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Cloning and characterization of *NYD-OP7*, a novel deltamethrin resistance associated gene from *Culex pipiens pallens* ☆

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Abstract

One mosquito *opsin* gene, *NYD-OP7*, has been cloned from *Culex pipiens pallens*. An open reading frame (ORF) of 1116 bp was found to encode a putative 371 amino acids protein which exhibits high identity with opsins from *Aedes* and *Anopheles* mosquitoes. Transcript expression of *NYD-OP7* was determined by real-time PCR in all life stages of deltamethrin-susceptible and -resistant strains of the *Culex* mosquito. The results demonstrated that this gene is expressed at all developmental stages, and it is expressed predominantly at the pupae and adult stages. Meanwhile, in pupae and adults, *NYD-OP7* is overexpressed in deltamethrin-resistant strain than in -susceptible strain. Importantly, stable expression of *NYD-OP7* in the mosquito C6/36 cells can confer moderate deltamethrin resistantce. Our study provides the first direct evidence that increased expression of an *opsin* gene may play some role in the development of deltamethrin resistantce in *Cx. pipiens pallens*.
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Keywords: *Culex pipiens pallens*; Opsin; Reverse transcription PCR; Transfection; Deltamethrin resistantce

1. Introduction

Opsins, the visual pigments of animal photoreceptors, belong to the G-protein coupled receptor family which is characterized by a seven-helix transmembrane topology and by the ability to activate heterotrimeric G-proteins [1–3].

Since the first sequence of an opsin, bovine rhodopsin, was determined by conventional protein sequencing and cDNA sequencing, more than 1000 opsins have been identified [4,5]. In invertebrates, some photoreceptor opsins have been well-characterized, both in terms of their sequences and cellular functions [1]. When sequence alignments are made of the invertebrate and vertebrate opsins, it is evident that individual sequence may be quite different, but the tertiary structure appears conserved [5,6].

Insecticide resistance is a major obstacle to the control of vector-borne diseases [7–9]. Many studies have suggested that the insecticide resistance phenotype evolves rapidly based on the selection of major effect genes [10,11]. However, recent genome-wide transcription profiling indicated that a broader range of genes may be involved and that insecticide resistance may be more complex than previously considered [12–14].

As part of an ongoing study of insecticide resistance in *Cx. pipiens pallens*, we have employed suppression subtractive hybridization (SSH) and cDNA microarray to identify differentially expressed genes between deltamethrin-susceptible and -resistant strains of *Cx. pipiens pallens*. *Opsin* is one of only ten genes that showed over-expression in deltamethrin-resistant strain [15]. In the present study, we isolated and sequenced the complete sequence of this gene and

termed it *NYD-OP7*[2]. We then characterized the expression pattern of *NYD-OP7* gene in different developmental stages of *Cx. pipiens pallens*. In addition, we investigated the role of *NYD-OP7* in deltamethrin resistance by transfecting this gene into the mosquito C6/36 cells and tried to determine if over-expression of *NYD-OP7* can change the sensitivity of the cells to deltamethrin.

2. Materials and methods

2.1. Mosquito strains

The deltamethrin-susceptible strain of *Cx. pipiens pallens* was obtained from the Shanghai Insect Institute of the Chinese Academy of Sciences and was maintained in our laboratory. The mosquitoes had never been exposed to any insecticides and the median lethal concentrations (LC_{50}) of deltamethrin (Roussel Uclaf, France) was 0.0008 mg/L. The strain was reared and bred at 25–27 °C in a 16 h light/8 h dark photoperiod. The deltamethrin-resistant strain was derived from the susceptible early fourth instar larvae by selection with deltamethrin for 10 generations until the resistant ratio (LC_{50} of filial generation/LC_{50} of parental generation) reached 400 [16].

2.2. Construction of cDNA library

Total RNA were extracted from 300 adult females of resistant strain using the RNeasy maxi kit (Qiagen, Germany) according to the manufacture's instructions. The integrity of total RNA was determined by denaturing agarose gel electrophoresis and the yield and purity of total RNA was estimated by spectrophotometry. The poly (A+) mRNA was purified from total RNA with the PolyA-tract mRNA isolation system (Promega, USA), and 2 µg polyA mRNA was used for cDNA library construction with λExcell/*Not*I/*Eco*RI/CIP vector system (Pharmacia, USA) following the manufacturer's protocols. The cDNA library contained approximately 5×10^5 clones and was amplified.

2.3. Cloning and sequencing of NYD-OP7 cDNA

To isolate the full-length of culex *opsin* gene, we used the standard SP6 and T7 library vector primers and the primers designed based on the sequence of the SSH fragment we reported previously (GenbBank Accession No. BE247812). The sequence of the oligonucleotide primers was: forward 5′-GTCGCTCTGGTGACCATTTC-3′; reverse 5′-CACGATCGGGGTTGTAGACAG-3′. PCR conditions were: initial denaturation at 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s with final 10 min extension at 72 °C. The PCR products were separated by 1% agarose gel electrophoresis and purified using a QIA quick Gel extraction kit (Qiagen, Germany). Products were then cloned into the pGEM-T easy vector (Promega, USA) and sequenced at Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Then the sequences of above three fragments were assembled to generate a putative full-length cDNA of opsin. A pair of PCR primers: forward 5′-TTAAGTCTGAAGTAGTTC-3′ and reverse 5′-CTAGCAAAGATCAGCAATC-3′ were designed for the amplification of full-length cDNA according to the assembled sequence of opsin. PCR was carried out using LA *Taq* polymerase (TaKaRa, Japan) using the following protocol: 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s with final 10 min extension at 72 °C. Then the PCR product was purified, cloned into the pGEM-T easy vector and sequenced.

