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Cloning and characterization of ribosomal protein S29, a deltamethrin resistance associated gene from *Culex pipiens pallens*

Haibo Sun · Linchun Sun · Ji He · Bo Shen · Jing Yu · Chen Chen · Mifang Yang · Yan Sun · Donghui Zhang · Lei Ma · Changliang Zhu

Abstract The 40S ribosomal protein *S29* (*RPS29*) was shown differentially expressed in deltamethrin (DM)-susceptible and resistant *Culex pipiens pallens* previously. Here, we cloned *RPS29* from this mosquito. An open reading frame (ORF) of 171 bp was found to encode a putative protein with 56 amino acid residues, which shares more than 90% identities with other mosquito RPS29 genes. Moreover, quantitative real-time PCR analysis demonstrated that the transcriptional level of RPS29 was significantly up-regulated both in DM-resistant *Cx. p. pallens* and C6/36 cells compared with the susceptible ones. Over-expression of RPS29 showed a slight decrease of cell viability in DM-susceptible C6/36 cells under DM treatment and knockdown of RPS29 by siRNA comparably increased cell viability in DM-resistant cells. Our study provides evidence that RPS29 might be associated with fitness cost of DM resistance in mosquitoes for the first time.

H. Sun · L. Sun · B. Shen (✉) · J. Yu · C. Chen · M. Yang · Y. Sun · D. Zhang · L. Ma · C. Zhu (✉)
Department of Pathogen Biology, Nanjing Medical University, Nanjing, Jiangsu 210029, People's Republic of China
e-mail: shenbo@njmu.edu.cn

C. Zhu
e-mail: clzhu@njmu.edu.cn

H. Sun · L. Sun · B. Shen · J. Yu · C. Chen · Y. Sun · D. Zhang · L. Ma · C. Zhu
Key Laboratory of Modern Pathogen Biology of Jiangsu Province,
Nanjing, Jiangsu 210029, People's Republic of China

J. He
State Key Laboratory of Monitoring and Detection for Medical Vectors, Xiamen Entry–Exit Inspection and Quarantine Bureau, Xiamen, Fujian 361012, People's Republic of China

Introduction

Mosquito is one of the most medically important vectors, which transmits reemerging insect-borne diseases such as malaria (Snow et al. 2005), dengue fever (Gubler 2002), yellow fever (Barrett and Higgs 2007), and West Nile fever (Kostiukov et al. 1986). Chemical control has been used since the 1950s to control disease-spreading insect vectors including mosquitoes (Hemingway et al. 2006). Deltamethrin (DM), an important synthetic pyrethroid insecticide, is used to kill insects by stimulating their nervous system, a similar mode to DDT (Hemingway et al. 2004). DM is widely used in bed nets impregnation and indoor residual spray to manage insect-borne diseases (Van Bortel et al. 1996). Unfortunately, continuous and excessive application of DM has accelerated the development of insecticide resistance in many species including *Cx. p. pallens* (Cui et al. 2006; 2007), which has become the major obstacle for controlling vector-borne diseases (Hemingway and Ranson 2000).

Insecticide resistance is a complicated polygenic inheritance, and many genes had been identified to be associated with it (Hemingway et al. 2004). Recently, a large-scale transcriptional profiling based on suppression subtractive hybridization (SSH) combined with cDNA microarray has been completed and many DM resistance associated genes

have been identified in *Culex* mosquitoes (Wu et al. 2004; Yang et al. 2008; Zhang et al. 2010). One of the differentially expressed genes was a homolog to *Aedes aegypti* RPS29 (GenBank ID BE247828).

Ribosomal proteins are mainly responsible for protein synthesis in organisms (Zimmermann 1995) and are highly conserved from yeasts to mammals (Wool et al. 1995; Lecompte et al. 2002; Klein et al. 2004). Ribosomal protein S29 (RPS29) is a component of the 40S small ribosome, which contains a zinc finger-like domain (Chan et al. 1993). In mammals, RPS29 is up-regulated in the apoptotic thymocytes and over-expression of RPS29 induces apoptosis in cancer cells and sensitizes cells to chemotherapy (Khanna et al. 2000, 2003). RPS29 has also been shown to induce cell quiescence and regulate many anti-tumor protein expressions (Kondoh et al. 1996; Coppock et al. 2000; Khanna et al. 2003). However, no report about the relationship between RPS29 and insecticide resistance has been seen.

In this study, we cloned full-length cDNA of *RPS29* from *Cx. p. pallens* and open reading frame (ORF) of RPS29 from *Aedes albopictus*. Quantitative real-time PCR was conducted to check the expression of RPS29 in DM-susceptible and DM-resistant C6/36 cells and *Cx. p. pallens*. In addition, to further investigate the role of RPS29 in DM resistance, plasmid transfection and siRNA were used to regulate RPS29 expression, and cell viability analysis was assessed.

