Transcriptome analysis in ovary of female banana shrimp, *Fenneropenaeus merguiensis*: A functional study of *nudel* as a potential gene involved in the ovarian development

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Abstract

Control of ovarian maturation is an important one of problems in the shrimp aquaculture. Several genes involved in control of crucial ovarian development process like vitellogenesis in female crustaceans. In this study, the ovary at each ovarian developmental stage of female banana shrimp, *Fenneropenaeus merguiensis*, was analyze for its transcriptome with Illumina HiSeq2500 platform. The *nudel (ndl)* was identified for study of characterization and the role of *FmNudel* in vitellogenesis of female banana shrimp. The result suggested that the *FmNudel* expression was significantly highest in ovary compared to other tissues. Its expression was significantly increased in previtellogenic stage (stage 0), then dropped in the other stages. The silencing of *FmNudel* was performed by *nudel*-dsRNA injection on day 10 resulted in significant decrease of *vitellogenin* (*Vg*) expression in ovary.

Keywords *Fenneropenaeus merguiensis*, ovarian development, transcriptome

Introduction

Shrimp is one of the widely consumed seafood products all over the world having high economic value (FAO, 2020). However, in recent years the shrimp aquaculture has been interrupted with several problems involved shrimp reproduction. In shrimp aquaculture, the eyestalk ablation was widely used to rapidly stimulate ovarian maturation in female shrimp (Uawisetwathana et al., 2011) because the eyestalks of crustaceans are the source of the gonad-suppressive hormone such as a gonad inhibiting hormone (GIH) (Treerattrakool et al., 2008) but the technique leads to an eventual loss in egg quality, high mortality in spawner

(Benzie 1998) and risks to impact animal cruelty laws. Therefore, this technique should be replaced with the more effective methods to induce shrimp ovarian maturation.

An understanding of molecular pathway for control of ovarian maturation is necessary. There were several factors involved in the ovarian developmental process. For example, GIH-specific double-stranded RNA in *Penaeus monodon* increased *vitellogenin (Vg)* expression level and led to ovarian maturation and eventual spawning (Treerattrakool et al., 2008, 2011). Serotonin and estradiol and progesterone in *P. monodon* (Wongprasert et al., 2006; Merlin et al., 2015) and insulin-like receptor in *Macrobrachium rosenbergii*, (Sharabi, 2001) could induce ovarian maturation.

In this study, we aimed to identify an ovarian-regulating gene using RNA sequencing and bioinformatic analyses in female banana shrimp, *Fenneropenaeus merguiensis*. The differential gene expression of putative gene during ovarian developmental stages was also investigated. The putative ovarian-regulating gene were characterized for its expressions, a candidate gene was studied for its role in the control of vitellogenesis using RNA interference.

Objectives

1. To identify and characterize a candidate gene involved in ovarian development in female banana shrimp.

2. To study an ovarian stimulating function of the *nudel* gene in female banana shrimp.

Research Scopes

1. Transcriptome from shrimp ovary was identified by RNA sequencing and bioinformatics.

2. Differential gene expression was analyzed.

3. A candidate gene, *nudel*, was characterized and studied for its role in ovarian development.

Methodology

1. Animals and acclimatization

Mature female banana shrimp were reared in 30 ppt seawater for 2-3 days before used.

2. RNA sequencing (RNA-seq) and analysis

The ovaries isolated from female shrimp at different stages of ovarian development including previtellogenic (stage 0), early vitellogenic (stage 1), late vitellogenic

(stage 2), and mature stages (stage 3) based on ovarian histology and color (Ayub and Ahmed, 2002). The 3 ovaries from individual shrimp at each stage were pooled and sent to analyze for its transcriptome with the Illumina HiSeq2500 platform (Novogene, Hongkong). The raw reads were filtered to remove the reads containing adapters or reads of low quality. The clean reads were further *de novo* assembled by Trinity software (Grabherr et al., 2011). CD-hit (Fu et al., 2012) performed hierarchical clustering to remove transcripts' redundance.

