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Short Communication

Molecular and functional analysis of the insulin-like peptides gene in the oriental river prawn *Macrobrachium nipponense*

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ABSTRACT

The insulin-like peptide (ILP) family is a group of evolutionarily conserved proteins that control body size and organ growth in metazoans. In the current study we describe, for the first time, the *Mn-ILP* gene in the oriental river prawn *Macrobrachium nipponense*. Full-length of the *Mn-ILP* cDNA was 1630 bp, encoding 174 amino acids. The deduced amino acid sequence of *Mn-ILP* had the typical features of ILP proteins, including two cleavage sites and six conserved cysteines. To define the function of *Mn-ILP*, the expression of the *Mn-ILP* gene in different growth stages of prawns of both sexes, in male prawns of different sizes, and in prawns at different stages of the molt cycle was analyzed by qRT-PCR. *Mn-ILP* expression was significantly higher 1) in the rapid growth stage than in the other stages of male prawns; 2) in the normal growth stage than in the gonad development stage of female prawns; 3) in big male prawns than in small male prawns; and 4) in the intermolt stage than in the other stages of the molt cycle in prawns of the same size. Further, silencing *Mn-ILP* expression by RNAi effectively slowed down the growth speed of *M. nipponense*. Thus, *Mn-ILP* appears to have an important role in the growth and development process of *M. nipponense*.

1. Introduction

Growth and development are fundamental biological processes in animals. The growth process is influenced by many factors, including nutrition, environment, temperature, population density, and heredity (Emlen et al., 2012). Genes are important internal factors influencing growth and development. The insulin-like peptides (ILPs) and insulin-like growth factors (IGFs) are evolutionarily conserved proteins that control body size and organ growth in metazoans (Chandler et al., 2015; Perillo and Arnone, 2014). In vertebrates, growth is primarily mediated through the growth hormone (GH)-*IGFs* system (Lindsey and Mohan, 2015; Reindl and Sheridan, 2012), whereas in invertebrates the process is mainly regulated by *ILPs* (Nässel and Broeck, 2015). Seven *ILPs* (*ILP1-7*) have been identified in *Drosophila melanogaster*. While all seven possess the ability to promote growth, *ILP2* appears to be the most potent (Grönke et al., 2010).

In recent years *ILPs* have also been reported in crustaceans. The insulin-like androgenic gland (*IAG*) hormone is the most widely-studied *ILP* gene so far. *IAG* is believed to be the sex-determining gene in crustaceans (Ventura et al., 2011; Ventura et al., 2012b). Full and functional sex reversal can be achieved by *IAG* silencing in *Macrobrachium rosenbergii* (Lezer et al., 2015; Ventura et al., 2012a). Another gene, *Sv-ILP1*, has been reported in *Sagmariasus verreauxi*, but its biological functions are not yet known (Chandler et al., 2015). *Tj-ILP1*, which is reported in *Tigriopus japonicus*, may be associated with food intake and assimilation (Min et al., 2015).

Because of the presence of an exoskeleton, crustaceans undergo multiple moltings in their lifetime, with the body being heavier and longer after each molting. Most studies on growth and development of crustaceans have focused on the molting mechanism (Chang and Mykles, 2011; Naya et al., 2016; Yuan et al., 2016). Whether the *ILPs* regulate growth and development in crustaceans is not yet known.

The oriental river prawn *Macrobrachium nipponense* is an important commercial decapod crustacean belonging to the Palaemonidae family. It is widely distributed in freshwater and in low salinity estuarine regions in East Asian countries (Li et al., 2015b). Due to its high nutritive value, *M. nipponense* is farmed on a large scale in China. In 2017 the annual production was approximately 240,739 tons, worth almost 20

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billion RMB (Bureau of Fishery, Ministry of Agriculture, P.R.C., 2018). Better understanding of the molecular mechanisms involved in *M. nipponense* growth and of key growth-related genes would be useful for improving aquaculture performance.

In this paper we report for the first time the full-length cDNA sequence of the *Mn-ILP* gene identified from *M. nipponense*. We also investigated *Mn-ILP* expression in different tissues and at several growth phases in both sexes, in male prawns of different sizes, and in different stages of the molt cycle. In addition, we analyzed its function, using RNA interference (RNAi) technology. These data will improve understanding of the molecular mechanisms by which *Mn-ILP* regulates growth and development in crustaceans.

