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Identification and characterization of a mitochondrial unfolded protein response transcription factor ATFS-1 in *Litopenaeus vannamei*

Yong-Gui Chen [a, c], Hai-Tao Yue [b, c], Ze-Zhi Zhang [b, c], Feng-Hua Yuan [b, c], Hai-Tao Bi [b, c], Kai Yuan [b, c], Shao-Ping Weng [b, c], Jian-Guo He [a, b, c], Yi-Hong Chen [a, c, *]

[a] Key Laboratory of Marine Resources and Coastal Engineering in Guangdong Province, School of Marine Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou, 510275, PR China
[b] School of Life Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou, 510275, PR China
[c] State Key Laboratory for Biocontrol, Sun Yat-sen University, 135 Xingang Road West, Guangzhou, 510275, PR China

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ABSTRACT

A mitochondrial specific stress response termed mitochondrial unfolded protein response (UPR^{mt}) is activated in responding to disturbance of protein homeostasis in mitochondria. The *activating transcription factor associated with stress-1* (designated as *ATFS-1*) is the key regulator of UPR^{mt}. To investigating the roles of *ATFS-1* (*LvATFS-1*) in *Litopenaeus vannamei* mitochondrial stress remission and immunity, it's full length cDNA was cloned. The open reading frame of *LvATFS-1* was 1, 557 bp in length, deducing to a 268 amino acids protein. *LvATFS-1* was highly expressed in muscle, hemocytes and eyestalk. Subcellular location assays showed that N-terminal of LvATFS-1 contained a mitochondrial targeting sequence, which could directed the fused EGFP located to mitochondria. And the C-terminal of LvATFS-1, which had a nuclear localization signal, expressed in nucleus. The *in vitro* experiments verified that LvATFS-1 could reduced the level of intracellular reactive oxygen species (*ROS*). And results of real-time RT-PCR indicated that LvATFS-1 might scavenge excess ROS via ROS-eliminating genes regulation. Reporter gene assays showed that LvATFS-1 could upregulated the expression of the *antimicrobial peptide* genes in *Drosophila* Schneider 2 cells. Results of real-time RT-PCR showed that *Vibrio alginolyticus* or white spot syndrome virus (WSSV) infection induced the expression of *LvATFS-1*. And knocked-down *LvATFS-1* by RNAi resulted in a higher cumulative mortality of *L. vannamei* upon *V. alginolyticus* or WSSV infection. These results suggested that LvATFS-1 not only rolled in mitochondrial specific stress responding, but also important for *L. vannamei* immunologic defence.

1. Introduction

Mitochondria are important organelles in eukaryotic cells, which works at cellular energy balance, Ca^{2+} homeostasis, and regulates apoptosis [1,2]. Mitochondrial protein homeostasis is essencial for mitochondria function [3]. To maintain mitochondrial protein homeostasis, the newly synthesized polypeptides asked for folding and assembling accurate in this organelle [4]. And mitochondrial chaperones play an important role in this process [5]. The expression of chaperones relates to the state of mitochondrial protein homeostasis [6]. And the maladjustment of mitochondria functions that caused by extracellular environment might result in the accumulation of unfolded proteins or misfolded proteins, which beyond the mitochondrial chaperone's capacity, and induce mitochondrial unfolded protein response (UPR^{mt}) [7]. UPR^{mt} then upregulates mitochondrial chaperones and other correlation factors to restoring mitochondrial homeostasis.

As reported, activating transcription factor associated with stress-1 (ATFS-1) worked at sensing of mitochondrial stress and mitochondrial–nucleus communication upon UPR^{mt} [8,9]. When mitochondria under homeostasis, ATFS-1 was imported into mitochondria, and degraded by special proteases. When mitochondria stress occurred, the mitochondrial import efficiency of ATFS-1 reduced, and a small percentage of ATFS-1 remained in the cytosol, and entered to the nucleus [8]. Then ATFS-1 bound to the promoter region of its target genes, and upregulated the transcription of these genes to restoring mitochondrial homeostasis

* Corresponding author. State Key Laboratory for Biocontrol, Sun Yat-sen University, 135 Xingang Road West, Guangzhou, 510275, PR China.
E-mail address: chenyh6@mail.sysu.edu.cn (Y.-H. Chen).