Materials and methods

Cells and mosquitoes

The *Ae. albopictus* C6/36 cell line were obtained from the China Center for Type Culture Collection. It was derived from pooled newly hatched *Ae. albopictus* larvae, which is often used in gene function study. The cells are susceptible to a range of mosquito-borne viruses. The DM-resistant C6/36 cells were selected in our laboratory with DM in a dose-dependent manner for more than 70 generations. The median lethal concentrations (LC_{50}) of the DM-resistant cells were 1,256.8 mg/l, 8.9-fold higher than 141.2 mg/l of the DM-susceptible cells. Cells were cultured in DMEM/High Glucose media which contains 10% of fetal calf serum and 1% of penicillin–streptomycin and incubated in a 5% CO_2-humidified incubator at 28°C. The DM-susceptible strain of *Cx. p. pallens* was obtained from the Shanghai Insect Institute of the Chinese Academy of Sciences and maintained in our laboratory. The mosquitoes had never been exposed to any insecticide and the LC_{50} of DM was 0.008 mg/l. The DM-resistant stain was selected with DM from DM-susceptible strain for more than 10

generations to reach a 400-fold-high resistance (Li et al. 2002). Both the susceptible and resistant strains were reared at 25–27°C in a 16-h light/8-h dark photoperiod.

RNA extraction and cDNA synthesis

Total RNA were extracted from mosquito cells and larvae using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The contaminating genomic DNA was removed by DNase I treatment. The quality of total RNA was determined by denaturing agarose gel electrophoresis and the yield was estimated by spectrometry. The cDNA was reverse synthesized from 1 μg of total RNA (20 μl reaction) using the PrimeScript™ RT reagent Kit (Takara, Shiga, Japan), according to the manufacturer's instructions.

Cloning and sequencing of RPS29 cDNA

To clone the full-length cDNA of *RPS29* in *Cx. p. pallens*, rapid amplification of 5′ cDNA ends (5′-RACE) and 3′ cDNA ends (3′-RACE) were carried out using the Clontech SMART™ RACE cDNA Amplification Kit (Takara). The specific primer 5′-CATGGGTTTCGCTGATTTGTGG TACTCGC-3′ (5′-RACE) and 5′-TCCTTGGCGTATTCTCG GAAGCACTGACG-3′ (3′-RACE) were designed based on the EST sequence (GenBank ID BE247828) reported previously. The sequence of 5′-RACE and 3′-RACE adaptor primers supplied by the kit was 5′-CTAATACGACTCACTA TAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ and 5′-CTGATCTAGAGGTACCGGATCC-3′, respectively. The fragment was separated by 1% agarose gel electrophoresis and purified using a quick gel extraction kit (Qiagen), and then it was ligated into the pGEM-T easy vector (Promega, WI, USA). The ligation mixture was transformed into *Escherichia coli* Top10 cells and the cells were then streaked on LB plates containing ampicillin (100 μg/ml), IPTG (400 μg/ml), and X-Gal (200 μg/ml). The positive colonies were selected and confirmed by PCR. Plasmid DNA was extracted using a plasmid mini kit (Qiagen) and sequenced at Shanghai Invitrogen Biotechnology company (Invitrogen, Shanghai, China). The sequencing results of 5′- and 3′-RACE were then assembled to generate a putative full-length cDNA of *RPS29*.

To clone the full ORF of *RPS29* from *Ae. albopictus*, degenerate primers, 5′-ATGGGTTTCGCTGATYTSTGG-3′ and 5′-TTAATCCAGCTTCCKGAAGCC-3′, were designed by sequence alignment for the generation of full CDS fragment of *RPS29*. The PCR was carried out using Ex taq kit (Takara) and the product was separated by 1% agarose gel electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen). The purified PCR product was sequenced at Shanghai Invitrogen Biotechnology Company.

Sequence alignment and phylogenetic analysis

The standard protein–protein BLAST program (BLASTP, http://blast.ncbi.nlm.nih.gov) was used to search for sequences in the SWISSPROT databases with similarities to the sequences of *RPS29*. 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 5.0 program. The sequences included in our analysis for sequence alignment and phylogenetic tree analysis were as follows: *Cx. p. pallens*, EF990189.2; *Cx. quinquefasciatus*, AF266749.1; *Ae. aegypti*, XM_001652883.1; *Armigeres subalbatus*, EU206018.1; *Anopheles gambiae*, XM_321509.4; *Anopheles darling*, EU934272.1; *Drosophila melanogaster*, NM_141689.2; *Anopheles funestus*, EZ973369.1; *Ae. albopictus*, EU600292.1; *Curculio glandium*, AM040020; and *Bombyx mori*, NM_001043814.1.

PCR and quantitative real-time PCR analysis

For 5′- and 3′-RACE, PCR conditions were as follows: initial five cycles at 94°C for 30 s, 72°C for 3 min, followed by five cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 3 m, with a final 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min. PCR conditions for all the other assays were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 40 s, 58°C for 40 s, and 72°C with a final 10-min extension at 72°C. Quantitative real-time PCR was performed on the ABI PRISM 7300 (Applied Biosystems, CA, USA) using Light Cycler FastStart DNA Master SYBR Green I (Rockford, IL, USA) according to the manufacturer's instructions. The primer sequences used in quantitative real-time PCR are listed in Table 1. The 20-μl PCR mixture contained SYBR Green PCR Master mix, forward and reverse primers, and diluted cDNA. A melting curve program was run immediately after the PCR program and the data were analyzed with a 7300 System SDS Software v1.2.1 (Applied Biosystems). 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 and 60°C for 1 min. β-Actin was used as the internal control and the threshold cycle (Ct) values were used to calculate relative expression levels of each sample using Relative Expression Software Tool 2008 (REST) (Pfaffl et al. 2002). The expression level of RPS29 in control group was considered as background 1. For each assay, all analysis was repeated three times using independent purified RNA samples.

