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Cloning and characterization of 60S ribosomal protein L22 (*RPL22*) from *Culex pipiens pallens*

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ABSTRACT

The 60S ribosomal protein L22 (GenBank accession no. EF990190) was cloned from *Culex pipiens pallens*. An open reading frame (ORF) of 447 bps was found to encode a putative 148 amino acids protein which shares 90% and 80% identity with *RPL22* genes from *Aedes aegypti* and *Anopheles gambiae* respectively. Real-time quantitative PCR analysis demonstrated that the transcription level of *RPL22* in deltamethrin-resistant strain was 2.57 folds higher than in deltamethrin-susceptible strain of *Cx. pipiens pallens*. Overexpression of *RPL22* in C6/36 cells showed that the deltamethrin-resistance was decreased in C6/36-RPL22 cell compared to the control. The mRNA level of cytochrome P450 6A1 (*CYP6A1*, GenBank accession no. FJ423553) showed that *CYP6A1* was down-regulated in the C6/36 transfected with *RPL22* (C6/36-RPL22) cells, suggesting that *CYP6A1* was repressed by RPL22. Our study provides the first evidence that *RPL22* may play some role in the regulation of deltamethrin-resistance in *Cx. pipiens pallens*.

1. Introduction

Many new and reemerging mosquito-borne diseases threaten the public health, such as malaria, dengue fever, yellow fever, filariasis, West Nile fever, LaCrosse Encephalitis, Western Equine Encephalitis, Eastern Equine Encephalitis, and St. Louis Encephalitis. Chemical control is a major method to manage the mosquito-borne diseases. Pyrethroid is a kind of synthetic insecticides which kills insects by strongly exciting their nervous system, a similar mode of action to DDT. Deltamethrin is a fourth generation synthetic pyrethroid pesticide which is commonly used for the impregnation of bed nets and indoor residual spray to help control the transmission of insect-borne diseases. The low toxicity of deltamethrin to mammals and birds and their limited soil persistence has encouraged the wide-spread and increasing use of deltamethrin. But under the natural selection, excessive and continuous application of insecticides has caused the development of insecticide resistance, which has become the major obstacle to controlling the insect-borne diseases. Consequently, the mosquito-borne diseases are now resurgent.

Insecticide resistance is a phenomenon of polygenic inheritance. A number of insecticide resistance associated genes such as cytochrome P450, esterases, GST, knockdown resistance (*Kdr*), and so on, had been identified. To investigate the deltamethrin resistance in *Cx. pipiens pallens*, we had employed suppression subtractive hybridization (SSH) and cDNA microarray to identify differentially expressed genes between deltamethrin-susceptible and -resistant strains of *Cx. pipiens pallens* in our previous experiments, and 13 deltamethrin resistance-related genes were isolated (Wu et al., 2004). One of the highly expressed genes in deltamethrin-resistant strain (DR-strain) was a *RPL22*-homolog.

RPL22 is located in 60S ribosomal subunit, and constitutes part of the peptide exit tunnel. RPL22 and RPL4 form a constriction that results in the narrowest passage in the tunnel. This exit tunnel interacts with nascent translation products and functions as a discriminating gate and may control the nascent chain elongation (Nakatogawa and Ito, 2002; Berisio et al., 2003). But the correlation between the function of RPL22 and insecticide resistance has not been reported.

To elucidate whether RPL22 is involved in deltamethrin resistance, we quantified expression difference between DR-strain and deltamethrin-susceptible strain (DS-strain) of *Cx. pipiens pallens* by real-time quantitative PCR. In addition, we cloned full-length *RPL22* from *Cx.*

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pipiens pallens, and constructed the expression plasmid of *RPL22*, stably transfected C6/36 cells with this *RPL22*-expressing construct. Viability of C6/36-RPL22 cells in the presence of deltamethrin was compared with control cells to observe variance of deltamethrin resistance of these cells. Because *CYP6A1* is an important insecticide resistant gene (Andersen et al., 1994), we also analyzed the mRNA levels of *CYP6A1* between the C6/36-RPL22 cells and the control when RPL22 was overexpressed.

2. Materials and methods

2.1. Mosquito strains

DS-strain and DR-strain of *Cx. pipiens pallens* were obtained from the Shanghai Insect Institute of the Chinese Academy of Sciences and was maintained in our laboratory which was reared at 28 °C with 70–80% humidity and a constant light/dark cycle (14 h:10 h). DR-strain was selected with deltamethrin from DS-strain for more than 10 generations to reach a 400 fold resistance (Li et al., 2002).

2.2. RNA extraction and cDNA synthesis

Total RNA were extracted from 4th instar larvae 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.

2.3. Cloning and sequencing

To clone the full length of *RPL22*, rapid amplification of 3′ cDNA ends (3′-RACE) and rapid amplification of 5′ cDNA ends (5′-RACE) were carried out with the BD SMART™ RACE cDNA Amplification Kit. The specific primers of 3′-RACE and 5′-RACE were designed based on the EST sequence reported previously (GenBank accession No. BE247832). The specific primer sequences of 3′-RACE and 5′-RACE were 5′-GAAGAAGGTCGCCGCTGTCAAAGTG-3′ and 5′-CGACCGCAGGTGCTCCTTCTTTTT-3′ respectively. The sequence of 3′-RACE and 5′-RACE adaptor primers supplied by the BD SMART™ RACE cDNA Amplification Kit were 5′-CTGATCTAGAGGTACCGGATCC-3′ and 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ respectively. The PCR products were separated by 1% agarose gel electrophoresis and purified using a QIA quick Gel extraction kit (Qiagen, Germany). Products were then ligated into the pGEM-T easy vector (Promega, USA) overnight at 16 °C, and the ligation products were transformed into *E. coli* DH5α competent cells and cultured in the LB plate containing ampicillin, IPTG and X-Gal. White colonies were selected and confirmed by PCR. Plasmids DNA were extracted using a plasmid mini kit (Qiagen) and sequenced at Shanghai Invitrogen Biotechnology Co., Ltd. (Shanghai, China). Then the sequences of above 2 fragments were assembled to generate a putative full-length cDNA of *RPL22*.

