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2 Establishment of lysozyme gene RNA interference system and its
3 involvement in salinity tolerance in sea cucumber (*Apostichopus*
4 *japonicus*)

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ABSTRACT

The lysozyme gene was silenced using RNA interference (RNAi) to analyze the function of lysozyme in sea cucumber under salt stress. The interfering efficiency of four lysozyme RNAi oligos ranged from 0.55 to 0.70. From the four oligos, p-miR-L245 was used for further interfering experiments because it had the best silencing efficiency. Peristomial film injection of p-miR-L245 (10 μ g) was used for further interfering experiments. The lowest gene expression, determined by RT-PCR assay, in muscle, coelomic fluid, and parapodium occurred 48 h after p-miR-L245 injection, while that of body wall and tube foot was 96 h and 24 h, respectively. Lysozyme activity in muscle and body wall was significantly lower than the controls. The lowest lysozyme activity in muscle, body wall and parapodium, was found at 48, 72, and 48 h, respectively, which was consistent with the transcription expression of lysozyme. The lowest point of lysozyme activity was at 96 h in coelomic fluid and tube foot, which was laid behind lysozyme expression in transcription level. The expression profile of the lysozyme transcription level and lysozyme activity in the body wall and tube foot increased at 12 h after p-miR-L245 injection before the interference effect appeared. NKA gene expression was expressed at a high level in the positive control (PC) and negative control (NC) groups at 12, 24, and 48 h, while NKA was expressed at low levels in the lysozyme RNAi injection group at 12 and 24 h. The level of NKA gene expression recovered to the level of the PC and NC group at 48, 72, and 96 h after the lysozyme RNAi injection. NKCC1 gene expression was high in the PC and NC groups at 96 h, while the NKCC1 was expressed at a low level 96 h after lysozyme RNAi injection. The results suggest that lysozyme decay involves NKA and NKCC1 gene expression under salinity 18 psu. The K^+ and Cl^- concentration after lysozyme RNAi injection was lower than in the PC and NC group.

Keywords: Sea cucumbers, Lysozyme, RNA interference, Salt stress

1. Introduction

The sea cucumber *Apostichopus japonicus* is an important aquaculture species, which is widely distributed in coastal areas of tropical and subtropical oceans, including Japan, China, Korea, and Far Eastern Russia. Sea cucumber is considered to be one of the most valuable species in the aquaculture markets in East and Southeast Asian countries because of its potential as an edible delicacy as well as a traditional Chinese medicine [1, 2]. An increasing demand to supply the global market has led to the large-scale cultivation of sea cucumbers in northern China and Japan [2-4]. The sea cucumber in general can survive in a range of salinities (18–39 psu) and is cultured in the intertidal zone. It is often exposed to a large fluctuation of salinity in its natural habitat due to water exchange, evaporation and precipitation [5, 6]. The optimal salinity for *A. japonicus* growth is reported as 29–32 psu and the best survival rates of sea cucumber juveniles are observed in salinities of 32 psu [7]. Sharp salinity changes may affect the feed intake and higher energy utilization for osmoregulation resulting in poor sea cucumber growth, and can even lead to intestine discharge and death.

Lysozyme (LYZ, EC3.2.1.17), also known as muramidase or N-acetylmuramide glycanhydrolase, is an alkaline globulin named by Fleming in 1922 [8]. It is a member of the antibacterial peptide family, which is widely distributed in a range of phylogenetically diverse biota from bacteriophage to humans [9, 10]. It has been proposed that lysozymes of marine invertebrates have a wider range of activities than those of terrestrial invertebrates in order to cope with a greater range of bacterial strains and species in the marine environment [11]. Studies show that transcripts of immune-related shrimp genes are affected by environmental stressors [12]. Significant up-regulation of lysozyme was observed in shrimp in response to stress with the highest levels in gill tissues [13]. Studies have indicated that the genes of immune-related proteins in the shrimp, *Penaeus monodon*, such as crustin-like antimicrobial peptide, antiviral and antimicrobial peptides, lysozyme, serine protease, anti-lipopolysaccharide factor, RIG-I C-terminal domain-containing protein, C type lectin 4, mucin and peroxidase are differentially expressed in low salinity stress conditions [14]. Lysozyme gene transcription was found to be stimulated in biopsies of residuary gill filaments from Atlantic salmon in seawater compared with freshwater, which also indicates that salinity has an effect on the expression of lysozyme [15].