Construction of the insect expression plasmid

The ORF of RPS29 was amplified using a pair of specific primers: forward primers 5′-ATCATGGGTTTCGCT GATTTGTGG-3′ and reverse primers 5′-TTAATGATGAT GATGATGATGATCCAGCTTCCGGAAGCCAA-3′. ATC was added before ATGG to formed Kozak sequence in the forward primer (Kozak 1986), and for the later Western blot identification, we added ATGATGATGATGATGATG (6× his) in the reverse primer. The stop codon TAA was moved to the downstream of 6× his. The purified PCR product was ligated into the pIB/V5-His-TOPO vector (Invitrogen, CA, USA), and the ligation mixture was then transformed into *E. coli* TOP10 competent cells. Positive clones were identified by PCR with *RPS29* Q-PCR primers of *Cx. p. pallens* (Table 1). The accuracy of the expression plasmid pIB/S29-His was finally verified by sequencing.

Stable transfection of S29

The pIB/Ctrl and pIB/S29-His plasmid were stable transfected into DM-susceptible C6/36 cells using FuGENE® HD transfection reagent (Roche, IN, USA) according to the manufacturer's protocol. Briefly, 5×10^5 cells/well were seeded in 2 ml complete growth medium in a six-well plate overnight. At the time of transfection, cells achieved 80% confluency. For each well, plasmid DNA was diluted to a concentration of 2 μg/100 μl (0.02 μg/μl). One hundred microliters of diluted plasmid DNA was placed into sterile tube and then 6 μl of FuGENE® HD transfection reagent was pipetted 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 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 for selection of stable transfected cells using 20 mg/l of blasticidin, which would kill non-transfected cells within 2 weeks. Forty-eight hours post-transfection, cells were selected with 20 mg/l blasticidin (Invitrogen, CA, USA) for 1 week and the medium was

Table 1 Primer sequences used in quantitative real-time PCR

Gene name	Primers	Primer sequences (5′–3′)
Aedes albopictus		
RPS29	Forward	TGGGTTTCGCTGATCTCTGG
(EU600292.1)	Reverse	CCTGAAACATTGACGGCAAAT
β-Actin	Forward	CCAGGGTGTGATGGTCGGT
(DQ657949.1)	Reverse	ATAAACCGAACCGCAAGAG
Culex pipiens pallens		
RPS29	Forward	TGGGTTTCGCTGATTTGTGG
(EF990189.2)	Reverse	TCGGAAGCACTGACGGCATA
β-Actin		(XM_001844545.1)
	Forward	CCAGGGTGTGATGGTCGGT
Reverse		ATAAACCGAACCGCAAGAG

changed every 3–4 days. Eight days later, the medium was replaced with medium containing 10 mg/l blasticidin. The stable pIB/RPS29-His transfected cells were characterized by reverse transcription PCR (RT–PCR) and Western blotting. At the same time, stable cell line transfected with pIB/Ctrl empty vector (Invitrogen, CA, USA) as a negative control was also established using the same protocol described above.

Construction and transfection of siRNA

Corresponding to the ORF sequence of *RPS29* (GenBank ID EU600292.1) cloned from *Ae. albopictus* C6/36 cells, double-stranded siRNA molecules were designed and synthesized by GenePharma company (GenePharma, Shanghai, China). The sense sequences of *RPS29* siRNA and scrambled siRNA control were as follows: 5′-GGGAAUACGCCAAGGAUAUUTT-3′ and 5′-UUCUCC GAACGUGUCACGUTT-3′, respectively. The DM-resistant C6/36 cells were transfected with siRNA using FuGENE®HD Transfection Reagent (Roche, IN, USA) according to the manufacturer's protocol. siRNA (final concentration 40 nM) and transfection reagent (6 μl) were mixed in 100 μl of Opti-MEM (Invitrogen, CA, USA) in a sterile and RNase-free tube for 15 min at room temperature and added to cells. After incubation for 6 h at 28°C, the medium was replaced with fresh complete media. Twenty-four hours later, cells were collected for later assay.

Western blotting analysis

Proteins from wild-type and stable transfected C6/36 cells were extracted by RIPA lysis buffer (Beyotime, Jiangsu, China) according to the manufacturer's instructions, and concentrations were determined by BCA Protein Assay kit (Pierce, IL, USA). Forty micrograms of protein per lane was loaded in 15% Tricine–SDS–PAGE gel using the protocol previously described (Schagger 2006). The SDS–PAGE electrophoresis was run at 80 V for 30 min followed by 80 min at 100 V. Then the proteins were transferred to a PVDF membrane for 150 min at 150 mA using Trans-Blot SD Cell and Systems (Bio-Rad, CA, USA). The fusion protein was detected using anti-His monoclonal antibody (1:500; Abmart, Shanghai, China) and a horseradish peroxidase–conjugated goat anti-mouse secondary antibody (1:1,000; DAKO, Glostrup, Denmark). Detection was done with the BeyoECL Plus (Pierce) according to the manufacturer's instructions.