After the full length sequence was obtained, a pair of primers was designed as follows: forward primer: 5′-ATGGTTGAGAAGAAGGT-3′, reverse primer: 5′-TTACTCGGCATCGTCTTC-3′ used to amplify the ORF of *RPL22*.

2.4. Sequence alignment and phylogenetic tree

The standard protein-/protein BLAST sequence comparison programs (http://beta.uniprot.org/?tab=blast) were used to search for sequences in the SWISSPROT databases with similarities to the translated sequences of *RPL22*. Deduced amino acid sequences were aligned using the ClustalW2 computer program (http://www.ebi.ac.uk/Tools/clustalw2/ index.html). The phylogenic tree was constructed by the neighbor-joining method using the MEGA 3.1 program. The sequences of RPL22 included in our analysis were from *Culex quinquefasciatus, Aedes aegypti* (Nene et al., 2007), *Anopheles gambiae* (Holt et al., 2002), *Drosophila melanogaster* (Koyama et al., 1999), *Drosophila ananassae* (Clark et al., 2007), *Cotesia congregate* (Bezier et al., 2008), *Xenopus tropicalis* (Klein et al., 2002), *Spodoptera frugiperda* (Landais et al., 2003), and *Bombyx mori*.

2.5. Real-time quantitative PCR analysis

Real-time quantitative PCR was performed on the ABI PRISM 7300 (Applied Biosystems, USA) using *Power* SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's protocol. The PCR mixture contained 10 μL 2× *Power* SYBR Green PCR Master Mix, 0.8 μL 10 μM forward and reverse primer respectively, 4 μL cDNA, and 4.4 μL ddH_2O. The sequences of forward and reverse primer for RPL22 were 5′-CAACTCGGTCTCTTTCGAG-3′ and 5′-ATCGTCTTCCTCA-TCATCGT-3′ respectively, and the product size was 205 bps. Another pair of primers was used for β-actin: 5′-AGCGTGAACTGACGGCTCTTG-3′ and 5′-ACTCGTCGTACTCCTGCTTGG-3′ with a product size of 153 bps, and β-actin was used as an internal RNA control. For amplification, the following program was employed: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min. A melting curve program was run immediately after the PCR program and the data were analyzed with 7300 System SDS Software v1.2.1 (Applied Biosystems). The threshold cycle (Ct) values were used to quantify the target gene expression for each sample, and the relative expression levels of *RPL22* in DR-strain and DS-strain of *Cx. pipiens pallens* were calculated using Relative Expression Software Tool 2008 (REST) (Pfaffl et al., 2002). The expression level of *RPL22* in DS-strain was considered as background level, or 1. To verify reproducibility, the real-time quantitative PCR analysis was performed 3 times using independent purified RNA samples with three replicates for each sample.

2.6. Construction of the eukaryotic expression plasmid

The ORF of RPL22 was amplified using a pair of specific primers: forward primer: 5′-GG***GGTACC***ATCATGGTTGAGAAGAAGGT-3′, reverse primer: 5′-GG***ACTAGT***TTAATGGTGATGGTGATGATGCTCGGCATCGT-CTTC-3′. ATC was added before ATGG to formed Kozak sequence in the forward primer (Kozak, 1986; Sano et al., 2002), and for the later Western blotting identification, we added ATGGTGATGGTGATGATG (6× His) in the reverse primer. The stop codon TAA was removed to the downstream of 6× His. At the same time, to clone the ORF fragment into the pIB/V5-His expression vector, the forward primer had a *Kpn* I recognition site (GGTACC), and the reverse primer also had a *Spe* I recognition site (ACTAGT). The PCR product and the pIB/V5-His expression vector (Invitrogen, USA) were all digested by *Kpn* I and *Spe* I, then the two objective bands were purified with QIA quick Gel extraction kit (Qiagen) and ligated with T4 DNA ligase (NEB, USA). After transformed the *E. coli* DH5α competent cells and PCR identification with OpIE2 forward and OpIE2 reverse sequencing primers supplied by the InsectSelect™ BSD System kit, extracted the plasmid DNA and sequenced.