Previously, we cloned and sequenced cDNA of c-type lysozyme, constructed a recombinant expression vector and analyzed its expression in various tissues [7]. The results showed significant up- and down-regulation in transcription-level expression of lysozyme in different tissues of sea cucumber in response to salinity stress condition. The expression profiles in sea cucumbers indicated that salinity stress can affect lysozyme expression as a non-specific immune factor. The sea cucumber lacks a specific organ to adapt to sharp salinity changes, so its immune system and immune-related genes and proteins must cooperate to keep homeostatic balance. Like other invertebrates, sea cucumbers lack an adaptive immune system and rely on various innate immune responses [16], which trigger diverse humoral and cellular activities via signal transduction pathways [17]. Similar results were reported in *Eriocheir sinensis* and white shrimp under salinity stress [18]. RNA interference (RNAi) is a powerful genetic tool that has been used in numerous studies to knockdown a gene of interest to better understand gene function and is a powerful technique for screening whole genomes [19]. Consequently, to further understand how lysozyme is involved in the mechanism of salinity adaption in sea cucumber, potential RNAi sequences directed against lysozyme mRNA were designed using the Invitrogen Stealth™ RNAi BLOCK-iTTM online program. This study optimizes the dosage and position of RNAi for the interference and detection of lysozyme expression in salinity stress. In order to detect how lysozyme is involved in salinity tolerance, Na^+/K^+ -ATPase gene expression and enzyme activities after p-miR-L245 injection and Na-K-Cl cotransporter 1 (NKCC1) gene expression and Na^+ , K^+ , Cl^- ion concentrations were evaluated. The results supplied the technical support for the study of lysozyme function in the salinity adaptive system of sea cucumbers.

2. Materials and Methods

2.1 Sea cucumbers RNA interference

A. japonicus, with an average body wet weight of 21.66 ± 2.5 g (mean $\pm$ SE), were collected from Wafangdian Aquatic Farm, Dalian, P.R. China. The sea cucumbers were acclimated in three aquariums (900×750×600 mm) in Dalian Ocean University, Key Laboratory of Mariculture & Stock Enhancement in North China Sea, Ministry of Agriculture. Sea cucumbers used in the experiment were divided into two groups according to different salinity conditions (32 psu and 18 psu). Tap water was dechlorinated before use in the experiments by aerating for at least 24 h to

allow the chlorine to evaporate from the water. Low salinity level was achieved by reducing the salinity of sea-water 2 psu per day by adding freshwater. For high salinity levels, seawater crystals were added until the correct salinity was reached. Control animals were cultured at a salinity of 32 psu. One-half or two-thirds of the rearing water and sediment (faeces and uneaten food) were changed daily and remained stable at the required salinity during the acclimation period. During the experiment, sea cucumbers were fed once per day, at the rate of 3–5% body mass with a laboratory made formulated diet (a solution mixed from equal parts of *Sargassum thunbergii*, fish meal and sea mud). The salinity and other water parameters were measured using YSI multi-parameter water quality monitor (YSI incorporated, Ohio, USA). Throughout acclimation, temperature, pH and dissolved oxygen were measured as 24–25°C, 7.92–8.29, 3.76–5.56 mg/L, respectively.

2.2 Design and synthesis of oligo DNA sequence of *Lyz*

According to the design rule for RNAi, potential RNAi sequences directed against sea cucumber c-type Lysozyme mRNA (GenBank: KF773759.1) were designed using the Invitrogen Stealth™ RNA interference BLOCK-iTTM online program (<https://rnaidesigner.lifetechnologies.com/rnaiexpress/rnaiDesign.jsp>). Each RNAi sequence consisted of a 64-nucleotide oligo DNA sequence. Four different sets of RNAi sequences, including RNAi-1, 2 3, and 4 were synthesized by Shanghai Invitrogen Company (China), according to the sequences shown in Table 1. One negative sequence was synthesized to be used as a negative control (NC) RNAi (Table 1).

2.3. RNA interference vector and efficient expression vector construction and verification

The RNAi vectors were constructed and verified by annealing, connection, transformation, and sequencing. The oligo single-strand DNA annealing system contained 5 µl of top strand oligo and bottom strand oligo, 2 µl 10× oligo annealing buffer, and 8 µl ddH₂O. The annealing mixture were incubated at 95°C for 5 min, and then placed at room temperature for 20 min to form double-stranded DNA. The double-stranded DNA was annealed for 30 min. The connection system included 4 µl 5× ligation buffer, 2 µl pcDNA6.2-GW/EmGFP, 4 µl ds oligo (10 nM), 1 µl T4 DNA ligase (1 U/µl), and 9 µl ddH₂O. The connection products were transformed to DH5α

and incubated in LB medium at 37°C. The transformation product was amplified at 94°C for 5 min; 94°C for 30 s, 56°C for 30 s, 72°C for 40 s; 72°C for 7 min. The reaction system was 1 µl 10× PCR buffer, 1 µl dNTPs, 0.1 µl Taq DNA Polymerase, 0.5 µl transformation product, 0.5 µl M13F (-47)/M13R (-48), and 6.4 µl ddH₂O. In order to verify the sequence and RNAi vector, positive clones were sequenced by Invitrogen (Shanghai, China). The RNAi vectors were named p-miR-L92, p-miR-L245, p-miR-L473, p-miR-L586, and p-miR-Negative.

Table 1

RNA interference sequences of lysozyme in sea cucumbers.