Cell viability assay

Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was used to determine cell viability (DM-susceptible, DM-resistant,

pIB/RPS29-His stable transfected, pIB/Ctrl stable transfected, siS29 transfected, and siCtrl transfected cells) under DM treatments. Cell suspension (100 μl) was distributed (5,000 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. Cells were then treated with various concentrations of DM (final concentrations were 0, $10^{0.5}$, 10^1, $10^{1.5}$, 10^2, and $10^{2.5}$ mg/l). Sixty-seven hours later, 10 μl of CCK-8 solution was added to each well. Plates were incubated for 5 h, and the absorbance was measured at 450 nm using a microplate reader. DM was dissolved in DMSO and the final concentration of DMSO was 0.5% (v/v). The experiments were repeated three times.

Statistics

All results were means of three independent experiments. The data of quantitative real-time PCR was analyzed by hypothesis test. Other data were analyzed by Student's *t* test. Data were expressed as mean±SD. *p* values <0.05 are considered significant.
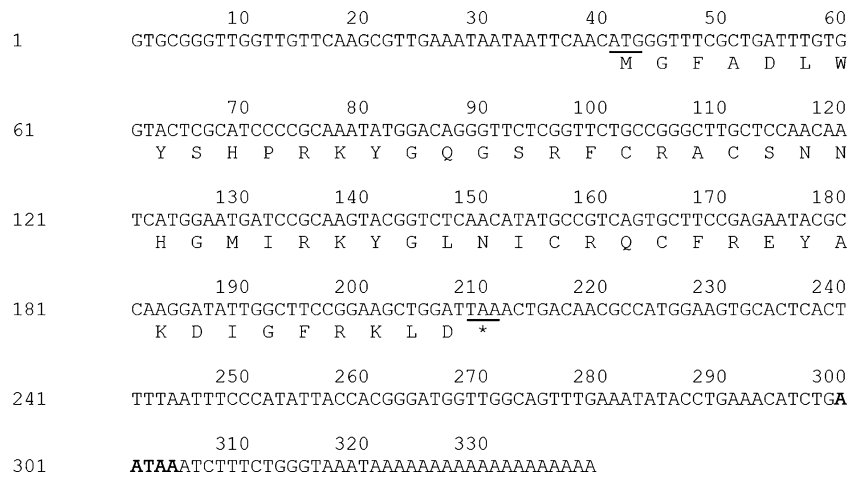
Results

Cloning the full-length cDNA of *RPS29* from *Cx. p. pallens*

The full-length cDNA of *RPS29* was amplified from *Cx. p. pallens* by RT–PCR using the 3′-RACE and 5′-RACE methods. One fragment of 191 bp was obtained from 3′-RACE and another 300 bp from 5′-RACE. They were assembled with the EST fragment (GenBank ID BE247828) and the cloned *RPS29* was 339 bp (GenBank ID EF990189.2). The ORF of *RPS29* is 171 bp which encodes a protein of 56 amino acids. The sequence analysis showed that a start codon "ATG" was at 41–43, a GC box like sequence "GCGGG" at 3–7, a TATA box like sequence "AAATAATAAT" at 26–35, an in-frame stop codon "TAA" at 209–211 with tailing signal sequence "AATAAA", and poly(A) at the 3′-untranslated region of the gene, which demonstrated the sequence is the full-length cDNA of *RPS29* (Fig. 1).

Sequence and phylogenetic analysis

The putative protein sequence of RPS29 deduced from the cDNA sequence shared 100% identities with *RPS29* of *Cx. quinquefasciatus*, *Ae. aegypti*, *Ae. albopictus*, and *Armigeres subalbatus* and 98% identities with *RPS29* of *An. gambiae*, *An. darling*, and *An. funestus*. An alignment by Clustal W2 software showed that the *RPS29* was conserved in different species (Fig. 2). The phylogenetic tree was shown using the neighbor-joining method, which

Fig. 1 The nucleotide and deduced amino acid sequences of *RPS29* from *Cx. p. pallens*. The deduced amino acid sequence is presented below the nucleotide sequence in *single letter*. The initial codon "ATG" and the termination codon "TAA" are *underlined*. The tailing signal sequence "AATAAA" in the 3′-untranslated region is in *bold letters*. GenBank ID: EF990189.2

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                10        20        30        40        50        60
1    GTGCGGGTTGGTTGTTCAAGCGTTGAAATAATAATTCAACATGGGTTTCGCTGATTTGTG
                                             M  G  F  A  D  L  W

                70        80        90       100       110       120
61   GTACTCGCATCCCCGCAAATATGGACAGGGTTCTCGGTTCTGCCGGGCTTGCTCCAACAA
      Y  S  H  P  R  K  Y  G  Q  G  S  R  F  C  R  A  C  S  N  N

               130       140       150       160       170       180
121  TCATGGAATGATCCGCAAGTACGGTCTCAACATATGCCGTCAGTGCTTCCGAGAATACGC
      H  G  M  I  R  K  Y  G  L  N  I  C  R  Q  C  F  R  E  Y  A

               190       200       210       220       230       240
181  CAAGGATATTGGCTTCCGGAAGCTGGATTAAACTGACAACGCCATGGAAGTGCACTCACT
      K  D  I  G  F  R  K  L  D  *

               250       260       270       280       290       300
241  TTTAATTTCCCATATTACCACGGGATGGTTGGCAGTTTGAAATATACCTGAAACATCTGA

               310       320       330
301  ATAAATCTTTCTGGGTAAATAAAAAAAAAAAAAAAAAAAA
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provided two kinds of information: branching pattern and branch length (Fig. 3). The branching pattern analysis showed the phylogenetic relationships of RPS29 between *Cx. p. pallens* and three other species. The results demonstrated that eight species of mosquitoes share a more common ancestry than other insect species.