2.7. 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 Eagle's minimum essential medium (EMEM, Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Sijiqing, China), 100 IU/mL penicillin, and 100 μg/mL streptomycin (P/S, Invitrogen). The cells were grown in a 5% CO_2-humidified incubator at 28 °C and were plated in a six-well plate. Transfection was performed using FuGENE® HD transfection reagent (Roche, USA) according to the

```
1       GCTTTTCACGTTTACCGTCAGGCTAGTGATATCCTGTTTGTGCAAATCGCTGGTGAAAAC
61      CAAGCTTTTCTAGCAGGATTTCAACACACACATCATGGTTGAGAAGAAGGTCGCCGCTGT
                                          M  V  E  K  K  V  A  A  V

121     CAAAGTGAAGCCCACCCCAGCTAATGGCGGAAAGGGCCAGAAGAAGCAGCTGCTCCGGGG
41        K  V  K  P  T  P  A  N  G  G  K  G  Q  K  K  Q  L  L  R  G

181     CAAGAATGTCCAGAAGAAAAAGAAGGAGCACCTGCGGTTCGGTGTCGACTGCACCAACAT
61        K  N  V  Q  K  K  K  K  E  H  L  R  F  G  V  D  C  T  N  I

241     CGCCGAGGACAACATTATGGATGTTGCCGACTTCGAGAAGTACCTGAAGGAGCGCTTCAA
81        A  E  D  N  I  M  D  V  A  D  F  E  K  Y  L  K  E  R  F  K

301     GGTTAACGGCAAGATCGGAAACCTCGGCAACTCGGTCTCTTTCGAGCGCCAGAAGATGAA
101       V  N  G  K  I  G  N  L  G  N  S  V  S  F  E  R  Q  K  M  K

361     GGTGTACGTCAACTCCGACGTTCACTACTCGAAGCGTTACCTCAAGTACTTGACCAAGAA
121       V  Y  V  N  S  D  V  H  Y  S  K  R  Y  L  K  Y  L  T  K  K

421     GTATCTGAAGAAGAACAGCCTGCGCGACTGGATCCGTGTTGTGTCCAACGACAAAGATCT
141       Y  L  K  K  N  S  L  R  D  W  I  R  V  V  S  N  D  K  D  L

481     GTACGAGCTGCGCTACTTCCGAATCAGCTCCAACGATGATGAGGAAGACGATGCCGAGTA
161       Y  E  L  R  Y  F  R  I  S  S  N  D  D  E  E  D  D  A  E  *

541     AACTCTGTTGTTTTATGATTCCCGGGAATGTGTACACGTCTGTGACGGTCGGAAGTGGTG
601     AATAAATGTCCAAGGTGGCCACATAGCCAAGAGGTCGCTTTGGAAAAACGGAAAAAAAAA
661     AAAAAAAAAAAAA
```

Fig. 1. The nucleotide and deduced amino acid sequences of *Cx. pipiens pallens RPL22*. The deduced amino acid sequence is presented below the nucleotide sequence in single letter code. The tailing signal sequence "AATAAA" and poly(A) in the 3′-untranslated region are in bold italic letters. The initial code "ATG" and the termination codon "TAA" are underlined. The asterisk denotes the stop codon. GenBank Accession No. EF990190.

manufacturer's protocol. Briefly, 5×10^5 cells/well were plated in 2 mL of complete growth medium in a six-well plate overnight, and achieved the desired density of >80% confluency at the time of transfection. For each well, dilute plasmid DNA with sterile water was added to a concentration of 2 μg plasmid DNA/100 μL (0.02 μg/μL). After placing 100 μL diluted plasmid DNA into sterile tube, the FuGENE® HD transfection reagent (6 μL) was pipeted directly into the tube and vigorously tapped for 2 s to mix the contents. The transfection reagent: DNA complex was incubated for 15 min at room temperature and then the transfection complex was added to the cells below the surface of the medium and swirled to ensure distribution over the entire plate surface. A kill curve was performed to test the cell line for sensitivity to 20 μg/mL blasticidin, which can kill cells within a week. 48 h post-transfection, the cells were selected with 20 μg/mL blasticidin (Invitrogen) for a week and the medium was changed every 3–4 days. 8 days later, the medium was replaced with medium containing 10 μg/mL blasticidin. The stable C6/36-*RPL22* cells were characterized by reverse transcription PCR (RT-PCR) and Western blotting. At the same time, stable cell line transfected with vector only pIB/V5-His (Invitrogen, USA) as control was established as described above.

2.8. RT-PCR analysis of RPL22 in the stable transfection cells

Total RNA was extracted from transfected cells using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was detected by Biophotometer (Eppendorf, Germany), and 2 μg total RNA was used in each RT-PCR. cDNA synthesized with M-MLV reverse transcriptase (Promega, USA) and random oligonucleotide primers according to the manufacturer's protocol was subsequently subjected to PCR. The *RPL22* specific primers were: 5′-GGGGTACCATCATGGTTGAGAAGAAGGT-3′ and 5′-GGACTAGTTTAATG-GTGATGGTGATGATGCTCGGCATCGTCTTC-3′ generating a product of 487 bp. The vector specific primers were: 5′-CGCAACGATCTGGTA-AACAC-3′ and 5′-GACAATACAAACTAAGATTTAGTCAG-3′ generating a fragment of 252 bp from C6/36-pIB/V5-his and a fragment of 729 bp from C6/36-RPL22. To confirm equal loading, PCR amplification of the β-actin gene of C6/36 cell was also done in parallel. The primers for β-actin were: 5′-CCACCATGTACCCAGGAATC-3′ and 5′-CACCGATCCA-GACGGAGTAT-3′ generating a product of 120 bp. Products were separated on 1% agarose gel and photographed under UV light.

2.9. Western blotting

Protein was extracted from stable transfected cells by RIPA lysis buffer (Beyotime, China) according to the manufacturer's instructions and concentrations were determined by BCA Protein Assay kit (Pierce, USA). 40 μg of protein per lane was loaded in 15% SDS PAGE gel. The SDS PAGE electrophoresis was run for 30 min at 80 V and 80 min at 100 V. Then the proteins were transferred to a PVDF membrane for 30 min at 10 mA with Trans-Blot SD Cell and Systems (Bio-Rad, USA). The fusion protein was detected using His·Tag® Monoclonal Antibody anti-His antibody (1:500, NovaGen, USA) and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000, Beyotime, China). Detection was done with the BeyoECL Plus (Beyotime, China) according to the manufacturer's instructions.