Oligo name	Oligo DNA sequence 5'-3'
12MR0080-1F	TGCTGTACATTGTCCAGTACAACCGTGT TTTGGCCACTGACTGACACGGTTGTTGGACAATGTA
12MR0080-1R	CCTGTACATTGTCCAACAACCGTGT CAGTCAGTGGCCAAAACACGGTTGTACTGGACAATGTAC
12MR0080-2F	TGCTGTACAATGACCAACTGAACCAGGTT TTTGGCCACTGACTGACCTGGTTCATGGTCATTGTA
12MR0080-2R	CCTGTACAATGACCATGAACCAGGTCAGTCAGTGGCCAAAACCTGGTTCAGTTGGTCATTGTAC
12MR0080-3F	TGCTGAAGAGTCGGAGTTAATCCCGT TTTGGCCACTGACTGACGGGATGTTCTCCGACTCTT
12MR0080-3R	CCTGAAGAGTCGGAGAATCCCGTCAGTCAGTGGCCAAAACGGGATGTTAACTCCGACTCTTC
12MR0080-4F	TGCTGTGTCCTTTCAGCACACTCCTGGT TTTGGCCACTGACTGACCAGGAGTGCTGAAAGGACA
12MR0080-4R	CCTGTGTCCTTTCAGCACACTCCTGGTCAGTCAGTGGCCAAAACAGGAGTGCTGAAAGGACAC
Negative-F	tgctgAAATGTACTGCGCGTGGAGACG TTTTGGCCACTGACTGACGTCTCCACGCAGTACATT
Negative-R	cctgAAATGTACTGCGTGGAGACGT CAGTCAGTGGCCAAAACGTCTCCACGCAGTACATTc

An efficient expression vector of lysozyme was also constructed with synthetic target sequence and the pMD18-T vector (TaKaRa, Japan). The target sequence lysozyme and pMD18-T vector were digested with *Hind*III/*Bam*HI restriction enzymes. T4 DNA ligase was used to ligate the target sequence lysozyme into the vector. The ligation product was transformed into DH5α and incubated in LB medium at 37°C. In order to verify the sequence and efficient vector, positive clones were sequenced by Invitrogen. The vector was named pcDNA3.1(+)-12GS0234.

2.4. RNAi vector and efficient expression vector transfection of target cells HEK293

Verified RNAi vectors were purified according to the instructions of a high-purity plasmid extraction kit (Invitrogen). HEK293 logarithmic growth phase cells were digested with 0.05%

trypsin and a single-cell suspension was grown to above 90% viability to carry out transfection experiments. The cells were lightly washed twice with serum-free Opti-MEM and then added to 1.5 ml Opti-MEM. The RNAi plasmids and negative control plasmid were diluted with 250 μ l Opti-MEM, and then 1 μ g high-expression vector was added. Lipofectamine 2000 reagent was also diluted with Opti-MEM and incubated for 5 min. The diluted RNAi plasmid and lipofectamine 2000 diluent were placed at room temperature for 20 min to form plasmid-lipofectamine compounds. The plasmid-lipofectamine compounds were added to cell wells and cultured for 24 h. A plasmid containing fluorescent protein gene was observed in order to verify that the HEK293 cells included efficient RNAi plasmid. To confirm the entry of RNAi into the cell, all samples were observed by fluorescence microscopy (BA400; Motic, China).

2.5. RNAi vector interference effect by qPCR in cells

Total RNAs were extracted from cells 48 h after transformation by transferring the collected tissue samples into a clean sterile tube containing 300 μ l RL buffer/30 mg of tissue. Then, total RNA was extracted by using a RNeasy pure Tissue Kit (Qiagen Biotech Co., China) according to the manufacturer's instructions. RNA concentration and integrity were estimated by NV3000 Spectrophotometer (SPECTRO, Germany). Then, total RNA was reverse transcribed using a PrimeScriptTM Real time PCR Kit (TaKaRa, Japan) for real time quantitative PCR (qRT-PCR) analysis. A pair of specific primers lyz-RT-F and lyz-RT-R (Table 2) was used to amplify a PCR product of 288 bp. In addition, cytb-F-real and cytb-R-real (Table 2) were used as control to amplify a 90 bp fragment. The RT-PCR was carried out in a total volume of 20 μ l, containing 10 μ l SYBR Premix Ex Taq (2 $\times$), 0.8 μ l each of lyz-RT-F (10 μ mol/L) and lyz-RT-R (10 μ mol/L) primer, 0.4 μ l ROX reference Dye II, 2 μ l 1:2 diluted cDNA, and 6 μ l DEPC-treated water. The PCR program was 95°C for 30 s, then 40 cycles of 95°C for 5 s and 60°C for 32 s, followed by 1 cycle of 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 65°C for 15 s. The cytb-F-real and cytb-R-real primers of a control gene for the replacement of specific primers were used as reference experiment. qRT-PCR data were analyzed with the ABI 7500 system SDS software (Applied Biosystems, USA). The comparative C_T method was used to analyze the relative expression level of LYZ. Using a transfection negative interference sample as control, gene expressions were analyzed and the interference effects were calculated. SPSS software (Ver 13.0)

was used to analyze the statistical data using one-way ANOVA and a $P < 0.05$ was significant.