2. Materials and methods

2.1. Prawns specimens and tissues analyzed

Oriental river prawns of both sexes, with wet weight of about 3.0 g each, were obtained from the Mi-He River in Shouguang, Shandong Province, China, and transferred to the laboratory for 1 week of acclimatization. Different tissues, i.e., eyestalk, muscle, hepatopancreas, heart, nerve cord, brain, testis, and ovary, were dissected and preserved in RNA Keeper Tissue Stabilizer (Vazyme Biotech Co., Ltd, Nanjing, China) for RNA extraction. Total RNA was isolated using RNAiso Plus Reagent (TaKaRa Bio Inc., Dalian, China) in accordance with the manufacturer's protocol. The concentration of RNA was measured using BioPhotometer (Eppendorf, Hamburg, Germany), and the integrity was checked by agarose gel electrophoresis.

2.2. Cloning and bioinformatics analysis of Mn-ILP

Total RNA extracted from hepatopancreas was subjected to 3′ and 5′ rapid amplification of cDNA ends (RACE) using SMARTer® RACE 3′/5′ Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The gene-specific 3′ and 5′ RACE primers (Table 1) were designed according to the candidate sequences identified from the hepatopancreas transcriptome of *M. nipponense* in our laboratory. The open reading frame (ORF) of *Mn-ILP* was further confirmed by the pair of primers Mn-ILPORFF and Mn-ILPORFR (Table 1) with a high fidelity Taq (TaKaRa Bio Inc., Shiga, Japan). Protein prediction was performed using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The signal peptide and potential endoproteinase arginine cleavage sites were predicted using ProP 1.0 Server (http://www.cbs.dtu.dk/services/ProP/).

2.3. Mn-ILP expression in different tissues

Total RNA was extracted from various tissues (eyestalk, muscle, hepatopancreas, heart, nerve cord, brain, testis, and ovary). The expression of *Mn-ILP* in different tissues was determined by quantitative real-time PCR (qRT-PCR). The Mn-ILPQF and Mn-ILPQR primers were designed to detect *Mn-ILP* expression in the above tissues. The arginine kinase (*AK*) gene was amplified in the same cDNA samples as a reference gene, using Mn-AKQF and Mn-AKQR primers (Chung, 2014; Chung et al., 2011; Huang et al., 2017) (Table 1). The system and procedure of qRT-PCR were as described in our previous study (Li et al., 2015a). Briefly, PCR reactions were performed in a total volume of 20 μL PCR mixture containing 1 μL cDNA, 8 μL SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 0.5 μL 10 μM of primers, and 10 μL of diethyl pyrocarbonate (DEPC)-water. The cycling protocol was as follows: initial incubation for 30 s at 95 °C, followed by 40 cycles of 10 s denaturation at 95 °C and 10 s extension at 60 °C. DEPC-water was used as the negative control. Each assay was performed in triplicate. The $2^{-\Delta\Delta CT}$ method was used to analyze the mRNA expression (Livak and Schmittgen, 2001).

2.4. Expression analysis related to its function

The expression of *Mn-ILP* were analyzed by qRT-PCR at different growth stages in prawns of both sexes, in male prawns of different sizes, and at different stages of the molt cycle. The growth stages of male prawns include the embryonic stage (ES); the rapid growth stage (RGS; body length: 1.0–4.5 cm, body weight: 0.02–2.0 g); the isokinetic growth stage (IGS; body length: 4.5–6.0 cm, body weight: 2.0–5.0 g); and the allometric growth stage (AGS; body length: 6.0–7.0 cm, body weight: 5.0–8.0 g) (Liu et al., 2003; Wen and Xie, 2014). The growth stages of female prawns include the normal growth stage (body length: 1.0–3.0 cm, body weight: 0.02–1.0 g) and the gonad development stage (body length: 3.0–4.0 cm, body weight: 1.0–2.0 g). The male and female growth stages were determined according to a previously published method (Liu et al., 2003; Wen and Xie, 2014). To examine how *Mn-ILP* expression varies with size, we selected male prawns in the same growth phase and separated them into two groups: big prawns (~5 g) and small prawns (~3 g). To determine variations in *Mn-ILP* expression during the molt cycle we used prawns of the same size (~3 g) but at different stages of the molt cycle, i.e., the postmolt (A/B), intermolt (C), premolt (D), and ecdysis (E) stages (Chan, 1988; Skinner, 1962).