RPS29 is up-regulated in DM-resistant cells and larvae

Quantitative real-time PCR was performed to assess the expressions of *RPS29* in cells and larvae. The results showed that the expression of *RPS29* was 6.82-fold higher in resistant cells compared with susceptible cells, and 2.93-fold higher in resistant larvae compared with susceptible larvae of *Cx. p. pallens* (Fig. 4). These results indicated that RPS29 might be associated with DM resistance in mosquitoes.

Characterization of over-expression and silencing of RPS29 in C6/36 cells

To confirm transfection and expression efficiency of exogenous *RPS29*, RT–PCR and Western blotting analysis

were conducted. RT–PCR results showed that an expected fragment (131 bp) was clearly observed only in cells transfected with pIB/RPS29-His vector (Fig. 5a). Furthermore, a band about 7 kDa was detected in pIB/RPS29-His cell lysate by a Western blotting analysis using anti-his antibody (Fig. 5b). Taken together, these results indicated that exogenous RPS29-His had been expressed in *RPS29*-transfected cells. To examine the knockdown efficiency of *RPS29*-siRNA, quantitative real-time PCR was performed. One of the three designed siRNAs significantly down-regulated the expression of *RPS29* (68.8% knocked down) compared with the scramble siRNA control (Fig. 5c).

RPS29 regulates DM sensitivity of mosquito cells

To investigate the relationship between RPS29 over-expression and DM resistance, DM-susceptible C6/36 cells (WT), pIB/S29-His, and pIB/Ctrl stably transfected DM-susceptible C6/36 cells were used for cell viability assay. The dose response of cell viability over a wide range of concentrations (0, $10^{0.5}$, $10^{1.0}$, $10^{1.5}$, $10^{2.0}$, and $10^{2.5}$ mg/l) of DM was measured using CCK-8 kit. pIB/S29-His

Fig. 2 Amino acid sequence alignment of *Cx. p. pallens* RPS29 with other species. *Asterisks* indicate identical amino acid and *dots* indicate similar amino acids. The *underlined* region showed a C4 zinc finger-like domain

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Cx. p. pallen          MGFADLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
Cx. p. quinquefasciatus MGFADLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
An. funestus           MGFANLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
An. gambiae            MGFANLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
An. darlingi           MGFANLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
Ae. aegypti            MGFADLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
Ar. subalbatus         MGFADLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
Ae. albopictus         MGFADLWYSHPRKYGQGSRFCRACSNNHGMIRKYGLNICRQCFREYAKDIGFRKLD
D. melanogaster        MGFATLWYSHPRKYGQGSRFCRACSNRHGLIRKYGLNICRQCFREYANDIGFKKLD
Cu. glandium           MGFQNIWYSHPRKYGQGSRSCRACSNRHGLIRKYGLNLCRQCFREYANDIGFKKLC
Bo. mori               MGHANIWYSHPRRYGQGSRSCRSCSNRHGLIRKYGLNICRQCFREYAHDIGFKKLD

                       **. :*****:***** **:***.**:*******:********:****:**
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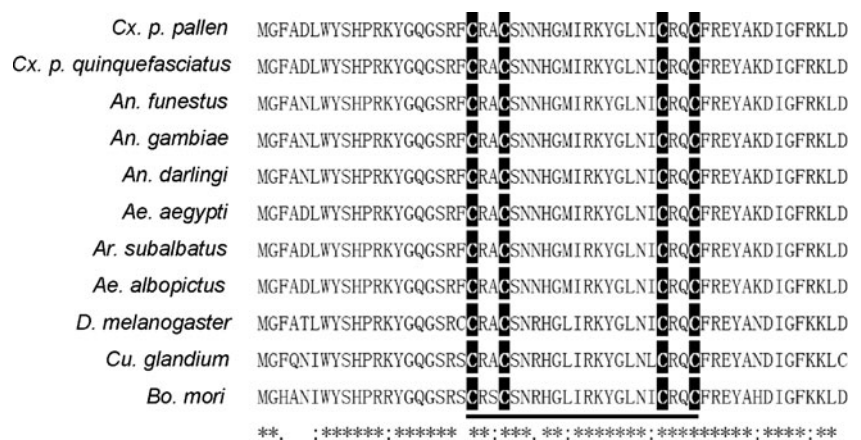
Fig. 3 Phylogenetic tree showed the relationships of RPS29 between *Cx. p. pallens* and other species. Species name and GenBank ID: *Cx. p. pallens*, EF990189.2; *Cx. quinquefasciatus*, AF266749.1; *Ae. aegypti*, XM_001652883.1; *Ar. subalbatus*, EU206018.1; *An. gambiae*, XM_321509.4; *An. darling*, EU934272.1; *D. melanogaster*, NM_141689.2; *An. funestus*, EZ973369.1; *Ae. albopictus*, EU600292.1; *Cu. glandium*, AF400188; and *B. mori*, NM_001043814.1
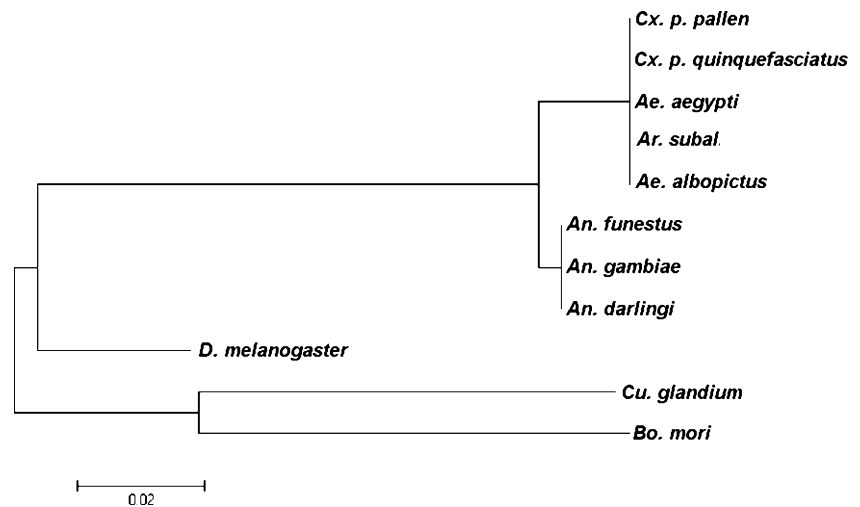

