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

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The 60S ribosomal protein L22 (GenBank accession no. EP990190) 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.

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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-bred 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.
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 resistant strains using the RNeasy mini kit (Qiagen, Germany). Products were then ligated into the pGEM-T easy (Promega, USA) and resistant strains using 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′-ATGGTGATGGTGATGCTCGGCATCGTCTTCCTTCCTTCCTTTTCTC-3′ respectively. The sequence of 3′- RACE and 5′-RACE adaptor primers supplied by the BD SMART™ RACE cDNA Amplification Kit were 5′-CTGAATTCATAGGACGGCCGC-3′ and 5′-CTTACAGCTACTATAGGCGACGCTGATCACCAGGAT-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′-GGGCTACCATATGGTTGAGAAGGTGCTCGGCATCGTCTTCCTCC-3′ and reverse primer: 5′-CTTACAGCTACTATAGGCGACGCTGATCACCAGGATG-3′. The PCR products were amplified using a pair of specific primers: forward primer: 5′-GGGCTACCATATGGTTGAGAAGG-3′, reverse primer: 5′-GAAGAAGGTCGCCGCTGTCAAAGTG-3′, and 5′-ATGGTGATGGTGATGCTCGGCATCGTCTTCCTTCCTTTTCTC-3′. 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.

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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 phylogenetic 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 congregata (Beziers 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 ddH2O. The sequences of forward and reverse primer for RPL22 were 5′-CAACCTCGGTCATCTTTGGAG-3′ and 5′-ATCGGTCATCCTCATGCT-3′ respectively, and the product size was 205 bps. Another pair of primers was used for j-actin: 5′-AAGTCGAAGCTGCCTCGGCTCT-3′ and 5′-ACTCTGTGCTACTCTCTTGCCTTG-3′ with a product size of 153 bps, and j-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′-GGGCTACCATATGGTTGAGAAGG-3′, reverse primer: 5′-GAAGAAGGTCGCCGCTGTCAAAGTG-3′, and 5′-ATGGTGATGGTGATGCTCGGCATCGTCTTCCTCC-3′. ATC was added before ATG to form Kozak sequence in the forward primer (Kozak, 1986; Sano et al., 2002), and for the later Western blotting identification, we added AATGGTGATGCTCGGCTCTG (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 pB/S-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 pB/S-V5-His expression vector (Invitrogen, USA) were all digested by Kpn I and Spe I, and 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 OpIIE2 forward and OpIIE2 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% CO2-humidiﬁed incubator at 28 °C and were plated in a six-well plate. Transfection was performed using FuGENEM™ HD transfection reagent (Roche, USA) according to the
FuGENE® HD transfection reagent (6 µL) was pipetted directly into the well. After placing 100 µL diluted plasmid DNA into sterile tube, the DNA was added to a concentration of 2 µg plasmid DNA/100 µL (0.02 µg/µL). For each well, dilute plasmid DNA with sterile water was pre-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 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 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.

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2.9. Western blotting

Protein was extracted from stable transfected cells by RIPA lysing 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-rabbit 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 transfected cells 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% CO₂-humidified incubator at 28 °C. Wells were then treated with 100 µL of various concentrations of deltamethrin (final concentrations: 0, 10⁻⁸, 10⁻⁹, 10⁻¹⁰, 10⁻¹¹, 10⁻¹² 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.
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′-GGCCCTCCAGCAGCATTCAT-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. 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.

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_558423; Cotesia congregata: AM492671; Xenopus tropicalis: BC150931; Spodoptera frugiperda: AF400188; Bombyx mori: AY769291.
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).

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. *p<0.001 compared with DS. DS-strain: deltamethrin-susceptible strain; DR-strain: deltamethrin-resistant strain.

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.

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 with the RPL22 gene. The His-tagged RPL22 was detected with a mouse anti-His tag antibody followed by a horseradish peroxidase conjugated goat anti-mouse secondary antibody.

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.

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.
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, 100.5 µg/ml, 101.0 µg/ml, 101.5 µg/ml, 102.0 µg/ml, and 102.5 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^{1.5} µg/ml, 10^{1.6} 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 (Feyerisen 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 enzyme in CYP6A1 to be a major cyclodiene epoxidase and multiple P450 forms were responsible for the elevated monoxygenase 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 Dro sophila (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 (Uyenoyma, 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. p. 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 overproduced 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).

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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