2.5. RNAi

Double-stranded RNA (dsRNA) was synthesized using a MEGAscript T7 Kit (Ambion Inc., Foster City, CA, USA). Primers used for RNAi were designed according to the *Mn-ILP* cDNA sequences, using SnapDragon-dsRNA Design. Templates for *in vitro* transcription were prepared by PCR, using gene-specific primers of ds-Mn-ILP-F and ds-Mn-ILP-R with the T7 polymerase promoter sequence at their 5′ ends (Table 1). The resulting dsRNA was dissolved in DEPC-treated water.

A long-term (1-month) RNAi experiment was performed to further investigate whether *Mn-ILP* was involved in the growth of *M. nipponense*. Adult prawns (wet weight 3.0 g) were divided into two groups: a *Mn-ILP* dsRNA-injected group (n = 30) and a DEPC-injected group (control group; n = 30). Prawns were injected twice a week for a month

Table 1
Universal and specific primers used in this study.

Primer name	Primer sequence 5′ → 3′	Purpose
Mn-ILP 5′ primer	GTCCAGAGGTTCTTCGTCACCCATGG	5′ RACE
Mn-ILP 3′ primer	CTGACAACTATACAGTACATTCTGCG	3′ RACE
Mn-ILPORFF	GAACAAAGTATGTGGGCATTTG	ORF
Mn-ILPORFR	TGTCAAGTATAAGACAGGGACG	ORF
Mn-ILPQF	GAAGTCTCCATCGCTCATCC	qRT-PCR
Mn-ILPQR	AGCGACGCTTCGGCTGCAGC	qRT-PCR
Mn-AKQF	GGTCCGTCAGCAGCTCGTCG	qRT-PCR
Mn-AKQR	TGATCCTGAGCTGATCCTCC	qRT-PCR
ds-Mn-ILP-F	TAATACGACTCACTATAGGGTCGACTGCGGCGACATTACG	RNAi
ds-Mn-ILP-R	TAATACGACTCACTATAGGGCGTTGCTGCAGCACTCCTCC	RNAi

Note: T7 polymerase promoter sequence is underlined.