Table 2

Primer sequences used in this study.

Primer	Sequence (5'-3')
lyz-RT-F	CAGCCTATCAGGAGAATGCGTGC
lyz-RT-R	CCGGTTGGTTTATCGAAACAACA
cytb-F-real	TGAGCCGCAACAGTAATC
cytb-R-real	AAGGGAAAAGGAAGTGAAAG

2.6. Optimization of injection position and the dosage of RNA interference for interference in sea cucumbers

To confirm the efficiency of the synthesized RNAi and to optimize the injection position, sea cucumbers used in the experiment were divided into two groups. The injection positions were in the body cavity and peristomial film. At 48 h after injection, muscle, coelomic fluid and tube foot of six sea cucumbers were collected for further RNA extraction. Different injection dosages were also optimized, so sea cucumbers used in the experiment were divided into five groups for optimizing the injection dosage including 0.5, 5, 10, 25, and 50 μg . Negative RNAi vectors were used as control. Three replications were set for each dosage. At 48 h after injection, muscle, coelomic fluid, and tube foot of six sea cucumbers were collected and frozen in liquid nitrogen for total RNA extraction. The relative expression level of lysozyme after RNAi injection was detected following the method described in Section 2.5.

2.7. Detection of lysozyme expression after p-miR-L245 interference

The sea cucumbers were injected with a 10- μg optimal dosage in the optimal position of peristomial film to detect the expression of lysozyme after p-miR-L245 injection. The sea cucumbers were collected at 12, 24, 48, 72, and 96 h after the p-miR-L245 injection under optimum salinity (32 psu). The negative controls were also sampled. The muscle, coelomic fluid, body wall, parapodium, and tube foot of six sea cucumbers were collected and frozen in liquid nitrogen for total RNA extraction and measurement of enzymatic activity. Parts of the

samples were used for total RNA extraction and cDNA synthesis as described in Section 2.5. Quantitative real-time PCR was used to test the relative expression level of p-miR-L245. The other tissues were used to measure lysozyme activity. The lysozyme activity of different tissues was measured using a biochemistry assay kit that was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.8. Detection of salinity tolerance after p-miR-L245 interference under salt stress

Based on preliminary experiments of sea cucumbers adaptation to salinity, two extreme salinities were selected (18 and 32 psu) in order to detect lysozyme involvement in salinity tolerance after p-miR-L245 interference. The lysozyme gene expression, Na^+/K^+ -ATP gene and NKCC1 gene expression, Na^+/K^+ -ATPase activity, and Na^+ , K^+ , Cl^- concentrations in coelomic fluid after p-miR-L245 interference under salt stress were detected. The groups included sea cucumbers in a control group (32 psu) and the aimed salinity (18 psu) sea water with a negative control for miR-L245. Each treatment group contained 30 sea cucumbers. After being transferred to pre-prepared seawater at 18 psu, three randomly selected sea cucumbers from each group were sampled at 0, 12, 24, 48, 72, and 96 h, respectively.

3. Results

3.1 Confirmation of RNA interference entry into cells

The positive clones of RNAi vector and efficient expression vector were sequenced to ensure that the lysozyme RNAi recombinant plasmid had been successfully constructed. The RNAi was treated with pcDNA6.2-GW/EmGFP vector with labelled green fluorescent protein, which was tested with fluorescence microscopy to ensure that the RNAi had been successfully introduced. Fluorescent images were taken 24 h after transformation in combination with light microscopy (Fig. 1). They showed that all cells treated with the various concentrations of RNAi had been successfully transfected by RNAi (Fig. 1).

233 **Fig. 1. Fluorescent images at 24 h after transformation by RNAi.** (A) HEK293 cells without transfection, (B)
234 EGFP expression of recombinant plasmid p-miR-L92, (C) p-miR-L245, (D) p-miR-L473, (E) p-miR-L586, and (F)
235 p-miR-Negative after transfection in HEK293 cells.

236 *3.2 The interference effect of RNAi in cells measured by qRT-PCR*

237 Using a transfection negative interference sample as a control, gene expressions were analyzed
238 and the interference effects were calculated. The expression of lysozyme was quantified by
239 qRT-PCR in the treated RNAi strains silenced by the specific RNAi and compared with those of a
240 NC and a positive control (PC, untreated strains). Four recombinant RNA interference plasmids
241 were successfully imported to cells. Target gene knockdown was clearly observed with the
242 gene-specific RNA interference, but there was no considerable change in lysozyme gene
243 expression following treatment with the p-miR-Negative (Table 3). The interfering efficiency of
244 four recombinant RNA interference cells ranged from 0.55 to 0.70, respectively, compared with
245 the untreated and scrambled RNAi-NC cells. The expression level of lysozyme in sea cucumbers
246 injected with p-miR-L473 showed the lowest level compared with p-miR-L92 and p-miR-L586
247 treatments. The p-miR-L245 showed the best silencing efficiency among these treatments.
248 Therefore, p-miR-L245 was used for further interfering experiments (Table 3).