2.4. Sequence alignment and phylogenetic tree

The standard protein-/protein BLAST sequence comparison (blastp; www.ncbi.nlm.nih.gov / BLAST/) and PSI-BLAST programs were used to search for sequences in the GenBank and SWISS-PROT databases with similarities to the translated sequences of *NYD-OP7* [17]. Sequence alignment and phylogenetic tree analysis using the neighbour-joining method was carried out by the CLUSTAL W program [18]. The opsins included in our analysis were: twelve sequences from *An. gambiae* genome [2], six sequences from *Drosophila melanogaster*, three sequences from *Manduca sexta*, six sequences from *Papilio glaucus* and two sequences from *Aedes aegypti* [6].

2.5. RNA extraction and cDNA synthesis

Total RNA were extracted from embryos, first, second, third and fourth instar larvae, pupae, and adults of both susceptible and resistant strains using the RNeasy mini kit (Qiagen, Germany) according to the manufacturer's protocol, and contaminant genomic DNA was removed by DNase I treatment. cDNA was synthesized from 2 µg of total RNA with M-MLV reverse transcriptase (Promega, USA) and random oligonucleotide primers according to the manufacturer's protocol.

2.6. Real-time PCR of NYD-OP7 in mosquito developmental stages

Aliquots of cDNA were amplified on an ABI PRISM 7300 (Applied Biosystems) using the SYBR green PCR kit (PE Applied Biosystems) with 25 µl reaction mixtures. The PCR mixture contained 1× SYBR PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM (each) dNTP, 0.2 µM primers, and 0.3 µl of *Taq* polymerase (2.5 U/µl). The primers used were: *NYD-OP7*: forward 5′-GTGGGCTTTCGCACTCTT-3′, reverse 5′-GTTCACGCATCTGTTCCTC-3′; β-actin: forward 5′-CGCTTCCTCGTCTACACTGG-3′, reverse 5′-GTGTTGGCGAACAGATCCTT-3′. For amplification, the following program was employed: 40 cycles of 95 °C for 15 s, 60 °C for

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[2] *Abbreviation used:* NYD, the abbreviation of the Chinese name of Nanjing Medical University.

1 min. A melting curve program was run immediately after the PCR program. Control measurement was done in the absence of reverse transcriptase to make sure that the level of genomic DNA contaminants was negligible.

Real-time PCR data were analyzed with a 7300 System SDS Software v1.2.1 (Applied Biosystems) to estimate transcript copy numbers for each sample. The raw threshold cycle (Ct) values were normalized against β-actin standard to obtain normalized Ct values, which were then used to calculate relative expression levels in the samples. The expression level of *NYD-OP7* in susceptible strain egg was considered as background level, or 1. To verify reproducibility, the real-time PCR analysis was repeated twice in independent experiments, with three replicates for each sample.

2.7. Recombinant insect expression vector construction

The 1116 bp *NYD-OP7* coding sequence, from the putative translational ATG to the end of the ORF, was amplified by PCR using the cDNA library as the template. Forward and reverse primers, containing at their ends, respectively, *EcoRI* and *NotI* restriction sites, were: forward 5′-CGGAATTCTGGCAGCTTTCGTTGAGCC-3′; reverse 5′-ACTAGCGGCCGCTGTACTTAAGCCTTCTCG-3′. The PCR product was ligated into the pGEM-T easy vector. Then the recombinant plasmid was digested with *SacII* and *NotI* and the purified fragment was ligated into the *SacII*–*NotI* linearized insect expression vector pIE1-3 (Novagen, USA), resulting in the pIE1-3/OP7 construct. The integrity of the construct was verified by restriction enzyme digestion and sequencing. The pIE1-3 vector is designed for constitutive expression of recombinant protein from the baculovirus *ie1* promoter in stably transfected insect cells [19]. To obtain stably transfected cells, a pIE1-3 recombinant should be cotransfected with pIE1-neo vector (Novagen, USA), which contains the Neo selectable marker under *ie1* control.

2.8. Cell culture and transfection

Mosquito C6/36 cells were obtained from China Center for Type Culture Collection (CCTCC, China). The cells were maintained in Eagle's minimum essential media (EMEM) (Invitrogen, USA) supplemented with non-essential amino acids, fetal bovine serum (10%) (Sijiqing, China), and antibiotics. The cells were grown in a 5% CO_2 humidified incubator at 28 °C. Transfection was performed using Cellfectin reagent (Invitrogen, USA) according to the manufacturer's protocol. Briefly, 4×10^5 cells/well were plated into a 6-well plate in growth medium without antibiotics and cultured overnight. Then the pIE1-3/OP7construct and the pIE1-3 vector only, were each separately cotransfected with pIE1-neo construct at a ratio 4:1 into C6/36 cells according to the manufacturer's protocol. After 24 h, the cells were selected with 300 μg/ml G418 (Calbiochem, Germany) for 2 weeks and the medium was changed every 4 days. Following selection, G418-resistant colonies were

isolated and expanded as individual clonal cell lines for subsequent analysis.

2.9. RT-PCR analysis

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol and the reverse transcript reaction was performed as above. The resulting cDNA preparation was subjected to PCR amplification using the forward primer specific to *NYD-OP7* (5′-GTGGGCTTTCGCACTCTT-3′) and the pIE1-3 vector-specific HRAS reverse primer (5′-AAGATTAGCGAC GCTGCT-3′). Reaction conditions were as follows: 94 °C for 5 min; 30 cycles (25 cycles for β-actin) of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 40 s; final extension at 72 °C for 10 min. To confirm equal loading, PCR amplification of the β-actin gene was also done in parallel. The primers used for β-actin PCR amplification were: forward 5′-CACCAGGGT GTGATG GTCGG-3′, reverse 5′-CCACCGATCCAGAC GGAGT-3′. The PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide, and photographed under UV light.