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>Mn-ILP
TTTAGACTTGATGTTGATCGCTTCTTTTAAATGAGGGGAAGCGATGACGTGATATTTTAC  60
CTTTGTTTTGTGTTTGTTTTGAGTTAAACGAGATCACAACGAACAAAGTATGTGGGCATT  120
TGAAGTGTATTCTGACTGCTAGAAGAGAGTCTGCTCTTGTGCTCACTCGTAACACTGCAT  180
                  L  L  E  E  S  L  L  L  C  S  L  V  T  L  H   15 aa
CTCCCACAACTTTCTTCAAGCTACGAGATCGAATGCCTCTCCGTTGACTTCGACTGCGGC  240
  L  P  Q  L  S  S  S  Y  E  I  E  C  L  S  V  D  F  D  C  G   35 aa
GACATTACGAACACCTTCGCCTCCGTCTGCCTGAGACACAACAACTACATCGAACCAGGA  300
  D  I  T  N  T  F  A  S  V  C  L  R  H  N  N  Y  I  E  P  G   55 aa
CCCGCCTACATCTCCAAACAGAGAAGATCTGCTGACAACTATACAGTACATTCTGCGAAG  360
  P  A  Y  I  S  K  Q  R  R  S  A  D  N  Y  T  V  H  S  A  K   75 aa
TCTCCATCGCTCATCCACCCAAGAGCTACCCACTTGTCCATGGGTGACGAAGAACCTCTG  420
  S  P  S  L  I  H  P  R  A  T  H  L  S  M  G  D  E  E  P  L   95 aa
GACGAAGAACCTCTGAAGGCATTGGAGGTGGAGGAGGAGATTCAACACACGACGCTGAGC  480
  D  E  E  P  L  K  A  L  E  V  E  E  E  I  Q  H  T  T  L  S   115 aa
CGGGAAGAAGCGAACAAGATGCTGCAGCCGAAGCGTCGCTTCCGGAGGGACGGAGTGAGG  540
  R  E  E  A  N  K  M  L  Q  P  K  R  R  F  R  R  D  G  V  R   135 aa
AAAACTCCGAGGGAGGAGTGCTGCAGCAACGCCTCCTTCAGACGCTGCAGCTTCGAGGAA  600
  K  T  P  R  E  E  C  C  S  N  A  S  F  R  R  C  S  F  E  E   155 aa
GTGGCCGAATATTGCATTGAACTGCGTCCCGGCGTCAACACCTGCGACATCTCCAGGTAG  660
  V  A  E  Y  C  I  E  L  R  P  G  V  N  T  C  D  I  S  R  *   174 aa
GAGGTCGGAAGGATCATCCCGTCCCTGTCTTATACTTGACATGAGATGTTTCAAGTCGAT  720
TCACAGTCTTCGAGATTGCAACATGACTTTCGTGATGTAGAATGACCTACTAAAGCTGTC  780
TTTTGTCTTTCCTCACAGTCAACTGAAATATTTTTTGCCTTACCCTTCCTTACCTTCAGC  840
TAAATTAATCTTTTGTCTCAGCTTTAAATTCAATATTCGTCTGCCTTTACCTTTAGTTCA  900
GCCAATAGCTCCTGTCTATCTTGCCATTAACTTTCACAAAGGTTTGTGTTGTCTTACCCT  960
CGGCTGAAACTTTCGATTGTCCCACCTTCATCTTCCACTGAATCTTTCTTTTGACTTCCG  1020
CTACTTTCTTTAGTCACACTCTTTTTCAGCTAGGGATTTGTTTTGTTTTTTTGACACTTT  1080
TACCTTCAGCTAAAGGTTCCTTTTACCCTTGCATGTAACTAAAAGTTCCTTTTGTCTCAG  1140
CCTTGCCTTCAGTCAAAGGTTCCTTTTGTCTCAGCCTTGTCTTCAGCTAAAGGTTACTTT  1200
TACCCTTGCATTCAGCTAAAGGTTCCATTTGTCACAGCCTTGCCTTCAGCTAAAGGTTCC  1260
TTTTACCCTTGCATTCAGCTAAAGGTTCCTTTTGTCTCAGCCTTGCCTTCAGCTAAAGGT  1320
TCCTTTTAACTCAACCTTGCATTCGGCAAATGGTTCCTTTTATTTCAGCCTTGCCACCAG  1380
CTAAATGTTCCTTTTGTCTGAACCTTGCCTTCAGCTAAACTCCCGTTTATCTTTAGCTAA  1440
AGTTTACTTTTACTAGACCCTTCCCAATCGTTCTAATGTTTTACCCTTACACGCAACAGC  1500
ATCTAGACATTTCAGAACATTAAACATCTTGCATTATTATTGGTGATTCTTGTCAACGTT  1560
TCCGAAAAATTGTTCGATACAGCAGTTATTCGTCAAATAAATGCTTTTGAGACTACCGAA  1620
AAAAAAAAAA                                                     1630
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Fig. 1. The complete cDNA and protein sequences of Mn-ILP. The full-length cDNA sequence comprises 1630 bp. The open reading frame (ORF) encodes a deduced protein of 174 amino acids. The putative signal peptide is shown by a dashed line, the start codon (CTG) is underlined, and the stop codon (TAG) is underlined and indicated by an asterisk. The predicted cleavage sites are marked with squares, and the putative polyadenylation site (AATAAA) is indicated by a grey background.

with either dsRNA (5 μg/g body weight) or DEPC. At the end of 1 month, hepatopancreas were collected from prawns in both groups for RNA extraction. The expression of *Mn-ILP* mRNA was determined by qRT-PCR. Molts were recorded daily. Body weight was recorded at the end of the experiment.

3. Results

3.1. Isolation of full-length Mn-ILP cDNA

The full-length cDNA encoding *Mn-ILP* from *M. nipponense* was obtained by 3′ and 5′ RACE. The total length of the *Mn-ILP* was 1630 bp. It consisted of a 135-bp 5′ untranslated region (UTR), a 525-bp ORF encoding a deduced Mn-ILP prepropeptide of 174 amino acid residues, and a 970-bp 3′-UTR including a poly-A signal sequence (Fig. 1). ORFfinder showed that the start codon of *Mn-ILP* is CUG. The deduced protein of 174 amino acids was predicted to encode a signal peptide (22 amino acids), a B chain (40 amino acids), a C peptide (68 amino acids), and an A chain (44 amino acids). Two putative cleavage sites, KQRR and RRFR, were located between the B chain and the C peptide and between the C peptide and the A chain, respectively. The putative Mn-ILP protein sequence-predicted B and A chains with six conserved cysteine residues likely involved in three disulfide bridges (two interchains: Cys_{34} and Cys_{143}, Cys_{45} and Cys_{160}; and one intrachain bridge: Cys_{142} and Cys_{151}). These key features were consistent with criteria used for identification of ILP (Chandler et al., 2015), and so we termed the conceptually translated protein Mn-ILP. The protein contained two additional cysteine residues (Cys_{27} and Cys_{170}).