3. Gene functional annotation and differentially expressed gene (DEG) analysis

The databases such as NR (the NCBI non-redundant protein), Swiss-Prot, and COG (/KOG were used to annotate the function of the transcriptomeIn addition, the NT (the NCBI non-redundant nucleotide), KEGG, PFAM, and GO (gene ontology) databases were also used. Furthermore, differential gene expression was analyzed using Bowtie, RSEM, and DEGseq software with thresholds of log2-foldchange ≥ 1 for up-regulation or ≤ -1 for down-regulation, and q-value < 0.005 (Li and Dewey, 2011; Wang et al., 2010).

4. RNA extraction and cDNA synthesis

Total RNA extraction was performed using a Trizol reagent (Ambion) according to the manufacturer's protocol. The 1 µg of total RNA was treated with DNase I. The reaction was at 37 °C for 20 min, then the 0.5 µl of 50 mM EDTA was added to the mixture before heating at 65 °C for 5 min. The DNase I-treated RNA was used as a template to construct first stranded cDNA with oligo dT- primer using the Viva cDNA synthesis kit (Vivantis). The profile was at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 10 min. The cDNA was kept at -20 °C until used.

5. Quantitative realtime PCR (qRT-PCR) analysis

The gene expression was determined by qRT-PCR analysis using a realtime PCR. The PCR mixture was composed of 1X KAPA SYBR® FAST Universal mastermix (Kapa Biosystems), 0.2 µM primer pair, and cDNA. The reaction was at 95 °C for 2 min, 40 cycles of 95 °C for 15 sec, and 60 °C for 30 sec. Then, it was heated at 95 °C for 1 min, 60 °C for 30 sec, and 95 °C for 30 sec. Elongation factor 1 α gene (EF1α) was used as an internal control. The normalized fold of the gene expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primer efficiency used in qRT-PCR was about 95-100 % according to the amplification with the serial dilution of the cDNA and calculated by the equation of efficiency = $[10^{(-1/slope)}] - 1]$.

6. Determination of the candidate gene expression in shrimp tissues and ovarian developmental stages

The different tissues including eyestalk, brain, thoracic ganglia, gill, hepatopancreas, muscle, and ovary were isolated from 2 previtellogenic shrimp. Also, the

ovary and hepatopancreas were isolated from 3-7 individual shrimps at each ovarian development stage. Those tissues samples were extracted for their total RNA and subsequently used to synthesize for cDNA as mentioned in 4. The relative quantification of gene expression was evaluated by qRT-PCR analysis as described above.

7. Construction and expression of the gene-specific dsRNA

The recombinant plasmid carrying the inverted repeat sequence of *nudel* was constructed and used to express for *nudel* specific dsRNA (dsNudel). The nucleotides corresponding to a region of the *nudel* sequence, including stem and stem-loop fragments were amplified by PCR with specific primers using the ovarian cDNA as a template. Their fragments were cloned in pET28a plasmid as the expression vector. The sequence was confirmed by DNA sequencing. Afterward, the recombinant plasmid was further transformed in *Escherichia coli* HT115. The bacteria was cultured in LB broth at 37 °C, 180 rpm for overnight. Then, the bacterial starter was inoculated in a new LB broth and cultured until OD_{600} was 0.4-0.8. The bacterial was added with 0.2 mM IPTG and then incubated for 2-3 hours. The bacterial cells were harvested and extracted by the ethanol method (Posiri et al., 2013). The dsRNA was confirmed for its dsRNA features by RNase digestion assay. In addition, a green fluorescent protein (GFP) from phMGFP plasmid was used to construct GFP-specific dsRNA as a non-specific dsRNA.

8. Effect of the candidate gene knockdown on vitellogenesis in female shrimps

Previtellogenic female shrimps were injected with the 3 µg/ g body weight of dsNudel or dsGFP. The 5-8 shrimps were used in each group. On days 7, the shrimps in each group were injected with the same concentration of the dsRNA and cultured for next 3 days. On day 10, their ovaries were determined for *Nudel* and *vitellogenin* expression by qRT-PCR.

9. Data analysis

All results from qRT-PCR analysis were statistically analyzed with one-way ANOVA followed by pair-wise comparison by Scheffe' s method. The difference and relationship between groups were considered statistically significant at a p-value < 0.05.