2.10. Cell proliferation ($[^3H]$-TdR) assay

$[^3H]$Thymidine (1 mCi/ml, Shanghai Institute of Applied Physics, Chinese Academy of Sciences) was used to determine the proliferation activity of stable C6/36 transfectants. Cells in exponential growth phase were trypsinized and resuspended in growth media. Cells were plated in 96-well plates (5000 cells/well) and cultured 4 h to allow for cell attachment. Cells were then treated with various concentrations of deltamethrin (0, 10, 15, 20, 25, 30, 40, 80, and 120 μM). After incubation for 72 h, $[^3H]$thymidine (0.5 μCi/ well) was added, and cultures were incubated for a further 16 h. Incorporation of $[^3H]$thymidine into DNA was measured using an Wallac 1414 liquid scintillation counter (WALLAC, Finland). Data were gathered as counts/min, representing the fraction of cells that contained newly synthesized DNA. Inhibition of proliferation was determined by comparing the level of $[^3H]$thymidine uptake with controls. Three independent experiments were done in quadruplicate wells. Deltamethrin was dissolved in DMSO and the final concentration of DMSO in the medium is 0.1%.

2.11. Statistics

Data were analyzed by Student's *t* test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Isolation full-length of the Cx. pipiens pallens NYD-OP7 gene

To isolate *opsin* gene from the *Cx. pipiens pallens*, we performed a RT-PCR screen of cDNA library using the

standard SP6 and T7 library vector primers and primers designed based on the sequences we reported previously (GenBank Accession No. BE247812). The sequences we got were assembled to generate a putative full-length cDNA of *opsin*. Then a pair of PCR primers was designed for the amplification of full-length cDNA. Sequencing of the amplified transcript led to the identification of a novel *opsin* gene which we termed *NYD-OP7* (GenBank Accession No. AY749413). The *NYD-OP7* cDNA has an open reading frame of 1116 bp, encoding 371 amino acids (Fig. 1).

3.2. Sequence and phylogenetic analysis

Alignment of the deduced NYD-OP7 amino acid sequence with *Ae. aegypti* and *An. gambiae* opsins revealed sequence identities of 87% with Ops1, 86% with GPRop1-4, 83% with Ops2, 79% with GPRop6, and 63% with GPRop5 (Fig. 2). The newly isolated opsin displays typical hallmarks of rhodopsins, such as a highly conserved lysine residue (Lys-317) in the seventh transmembrane region, to which the retinal chromophore is attached; putative phosphorylation

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  1 - TTAAGTCTGAAGTAGTTCGTTCTAGGACGATTTTCTTCAGCTTAACTTAACCGTTCGAAA - 60

 61 - CTTAGTGAAACCCGCTCTAGACGCTGTGAGATTATTACTTTTTAAAAGTAACATCACCAA - 120

121 - TCGGAAGCATGGCAGCTTTCGTTGAGCCGCATTTCGACGCCTATCAGGCGGGTAATGGAA - 180
    -           M   A   A   F   V   E   P   H   F   D   A   Y   Q   A   G   N   G   N

181 - ACATGACCGTCGTCGATATGGTATCCCCGGATATGTTGCACATGGTTCACCCTCACTGGA - 240
    -  M   T   V   V   D   M   V   S   P   D   M   L   H   M   V   H   P   H   W   N

241 - ATCAGTTCCCGCCGATGAACCCGCTGTGGCACTCGATCCTCGGATCTGCCATCTTCTGTT - 300
    -  Q   F   P   P   M   N   P   L   W   H   S   I   L   G   S   A   I   F   C   L

301 - TGGGAATGGTCTCGATGATTGGCAACGGTTGTGTCATTTCGATCTTCACCAGCACCAAGT - 360
    -  G   M   V   S   M   I   G   N   G   C   V   I   S   I   F   T   S   T   K   S

361 - CACTCAAAACCCCGTCCAACCTGCTAGTGGTCAATCTAGCCTTCTCCGATTTCCTGATGA - 420
    -  L   K   T   P   S   N   L   L   V   V   N   L   A   F   S   D   F   L   M   M

421 - TGTTCACGATGGGACCGCCGATGGTGATCAACTGCTGGCATCAGACCTGGAGCTTCGGAC - 480
    -  F   T   M   G   P   P   M   V   I   N   C   W   H   Q   T   W   S   F   G   P

481 - CTTTCGCTTGCGAACTGTACGCCTGCCTGGGATCGCTGTTCGGCTGTGCCTCGATCTGGA - 540
    -  F   A   C   E   L   Y   A   C   L   G   S   L   F   G   C   A   S   I   W   T

541 - CCATGACTTTGATTGCGTTTGACCGATACAATGTTATCGTGAAGGGTCTTGCCGCCAAGC - 600
    -  M   T   L   I   A   F   D   R   Y   N   V   I   V   K   G   L   A   A   K   P

601 - CAATGACCAACAGTGGTGCTATGGTCAAGATCCTGCTCGTGTGGGCTTTCGCACTCTTCT - 660
    -  M   T   N   S   G   A   M   V   K   I   L   L   V   W   A   F   A   L   F   W

661 - GGACTCTGGCCCCATTCTTCGGATGGAACCGATATGTCCCAGAGGGTAACGTGACTGCCT - 720
    -  T   L   A   P   F   F   G   W   N   R   Y   V   P   E   G   N   V   T   A   C

721 - GCGGAACTGGCTACCTGACCCAGACCTGGTTGAGCCGCTCGTACATAATCGTTTACGCCG - 780
    -  G   T   G   Y   L   T   Q   T   W   L   S   R   S   Y   I   I   V   Y   A   V

781 - TCTTCGTCTACTGGCTGCCTCTGCTCACCATCATTTACTCGTACACGTTCATCCTGAAGG - 840
    -  F   V   Y   W   L   P   L   L   T   I   I   Y   S   Y   T   F   I   L   K   A