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through distinct ways, by enhancing the protein folding capacity, or scavenging excess ROS [10]. Recently, research also showed that ATFS-1 induced not only mitochondrial protective genes, but also immune genes, such as *lysozyme* and *antimicrobial peptides* (*AMPs*), and contributed to pathogen resistance in *Caenorhabditis elegan* [11,12].

Shrimp diseases were caused by multiple factors, such as bacteria infection, viruses infection or environmental stress [13]. As reported, in some other species, these factors all might lead to UPR^{mt} [14]. In this study, we cloned of the *LvATFS-1* in *Litopenaeus vannamei*. Reporter gene assay indicated that it upregulated *AMPs* in *Drosophila* Schneider 2 (S2) cells. Knocked-down LvATFS-1 resulted in cumulative mortality increasing upon white spot syndrome virus (WSSV) or *Vibrio alginolyticus* challenged. And it also rolled in eliminating reactive oxygen species (ROS). These results suggested that LvATFS-1 involved in both UPR^{mt} as well as immunologic defence in *L. vannamei.*

2. Materials and methods

2.1. Cloning of LvATFS-1 from L. vannamei

Based partial cDNA sequence of an *ATFS-1* in *L. vannamei* (Genbank associated No. FE111782) was retrieved from GenBank (http://ncbi.nlm.nih.gov). The full-length cDNA of *LvATFS-1* was further identified by rapid amplification of cDNA ends (RACE) PCR. The RACE cDNA templates were prepared using the BD SMART RACE cDNA Amplification Kit (Clontech, Japan). The *LvATFS-1* 5′RACE1, *LvATFS-1* 3′RACE1 together with Universal Primer Mix (UPM) were used for the first round RACE-PCR. The *LvATFS-1* 5′RACE2, and *LvATFS-1* 3′RACE2 primers together with UPM were used for the second round RACE-PCR. The primer sequences were listed in Table 1. The PCR protocol was: denaturation at 94 °C for 3 min, 7 cycles of 98 °C for 10 s, 68 °C for 30 s (decreasing by 1 °C per cycle), and 72 °C for 40 s, followed by 34 cycles of 98 °C for 10 s, 60 °C for 30 s, 72 °C for 40 s, and final extension at 72 °C for 5 min. The PCR products were cloned into pMD-18 vector (TaKaRa, Japan) and subsequently sequenced (ABI PRISM, Applied Biosystems, USA).

2.2. Bioinformatics analysis

Protein domains were predicted using the SMART program (http://smart.embl-heidelberg.de/). The mitochondrial targeting sequence was predicted with MITOPROT program (http://ihg.gsf.de/ihg/mitoprot.html). And the nuclear localization signal was predicted with PSORT II Prediction program (http://psort.hgc.jp/form2.html) [15].

2.3. Detection of the tissue distribution of LvATFS-1 in L. vannamei

Shrimps were sacrificed, and their hemocytes, hepatopancreas, gills, muscles, intestines, epidermis, eyestalks, stomach, pyloric caecum, nerves and heart were collected. Total RNA was extracted from the tissue samples and subsequently reverse transcribed into cDNA using PrimeScript™ RT reagent Kit With gDNA Eraser (Perfect Real Time) (TaKaRa). Real-time RT-PCR assays were performed at a volume of 10 μL comprised of 1 μL of 1:10 cDNA diluted with ddH_2O, 5 μL of 2 × SYBRGreen Master Mix (Takara, Japan), and 250 nM of each primer. The cycling parameters were 95 °C for 2 min to activate the polymerase, followed by 40 cycles of 95 °C for 15 s, 62 °C for 1 min, and 70 °C for 1 s. Cycling ended at 95 °C with 5 °C/s calefactive velocity to create the melting curve. Fluorescence measurements were taken at 70 °C for 1 s during each cycle. Expression levels of *LvATFS-1* were calculated using the Livak ($2^{-\Delta\Delta CT}$) method after normalization to *L. vannamei EF-1a* (GenBank accession no. GU136229).