Results

1. Sequencing analysis, functional annotation, and DEG analysis

A total of 32,176 transcripts were obtained from *de novo* assembly of raw reads using Trinity. After hierarchically clustered and removed for the redundant transcripts, the 32,160 longest transcripts of each cluster were obtained with assembly statistics (Table 1). Then, the unigenes were annotated against the NR, NT, Swiss-Prot, KOG, KEGG, PFAM, and GO databases. The result showed that the most unigenes were annotated with the NR, GO,

and PFAM databases (Figure 1A). In addition, a total of 241 unigenes were differentially expressed between ovarian developmental stages as shown in Figure 1B. Some genes can express in more than one group. The number of up-regulated genes in compared stages of 3 and 1, 2 and 0, and 2 and 1, were higher than other groups. On the other hand, the number of down-regulated genes in compared stages of 2 and 0, 2 and 1, and 3 and 2, were higher than other groups (Figure 1B).

Table 1: Summary statistics for the *de novo* assembly of the *F. merguiensis*'s transcriptome.

Transcriptome assembly statistics	
Number of transcripts	32,176
Number of unigenes	32,160
• GC (%)	43.12
• Longest contig length (bp)	16,137
• Shortest contig length (bp)	201
• Average contig length (bp)	1,485
• Contig N50 (bp)	6,988
• Total bases	47,775,273

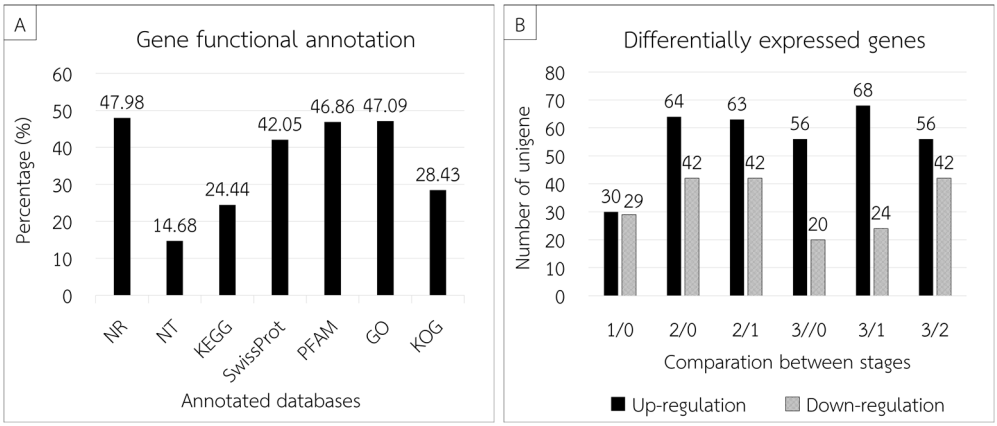

Figure 1: The percentage of unigenes successfully annotated in the databases (A). The number of genes which were uniquely expressed differentially between the stages and some genes expressed in two or more groups (B).

2. Expression profiles of the *nudel* in ovarian developmental stages and tissues

In this study, the *nudel* was selected as a candidate gene due to its expression level was a one of the highest differential expression among ovarian developmental stages. Its expression was up-regulated in mature stage 3 compared with previtellogenic stage. In

addition, its role is involved in reproductive processes such as dorsal/ventral axis, regulation of Toll signaling pathway, and egg activation. The *nudel* expression was determined in various tissues. The result showed that the *nudel* expression was the significantly highest only in ovary compared to other tissues (Figure 2A), suggesting its specific function in gonads. According to its expression in ovary, the ovarian tissues at each ovarian development stage were determined by qRT-PCR. The result showed that the *nudel* expression was significantly high in stage 0 and rapidly low in stage 1. Then, it slightly increased in stage 2 and gradually decreased in stage 3. However, the *nudel* expression from qRT-PCR was not similar profile to the prediction of DEG derived from transcriptome analysis (Figure 2B).