841 - CTGTCTCTGCCCACGAGGAACAGATGCGTGAACAGGCCAAGAAGATGAACGTTGCCTCGC - 900
    -  V   S   A   H   E   E   Q   M   R   E   Q   A   K   K   M   N   V   A   S   L
```

Fig. 1. The nucleotide and deduced amino acid sequences of *Cx. pipiens pallens NYD-OP7*. The deduced amino acid sequence is presented below the nucleotide sequence in single letter code. The predicted seven transmembrane segments are underlined. The putative polyadenylation signals in the 3′-untranslated region are also underlined (thick lines). The two cysteine residues in the first and second extracellular loops are boxed. The 317th K-residue is shown in bold. Potential sequences for G-protein binding sites, DRY and QAKKMNV, as mentioned in Section 3, are indicated by open boxes. Potential glycosylation sites in the N terminals and HEE motif are indicated by shaded boxes. The asterisk indicates the stop codon.

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901 - TGCGATCGTCGGAAGCTCAACAGACCAGCGCTGAGATCAAGCTGGCCAAGGTCGCCCTCG - 960
      -  R  S  S  E  A  Q  Q  T  S  A  E  I  K  L  A  K  V̲ ̲A̲ ̲L̲ ̲V̲
961 - TGACCATTTCCCTGTGGTTCATGGCCTGGACTCCATACCTGGTCATCAACTTCACCGGAA - 1020
      -  T̲ ̲I̲ ̲S̲ ̲L̲ ̲W̲ ̲F̲ ̲M̲ ̲A̲ ̲W̲ ̲T̲ ̲P̲ ̲Y̲ ̲L̲ ̲V̲ ̲I̲ ̲N̲ ̲F̲ ̲T̲ ̲G̲  I
1021 - TCTTCAAGGCTGCCCCGATCAGCCCACTGGCCACCATCTGGGGATCGCTGTTCGCCAAGG - 1080
      -  F  K  A  A  P  I  S  P  L̲ ̲A̲ ̲T̲ ̲I̲ ̲W̲ ̲G̲ ̲S̲ ̲L̲ ̲F̲ ̲A̲ **K̲** ̲A̲
1081 - CCAACGCTGTCTACAACCCGATCGTGTACGGAATCAGCCATCCGAAGTACCGTGCTGCTC - 1140
      -  N̲ ̲A̲ ̲V̲ ̲Y̲ ̲N̲ ̲P̲ ̲I̲ ̲V̲ ̲Y̲ ̲G̲ ̲I̲  S  H  P  K  Y  R  A  A  L
1141 - TGTACCAGCGGTACCCAGGACTGTCGTGCCAGTCAGACAACTCTCCCAACGACACCCAGT - 1200
      -  Y  Q  R  Y  P  G  L  S  C  Q  S  D  N  S  P  N  D  T  Q  S
1201 - CGGTTGCTTCCGGGGCGACGACCGCCTCCGACGAGAAGGCTTAAGTACAAAGGTGAGATA - 1260
      -  V  A  S  G  A  T  T  A  S  D  E  K  A  *
1261 - GGACAAACACGGTGATATTTTGGCAATGATTGCTGATCTTTGCTAGAAAAAAAAAATTAG - 1320
1321 - ATAGAAAAAATAGAAGCAAATGA̲A̲A̲T̲A̲A̲TCAATGGATTAAAACGGAGAAA̲A̲A̲T̲A̲A̲TAAAA - 1380
1381 - GAAGAGGTCTAGTAAAAGACAGAGTAAAAGAGACTCAAAAAGTAAAAGAAACCTCCT    - 1437
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Fig. 1 (*continued*)

sites (Ser, Thr) near the C-terminus; putative *N*-glycosylation sites (Asn-Xaa-Ser/Thr) near the N-terminus, and two cysteine residues (Cys-121 and Cys-198) in the first and second extracellular loop, which may form a disulphide bond. NYD-OP7 contains a DRY sequence at the junction of helix III and the second intracellular loop that is very similar to the ERY sequence found in bovine rhodopsin. This sequence contains a charged pair that is required for transducin activation [20,21]. Although dissimilar from the vertebrate opsins, the sequence QAKKMNV found in the N-terminal half of cytoplasmic loop 3 is very highly conserved among the invertebrate opsins and is located within a region of the protein that is also required for transducin activation [22]. Most insect rhodopsins have a His-Glu-Lys (HEK) motif at the beginning of a conserved region in the third cytoplasmatic loop, and it is implicated in rhodopsin–G-protein interaction. Interestingly, the HEK motif is modified to His-Glu-Glu in NYD-OP7. Other insect rhodopsins in which the lysine residue of the HEK motif is substituted include two *M. sexta* rhodopins [23], two *An. gambiae* rhodopsins [2], one calliphora rhodopsin [24] and one *Ae. aegypti* opsin [6].

Fig. 3 shows the molecular phylogenetic relationship between NYD-OP7 and some other insect opsins. Results showed that the NYD-OP7 sequence is most closely related to the *Ae. aegypti* Ops1, and it clearly belongs to a group comprising the known arthropod opsins.

3.3. Expression profile of the NYD-OP7 gene

The expression of the *NYD-OP7* gene was analyzed by real-time PCR amplification of the corresponding mRNA from different *Cx. pipiens pallens* developmental stages. As shown in Fig. 4, *NYD-OP7* is expressed at significantly high levels in pupae and adults, whereas expression is very low in the egg, first, second, third and fourth instar larvae stages. Interestingly, in pupae and adults, the expression of *NYD-OP7* is significantly higher in resistant strain than in susceptible strain.

3.4. NYD-OP7 expression and deltamethrin sensitivity analysis of pIE1-3/OP7 transfected C6/36 cells

To elucidate the contribution of the *NYD-OP7* gene product to deltamethrin resistance, the mosquito C6/36 cells were transfected with the pIE1-3/OP7 insect expression vector. After G418 selection, one stably transfected *NYD-OP7* subclone was obtained. Analysis of this transfectant demonstrates a higher expression of *NYD-OP7* gene as compared with the empty vector transfectant control (Fig. 5). These subclones did not show any growth morphological changes and then they were examined for deltamethrin sensitivity by [^{3}H]-TdR assay. As shown in Fig. 6, *NYD-OP7* transfected C6/36 cells are relatively resistant to deltamethrin at the concentrations of 15, 20 and 25 μM ($p < 0.05$). Meanwhile, a slight increase of cell viability was also observed at 30 μM deltamethrin but without statistical significance ($p = 0.0744$).

4. Discussion

Opsins are membrane proteins with molecular masses of 30–50 kDa that are related to the protein moiety of the photoreceptive molecule rhodopsin; typically they are considered to act as light sensors in animals, but there are still many unanswered questions concerning the functions of different opsins [5,25–28]. *Opsin* gene has been cloned in various invertebrates, such as *D. melanogaster* [29],

An. gambiae [2], *Ae. aegypti* [6], *M. sexta* [23], *P. glaucus* [30] and aphid [31].

In this experiment, we studied a novel cDNA encoding opsin (*NYD-OP7*) isolated from *Cx. pipiens pallens*. Considering the amino acid sequence of this gene, the translated protein is expected to have a seven-transmembrane structure, and it has all the conserved residues required for a functional opsin protein. This feature and the amino acid

identity of NYD-OP7 with known opsins suggest that it encodes a functional opsin protein. We have compared the amino acid sequence of the novel opsin with those of other insect opsins. The phylogenetic analysis indicates that NYD-OP7 is a member of the group including *Ae. aegypti* Ops1 and *An. gambiae* GPRop1-3. Real-time PCR analysis revealed the expression of *NYD-OP7* at all developmental stages both in deltamethrin-susceptible and -resistant