2.10. Cytotoxicity assay

Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was used to determine the deltamethrin resistance of stable C6/36 transfectants under deltamethrin treatments. Cell suspension (100 μL) was distributed (5000 cells/well) in a 96-well plate and plates were pre-incubated for 24 h in a 5% CO_2-humidified incubator at 28 °C. Wells were then treated with 100 μL of various concentrations of deltamethrin (final concentrations: 0, $10^{0.5}$ μg/mL, $10^{1.0}$ μg/mL, $10^{1.5}$ μg/mL, $10^{2.0}$ μg/mL, and $10^{2.5}$ μg/mL) . After another 72 h, 10 μL of CCK-8 solution were added to each well. Plates were incubated for 4 h in the incubator, then the absorbance was measured at 450 nm using a microplate reader. Deltamethrin was dissolved in DMSO (Sigma, USA) and the wells of various concentrations of deltamethrin had same final concentration of DMSO. Three independent experiments were done.

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Culex pipiens pallens                 ----------------------------------------------------------------------------------------------------
Culex quinquefasciatus                ----------------------------------------------------------------------------------------------------
Aedes aegypti                         ----------------------------------------------------------------------------------------------------
Anopheles gambiae                     ----------------------------------------------------------------------------------------------------
Drosophila melanogaster               MAPTAKTNKGDTKTAAAKPAEKKAAPAAAAAKGKVEKPKAEAAKPAAAAAKNVKKASEAAKDVKAAAAAAKPAAAKPAAAKPAAASKDAGKKAP-AAAAPKKDAKAAAAPAPAKAAPAKK 119
Drosophila ananassae                  MAPTAKTNKGDTK-AAAKPAEKKAAP-AAAAKGKVEKPKAEAAKPAAAAAKNVKKAPEAAKDVK-AAAAAKPAAAKPAAAKPAPA-KDAGKKAPAAAAAPKKDAKAAAP--AKAAAPAKK 114
Cotesia congregata                    ----------------------------------------------------------------------------------------------------
Xenopus tropicalis                    ----------------------------------------------------------------------------------------------------
Spodoptera frugiperda                 ----------------------------------------------------------------------------------------------------
Bombyx mori                           ----------------------------------------------------------------------------------------------------

Culex pipiens pallens                 ----------------------------------MVEKKVAAVKVKP-TPANGGKGQKKQLLRGKNVQKKKKEHLRFGVDCTNIAEDNIMDVADFEKYLKERFKVNGKIGNLG-NSVS 82
Culex quinquefasciatus                ----------------------------------MVEKKVAAVKVKP-TPANGGKGQKKQLLRGKNVQKKKKEHLRFGVDCTNIAEDNIMDVADFEKYLKERFKVNGKIGNLG-NSVS 82
Aedes aegypti                         ----------------------------------MAPVVKAVASVKTKPGKDAKGGKQTKKQLLRGKNAQKKKKEHLRFGVDCTNIAEDNIMDVADFEKYLKERFKVNGKTGNLG-NSVS 85
Anopheles gambiae                     ----------------------------------MSPVEKSVNAVKTKP-----AAQKPAQKNVLRGKNPIKKKREHLRYGIDCTNIAEDNIMDVADFEKYLKERFKVNGKAGNLG-SNVS 81
Drosophila melanogaster               AASTPAAAPPAKKAAP-AKAAAP-AAAAPAP-AAAAPAVAKP-APKPKAKAAPAPSKVVKKNVLRGKG-QKKKKVSLRFTIDCTNIAEDSIMDVADFEKYIKARLKVNGKVNNLG-NNVT 233
Drosophila ananassae                  AASTPAAAPPAKKAAPAAKAAAPAAAAAPAPAAAAAPAAAKPAAPKPAKAAPAPSKVVKKNVLRGKG-QKKKKVSLRFTIDCTNIAEDSIMDVADFEKYVKARLKVNGKVNNLG-NNVT 232
Cotesia congregata                    ----------------------------------MAPQAKISTKKPVP-----KKTGAPKKNQLRGKA--QKKKVSLSFTIDCTHPVEDNIMDVASFDKYMKERIKINGKTGNLG-NLVN 78
Xenopus tropicalis                    ----------------------------------------------------MAPVKKTVTKGSK---KKKQLLKFTLDCTHPVEDGIMDAANFEQFLHDRIKVNGKVGNLGGGVVS 81
Spodoptera frugiperda                 ----------------------------------MAVAKKPVAKKAQLHQK-SGKKGVKGGKIRGKG--IKRKINLKFMIDCTHPAEDSILDVGNFEKYLKERVKVEGKTNNLG-NHVV 81
Bombyx mori                           ----------------------------------MAVTKKPVAKKAQLHQK-TGKKGVKGGKIRGKG--IKRKINLKFTIDCTHPAEDSILDVGNFEKYLKEHVKVEGKTNNLS-NHVV 81
                                                                                                                :    :*.    *:: * : :***: .**.*:*...*::::: :.*::** .**. . *