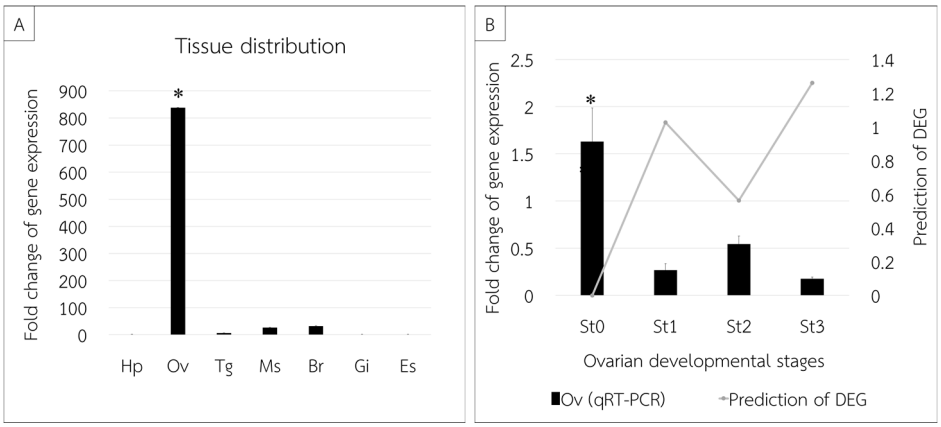

Figure 2: The *nudel* expression in various tissues (A) and ovarian tissue during ovarian developmental stages (B) were performed by qRT-PCR. The tissues including thoracic ganglia (Tg), muscle (Ms), eyestalk (Es), brain (Bn), gills (Gi), hepatopancreas (Hp), and ovary (Ov). The ovarian development stages classified into 4 stages including previtellogenic (st0), early vitellogenic (st1), late vitellogenic (st2), and mature stages (st3). Bars and error bars were means and SEM, respectively. An asterisk indicated significant differences between groups analyzed by one-way ANOVA (p<0.05).

3. Production of the *nudel* specific dsRNA

The inverted repeat nucleotide sequence of *FmNudel* and *GFP* were cloned in the pET28a plasmid, and subsequently transformed in *E. coli* HT115 (DE3). Both dsNudel and dsGFP were produced in bacterial system. For the determination of their feature, their extracted total RNAs were treated using RNase digestion. The RNase A and RNase III specifically digested ssRNA and dsRNA, respectively. At the results, both dsNudel and dsGFP were degraded with RNase III but not with RNase A (Figure 3). The result indicated that that both RNAs were a double-stranded structure.

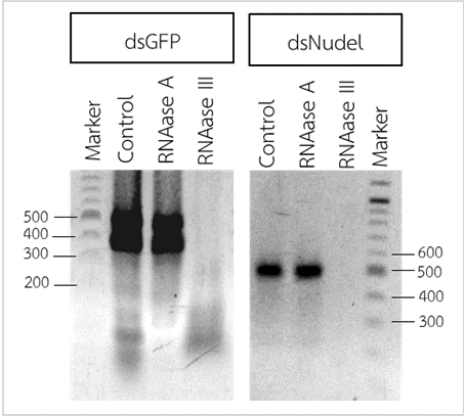

Figure 3: The production and RNase digestion of dsNudel and dsGFP. Both dsNudel and dsGFP produced from *E. coli* were digested with RNase A or RNase III. The result of the digestion was analyzed by agarose gel electrophoresis. The left and right lanes were a DNA ladder.

4. Effect of *FmNudel* silencing on vitellogenesis in female *F. merguiensis*

The previtellogenic female shrimps were injected with dsNudel or dsGFP. On 10 days, their ovarian tissue was isolated and determined the effect of *nudel* knockdown on the expression of *nudel* and *vitellogenin* (*Vg*) by qRT-PCR. The result showed that the *nudel* expression was significantly decreased approximately a 2-fold in dsNudel-injected shrimp compared with the dsGFP-injected shrimp, indicating the suppression of *nudel* expression by dsNudel. Interestingly, *Vg* expression was significantly dropped approximately 10-fold in *nudel* knockdown shrimp when compared with dsGFP-injected shrimp. The result suggested that the *nudel* expression involved in control of the *Vg* expression (Figure 4).

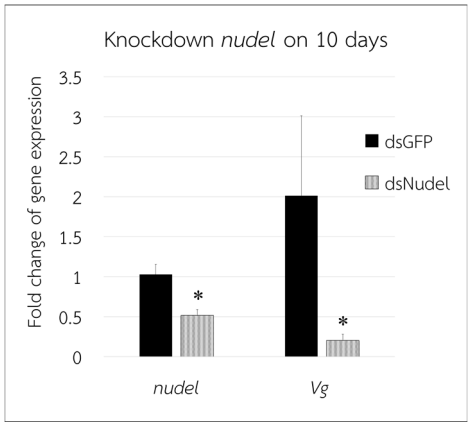

Figure 4: Effect of *FmNudel* knockdown on *Vg* mRNA expression on day 10. The ovary was determined for *FmNudel* and *Vg* expressions by qRT-PCR. Bars and error bars were means and SEM, respectively. An asterisk indicated significant differences between groups analyzed by one-way ANOVA ($p<0.05$).