2.4. Subcellular localization of LvATFS-1

Enhanced green fluorescent protein (*EGFP*) was cloned into pIZ-V5/His vector (Invitrogen, USA), named pIZ-EGFP. And then the *Caenorhabditis elegans* ATFS-1 (1–100aa, CeATFS-1N), LvATFS-1 (1–E90 aa, LvATFS-1N), LvATFS-1 (91–268 aa, LvATFS-1C) and the full-length of LvATFS-1 were cloned into pIZ-EGFP. Primers were listed in Table 1. S2 cells were transfected with the fusion plasmid pIZ-LvATFS-1N-EGFP, pIZ-LvATFS-1C-EGFP or pIZ-CeATFS-1N-EGFP for 48 h. For investigation the subcellular localization of LvATFS-1N, cells were washed twice with PBS, and stained with 200 nM MitoTracker® probe (Life Technologies, USA) for 15 min at 37 °C. Then the cells were washed twice with PBS and stained with 2 g/mL Hoechst 33258 (Beyotime, China) for 10 min at room temperature. After two further washes with PBS, coverslips were mounted on glass slides with 2 μL antifade solution (Beyotime, China). And for investigating the subcellular localization of LvATFS-1C, cells just stained with 2 g/mL Hoechst, but not MitoTracker® probe. The Fluorescent signals were examined by using a confocal laser scanning microscope (Leica TCS SP5).

2.5. Dual-luciferase reporter assays

Expression vector for the LvATFS-1 (91–268 aa, LvATFS-1C) was constructed using pAc5.1/V5-His B vector (Invitrogen, USA). The primers designed for LvATFS-1C were listed in Table 1. The PCR products were digested with the same restriction enzymes, and subsequently ligated into the expression vector. The reporter gene vectors, pGL3Metchnikowin (pGL3Mtk), pGL3Cecropin A (pGL3CecA) pGL3Attacin A (pGL3AttA), pGL3Penaeidin 4 (pGL3Pen4) and pGL3Drosomycin (pGL3Drs) were constructed by inserting the corresponding antimicrobial peptides genes promoter regions into the pGL3Basic. S2 cells were maintained, transfected and harvested as described before [16]. The firefly and renilla luciferase activities were measured using the Dual-luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. All assays were performed in three independent transfections.

2.6. Synthesis of double-stranded RNAs

The DNA templates of *LvATFS-1* were prepared by PCR using primers DsRNA-LvATFS-1-T7-F/DsRNA-LvATFS-1-R and DsRNA-LvATFS-1-F/DsRNA-LvATFS-1-T7-R; the DNA templates of *EGFP* were prepared by PCR using primers DsRNA-EGFP-T7-F/DsRNA-EGFP-R and DsRNA-EGFP-F/DsRNA-EGFP-1-T7-R (Table 1). Subsequently, the products were used as templates for the sense and antisense RNA strands, and subjected to *in vitro* transcription using T7 RiboMAX™ Express RNAi System (Promega) following the technique manual. After the *in vitro* transcription, the single-stranded RNA were subjected to annealing by mixing equal volumes of complementary RNA reactions together and incubating at 70 °C for 10 min, then slowly cool to room temperature (~20 min). Using the RNase Solution and RNase-Free DNase, we removed any remaining single-stranded RNA and the template DNA. Then the dsRNA were subjected to purification by adding 0.1 volume of 3 M Sodium Acetate (pH 5.2) and 2.5 volumes of 95% ethanol. Carefully pour off the supernatant, and wash the pellet with 0.5 ml of cold 70% ethanol. Air-dry the pellet for 15 min at room temperature, and resuspend the RNA sample in Nuclease-Free Water. The length of dsLvATFS-1 was 501 bp, and dsEGFP was 504 bp.