Culex pipiens pallens                 FERQKMKVYVNSDVHYSKRYLKYLTKKYLKKNSLRDWIRVVSNDKDLYELRYFRISSN-DDEEDDAE 148
Culex quinquefasciatus                FERQKMKVYVNSDVHYSKRYLKYLTKKYLKKNSLRDWIRVVSNDKDLYELRYFRISSN-DDEEDDAE 148
Aedes aegypti                         FERQKMKVYVNSDVQYSKRYLKYLTKKYLKKNSLRDWIRVVSNDKDLYELRYFRISSN-DDEEEDNE 151
Anopheles gambiae                     FERQKMKVYVNSDVHFSKRYLKYLTRKYLKKNSLRDWIRVVSNDKDLYELRYFRISSN-DDDEEENE 147
Drosophila melanogaster               FERSKLKLIVSSDVHFSKAYLKYLTKKYLKKNSLRDWIRVVANEKDSYELRYFRISSN-DDEDDDAE 299
Drosophila ananassae                  FERSKLKLHVSSDVHFSKAYLKYLTKKYLKKNSLRDWIRVVANEKDSYELRYFRISSN-DDEDDDAE 298
Cotesia congregata                    IERNKNKLIINSDTELSKRYLKYLTKKFLKKHKLRDWLRVVSKDKNTYELRYFQITSQEDDDEEDAD 145
Xenopus tropicalis                    IERSKSKITVSSEVPFSKRYLKYLTKKYLKKNNLRDWLRVVANSKESYELRYFQINQD-EEEEEDED 128
Spodoptera frugiperda                 IARDKTKISINADIPFSKRYLKYLTKRYLKKNNLRDWLRVVASAHDSYELRYFNINAD-SDNEDNED 147
Bombyx mori                           VARDKTKVAITADIPFSKRYLKYLTKRYLKKNNLRDWLRVVASAHDAYELRYFNINAD-SDNEDNED 147
                                      . *.* *: :.::   ** ******:::***:.****:***:. :: ******.*. : .::::: :
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Fig. 2. Amino acid sequence alignment of *Cx. pipiens pallens* RPL22 and other species of RPL22. Asterisks indicate identical amino acid and dots indicate similar amino acids. The conserved domains on RPL22 are in italic letters.

2.11. Detection of CYP6A1 expression by real-time quantitative PCR analysis

As described above, real-time quantitative PCR was also performed to detect the various level of *CYP6A1* mRNA between C6/36-RPL22 and C6/36-pIB/V5-his cells (as control). The sequences of forward and reverse primer for *CYP6A1* were 5′-GGCCTCCAGCAGCATTCAT-3′ and 5′-TCACGATGCATGGACCAGAT-3′ respectively. The sequences of β-actin gene of C6/36 cell as an internal RNA control were: 5′-CCACCATGTACCCAGGAATC-3′ and 5′-CACCGATCCAGACGGAGTAT-3′. The expression level of *CYP6A1* in control was considered as background level, or 1. To verify reproducibility, the real-time quantitative PCR analysis was repeated twice using independent purified RNA samples with three replicates for each sample.

2.12. Statistics

The real-time PCR data was analyzed by hypothesis test. Other data were analyzed by Student's *t* test. A value of $p<0.05$ was considered significant.

3. Results

3.1. Cloning the full length RPL22 gene from Cx. pipiens pallens

The full length of *RPL22* was amplified *Cx. pipiens pallens* by RT-PCR with 3′-RACE and 5′-RACE. One fragment of 571 bps was obtained from 3′-RACE and another 278 bps from 5′RACE. They were assembled with the

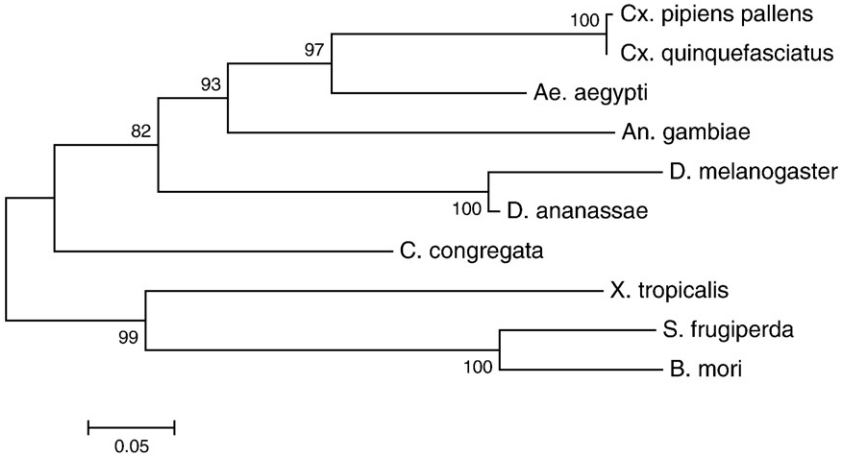

Fig. 3. Phylogenetic relationships of RPL22 among *Cx. pipiens pallens* and some other species. Species name and GenBank Accession No.: *Culex pipiens pallens*: EF990190; *Culex quinquefasciatus*: XM_001846404; *Aedes aegypti*: XM_001658595; *Anopheles gambiae*: XM_558423; *Drosophila melanogaster*: AF080131; *Drosophila ananassae*: XM_001966493; *Cotesia congregata*: AM492671; *Xenopus tropicalis*: BC159391; *Spodoptera frugiperda*: AF400188; *Bombyx mori*: AY769291.