249 **Table 3**

250 The silence efficiency of target genes with transfection by qRT-PCR detection.

RNA	Ct	ΔCT	$\Delta\Delta CT$	$2^{-\Delta\Delta CT}$	interfering efficiency
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interference					
name					
p-miR-L92	12.95	-7.37	1.39	0.38	0.62
p-miR-L245	13.40	-7.00	1.75	0.30	0.70
p-miR-L473	13.04	-7.61	1.14	0.45	0.55
p-miR-L586	13.28	-7.43	1.32	0.40	0.60
p-miR-Negative	11.96	-8.75	0.00	1.00	
HEK293	27.45				

251

252 3.3 Optimizing inhibitory concentration and position

253 The expression levels of lysozyme in sea cucumber injected with p-miR-L245 at different
254 positions are shown in Fig. 2. The results show lysozyme-specific RNAi affected by injection
255 position. Compared with body cavity and peristomial film injection, the expression of lysozyme in
256 the treated RNAi strains had different silencing efficiency. The results indicated that the
257 expression of lysozyme in injected peristomial film showed better silencing efficiency in three
258 tissues ($P \leq 0.05$). Therefore, peristomial film injection was used for further interfering experiments.
259 The comparison of lysozyme transcription levels in sea cucumbers from five groups at different
260 concentrations is shown in Fig. 3. The diagram shows that lysozyme-specific RNAi reduced the
261 expression of the lysozyme gene in a manner directly related to RNAi concentration. The
262 expression levels of lysozyme in the 10 μg RNAi group were significantly lower than that of 0.5
263 μg , and 5 μg concentration group in different tissues ($P \leq 0.05$) (Fig.3). No significant difference
264 was found among the 10, 25, and 50 μg groups. Therefore, 10 μg RNAi concentrations were used
265 as optimal dosage for further interfering experiments.

266

Fig. 2. The relative expression levels of lysozyme after p-miR-L245 is injected at different positions.

Fig. 3. The relative expression levels of lysozyme with different concentrations of p-miR-L245.

3.4 Effect of p-miR-L245 on lysozyme expression and enzyme activity under normal salinity

The sea cucumbers were injected with an optimal dosage 10 μg of p-miR-L245 and in an optimal peristomial film position to detect the expression of lysozyme. The expression of lysozyme was quantified by RT-PCR in the silenced strains by the specific p-miR-L245 and compared with those of the NC and PC. The expression of lysozyme after p-miR-L245 injection in sea cucumbers is shown in Fig. 4. Different tissues showed different silencing efficiency after p-miR-L245 injection, respectively. The expression of lysozyme in muscle and in coelomic fluid maintained a reduced silencing effect during the 96-day experiment. The best silencing effect of lysozyme appeared at 72 h in coelomic fluid, while that in muscle was at 48 h. The expression profile in body wall indicated decay expression level except at 12 h. The best silent effect in all five tissues was at 48 h after p-miR-L245 injection. The silencing effect in parapodium and in the tube foot weakened at 96 h. These results indicate that the interference efficiency of RNAi may be related to tissue specificity and the best interference effect was in the muscle and in coelomic fluid of sea cucumbers. The best interference time appeared at 48 h after interference.

In order to know the expression of lysozyme at the protein level, lysozyme activity analysis was performed (Fig. 5). On the whole, the lysozyme activities were reduced by RNAi introduction. Especially, lysozyme activities in the muscle and body wall were significantly lower than the NC and PC. The lowest points of lysozyme activities were at 48 h in the muscle, 72 h in the body wall and 48 h in the parapodium. The lowest transcription expression of lysozymes was present at 48 h in the muscle, 72 h in the body wall, and 48 h in parapodium (Fig. 4). The results indicated that p-miR-L245 interference effects the time of lysozyme translation levels almost in accordance with the transcription levels in muscle, body wall, and parapodium. The lysozyme activities in different tissues show different profiles. The lysozyme activities in coelomic fluid and parapodium were significantly lower than the control groups during the whole experiment. Lysozyme activity decreased obviously in coelomic fluid during the 96-h experiment; the lowest point appeared at 96 h, which was the time point that it obviously lagged behind the expression of lysozyme RNA (Fig. 4). The lowest lysozyme activity points ranged from 24 h to 96 h in different tissues, which indicated that the optimal interfering time of lysozyme were 24–96 h in sea cucumbers translation levels.

Interestingly, the expression profile of lysozyme transcription levels in the body wall and tube foot increased at 12 h after p-miR-L245 injection before the interference effect appeared. An increasing situation was also found in lysozyme activity. The lysozyme activities after p-miR-L245 injection in four tissues increased at 12 h and subsequently maintained at a low level. The lysozyme activities in muscle, body wall, and tube foot increased at 12 h after p-miR-L245 injection before the interference effect appeared. These results indicate that the lysozyme activity changes in the translation level were almost consistent to the expression of lysozyme in the transcription level after RNAi. MicroRNAs of lysozyme oscillate between repression and activation in the transcription and translation levels in these tissues.