transfected cells are more susceptible to DM at the concentrations of $10^{1.5}$ mg/l, $10^{2.0}$ mg/l, and $10^{2.5}$ mg/l compared with the WT and pIB/Ctrl groups (Fig. 6a, *$p<0.05$). Since *RPS29* is up-regulated in DM-resistant C6/36 cells, we further transfected RPS29-targeted siRNA in these cells and then conducted cell viability analysis for a reverse authentication. Compared with the siCtrl groups, *RPS29* knockdown significantly enhanced cell viability in the presence of DM with concentrations of 2, 40, 80, 160, and 320 mg/l. We noted in several concentration points that cell viability in siS29 groups was lower than WT groups (Fig. 6b). We proposed that the siRNA molecules transfection might have some non-specific toxicity to cells.

Discussion

Chan et al. have discovered a C-X2-C-X14-C-X2-C (C4) zinc finger-like motifs in rat RPS29 (Chan et al. 1993). Kondoh et al. further showed that the C4-type domain of RPS29 indeed has zinc binding activity (Kondoh et al. 1996). We also found this zinc finger-like motif at the same position (21–42) in mosquitoes (Fig. 2). Zinc fingers are small protein domains which recruit one or more zinc ions to help stabilize the domain (Krishna et al. 2003). Zinc finger proteins perform numerous functions in various cellular activities, such as DNA damage repair, transcription, signal transduction, cell proliferation, and apoptosis

