises to a maximum in the late larval stage, drops in the pupal stage, and rises again in both adult males and females [28]. *CYP6B* genes are specifically expressed in larval stages [16], and *CYP6D1* is regulated specifically in the adult stages [15]. Because of this variability, we determined the expression profile of *CYP6F1* mRNA during the mosquito life cycle. Our results demonstrated that the levels of *CYP6F1* expression are developmentally regulated. *CYP6F1* expression was highest in 4th instar larvae. If the function of *CYP6F1* is in xenobiotic metabolism, the overexpression of the gene in 4th instar larvae may be indicative of an adaptive ability of the larvae to metabolize xenobiotics upon exposure. The adaptive regulation of insect P450s in response to an exposure to toxins over the course of development has previously been reported [16].

Increased amounts of cytochrome P450, particularly in the *CYP6* family, and increases in cytochrome P450 activity have long been considered to be associated with many cases of pyrethroid resistance [7,8,11,25,29]. Therefore, it would be interesting to determine whether *CYP6F1* also contributes to resistance. In the present study, we found higher band intensities in the resistant strain than in the susceptible strain. Although we have no direct evidence at present to show that *CYP6F1* is involved in deltamethrin resistance, our results suggest that *CYP6F1* is a good candidate for future studies of pyrethroid resistance.

The cytochrome P450 genes are a large family, which is involved in a wide variety of metabolic functions. In insects, these enzymes play roles in key processes ranging from host plant utilization to xenobiotic resistance [3]. Recently, DDT-R was found to be associated with overtranscription of the P450 gene *CYP6G1* in three *Drosophila melanogaster* strains [30], and induced 10 to 100 times as much mRNA in resistant strains than seen in susceptible strains. Transgenic analysis of *CYP6G1* showed that overtranscription of this gene alone is both necessary and sufficient for resistance. Resistance and upregulation in *Drosophila melanogaster* populations are associated with a single *CYP6G1* allele that has spread globally [12]. To confirm that *CYP6F1* is involved in insecticide resistance, it is necessary to demonstrate that

P450 is able to detoxify or sequester pyrethroid insecticides. Therefore, we constructed an expression vector directing the overproduction of *CYP6F1* mRNA and protein in transfected cells and created stable expression cell lines. *CYP6F1*-transfected cells had greater resistance to deltamethrin than did control cells. Although the protection was not complete, our findings provide evidence that overexpression of *CYP6F1* may be associated with insecticide resistance.

In summary, we have cloned the first *CYP6F1* gene known from *Cx. pipiens pallens*. Based on the characteristics of the gene, it is a member of the P450 *CYP6* family. The convergence of data in the present study suggests that *CYP6F1* may confer some deltamethrin resistance in mosquitoes. Research carried out to date has provided a basis for further studies on the gene function associated with insecticide resistance, which will improve our understanding of the molecular basis of P450-mediated resistance in *Cx. pipiens pallens*.

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