Discussion

In this study, the *nudel* was most expressed in ovary in female banana shrimp (Figure 2A), similar to the *nudel* (*ndl*) was reported to be female-specific gene in the ovaries of the *Drosophila* (Hong and Hashimoto, 1996) and shrimp, *Marsupenaeus japonicus* (Callaghan et al., 2010). Furthermore, the *nudel* expression profile during the ovarian development in ovary was significantly increased in previtellogenic stage (st0) compared with other stages (Figure 2B). However, the *nudel* expression from prediction of DEG and from qRT-PCR analysis during the ovarian development was not similar profile (Figure 2B) because our transcriptome having only one biological replicate, this may be provided some unstable data. In addition, the *nudel* was determined the ovarian stimulating role using *FmNudel* injection in shrimp. After the injection 10 days, the *Vg* expression was significantly decreased only in ovaries (Figure 4). These results indicated that *FmNudel* plays roles in the ovarian developmental processes and stimulated vitellogenesis in ovary.

The ovarian developmental role of *nudel* gene in crustacean has not been reported so far. This study may be the first report of *nudel* for ovarian development in shrimp. However, some studies especially in *Drosophila* have been reported that the *nudel* are expressed in ovarian follicle cells and encoded a multi-functional protein with serine protease domain. It requires for the activation of the Toll signaling pathway associated with determination of dorsoventral axis in early embryogenesis. Also, the *nudel* involves in a role in structural integrity of the membrane which is a cross-linking of the vitelline membrane between the follicle cell layer and the embryo (Hong and Hashimoto, 1996; LeMosy et al., 1999; LeMosy and Hashimoto, 2000; Turcotte and Hashimoto, 2002).

Conclusions

A total of 32,160 unigenes obtained transcriptome analysis were annotated against the NR, NT, Swiss-Prot, KOG, KEGG, PFAM, and GO databases. The unigenes was also analyzed for differential expression level giving a total of 241 differential expression gene. The *nudel* was selected as a candidate gene due to its analysis involved in reproductive processes, had a high expression level, and expressed for up-regulated gene. In experiments, the characterization and functional of *nudel* were investigated in female banana shrimp. The *nudel* was a high expression in ovarian tissue and significantly increased in previtellogenic stage (st0) during ovarian development in ovary. The silencing of *FmNudel* affected the decrease of Vg expression in ovary.

Supplement table: Oligonucleotides used in this study

Primer name	Forward sequence (5'→3')	Purpose
oligo-dT (PRT)	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTT	cDNA synthesis
qFm-Vg-F	TCCATCTGCAGCACCAATCTTCGC	qRT-PCR
qFm-Vg-R	GCAACAGCCTTCATTCTGATGCCA	qRT-PCR
qFm-EF1α-F	GAACTGCTGACCAAGATCGACAGG	qRT-PCR
qFm-EF1α-R	GAGCATACTGTTGGAAGGTCTCCA	qRT-PCR
Stem-GFP-F	GCG-XbaI-AGCAGACTATGGACCTGACC	Cloning
Stem-GFP-R	GCT-NdeI-ATCTTCTCGGTACTGGGCTC	Cloning
Stem-loop-GFP-F	AA-NotI-AGCAGACTATGGACCTGACC	Cloning
Stem-loop-GFP-R	GCT-NdeI-ACGAAGTGGTAGTCGGGAAG	Cloning
Stem-Nudel-F	GGG-XhoI-GAAAACTGTCCTCCGCCGAT	Cloning
Stem- Nudel -R	GGG-HindIII-ACGTCTAGCAACCCGT	Cloning
Stem-loop- Nudel -F	GGG-XbaI-GAAAACTGTCCTCCGCCGAT	Cloning
Stem-loop- Nudel -R	GGG-HindIII-CACCCAATTCGTGCAAAGGC	Cloning

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