Fig. 4 Quantitative real-time PCR analysis for investigation of RPS29 expressions in DM-resistant and -susceptible mosquitoes and cells. **a** mRNA level of RPS29 in DM-resistant strain and DM-susceptible strain of *Culex pipiens pallens*. **b** mRNA level of RPS29 in DM-resistant C6/36 cells and DM-susceptible C6/36 cells. Results are expressed as mean± SD. The relative expression of *RPS29* in DM-susceptible group was considered as background 1 ($n=3$; *$p<0.05$, **$p<0.01$ vs. control). The experiment was repeated three times. *DS-Strain* DM-susceptible strain, *DR-Strain* DM-resistant strain, *DS cells* DM-susceptible cells, *DR cells* DM-resistant cells
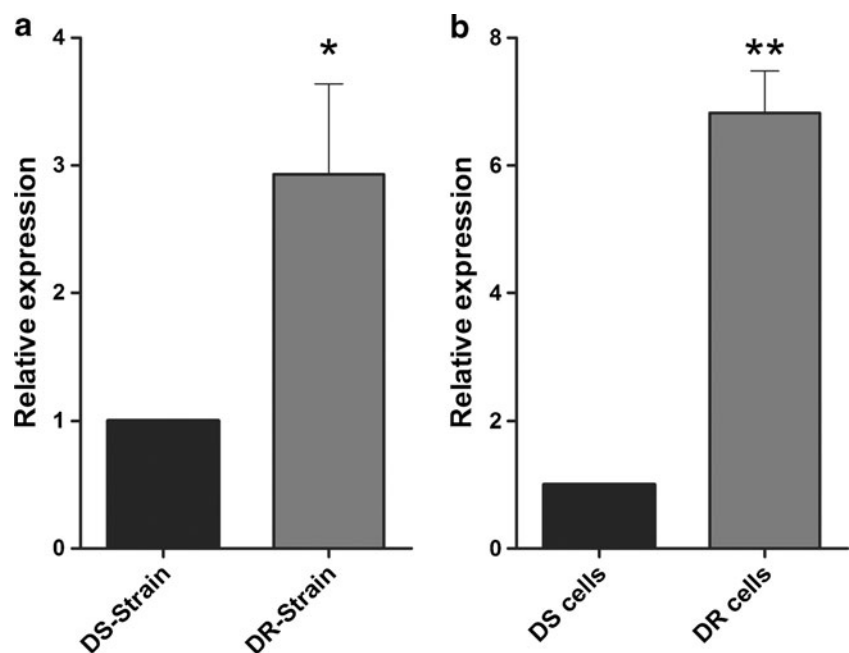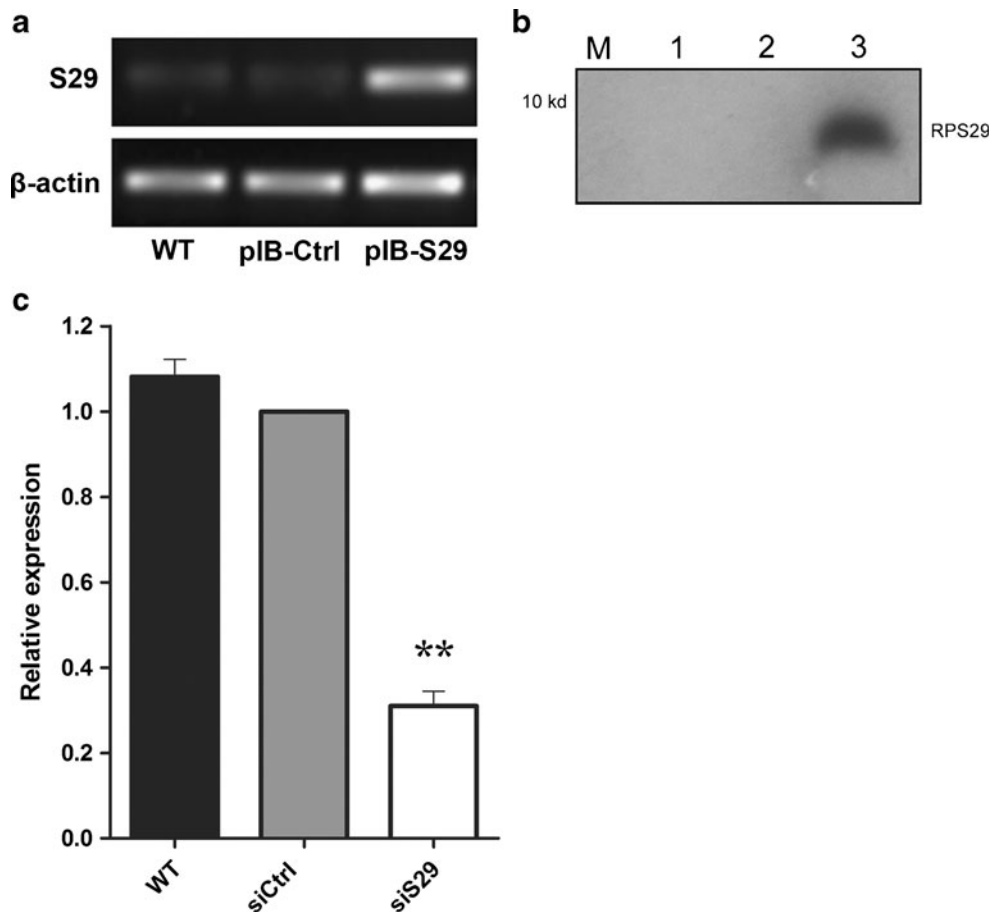
Fig. 5 Characterization of stable transfection of pIB/S29-His plasmid and knockdown efficiency of S29-targeted siRNA. **a** RT–PCR characterization of over-expression of exogenous *RPS29* in DM-susceptible C6/36 cells. Total RNAs (1.0 μg) from wild-type, C6/36-pIB/Ctrl, and C6/36-pIB/S29-His cells were analyzed by RT–PCR with primers (primers for *Culex pipiens pallens*, see Table 1) for detecting exogenous expression of RPS29. β-Actin was used as an internal loading control. **b** Western blot analysis of RPS29 expression in cells. The His-tagged RPS29 was detected with a mouse anti-His tag antibody followed by a horseradish peroxidase conjugated goat anti-mouse secondary antibody. *M* protein markers, *1* wild-type cells, *2* pIB/Ctrl stable transfected cells, *3* pIB/S29-His stable transfected cells. **c** Quantitative real-time PCR analysis of knockdown efficiency of *RPS29* in DM-resistant C6/36 cells. Total RNAs (1.0 μg) from wild-type, siCtrl transfected, and siS29 transfected cells were analyzed by Q-PCR (primers for *Aedes albopictus*, see Table 1) for detecting RPS29 expression. β-Actin was used as an internal control. $n=3$; **$p < 0.01$, vs. control (WT and siCtrl). The experiment was repeated three times

(Krishna et al. 2003). C4-type zinc fingers are found in many well-characterized families of nuclear receptors (Schwabe and Rhodes 1991) such as TFIIIA (Brown 1984; Gronemeyer and Laudet 1995). Besides, HDM2, as an important negative regulator of the p53 tumor suppressor, also has the C4-type domain (Lindstrom et al. 2007). Based on these reports, it is reasonable to propose that the C4-type domain might be important for RPS29 to bring its extra-ribosomal role.

RPS29 performed its extra-ribosomal role depending on its C4-type domain (Kondoh et al. 1996; Coppock et al. 2000; Khanna et al. 2000, 2003). Moreover, our newly cloned RPS29 shared more than 98% identities to other five mosquito species, 84% to three other insects and even 77% to homo species. This highly conserved characteristic indicates that RPS29 homolog might have similar functions in all species.