Fig. 4. The relative expression profiles of lysozyme by qRT-PCR in different tissues after specific p-miR-L245 decay

Fig. 5. The lysozyme activity in different tissues after specific p-miR-L245 decay

3.5 *NKA, NKCC1 gene expression and ion concentration after lysozyme p-miR-L245 decay*

In order to detect lysozyme activity involved in salinity tolerance, Na⁺/K⁺-ATPase gene expression and enzyme activities after p-miR-L245 injection and Na-K-Cl cotransporter 1 (NKCC1) gene expression, Na⁺, K⁺, Cl⁻ ion concentration were evaluated under a salinity of 18 psu. The NKA, NKCC1 gene expression and ion concentration after lysozyme p-miR-L245 decay is shown in Figures 6–10. The lysozyme gene expression maintained a low level during the experiment 96 h under a salinity of 18 psu (Fig. 6). NKA gene expression was at high level in PC and NC groups at 12, 24, and 48 h, while NKA showed a lower level of expression in the lysozyme RNAi injection group at 12 and 24 h. The level of NKA gene expression recovered to the level of the PC and NC group at 48, 72, and 96 h in the lysozyme RNAi injection group. The results suggest that NKA gene expression was affected by lysozyme interference, especially at 12 and 24 h. The lysozyme gene expression maintained a low level during the 96-h experiment under a salinity of 18 psu. NKCC1 gene expression was higher in the PC and NC group at 96 h, while NKCC1 had low levels of expression in the lysozyme RNAi injection group at 96 h (Fig. 7). The results suggest that NKCC1 gene expression was significantly affected by lysozyme decay.

The Na⁺ concentration in the lysozyme RNAi injection group had no significant effect in the PC and NC group (Fig. 8). The K⁺ concentration and Cl⁻ in the lysozyme RNAi injection group was lower than in the PC and NC group (Figs. 9 and 10). The lowest K⁺ concentration in the lysozyme RNAi injection group was at 24 h, while the lowest Cl⁻ concentration in lysozyme RNAi injection group was at 12 h.

Fig. 6. The relative expression level of the NKA and lysozyme genes after p-miR-L245 in coelomic fluid under salinity 18 psu

Fig. 7. The relative expression level of NKCC1 and lysozyme gene after p-miR-L245 in coelomic fluid under salinity 18 psu

Fig. 8. The Na⁺ concentration after p-miR-L245 in coelomic fluid under salinity 18 psu

Fig. 9. The K⁺ concentration after p-miR-L245 at coelomic fluid under salinity 18 psu

Fig. 10. The Cl^- concentration after p-miR-L245 at coelomic fluid under salinity 18 psu

4. Discussion

Over the past decade, high-throughput transcriptome analysis has revealed that about 90% of the human genome is actively transcribed, with protein-coding genes accounting for only around 2% of the human genome [20]. It is estimated that the expression of up to 60% of all protein coding genes are under the control of RNA interferences [21]. The RNA interferences are short RNA sequences that negatively manipulate gene expression by pairing with partially or fully complementary target sequences of mRNAs to accelerate mRNA degradation and prohibit translation. These RNA interferences are involved in various biological processes, such as cell proliferation, development, differentiation and metabolism [22, 23]. Now, RNAi is a powerful genetic tool that has been used in numerous studies to knockdown genes of interest. New RNAi-based approaches for gene-specific silencing are being used to better understand gene function as a powerful technique for screening whole genomes. RNAi methodology and application has been reported for several aquatic animals, including *Pacific white shrimp*, red swamp crayfish, zebrafish, grass carp, yellow croaker, tongue sole, and sea cucumbers [24-32]. Accumulative evidence has indicated that RNA interferences modulate target gene expression at the mRNA or protein level, and a given mRNA can be targeted by several RNA interferences.

370 However, scarce information is available concerning the modulation of a specific target through
371 different RNA interferences at different levels, particularly in non-model invertebrates.

372 On the basis of these studies, we used RNAi to silence the lysozyme gene in sea cucumbers in
373 order to determine the precise function of lysozyme. The lysozyme expression and activities were
374 significantly reducing by RNAi-mediated gene silencing in sea cucumbers (Figs. 4 and 5). Some
375 studies indicate that the antimicrobial peptide (AMP) gene expression of LvIAP1, LvIAP2, and
376 LvIAP3 were significantly knocked down by dsRNA-mediated gene silencing [33] (Wang et al.,
377 2013). In the gills of LvIAP1- or LvIAP3-silenced shrimp, the expression of WSSV VP28 was
378 significantly higher than that of the dsGFP control group, suggesting that LvIAP1 and LvIAP3
379 may play protective roles in host defense against WSSV infection [34]. Dong et al. (2011) [25]
380 reported that RNAi experiments demonstrated that silencing peroxinectin significantly reduced the
381 survival rate of red swamp crayfishes after challenge with *Aeromonas hydrophila*, which indicated
382 that peroxinectin was important for *Procambarus clarkii* to survive *A. hydrophila* infection. Zhang
383 et al. (2015) [32] observed that both miR-137 and miR-2008 targeted and modulated the
384 transcription and translation of this gene, respectively. With regards to these results, RNA
385 silencing technology could be used as an efficient means to study gene function and improve the
386 immunity capacity of sea cucumbers.