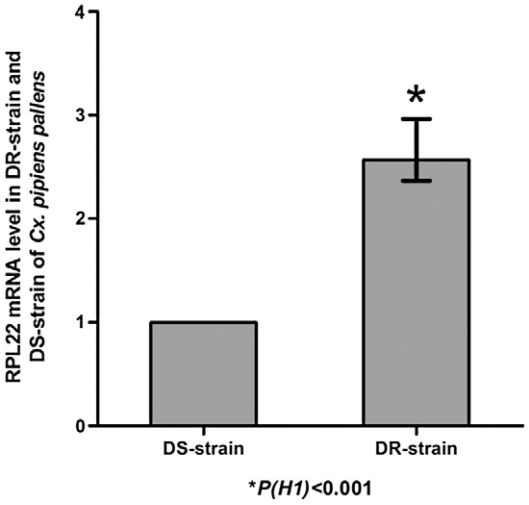

Fig. 4. mRNA level of *RPL22* in DR-strain and DS-strain of *Cx. pipiens pallens*. Results are expressed as mean ± standard error. The relative expression of *RPL22* in DS-strain was considered as background level or 1. *$p<0.001$ compared with DS. DS-strain: deltamethrin-susceptible strain; DR-strain: deltamethrin-resistant strain.

EST fragment and *RPL22* was finally identified with 673 bps (GenBank accession no. EF990190). The ORF of *RPL22* is 447 bps and encodes a 148 amino acids protein. Start codon ATG is found to be nucleotides 95–97 of the gene and an in-frame stop codon TAA is 539–541 with tailing signal sequence "AATAAA" and poly(A) present at the 3′-untranslated region, indicating the sequence is the full length of *RPL22* mRNA (Fig. 1).

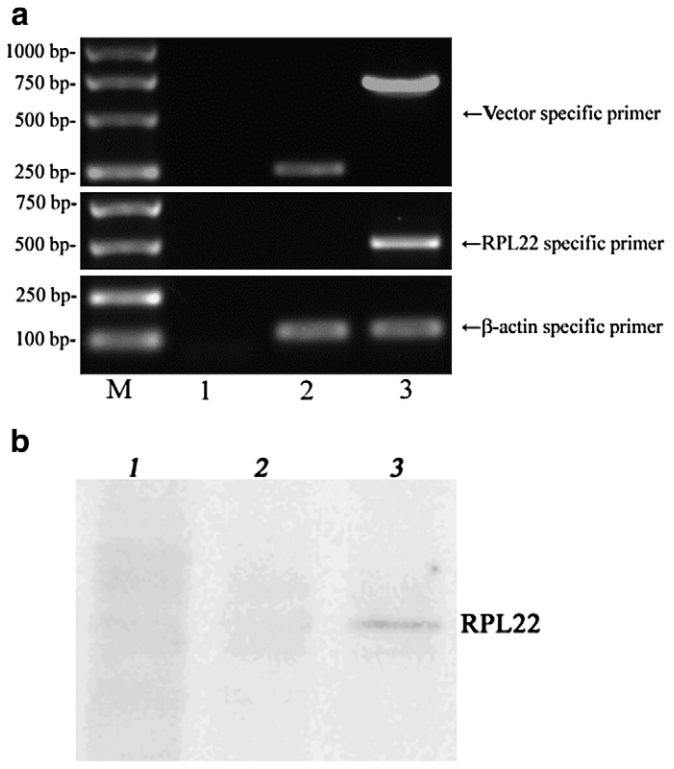

Fig. 5. a. Analysis of transcription and expression of transfected cells. (M) Marker; (1) No template control; (2) C6/36-pIB/V5-His (vector control); (3) C6/36-RPL22. Total RNAs (2.0 μg) from C6/36-pIB/V5-His and C6/36-RPL22 were analyzed by RT-PCR with primers for exogenous RPL22 (consisting of a pair of Vector-specific primers and a pair of RPL22-specific primers) and β-actin was used as an internal control. b. Western blot analysis of RPL22 expression in transfected cells. (1) C6/36; (2) C6/36-pIB/V5-His; (3) C6/36-RPL22. The His-tagged RPL22 was detected with a mouse anti-His tag antibody followed by a horseradish peroxidase conjugated goat anti-mouse secondary antibody.

3.2. Sequence and phylogenetic analysis

The putative protein sequence of RPL22 deduced from the cDNA sequence shared 100%, 90% and 80% identities with RPL22 of *Cx. quinquefasciatus*, *Ae. aegypti* and *An. gambiae*, respectively. And alignment by Clustal W2 software showed the conservation of RPL22 in different species (Fig. 2). The sequence from 35 to 146 amino acids (italic letters in Fig. 2) belongs to the ribosomal L22e protein superfamily. The phylogenetic tree that I had shown by using the neighbor joining method provide two kinds of information: branching pattern and branch length (Fig. 3). The branching pattern showed the phylogenetic relationships of RPL22 among *Cx. pipiens pallens* and some other species. It showed that *Cx. pipiens pallens*, *Cx. quinquefasciatus* and *Ae. aegypti* share more common ancestry than the others. In the tree above, *Cx. pipiens pallens* and *Cx. quinquefasciatus* share the most recent common ancestry. Thus, of the species in the tree, *Cx. pipiens pallens* and *Cx. quinquefasciatus* are the most closely related. The branch length of the line leading from the *Cx. pipiens pallens* + *Cx. quinquefasciatus* common ancestry to *Cx. pipiens pallens* is similar to *Cx. quinquefasciatus*. This is intended to represent the accumulation of a similar amount of change. And these results was according to the previous report that *Cx. pipiens pallens* and *Cx. quinquefasciatus* were two subspecies of *Cx. pipiens* complex.

3.3. Expression profile of the RPL22 gene

To confirm the result from the cDNA microarray, real-time PCR was performed. The relative expression of *RPL22* in DS-strain was considered as background level or 1 and the result showed that *RPL22* exhibited 2.57 folds higher level of transcription in the DR-strain than in the DS-strain. This result suggests that *RPL22* expression is up-regulated in the DR-strain (Fig. 4).