We found that mRNA level of RPS29 was both up-regulated in DM-resistant mosquitoes and cells, while our results suggested that RPS29 plays a negative role in the development of DM resistance in mosquito cells. It seems to be an interesting paradox that the up-regulation of *RPS29* has a negative effect on DM resistance, while this genetic feature is retained together with the DM resistance phenotype in mosquitoes.

As we have known, DNA structural variation is one of the most important bases for establishing a new phenotype (Eklov and Svanback 2006). There is a common problem
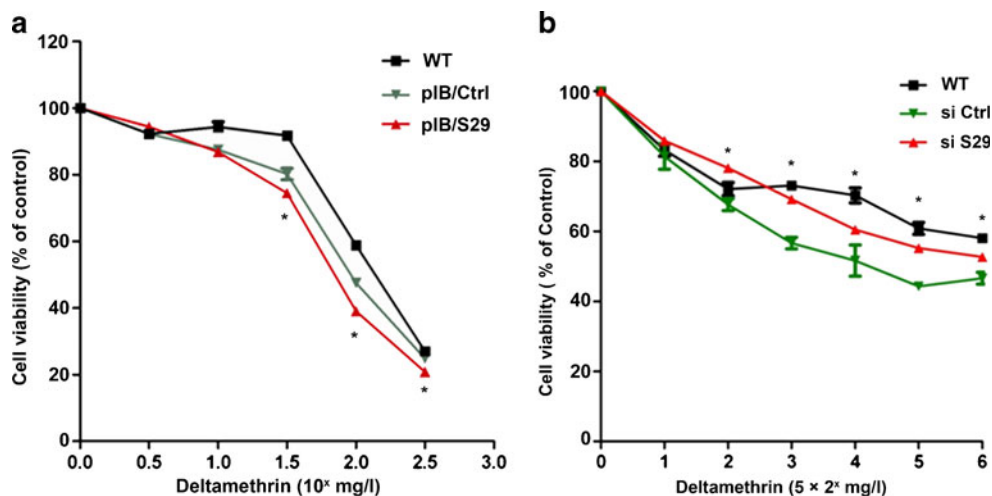
Fig. 6 RPS29 regulated cell viability of mosquito cells under DM stress. **a** Over-expression of RPS29 reduced cell viability in DM-susceptible C6/36 cells. Wild-type and transfected cells were treated with DM at the indicated concentrations, and viable cells were measured by CCK-8 after 72 h of treatment. The percentage of viable cells is shown relative to the control (absorbance value of 0 mg/l). $n=3$; $*p<0.05$, vs. control (pIB/Ctrl). The experiment was repeated three times and showed the same pattern. **b** siRNA mediated RPS29 silencing increased cell viability in DM-resistant C6/36 cells. Wild-type and transfected cells were treated with DM at the indicated concentrations, and the percentage of viable cells was measured by CCK-8 after 72 h of treatment. The percentage of viable cells is shown relative to the control (absorbance value of 0 mg/l). $n=3$; $*p<0.05$, vs. control (siCtrl). The experiment was repeated three times

that insects resistant to insecticides are less fit in the insecticide-free environment, a phenomenon called the fitness cost of resistance (Shi et al. 2004; Marrelli et al. 2006; Babiker 2009). Theoretically, the resistance resulting from genomic variations damages some structure, which makes them less effective at what it was designed for. There are many examples of fitness cost of insecticide resistance in *Culex* mosquito involving both knockdown resistance and metabolic resistance. For example, it has been reported that emergence percentage of the susceptible strain was significantly greater than that of the Kdr-resistant strains in an insecticide-free environment (Berticat et al. 2008). AChE1-resistant strains were associated with a longer development time and shorter wing length (Bourguet et al. 2004).

Many reports indicate that insecticides may cause genomic variations of organisms. Insecticides have been shown to hasten the evolution of resistance by increasing mutation frequencies (Gressel 2010). In addition, DM could induce chromosome aberrations, and micronuclei and sperm abnormalities in mice (Bhunya and Pati 1990). DM has also been shown to cause DNA damage and induce apoptosis in a variety of cells (El-Gohary et al. 1999; Wu and Liu 2000). Taken together, we hypothesize that DM selectively induces a constitutively up-regulated RPS29 through genetic variation.

The structural variations in DNA such as mutations and chromosome aberrations seem to be a "double-edged sword". In an environment containing insecticides, own resistance phenotype is essential for mosquitoes' survival. Once they live in an insecticide-free environment, the genotype of resistance is meaningless to them. As shown in this study, RPS29 is critical for mosquito under DM stress. But when the defects of genomic variations emerged, over-expressed genes such as RPS29 become the price of resistance fitness. This consequence prevents the transmission of resistant strains to a wild environment which makes the strategy of rotation of insecticides valuable for vector control (Rowland 1991).

We previously reported that RPL39 and RPL22 are associated with DM resistance in mosquitoes (Tan et al. 2007; He et al. 2009). Including RPS29, all the three ribosomal proteins are up-regulated in DM-resistant *Culex* mosquitoes. These results indicate that ribosomal proteins and ribosome stability might be involved in DM resistance. To our interest, over-expression of RPL22 decreases the cell viability in the presence of DM. However, over-expression of RPL39 seems to increase DM resistance. The underlying mechanisms call for further investigation.

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