```
CpNYD-OP7  --------------------------MAAFVEPHFDAYQAG-NGNMTVVDMVSPDMLHMVHPHWN  38
AaOps1     --------------------------MAAFVEPHFDAWQAA-GGNLTVVDKVPPEMLHMIHPHWN  38
AaOps2     --------------------------MAAFVAPHFDAWQS--RGNMTVVDKVPPEMLHMVHPHWN  37
AgGPRop2   --------------------------MAAFVEPHFDAWTQS-GGNMSVVDKVPPEMLHMVHPHWN  38
AgGPRop4   -----------MLKTEVLIFPDMAAFVEPHFDAWTQS-GGNMSVVDKVPPEMLHMVHPHWN      49
AgGPRop3   --------------------------MAAFVEPHFDAWTQS-GGNMSVVDKVPPEMLHMVHPHWN  38
AgGPRop1   --------------------------MAAFVEPHFDAWTQG-SGNMSVVDKVPPEMLHMVHPHWN  38
AgGPRop6   SVDNRTPLCLVVGNCQRRETEMAAFAEPHFSAWTQTVVSNVTVVDKVPPEMLHMVDAHWY      60
AgGPRop5   -----------MMDHRPVGIFGPKSPQALTWTIS-VANLTVVDKVPPEMLHLVDTYWY        46
                       :  .   *:   ::      .*::*** *.*:***::..:*
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CpNYD-OP7  QFPPMNPLWHSILGSAIFCLGMVSMIGNGCVISIFTSTKSLKTPSNLLVVNLAFSDFLMM  98
AaOps1     QFPPMNPLWHSILGFAIFVLGMVSMIGNGFVMSIFTSTPSLRTPSNLLVVNLAFSDFLMM  98
AaOps2     QFPPMNPLWHSILGFAIFVLGVVSMLGNGCVIYIFTGTRSLRTPSNLLVVNLAFSDFFMM  97
AgGPRop2   QFPPMNPLWHSILGFAIFMLGMISMTGNGCVMYIFTNTKSLRTPSNLLVVNLAFSDFFMM  98
AgGPRop4   QFPPMNPLWHSILGFAIFMLGMISMTGNGCVMYIFTNTKSLRTPSNLLVVNLAFSDFFMM  109
AgGPRop3   QFPPMNPLWHSILGFAIFMLGMISMTGNGCVMYIFTNTKSLRTPSNLLVVNLAFSDFFMM  98
AgGPRop1   QFPPMNPLWHSILGFAIFMLGMISMTGNGCVMYIFTNTKSLRTPSNLLVVNLAFSDFFMM  98
AgGPRop6   QFPPMNPLWHSILGFAIFVLGVVSIIGNGCVIYIFTNTKALRTPSNLLVVNLAFSDFLMM  120
AgGPRop5   QFPPLETKWHAALATTIGLLALISIVGNGCVILIFSSTKGLRTPSNLMVINLAFADFMMM  106
           ****::. **: *. :*  *.::*: *** *: **:.* .*:*****:*:****:**:**
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CpNYD-OP7  FTMGPPMVINCWHQTWSFGPFACELYACLGSLFGCASIWTMTLIAFDRYNVIVKGLAAKP  158
AaOps1     FTMGPPMVINCWHETWVFGPFACEVYACLGSLFGCASIWTMTMIAFDRYNVIVKGLAAKP  158
AaOps2     FTMGPPMVINCWHETWVLPPFACELYAMFGSLFGCASIWTMTMIAFDRYNVIVKGLSAKP  157
AgGPRop2   FTMGPPMVINCWHETWTFGPFACELYAMLGSLFGCASIWTMTMIAFDRYNVIVKGLAGKP  158
AgGPRop4   FTMGPPMVINCWHETWTFGPFACELYAMLGSLFGCASIWTMTMIAFDRYNVIVKGLAGKP  169
AgGPRop3   FTMGPPMVINCWHETWTFGPFACELYAMLGSLFGCASIWTMTMIAFDRYNVIVKGLAGKP  158
AgGPRop1   FTMGPPMVINCWHETWTFGPFACELYAMLGSLFGCASIWTMTMIAFDRYNVIVKGLAGKP  158
AgGPRop6   FTMAPPMVINCWHETWVFGPFACELYAMLGSLFGCASIWTMTMIAFDRYNVIVKGLAGKP  180
AgGPRop5   FTMAPPLIINSYHETWVFGPLMCEIYGMFGSLSGCVSIWSMTMIAFDRYTVIVKGLSAKP  166
           ***.**::**.:*:** : *: **:*.  :*** **.***:**:*****.******:.**
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CpNYD-OP7  MTNSGAMVKILLVWAFALFWTLAPFFGWNRYVPEGNVTACGTGYLTQTWLSRSYIIVYAV  218
AaOps1     LTNNGAIVRILGIWAFALFWTLAPFFGWNRYVPEGNMTACGTDYQTQTWLSRSYIIIYAI  218
AaOps2     LTNNGALLSILGIS-FAGFWTLAPFFGWNRYVPEGNMTACGTDYLTTTWLNRSYIIVYAI  216
AgGPRop2   MTNNGALLRILGVWVFALFWTLAPLFGWNRYVPEGNMTACGTDYLTQTWLSRSYIIIYAI  218
AgGPRop4   MTNNGALLRILGVWVFALFWTLAPLFGWNRYVPEGNMTACGTDYLTQTWLSRSYIIIYAI  229
AgGPRop3   MTNNGALLRILGVWVFALFWTLAPLFGWNRYVPEGNMTACGTDYLTQTWLSRSYIIIYAI  218
AgGPRop1   MTNNGALLRILGVWVFALFWTLAPLFGWNRYVPEGNMTACGTDYLTQTWLSRSYIIIYAI  218
AgGPRop6   MTNNGALLRILGIWAFSLLWTLAPLFGWNRYVPEGNMTACGTDYLSQDFTSRSYILIYSG  240
AgGPRop5   LTYVGSVMRILFVWANSLVWTLAPLFGWNRYVPEGNMSACGTDYLSKDWISVSYIYAYSV  226
           :*  *::: ** :    . .*****:***********::****.* :   :. *** *:
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Fig. 2. Amino acid sequence alignment of *Cx. pipiens pallens* NYD-OP7, *Ae. aegypti* opsins and *An. gambiae* opsins. Asterisks indicate indentical amino acid and dots indicate similar amino acids. *Abbreviations* and GenBank Accession Nos.: *An. gambiae* (Scaffold Nos., GPRop1 Rh6 AAAB01008987 gi|19612317: 15485449- 15486561,GPRop2 Rh6 AAAB01008987 gi|19612317:15548577-15547465,GPRop3 Rh6 AAAB 01008987 gi|19612317:15549903-15551016,GPRop4 Rh6 AAAB01008987 gi|19612317:1554 8608- 15547465,GPRop5 Rh6 AAAB01008987gi|19612317:15552323- 1555 3525,GPRop6 Rh6 AAAB01008987 gi|196123 17: 15555428 - 15556641), *Ae. aegypti* Ops1 and Ops2 sequences are published in Graf et al. [6].

