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Cloning and characterization of myosin regulatory light chain *(MRLC)* gene from *Culex pipiens pallens*

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Myosin regulatory light chain (*MRLC*) (GenBank accession no. DQ140391) was cloned from *Culex pipiens pallens.* An open reading frame (ORF) of 630 bps was found to encode a putative 210 amino acids protein which shows 73% similarity with myosin regulatory light chain of *Gryllotalpa orientalis*. Real-time quantitative PCR analysis demonstrated that the transcription level of *MRLC* in deltamethrin-resistant strain (DR-strain) was 4.08-fold higher than in deltamethrin-susceptible strain (DS-strain) of *C. pipiens pallens*. Over-expression of *MRLC* in *Aedes albopictus* C6/36 cells conferred protection against deltamethrin based on tritiated methyl tritiated thymidine (^{3}H-TdR) incorporation assay. These results indicate that *MRLC* may be a potential cause of deltamethrin resistance in *C. pipiens pallens*.

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1. Introduction

Many insect-borne diseases threatening public health such as malaria (Butler 2007), dengue fever (Phuc et al., 2007), yellow fever (Barnett 2007), filariasis (Carme 2007), encephalitis, West Nile fever (Takasaki 2007) and chikungunya (Goonaratna 2007) are transmitted by mosquitoes. Use of synthetic insecticides has dramatically reduced the risk of insect-borne diseases. To date, four chemically different classes of synthetic insecticides have been used to control insect-borne diseases: organochlorines (that are now banned in most countries), organophosphates, carbamates and pyrethroids (Zaim et al., 2002). Pyrethroids have been used extensively because they rapidly kill insects and have low adverse effects on humans (Titchener et al., 1980; Katsuda et al., 2008). But frequent application of insecticides has resulted in a rapid increase in resistance, and this resistance has accelerated disease transmission(Ecobichon 2001). Management of insecticide resistance is crucial and should be considered as one of the most challenging issue in modern applied entomology.

Resistance mechanisms in mosquitoes have been extensively studied, and can be divided into two groups: metabolic resistance (degradation of the active ingredient by detoxification enzymes) and target-site resistance (mutations in the target proteins) (Hemingway et al., 2000, 2002, 2004). A number of insecticide resistance associated genes such as cytochrome P450 and carboxylesterase have been identified (Joussen et al., 2007). But insecticide resistance is a multi-gene phenomenon, our research focuses on these genes responsible for insecticide resistance. We have identified 16 EST sequences that are differentially expressed between deltamethrin-resistant strain (DR-strain) and deltamethrin-susceptible strain (DS-strain) of *Culex pipiens pallens* by employing suppression subtractive hybridization (SSH) and cDNA microarray. One of these 16 EST sequences was highly similar to *MRLC* gene (H.W. Wu et al., 2004).

Conventional myosin is built as homodimers of heavy chains each of which binds one essential light chain (ELC) and one regulatory light chain (RLC) (Geeves et al., 2005). The regulatory light chain binds competitively with Mg^{2+} or Ca^{2+} ions and undergoes phosphorylation by myosin light chain kinase (Nieznanski et al., 2003). The contraction of vertebrate striated muscle is regulated by Ca^{2+} binding to the troponin in the tropomyosin-troponin complex. There is compelling evidence that binding of Ca^{2+} to RLC may have a modulatory effect. During prolonged stimulation of muscle, Ca^{2+} can partially replace RLC bound Mg^{2+} (Holroyde et al., 1979). Podlubnaya et al. reported that synthetic myosin filaments undergo order-to-disorder transitions when transferred from Mg^{2+} to Ca^{2+} saturating RLC conditions (Podlubnaya et al., 2000a,b). Currently, no studies have examined the relationship between myosin regulatory light chain and insecticide resistance.

To elucidate if *MRLC* is involved in deltamethrin resistance, we cloned full-length *MRLC* from *C. pipiens pallens* into a mosquito expression plasmid, stably transfected C6/36 cells with this *MRLC*-expressing construct, compared *MRLC* expression level with control plasmid-transfected cell, and observed proliferation of these cells with deltamethrin treatments.

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2. Materials and methods

2.1. Mosquito strains

DS-strain of *C. pipiens pallens*, obtained from the Shanghai Insect Institute of the Chinese Academy of Sciences, were reared at 28°C in 70–80% humidity and constant light : dark photoperiod (14 h:10 h). DR-strain was selected with deltamethrin from DS-strain of early 4th instar larvae for more than 10 generations. The LC_{50} of DR-strain is 31.92 μM, 400-fold greater than the DS-strain (0.079 μM) (X.L. Li et al., 2002).

2.2. Cloning and sequencing

Total RNA was extracted from 4th instar larvae of *C. pipiens pallens* (≈20 mg) with TRIzol (Invitrogen) according to the manufacturer's instructions. 3′ RACE was performed with specific primer 5′-ACGGATTCTTCACGAACC-3′, which was based on the EST sequence reported previously, and the adaptor primer 5′-CTGATCTAGAGGTACCGGATCC-3′, which was supplied by the BD SMART™ RACE cDNA Amplification Kit. 5′ RACE was performed with special primer 5′-CCTGACCCCAGTGAGTGAGGGCATAAC-3′, which was also based on the EST sequence reported previously, and the adaptor primer 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′ , which was supplied by the BD SMART™ RACE cDNA Amplification Kit. The products were separated by 1% agarose gel electrophoresis and purified using QIAGEN quick Gel extraction kit (Qiagen, Germany), then cloned into the pGEM-T easy vector (Promega, USA) and sequenced at Shanghai Invitrogen Biotechnology Co. Ltd. (Shanghai, China). The sequences obtained from 3′, 5′RACE and EST fragment were assembled to generate a putative full-length cDNA. The open reading frame (ORF) was verified using following primers: 5′-TAACAGCCCAGCATCATGTCTGA-3′ and 5′-TTATTCTTCCTCTCCCTCCTCGG-3′.