3.2. Tissue distribution of Mn-ILP

The tissue distribution of *Mn-ILP* mRNAs were determined by qRT-PCR. *Mn-ILP* mRNA was detected in eight different tissues. As shown in Fig. 2, the *Mn-ILP* expression was significantly higher in the hepatopancreas than in other tissues. The lowest expression was in the eyestalk.

3.3. Functional analysis

In male prawns the expression of *Mn-ILP* was highest in the RGS, intermediate in the IGS and AGS, and lowest in the ES (Fig. 3A). In female prawns the expression of *Mn-ILP* mRNA was significantly higher

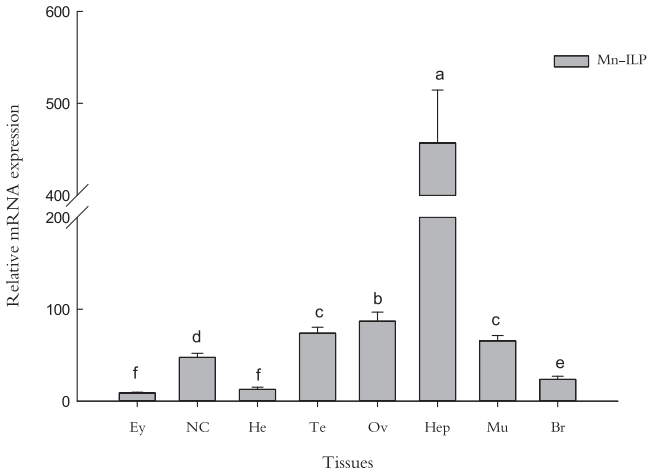

Fig. 2. The expression of *Mn-ILP* in different tissues. The tissues include: eyestalk (Ey), nerve cord (Nc), heart (He), testis (Te), ovary (Ov), hepatopancreas (Hep), muscle (Mu), and brain (Br). qRT-PCR data are shown as means ± SE (standard error). Significant differences in *Mn-ILP* mRNA levels in different tissues are represented by lowercase, respectively. Different letters indicate significant differences ($P < 0.01$).

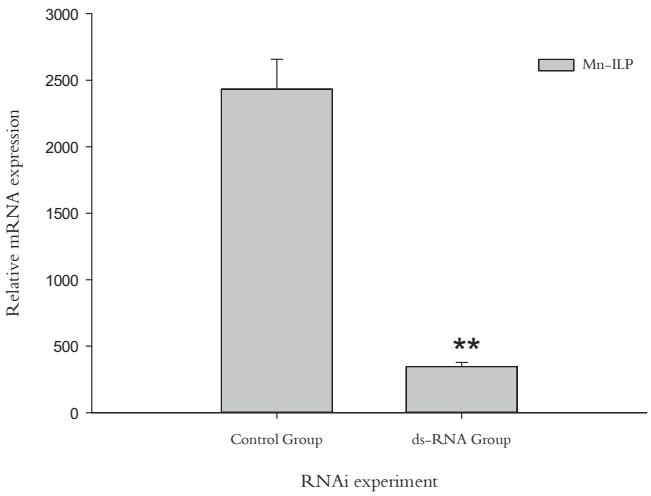

Fig. 4. *Mn-ILP* expression in dsRNA-treated and control prawns. Data from qRT-PCR are shown as means ± SE. $^{**}P < 0.01$.