3.4. Transfection and expression of RPL22 in C6/36 cells

To confirm transfection and expression were successful. RT-PCR and western blotting was employed. RT-PCR results showed that an expected product 487 bps was observed only in the cells transfected

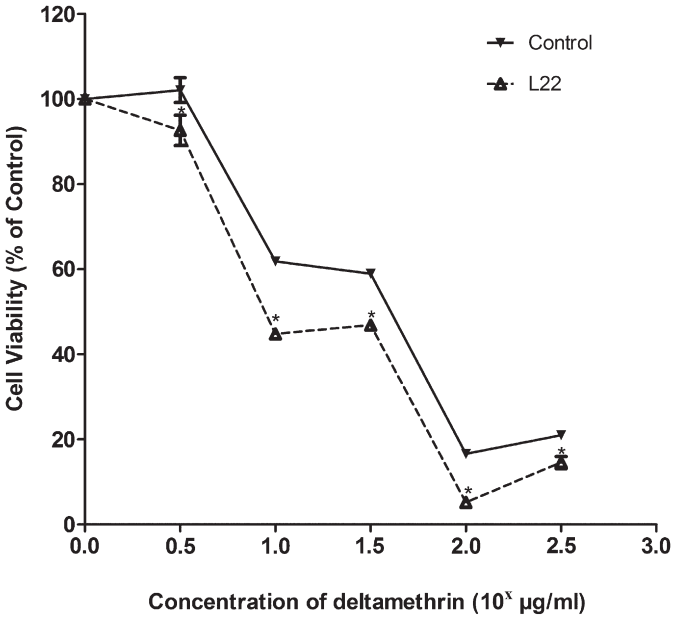

Fig. 6. Over-expression of RPL22 reduce deltamethrin resistance in C6/36 cells. C6/36-RPL22 cells were treated with deltamethrin at the indicated concentrations, and viable cells were measured after 72 h of treatment. The percentage of viable cells is shown relative to the control. *$p<0.05$ compared with control. The same experiment was done three times and showed the same pattern.

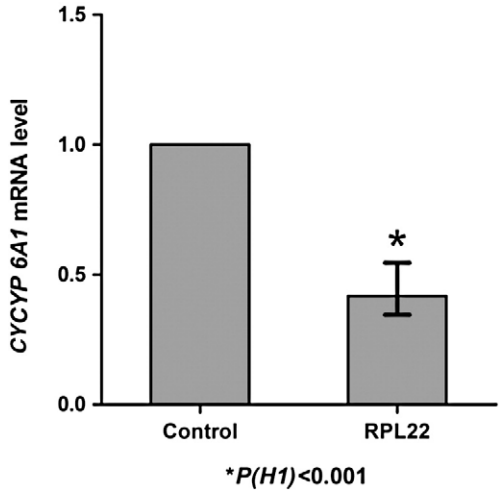

Fig. 7. Real-time quantitative PCR assay of *CYP6A1* in C6/36-RPL22 and control. Results are expressed as mean±standard error. The relative expression of *CYP6A1* in control was considered as background level or 1. *$p<0.001$ compared with C6/36-RPL22 cells.

with *RPL22* (Fig. 5a), and confirmed *RPL22* had been transcribed in the transfected cells. Western blotting analysis using anti-His antibody identified a protein of 17 kDa in the cells transfected with *RPL22* (Fig. 5b).

3.5. Deltamethrin sensitivity analysis of pIB/V5-his-RPL22 transfected C6/36 cells

To investigate the RPL22 overexpression in relation to deltamethrin resistance, C6/36 cell line stably transfected with pIB/V5-his-RPL22 and pIB/V5-his were used for this assay. The dose response of cell viability over a wide range of concentrations (0, $10^{0.5}$ μg/ml, $10^{1.0}$ μg/ml, $10^{1.5}$ μg/ml, $10^{2.0}$ μg/ml, and $10^{2.5}$ μg/ml) of deltamethrin was measured based on the cytotoxicity assay using CCK-8 kit. As shown in Fig. 6, C6/36-RPL22 cells are relatively more susceptible to deltamethrin at the concentrations of $10^{0.5}$ μg/ml, $10^{1.0}$ μg/ml, $10^{1.5}$ μg/ml, $10^{2.0}$ μg/ml, and $10^{2.5}$ μg/ml ($p<0.05$).

3.6. CYP6A1 mRNA level analysis

To explain the reason that RPL22 cause the cell more sensitivity to deltamethrin, mRNA level of insecticide-resistant gene *CYP6A1*was detected. The relative expression of *CYP6A1* in C6/36-pIB/V5-his cells was considered as background level or 1 and the result showed that *CYP6A1* mRNA level of C6/36-RPL22 cells had 2.39 folds lower than the control. This result suggested when RPL22 overexpressed, the expression of *CYP6A1* was down-regulated (Fig. 7).

4. Discussion

RPL22 is a component of 60S subunit of ribosome, which constitutes the peptide exit tunnel with ribosomal protein L4 (RPL4). The nascent peptides pass through the exit tunnel before they reach the extraribosomal environment. A number of nascent peptides interact with the exit tunnel and stall elongation at specific sites within their peptide chain. The tunnel constriction has also been implicated in peptide-mediated pausing (Nakatogawa and Ito, 2002; Cruz-Vera et al., 2005). Previous research had indicated that in bacteria, mutations in RPL22 and RPL4 mediate erythromycin resistance by perturbing the conformation of rRNA, and a variety of changes in these proteins could mediate macrolide resistance (Gregory and Dahlberg, 1999; Gabashvili et al., 2001; Zaman et al., 2007; Caldwell et al., 2008). Although RPL22 is associated with macrolide resistance, but no evidence has related RPL22 to insecticide resistance in present.