387 The results showed that there was a knockdown of lysozyme gene expression at all tested
388 concentrations of gene-specific RNAi (Fig. 3). Furthermore, comparison among the different
389 concentrations tested showed that there was less down-regulation at 0.5 and 5 µg, and greater
390 down-regulation at 10, 25, and 50 µg, the effect was statistically significant. Therefore, the extent
391 of down-regulation of lysozyme was dependent on the applied concentration of gene-specific
392 RNAi. One study found that the extent of cyp51A down-regulation was dependent on the applied
393 concentration of gene-specific siRNA and ranged from a 4-fold reduction in gene expression at 15
394 nM to a 10-fold reduction at 50 nM, compared with the expression level of the untreated positive
395 control [35]. Another study indicated that hairpin RNA-induced gene silencing was a valuable
396 genetic tool for functional analysis of essential genes in several species so that FgCYP51A gene
397 expression was reduced by approximately 96% in FgCYP51A in comparison to wild-type after
398 treatment with sterol demethylation inhibitor fungicides [36]. These studies showed that RNA
399 interferences at very low concentrations are powerful agents for mediating gene silencing, and this

might be due to the post-transcriptional degradation of the target mRNA [37].

Recent reports have shed more light into the complex mechanisms regulating RNAi function on target mRNAs. The expression profile of lysozyme in the body wall and tube foot increased at 12 h before the p-miR-L245 interference effect appeared; lysozyme activity also increased. The lysozyme activity in muscle, body wall, and tube foot also increased at 12 h after p-miR-L245 injection before the interference effect appeared (Figs. 4 and 5). Vasudevan et al. (2007) [38] also reported that microRNA can upregulate translation upon growth arrest conditions. Zhang et al. (2014) [30] performed iTRAQ-based proteomics analysis of pathogen-challenged sea cucumbers, showing that the inhibition of miR-137 increased the mRNA and protein expression levels of the target gene. Betaine-homocysteine S-methyltransferase increased at 24 h post-infection but decreased at 48 and 96 h post-infection. Some reports also indicate that the deregulation of RNAi expression can be a result of increased or decreased transcription due to an altered transcription factor activity: microRNA can indeed be either positively or negatively regulated by transcription factors [39]. More recently, some RNA interferences were reported to activate mRNA translation [40-43]. These results were consistent with our results. Related phenomena have also been observed in immature oocytes where the RNAi machinery is either in an inactive state [44] or even stimulates translation of target transcripts [45]. The authors thought that RNAi oscillates between repression and activation in coordination with the cell cycle [38]. Vasudevan et al. (2012) [46] thought RNA interferences have also been implicated in enhancing translation under specific cellular conditions, besides their primary functions in translational repression. The opposite function of RNA interferences has not been widely appreciated, partly because the mechanism involved has remained largely unclear [38]. We speculated that lysozyme RNAi shifts between repression and activation in transcription expression level and lysozyme activities may be related to an RNA interference role in response to organizational structure or cells. Another possibility was relative to the lysozyme function in the sea cucumbers immune system. The innate immune system is the first line of defense of sea cucumbers and lysozyme is an important member of the composition of sea cucumber innate immunity, which plays an important role in resistance to many kinds of pathogens and adverse environments. When p-miR-L245 plasmids are injected into sea cucumbers, the innate immunity of sea cucumber might need to mobilize the lysozyme to deal with the stimulation from the exterior. So, this situation resulted in the transcription expression

and enzyme activity of lysozyme increasing after RNAi. These speculations need further investigation in our future work.