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CpNYD-OP7    FVYWLPLLTIIYSYTFILKAVSAHEEQMREQAKKMNVASLRSSEAQQTSAEIKLAKVALV  278
AaOps1       FVYWTPLLTIIYSYTFILKAVSAHEAQMREQAKKMNVASLRSSEANQTSAEIKLAKVALV  278
AaOps2       FVYWTPLLTIIYSYTFILKAVSAHEKNMREQAKKMNVGSLRSSEAQQTSAEIKLAKVALV  276
AgGPRop2     FVYWLPLLTIIYSYTFILKAVSAHEKNMREQAKKMNVASLRTQEAQNTSTEMKLAKVALV  278
AgGPRop4     FVYWLPLLTIIYSYTFILKAVSAHEKNMREQAKKMNVASLRTQEAQNTSTEMKLAKVALV  289
AgGPRop3     FVYWLPLLTIIYSYTFILKAVSAHEKNMREQAKKMNVASLRTQEAQNTSTEMKLAKVALV  278
AgGPRop1     FVYWLPLLTIIYSYTFILKAVSAHEKNMREQAKKMNVASLRTQEAQNTSTEMKLAKVALV  278
AgGPRop6     FVYYLPLFSIIYSYIYIIQAVSAHEKNMREQAKKMNVASLRSQEAQNTSTEMKLAKVALV  300
AgGPRop5     FVYWLPLLLIIYCYTYILKAVSAHERNMREQAKKMNVASLRSSDASKTNAEIKLAKVALV  286
             ***: **: ***.* :*::****** :**********.***:.:*.:*.:*:********


CpNYD-OP7    TISLWFMAWTPYLVINFTGIFKAAPISPLATIWGSLFAKANAVYNPIVYGISHPKYRAAL  338
AaOps1       TISLWFMAWTPYLVINFTGIFKAAPISPLATIWGSLFARANAVYNPIVYGISHPKYRAAL  338
AaOps2       TISLWFMAWTPYLVINFTGIFKAAPISPLATIWGSLFPRANAVYNPIVYGISHPKYRAAL  336
AgGPRop2     TISLWFMAWTPYLVINFTGIFKAAPISPLATIWGSLFAKANAVYNPIVYGISHPKYRAAL  338
AgGPRop4     TISLWFMAWTPYLVINFTGIFKAAPISPLATIWGSLFAKANAVYNPIVYGISHPKYRAAL  349
AgGPRop3     TISLWFMAWTPYLVINFTGIFKAAPISPLATIWGSLFAKANAVYNPIVYGISHPKYRAAL  338
AgGPRop1     TISLWFMAWTPYLVINFTGIFKAAPISPLATIWGSLFAKANAVYNPIVYGISHPKYRAAL  338
AgGPRop6     TISLWFMAWTPYLVINYTGIFKAAPISPLATIWGSLFAKANAVYNPIVYGISHPKYRAAL  360
AgGPRop5     TITLWFMAWTPYLVINYAGIIDSSPISPLQTIWGSVFAKANAVYNPIVYGISHPKYRAAL  346
             **:**************::**:.::***** *****:*.:*********************