In our previous work, we found that *RPL22* was highly expressed in DR-strain (Wu et al., 2004). So the function of *RPL22* gene was further studied in this work. The full length of *RPL22* gene was cloned, sequenced and characterized from *Cx. pipiens pallens* by 5′-RACE and 3′RACE. A ribosomal L22e superfamily was found from the alignment of the putative protein sequence. Data from phylogenetic tree demonstrated that the phylogenetic relationship of RPL22 between *Cx. pipiens pallens* and *Cx. quinquefasciatus* was the most closest. These data could confirm that the full length of the gene we cloned was RPL22 of *Cx. pipiens pallens*. To determine the association between *RPL22* and insecticide resistance, cytotoxicity assay was performed on the mosquito C6/36 cell line which were stably transfected with *RPL22*. The results showed that the cell viability of *RPL22*-transfected cell was significantly lower than the control, suggesting that *RPL22* rendered the C6/36 cells to be more susceptible to deltamethrin. The condition that the gene highly expressed in the DR-strain and inhibited the resistance was found in our previous research. Among the 13 deltamethrin resistance-related genes identified in our previous experiments (Wu et al., 2004), *NYD-OP7*, *RPL39*, and myosin regulatory light chain (*MRLC*) which showed higher expression in DR-strain enhanced resistance to deltamethrin (Hu et al., 2007; Tan et al., 2007; Yang et al., 2008). On the other hand, glycogen branching enzyme (*GBE*), an upregulated gene in DR-strain, causes the cells to be more susceptible to deltamethrin (Xu et al., 2008). To find how RPL22 could reduce the resistance in the mosquito cell, the change of insecticide resistant gene was detected.

CYP6A1 was confirmed as one of the important insecticide-resistant genes, which was initially isolated and sequenced from insecticide resistant strain of *Musca domestica* (Feyereisen et al., 1989). Carino et al. (1994) found that both larvae and adults of the insecticide-resistant strain had significantly higher *CYP6A1* mRNA levels than susceptible larvae or adults of the same age (Carino et al., 1994). In larvae the difference reached 12 folds, and c. 10 folds in adults. Andersen et al. (1994) found that a periplasmically directed reductase supports monooxygenase reactions with *CYP6A1* in a reconstituted system, and suggested that *CYP6A1* is a major cyclodiene epoxidase and multiple P450 forms were responsible for the elevated monooxygenase activities in insecticide resistance (Andersen et al., 1994). In present study, the *CYP6A1* gene in C6/36-RPL22 cells was also detected, and the results showed that mRNA level of *CYP6A1* was lower than that of control. The result could elucidate why the C6/36-RPL22 cells were relatively more susceptible to deltamethrin compared to the control. It has also been reported that overexpression of RPL22 was involved in transcriptional repression in 7.46% (1007/13,500) genome-wide in *Drosophila* (Ni et al., 2006), and *CYP 9c1*, *CYP 4p1*and *GST* were among down-regulated genes. It is possible that *RPL22* is very important in the regulation of insecticide resistance, partly by the repression of *CYP6A1* expression.

It is very interesting that when *RPL22* was highly expressed in the DR-strain, the transcription of *CYP6A1* was repressed. And the cell transfected with *RPL22* was more susceptible. Some potential explanations were the following:

1. The insecticide selection pressures acting on the resistant genes, i.e. their selective advantage in the presence of insecticide and their disadvantage (resistance cost) in absence of insecticide, were already reported (Bourguet et al., 1997; Rodcharoen and Mulla, 1997). One possible mechanism by which resistance costs are generated is the disruption of metabolic equilibrium (Uyenoyama, 1986). Some genes, such as GSTs, Ests, and P450s, confer the insecticide resistance through protein overproduction, which is achieved either by gene up-regulated or gene amplification (Rooker et al., 1996). And protein overproduction of these detoxifying enzymes would be deleterious to the organisms. For

example, resistance allele E4 appears to impose strong deleterious effects such as reduced overwinter survival (Foster et al., 1996) and disruption of the life cycle (Blackman et al., 1996). Up-regulated RPL22 in resistant *Cx. pipiens pallens* might protect mosquitoes through repressing these resistance genes. In our experiment, there was only RPL22 had been up-regulated. The detoxifying enzymes, such as *CYP6A1*, had not been overproducted before the cell was treated with deltamethrin. Thus, overexpression of RPL22 could cause the cell to be more susceptible to deltamethrin.

2. Alternatively, the effect of RPL22 on resistance is dose-dependent. Below threshold, the effect is positively correlated with resistance. Above the threshold level, RPL22 may inhibit resistance. In the highly resistant mosquitoes (400 folds increase), RPL22 was only up-regulated 2.57 folds compared to the susceptible mosquitoes. In the present, overexpression of RPL22 causes cells to be more sensitive to deltamethrin. This result might be a toxicity effect of overexpression. Further studies on the relationship between RPL22 doses and insecticide resistance are needed to confirm this hypothesis.
3. Selection of highly resistant mosquitoes may alter expression of genes not directly associated with resistance. That is, RPL22 does not directly cause resistance to deltamethrin, but it is a by-product of selection for highly resistant mosquitoes (400 folds increase).
4. This result provides a cautionary tale on the interpretation of gene expression results from microarray studies and from laboratory selected highly resistant mosquito lines.

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