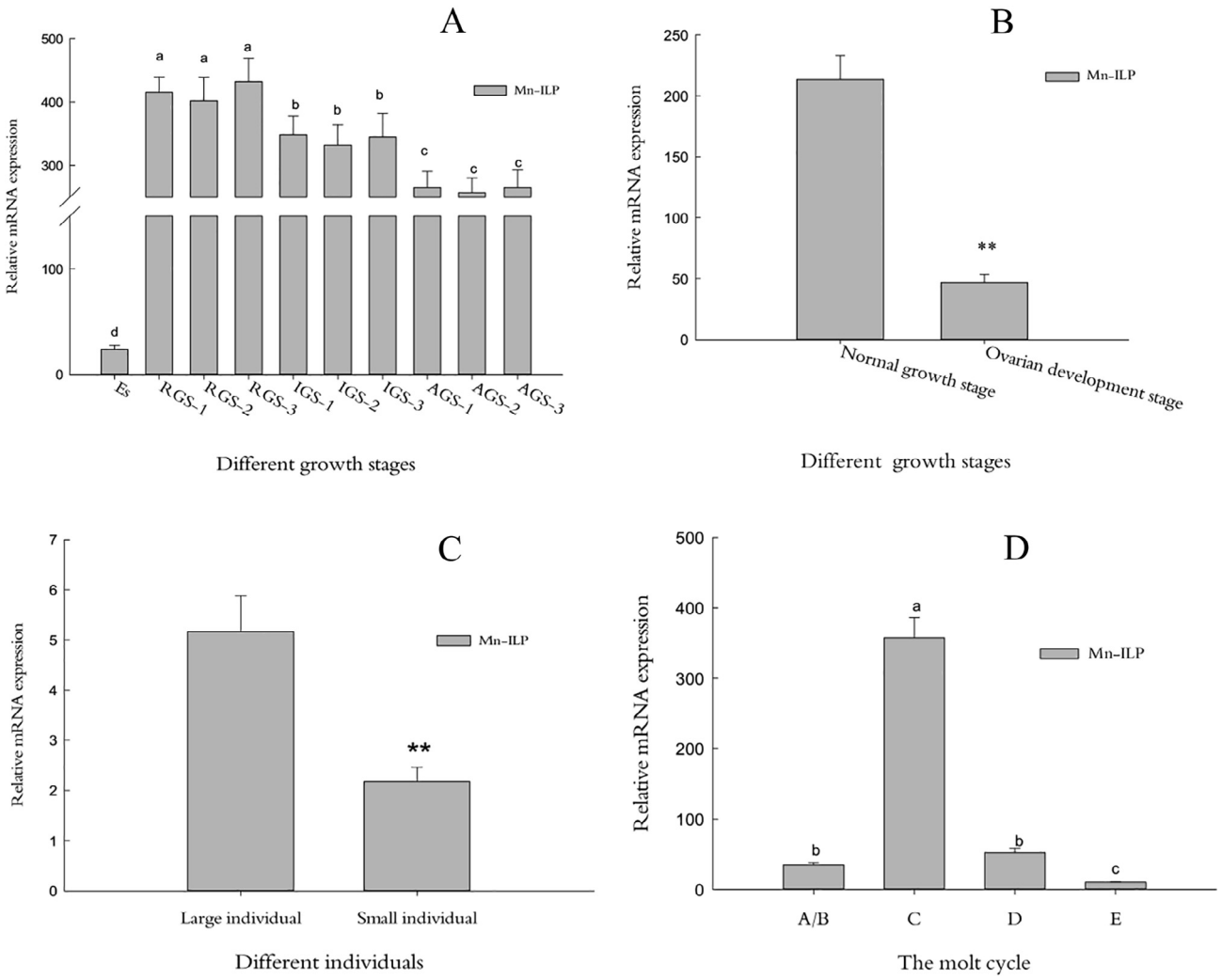

Fig. 3. The expression of *Mn-ILP*. (A) *Mn-ILP* expression at different growth stages in male prawns; (B) *Mn-ILP* expression at different growth stages in female prawns; (C) *Mn-ILP* expression in male prawns of different sizes; (D) *Mn-ILP* expression at different stages of the molt cycle. qRT-PCR data are shown as means ± SE (standard error). Significant differences in *Mn-ILP* mRNA levels are represented by lowercase, respectively. Different letters indicate significant differences ($P < 0.01$). $^{**}P < 0.01$.

in the normal growth stage than in the gonad development stage (Fig. 3B). *Mn-ILP* expression was significantly higher in big prawns than in small prawns (Fig. 3C). *Mn-ILP* expression was significantly higher in the intermolt stage than in other stages (Fig. 3D).