CpNYD-OP7    YQRYPGLSCQ-SDNSPN--DTQSVASGATTASDEKA--  371
AaOps1       YQRHPWLSCQDAQESSH--DNQSTVSGATTATEEKA--  372
AaOps2       TQKFPALSCTDAPAASNSDDNQSTVSGATTATDEKA--  372
AgGPRop2     YQKFPSLSCQDAPVDDG---QSVASGATQASDEKA--  370
AgGPRop4     YQKFPSLSCQDAPVDDG---QSVASGATQASDEKA--  381
AgGPRop3     YQKFPSLSCQDAPVDDG---QSVASGATQASDEKK--  370
AgGPRop1     YQKFPSLSCQDAPVDDG---QSVASGATQASDEKA--  370
AgGPRop6     YQKFPSLSCQDNSDDGQ---SVASAATGVSEEKPAA  393
AgGPRop5     YKRFPALQLRKENATDQ--NSVASCTTAADQCET--  378
             ::.* *.              *..* :*. : :
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Fig. 2 (*continued*)

strains and it appeared that *NYD-OP7* mRNA levels are significantly lower in *Cx. pipiens pallens* embryos and larvae than in older developmental stages. Whether the expression pattern reflects a function for *NYD-OP7* in eye development remains to be seen.

In previous study, we identified 16 genes that were differentially transcribed among the deltamethrin-susceptible and -resistant strains of *Cx. pipiens pallens* and as shown by microarray, one *opsin* gene is 3.06-fold overexpressed in the resistant strain [15]. In this paper, we also found that in pupae and adults, *NYD-OP7* is overexpressed in resistant strain than in susceptible strain, which shows that *NYD-OP7* may play a role in the deltamethrin resistance phenotype. However, it remains possible that *NYD-OP7* over-expression is an indirect consequence of acquired deltamethrin resistance. To determine whether *NYD-OP7* directly participates in the establishment of the deltamethrin resistance phenotype, we transfected this gene into the mosquito C6/36 cells and to determine if over-expression of *NYD-OP7* can change the deltamethrin sensitivity in C6/36 cells. The results showed that *NYD-OP7* transfected cells are relatively resistant to deltamethrin at low drug concen-

trations which suggests that *NYD-OP7* is directly involved in the development of deltamethrin resistance.

Previously, by using a small-scale microarray representing all *Drosophila* P450 genes, Daborn et al. and Le Goff et al. suggested that resistance to DDT in the field is monogenic and is due to the over-expression of a single P450 gene, *Cyp6g1* [10,32]. However, by using a detox chip and a genome-wide microarray of *An. gambiae*, David et al. and Vontas et al. found that many genes that belong to families not usually associated with insecticide resistance (such as peptidases, sodium/calcium exchangers and genes implicated in lipid and carbohydrate metabolism) were over-transcribed in the resistant strain [13,14]. Meanwhile, by using genome-wide microarray, Pedra et al. demonstrated that DDT-metabolic resistance in *Drosophila* is associated with over-expression of detoxification related genes but also over-expression of ion transport, signal transduction, RNA transcription, and lipid and sugar metabolism pathways[12]. Interestingly, they also reported that *Drosophila* UV-sensitive opsin (Rh3 and Rh4) and Blue-sensitive opsin (NinaE) were overexpressed in the DDT-resistant strain, suggesting that *opsin* gene may play some role in DDT-resistance [12].
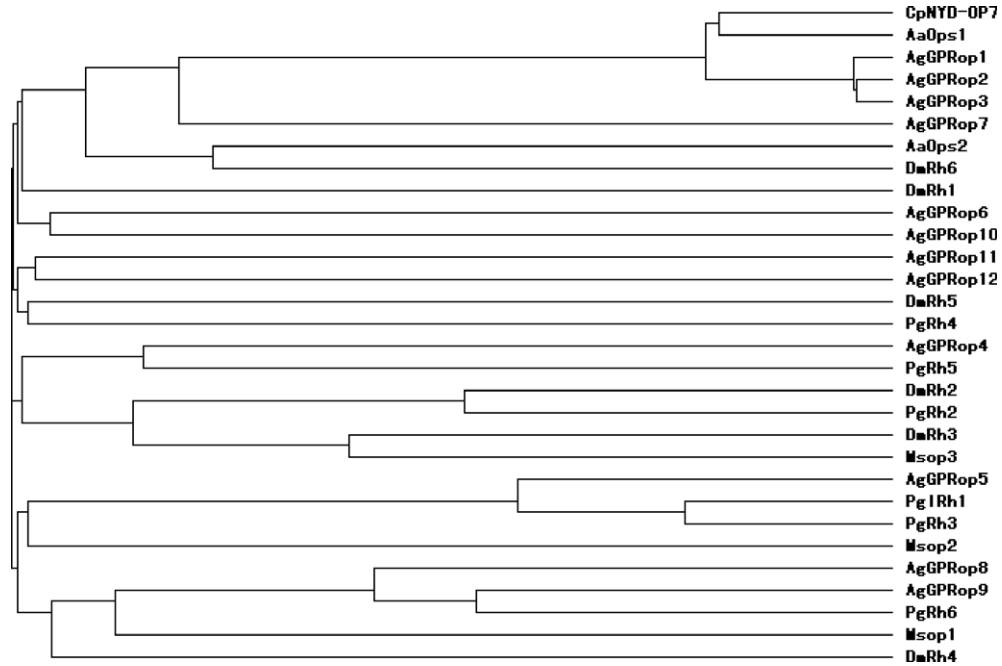
Fig. 3. Phylogenetic relationships between NYD-OP7 and some other known insect opsins. *Abbreviations* and GenBank Accession Nos.: *An. gambiae* (Scaffold Nos., GPRop7 Rh6 AAAB01008859 gi|19611897:9719568 -97 18344,GPRop8 Rh3, Rh4 AAAB01008960 gi|19612143: 3070327-13075141, GPRop9 Rh5 AAAB 01008980 gi|19612245: 12661961- 12660707,GPRop10 Rh7 AAAB01008807 gi|19611 723: 1869873 -1884336,GPRop11 - AAAB 01008859 gi| 19611897: 10064831-10075 16 9,GPRop12 -AAAB0100 8859 gi|19611897:10056116- 10044185); *D. melanogaster* (DmRh1, P06002; DmRh2, P08099; DmRh3,P04950; DmRh4,P08255; DmRh5, P91657; DmRh6, O01668); *M. sexta* (Manop1, L78080; Manop2, L78081; Manop3, AD001674); *P. glaucus* (PglRh1, AF077189; PglRh2, AF077190; PglRh3, AF067080; PglRh4, AF077193;PglRh5, AF077191; PglRh6, AF077192).
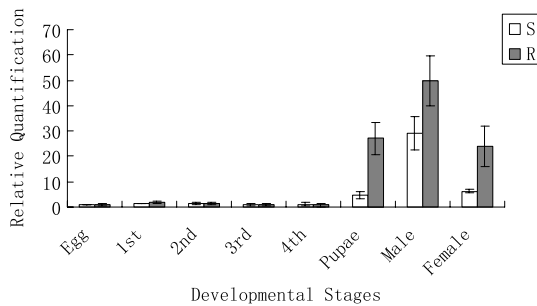
Fig. 4. Real-Time PCR analysis of the expression of *NYD/OP7* transcripts at different developmental stages of *Cx. pipiens pallens*. Results are normalized against the internal control *Cx. pipiens pallens* actin. The expression level of *NYD-OP7* in susceptible strain egg was considered as background level, or 1. S, deltamethrin-susceptible strain; R, deltamethrin-resistant strain.