2.3. Bioinformatics

The standard protein-protein BLAST sequence comparison and PSI-BLAST programs were used to search for sequences in the GenBank and Swiss-Prot databases with similarities to the translated protein of *MRLC*. Phylogenetic tree was carried out with the neighbor-joining method (Pirovano et al., 2008) Sequence alignment was analyzed by Clustal W2 programs (Larkin et al., 2007). The species included in our analysis were *Anopheles gambiae, Gryllotalpa orientalis, Lonomia oblique, Drosophila melanogaster, D. pseudoobscura, Apis mellifera, Caenorhabditis elegans, Ixodes pacificus, Homo sapiens, Pongo pygmaeus, Gallus gallus, Mus musculu, Sus scrofa, Felis catus, Rattus norvegicus, Oryctolagus cuniculus*. The isoelectric point , molecular mass, functional domain , signal peptide and cellular localization of MRLC was predicted by software ExPASy (http://us.expasy.org/tools/pi_tool.html), SMART (http://smart.embl-heidelberg.de/), Signal IP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and PSORT (http://psort.nibb.ac.jp/form2.html).

2.4. Real-time quantitative RT-PCR

Total RNA was extracted from 4th instar larvae of DR-strain and DS-strain *C. pipiens pallens* (≈20 mg) with TRIzol (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR was performed on ABI PRISM 7300 (Applied Biosystems) by using SYBR Green Power PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. The PCR mixture contained 10 μL 2 × SYBR Green Mix, 0.8 μL primers respectively, 2.0 μL cDNA and 6.4 μL ddH_2O. A pair of primers were used for MRLC: 5′-GGTGAGAAGTTCCGTTATGCC-3′and 5′-GGTTCGTGAAGAATCCGTTTA-3′ with a product of 170 bps. Another pair of primers was used for β-actin: 5′-AGCGTGAACTGACGGCTCTTG-3′ and 5′-ACTCGTCGTACTCCTGCTTGG-3′ with a product of 153 bps. The following PCR conditions were performed: 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 amplification fold of *MRLC* in DR-strain and DS-strain of *C. pipiens pallens* was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001). The real-time PCR analysis was repeated three times with independent samples.

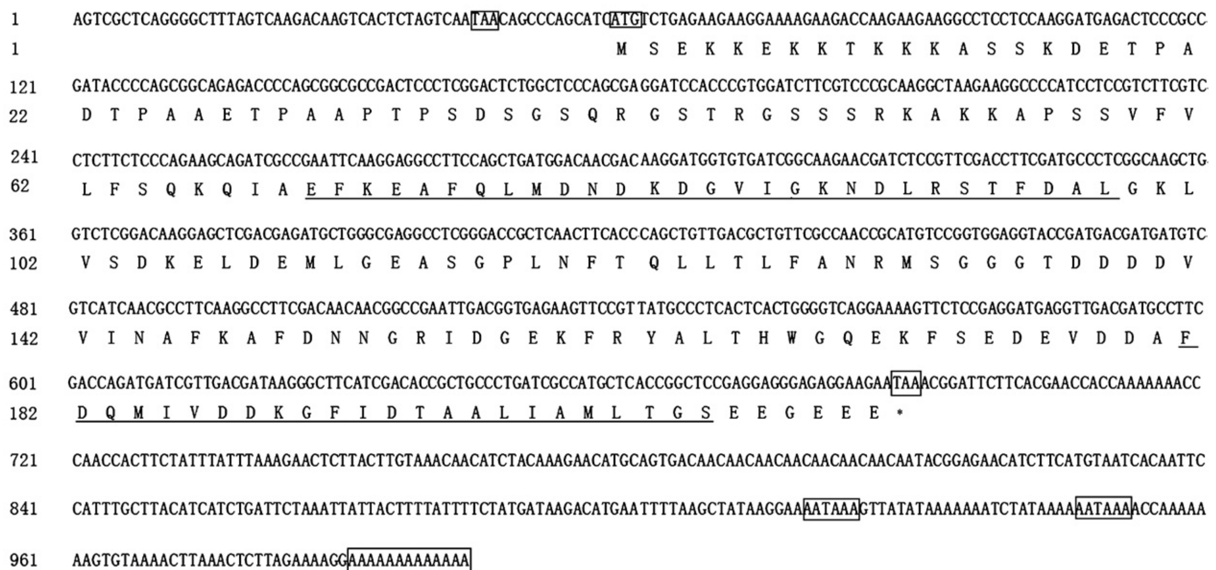

Fig. 1. The nucleotide and deduced amino acid sequence of *C. pipiens pallens MRLC*. The deduced amino acid sequence is presented below the nucleotide sequence in single letter code. The predicted "EF-hand" frames are underlined. The initial code "ATG", the termination codon "TAA", the tailing signals "AATAAA" and poly (A) in the 3′-untranslated region are in pane. The asterisk denotes the stop codon. GenBank accession no. DQ140391.

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