3.4. RNAi

In the RNAi experiments, *Mn-ILP* expression was effectively reduced by dsRNA injection. As Fig. 4 shows, the *Mn-ILP* expression in the *Mn-ILP*-dsRNA-injected group was significantly reduced ($P < 0.01$) to 14.2% of that in control-injected prawns. After a month-long silencing of *Mn-ILP* with dsRNA, the mean weight was significantly lower in the dsRNA-injected group than in the control group (3.67 ± 0.46 g vs. 4.57 ± 0.36 g; $P < 0.01$). Furthermore, the number of molted individuals in the *Mn-ILP*-dsRNA injected group and the control group were 11 and 25, respectively, indicating delayed molting in the *Mn-ILP*-dsRNA-injected group.

4. Discussion

In this study a putative *ILP* gene, termed *Mn-ILP*, was isolated and characterized from the oriental river prawn *M. nipponense*. As a member of evolutionarily conserved insulin-like peptides, Mn-ILP shares several common characteristics with ILP proteins in invertebrates, including 1) an RR cleavage site located at the end of the B-chain followed by an RxxR cleavage site at the beginning of the A-chain; and 2) two and four conserved cysteines located in the putative B- and A-chains, respectively (Chandler et al., 2015; Ikeya, 2001). As is common to all insulins, the signal peptide and C peptide of Mn-ILP prepropeptide may be cleaved off to give rise to the mature ILP protein, which has three disulfide bonds: two interchain bonds and one intrachain bond. These disulfide bridges enable the three-dimensional folding of the mature Mn-ILP protein (Ventura et al., 2011; Ventura et al., 2012b). The analogous protein structure of Mn-ILP and insulin suggested that they have a similar mechanism of action. Interestingly, two additional cysteine residues (Cys_{27} and Cys_{170}) were detected in the Mn-ILP backbone. Four disulfide bonds constructed by eight cysteine residues have been found in classical insulin-like family members in insects (Blundell and Humbel, 1980; Okuno et al., 2002). Furthermore, as a typical insulin-like peptide, IAG, with eight rather than six cysteine residues, has been reported in a decapod crustacean *Cherax quadricarinatus*, which shows that a fourth disulfide bond may be formed by two additional cysteine residues (Manor et al., 2004). Recent studies have shown that IAGs with eight cysteine residues are common in crustaceans (Alkalay et al., 2014; Banzai et al., 2012; Ma et al., 2013).These results suggest that an additional disulfide bond may be formed by Cys_{27} and Cys_{170} of Mn-ILP.

Usually eukaryotes utilize AUG as a start codon but, interestingly, *Mn-ILP* has CUG as its start codon. There is an increasing body of evidence indicating that AUG is by no means the only possible codon in eukaryotes; non-AUG initiation events are often used to generate or regulate proteins with key cellular functions (Kearse and Wilusz, 2017). Studies have shown that six alternative start codons (AUU, UUG, AUA, CUG, GUG, and ACG) are available in eukaryotes; this alternative translation initiation provides greater proteome diversity (Kozak, 1989; Lee et al., 2012). Interestingly, ILP diversity has been demonstrated in insects, the sister taxon to crustaceans (Shultz and Regier, 2000). *ILP1* and *ILP3* of *Anopheles gambiae* are arrayed in tandem on chromosome III and, very likely, arose by gene duplication, suggesting that two genes might originate from a eukaryotic operon, transcribed as polycistronic pre-mRNA and subsequently processed to individual transcripts (Krieger et al., 2010). Recently, non-AUG codons were also found in crustaceans. In the blue crab *Callinectes sapidus*, three *IAG* gene transcripts were separately identified in the androgenic gland (*CasIAG-ag*) (Chung et al., 2011), hepatopancreas (*CasIAG-hep*) (Chung, 2014), and ovary (*CasIAG-ova*) (Huang et al., 2017). The start codons of *CasIAG-hep* and *CasIAG-ova* were both UUG rather than AUG. Furthermore, the three *IAG* gene transcripts perform different biological functions: *CasIAG-ag* is involved in developing secondary characterization of males (Chung, 2014), *CasIAG-hep* is associated with carbohydrate metabolism (Chung, 2014), and *CasIAG-ova* is involved in the ovarian development of females (Huang et al., 2017). Thus, it is possible that *Mn-ILP* uses AUG as its start codon to regulate the growth and development of *M. nipponense*.