The RNAi biogenesis and function are more complex than previously expected. The lowest transcription expression of lysozymes was found at 48 h in muscle, 4 days in the body wall, and 48 h in parapodium (Figs. 4 and 5). The lowest point of lysozyme activities was found at 48 h in the muscle, 96h in the body wall and 48 h in parapodium. These results indicate that p-miR-L245 interference effects the time of lysozyme translation levels almost in accordance with the transcriptions level in muscle, body wall, and parapodium. The lysozyme RNA interference in the body wall, muscle and parapodium play a role without complex processing in transcription and translation. This simultaneous regulation might represent a highly efficient mechanism for the tight regulation of target proteins. The lowest point of lysozyme activities was found at 96h in coelomic fluid and tube foot, which was laid behind lysozyme expression in transcription level. Generally, the RNA interferences repress target gene expression in two main ways, mRNA degradation and translation inhibition. The mRNA degradation occurs through RNA interference-guided transcript cleavage in plants and deadenylation followed by mRNA decay in animals [47]. Translation inhibition by RNA interferences is poorly understood in plants and may entail multiple mechanisms in animals [48]. For animal RNA interferences, the degrees of contribution by the two modes of action to target gene regulation have been controversial [47]. Recent studies that examined the translation inhibition and mRNA decay activities of zebrafish and *Drosophila* RNA interferences with temporal resolution concluded that translation inhibition precedes target mRNA decay [49, 50]. The mechanistic details of RNA interference's function in repressing protein synthesis are not well understood. In addition, the results from the studies conducted in different systems have often been contradictory. It is difficult to conclude whether the reported discrepancies are artifacts of different experimental approaches or whether RNA interferences are indeed able to exert their repressive effects by disparate mechanisms [51-54]. From these results, we concluded that lysozyme gene RNA interference in sea cucumbers probably possessed different RNA interference processing in different tissues. Hence, it may be necessary to perform more experiments on the relationship between RNA interference and target genes.

In order to detect lysozyme involved in salinity tolerance, Na^+/K^+ -ATPase gene expression and

enzyme activities after p-miR-L245 injection and NKCC1 gene expression, Na^+ , K^+ , Cl^- ion concentration were evaluated under a salinity of 18 psu. Lysozyme gene expression maintained a low level during the 96-h experiment under this salinity (Fig. 6). NKA gene expression was higher in the PC and NC group at 12, 24, and 48 h, while NKA showed a low level in the lysozyme RNAi injection group at 12 and 24 h. The NKA gene expression after p-miR-L245 was significantly lower than PC and NC groups. The results suggest that NKA gene expression was affected by lysozyme interference, especially at 12 and 24 h. NKCC1 gene expression was high in the PC and NC group during the 96 h, while NKCC1 expression levels were low in the lysozyme RNAi injection group at 96 h (Fig. 7). These results suggested that the NKCC1 gene expression was significantly affected by lysozyme decay. The results indicate lysozyme may play a role in the salinity stress of sea cucumbers. It has been proposed that lysozymes of marine invertebrates have a wider range of activities than those of terrestrial invertebrates in order to cope with a greater range of bacterial strains and species in the marine environment [55]. The results showed that the lysozyme gene was significantly regulated in transcription-level expression in different tissues of sea cucumber in response to 72 h post salinity stress [7]. Studies showed that transcripts of immune-related shrimp genes can be affected by environmental stressors [56]. Significant up-regulation of lysozyme was observed in the gill tissues of shrimp [13]. Some studies indicated that immune-related proteins in the shrimp, such as crustin-like antimicrobial peptides, antiviral genes, antimicrobial peptides, relish, lysozyme, serine protease, anti-lipopolysaccharide factor, RIG-I C-terminal domain-containing protein, Ctype lectin 4, mucin and peroxidase genes were identified to be related to low salinity stress conditions. Lysozyme activity increased in eastern oysters as the NaCl concentration was increased from 0 to 0.1 M and then decreased from its maximum activity at 0.1 M NaCl with further increases in NaCl concentrations [14]. Lysozyme gene transcription in Atlantic salmon was stimulated in the residuary gill filaments after biopsy in both freshwater and seawater, which also indicated that salinity affected the expression of lysozyme [57]. A study in Nile tilapia suggested that environmental salinity also affects the amount of plasma lysozyme that can be detected [58], which indicates salinity can affect immune-related lysozyme expression. The sea cucumber lacks a specific organ to adapt to sharp salinity changes, so it needs to promote the cooperation of the immune system and immune-related genes and proteins for keeping homeostatic balance.

The level of NKA gene expression recovered to the level of the PC and NC groups at 48, 72, and 96 h in the lysozyme RNAi injection group (Fig. 6). The results infer that the expression of NKA may rebound after the inhibition of NKA by lysozyme RNAi or the suppression of the lysozyme gene is counteracted by the demand for NKA during salinity stress. Further experiments are needed to study the problem.

4. Conclusion

RNAi technology is an endogenous process for the post-transcriptional regulation of gene expression in sea cucumbers. Therefore, it is also a faster and simpler method to knock down genes compared with other routinely used approaches, and can be used for the analysis of gene function of sea cucumbers. Lysozyme gene silencing efficiency using RNAi was dependent on the applied concentration of the gene-specific RNAi. The p-miR-L245 interference effect time of lysozyme at the translation level was almost in accordance to the transcription levels in muscle, body wall, and parapodium. The lowest points of lysozyme activities in coelomic fluid and tube foot were laid behind lysozyme expression in transcription level. The different profiles of RNAi inferred that lysozyme RNAi systems had different processing in transcription and translation in different tissues. The Na^+/K^+ -ATPase activities in the experimental sea cucumbers after p-miR-L245 interference were significantly lower than in the PC and NC groups. The results indicate that lysozyme is involved in the salinity stress of sea cucumbers.

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