Fig. 5. RT-PCR analysis of *NYD/OP7* over-expression in C6/36 cells after stable transfection. Total RNAs (2.0 μg) from vector control (C6/36$_{pIE}$) and *NYD/OP7*-overexpressing clone (C6/36$_{OP7}$) were analyzed by RT-PCR with primers for exogenous *NYD/OP7* (consisting of a *NYD/OP7*-specific upstream and vector-specific downstream primers) and β-actin was used as an internal control. The products were 700 and 911 bp, respectively.

In the case of *NYD-OP7*, we hypothesized that as one member of G-protein coupled receptor family, NYD-OP7 may contribute to the phenotype of insecticide resistance by activating downstream G-protein and signal transduction pathway. It is also possible that the deltamethrin-resistant phenotype requires the coordinated expression of many different genes so that over-expression of individual gene is necessary, but maybe not sufficient, to generate a high resistance phenotype. It should also be mentioned that because of lack of a proper antibody, we cannot do western blot to confirm the over-expression of *NYD-OP7* in stably transfected cells at the protein level. So there is a possibility that the expression of *NYD-OP7* at the protein level is not
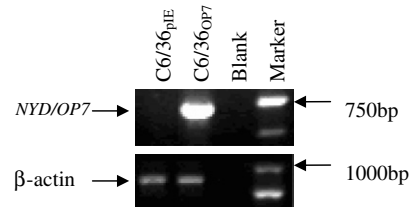
as high as the mRNA level which may be the reason for why high *NYD-OP7* transcriptional level expression only causes a modest increase in resistance. In a recent study, we have proved that the cotransfection of two genes (NYD-Tr and NYD-Ch) into C6/36 cells can induce deltamethrin resistance [33]. All these data suggest that insecticide resistance is more complex than we considered and we believe that further investigation should be done to fully elucidate the role that *NYD-OP7* has in insecticide resistance.

In summary, we have reported the cloning and identification of a novel *opsin* gene (*NYD-OP7*) from *Cx. pipiens pallens*. Based on the expression profiles and previous SSH and microarray results, this gene appears to have some role in deltamethrin resistance. Over-expression of *NYD-OP7* in mosquito C6/36 cells can confer moderate resistance to
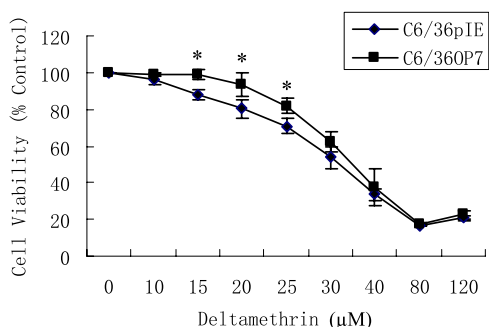
Fig. 6. Over-expression of *NYD/OP7* confers deltamethrin resistance in C6/36 cells. The relative cytotoxicity of deltamethrin in vector control clone (C6/36$_{pIE}$) and *NYD/OP7* overexpressing clone (C6/36$_{OP7}$) were assessed using the [^{3}H]-TdR assay. *$p < 0.05$ compared with vector control clone.

deltamethrin, suggesting that *NYD-OP7* directly participates in the development of deltamethrin resistance and the precise definition of its role awaits further investigation.

Acknowledgments

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