Gene expression is generally related to its function. To investigate the function of *Mn-ILP*, we analyzed the gene expression in different developmental stages of prawns. The life cycle of male *M. nipponense* is divided into three stages: RGS, IGS, and AGS. The fastest growth rate is in the RGS, followed by the IGS and then the AGS. The growth trend is in agreement with the expression of *Mn-ILP*. The life cycle of female prawns passes through two stages: a normal growth stage, which lasts till a body length of 3.0 cm is reached, followed by the gonad development phase during which there is slowing, or even arrest, of somatic growth. We found significantly higher *Mn-ILP* expression in the normal growth stage than in the gonad development stage. Furthermore, among male prawns, *Mn-ILP* expression was significantly higher in big prawns than in small prawns. These findings suggested that *Mn-ILP* played an important role in the growth and development of *M. nipponense*.

Crustaceans undergo a series of moltings in their life. A molt cycle includes four stages: the intermolt (C), premolt (D), ecdysis (E) and postmolt (A/B). With every molting, the body of the crustaceans gets longer and heavier. Somatic growth of crustaceans occurs mainly during the intermolt stage: the nutrients accumulate extensively in this stage, and muscles mass increases. For example, in *Litopenaeus vannamei*, fibers were fully expanded and fiber walls were completely swollen, and actin and myosin heavy chain concentrations reached a peak in the intermolt stage (Cesar et al., 2006). It is noteworthy that *Mn-ILP* expression was significantly higher in the intermolt stage compared to the other stages. The result further confirms the regulatory role of *Mn-ILP* in growth and development.

To further investigate the effects of *Mn-ILP* on the growth of *M. nipponense*, we used a loss-of-function molecular approach (RNAi) to silence the *Mn-ILP* gene *in vivo*. Month-long RNAi treatment resulted in significant lag in the rate of weight gain of the treated group relative to a control group, thus further demonstrating the effect of *Mn-ILP* on growth and development of *M. nipponense*.

Crustacean growth is a complex process regulated by chemical messengers, including ecdysteroids, sequiterpenoids, and peptide hormones. Periodic molting is stimulated by ecdysteroids. Synthesis of ecdysteroids by Y-organs is regulated by molt-inhibiting hormone and crustacean hyperglycemic hormone, peptides secreted by neurosecretory cells in the X-organ/sinus gland complex of the eyestalks (Jung et al., 2013). In addition, sesquiterpenoids may serve as regulators or modulators of growth, molting, and larval development in crustaceans. For instance, methyl myristate synthesized in the crustacean mandibular organ has been shown to play a role in controlling growth and molting in the spider crab, *Libinia emarginata* (Laufer et al., 2002). Elucidating the function of *Mn-ILP* will help us to understand the mechanisms by which growth is regulated in crustaceans. The general consensus is that growth and development in insects are regulated by the insulin signaling pathway via *ILP* activation (Nässel and Broeck, 2015). So far, eight ILPs have been identified in *Drosophila* (Ling et al., 2017); seven possess the ability to promote growth (Grönke et al., 2010). In insects, muscle is the main metabolic organ that responds to insulin. In *Drosophila,* insulin signaling can non-autonomously regulate muscle growth by modulating muscle-specific insulin receptors (InR) (Hyun, 2013). We recently used RNA sequencing technology and identified the *InR* gene, which encodes 1412 amino acids (data not shown). *Mn-ILP*, which we found to be highly expressed in hepatopancreas, is similar to *IGF* of vertebrates. We therefore speculate that the molecular mechanism of *Mn-ILP* may be similar to the insulin signaling pathway of vertebrates. Similar to several other *ILP*s in insects,

Mn-ILP may be a factor regulating ecdysteroid levels throughout the molt cycle, (Riehle and Brown, 1999; Ventura et al., 2009). It is possible that the ILP signaling pathway in crustaceans regulates cell number and size and thus influences organism size. Molting is directly related to muscle development and growth in crustaceans, and it is likely that when the exoskeleton restrains somatic growth ecdysteroids or sesquiterpenoids are activated to regulate the molting. However, further investigations are necessary to clarify the regulatory mechanisms.

In summary, we cloned the cDNA sequence of *Mn-ILP* and investigated its expression in different tissues, in different growth stages of prawns of both sexes, in the male prawns of different sizes, and in different stages of the molt cycle. We also confirmed the effects of *Mn-ILP* on the growth of *M. nipponense*. Our results provide basic information about the regulatory mechanism of growth and development in *M. nipponense*.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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