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Full-length normalization subtractive hybridization analysis provides new insights into sexual precocity and ovarian development of red swamp crayfish *Procambarus clarkii*

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ABSTRACT

The red swamp crayfish *Procambarus clarkii* is an important freshwater economic crayfish in Chinese inland due to its high market value and consumer demand. The sexual precocity in this species has received considerable attention in recent years because more and more individuals matured at small sizes, which devalues the commercial production. In this study, by the full-length normalization subtractive hybridization (FNSH) method we identified 16 differently expressed sequences between precocious and normal grown *P. clarkii* ovaries. Of the down-regulated sequences in precocious ovaries, one encodes a peptide which shared the highest identity to *C1q-like* family and contained a typical C1q domain at the C-terminal, thus it was named *PcC1q-like*. QRT-PCR analysis showed that *PcC1q-like* was transcribed specifically in ovary, with higher expression level in vitellogenic stages. *In situ* hybridization exhibited specific expression of *PcC1q-like* in follicular cells around the vitellogenic oocytes. Knockdown of *PcC1q-like* by injection of double strand RNA (dsRNA) resulted in disruption of follicular cells, decreased transcripts of *vitellogenin* (*Vg*) and *vitellogenin receptor* (*VgR*), and reduced progesterone content. These results suggest that *PcC1q-like* may promote ovarian development by stimulating the synthesis and transportation of vitellogenin into oocytes. These data provide insights into the molecular regulatory mechanisms of sexual precocity and ovarian development in crustaceans.

Statement of relevance: The red swamp crayfish *Procambarus clarkii* is one of the most important freshwater aquaculture species in Chinese inland. We screened 16 sequences that are potentially involved in sexual precocity of *P. clarkii*. One candidate was demonstrated to be a novel ovary-specific *C1q-like* gene of *P. clarkii*, thus named *PcC1q-like*. Knockdown of *PcC1q-like* resulted in disruption of follicular cells, decreased transcripts of *Vg* and *VgR*, and reduced progesterone, suggesting its essential role in vitellogenic ovarian development of *P. clarkii*.

1. Introduction

The red swamp crayfish *Procambarus clarkii* is considered as an important freshwater fishery resource and widely farmed in Chinese inland due to its flavor, high nutritive value and increasing commercial benefits. With the development of intensive culture, various problems have appeared in cultured populations, including sexual precocity. Sexually precocious *P. clarkii* has a low value due to its small size, low growth rate, poor survival, and decreased stress resistance and fertility (Wu et al., 2008; Zhou et al., 2011; Huang et al., 2015), which seriously restricts the sustainable development of this species. Therefore, understanding the mechanisms that regulate ovarian maturation and cause sexual precocity in female *P. clarkii* is crucial for improving the production of this species.

Mechanisms underlying precocity have not been well documented in crustaceans though they are thought to be influenced by both genetic and environmental factors. Studies on Chinese mitten crab *Eriocheir sinensis* show that temperature, salinity, water calcium concentration, stocking density and nutrient are associated with sexual precocity. High salinity, high temperature, high breeding density, and excess or lack of nutrients may lead to sexual precocity of this crab (He et al., 1999; Zhu et al., 1999; Li et al., 2011). In addition to environmental factors, intrinsic factors are also involved in precocity (Chen et al., 2003; Li et al., 2005; Li et al., 2011; Zhao and Lu, 2003). The liver pancreas index and liver fat content of the male and female crab in precocious puberty were significantly lower than those of the normal developmental male and female crab (Chen et al., 2003). A mixture of fish oil and soybean oil as a dietary lipid source has been demonstrated to be able to prevent precocity in juvenile *Macrobrachium nipponense* (Ding et al., 2014). Nevertheless, only a few scientific investigations have been dedicated to elucidating the molecular mechanisms underlying precocity in

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crustaceans. Using numbers of microsatellite markers isolated from the genome of *E. sinensis*, Qiu et al. (2016) constructed a first generation genetic linkage map and identified in both parent maps two quantitative trait loci (QTL) related to sexual precocity, strongly suggesting a genetic correlation with early maturation of the crab. By high-throughput sequencing of the ovaries of sexually precocious and normal sexually mature *M. nipponense*, Jiang et al. (2016) identified 9 key differentially expressed genes that may be related to sexual precocity, which involved in 'ovary development', 'ovarian steroidogenesis', 'peroxisome', 'glutathione metabolism' and 'fatty acid biosynthesis' pathways. These data dramatically improve understanding of molecular regulatory mechanism of sexual precocity of crustaceans.

C1q-domain-containing proteins have been shown to be members of TNF/C1q superfamily (Bodmer et al., 2002; Shapiro and Scherer, 1998; Kishore et al., 2004). Recent studies have revealed that they may not only perform complement functions in immune response, but also play a diverse range of non-complement functions by modulating non-immune cells, including nerve cells, follicular cells surrounding the oocyte and trophoblast cells (Csomor et al., 2007; Mei et al., 2008a; Bulla et al., 2008; Kouser et al., 2015). An ovary-specific C1q-like factor has been identified to be abundant in eggs of both *Carassius auratus* and *Carassius auratus gibelio* (Chen and Gui, 2004; Mei et al., 2008a), implying an important role of C1q-like in ovarian development.

The ovary is a multifunctional organ that plays a key role in reproduction and secretion of hormones for regulation of growth and development in female crustaceans (Racotta et al., 2003; Guan et al., 2014). Ovarian maturation is a complex process controlled by several factors, such as endocrine control, nutrition and environmental factors (Ibarra et al., 2007; Makkapan et al., 2011). The egg development and maturation occur in the ovary. However, the molecular mechanisms involved in regulating oogenesis and ovarian development in *P. clarkii* are still unclear. To investigate the molecular mechanisms of sexual precocity and ovarian development in female *P. clarkii*, we first constructed two-directed full-length normalization subtractive hybridization (FNSH) cDNA libraries and obtained 16 unique transcripts. Then we knocked down one candidate, which shares high identity with *C1q-like* family and found that it may play an essential role in ovarian development by promoting synthesis and transportation of vitellogenin. These data provide a basis to help elucidate the underlying mechanisms of sexual precocity and ovarian development in crustaceans.

2. Materials and methods

2.1. Sample preparation and RNA extraction

There were two groups of female experimental crayfish. One group was sexually precocious *P. clarkii* (SP) with gonado-somatic index (GSI) of 2.90 ± 0.06, body weight of 14.53 ± 0.78 g and body length of 8.00 ± 0.06 cm. The other group was normal grown *P. clarkii* (NG) with GSI of 0.59 ± 0.09, body weight of 16.71 ± 0.71 g and body length of 8.30 ± 0.21 cm. The crayfishes in these two groups were from the same parents and underwent the same growth period under different conditions. The SP crayfishes grew at 22 °C with the diet containing 50% fish meal and 10% soybean meal, while the NG animals were bred at 28 °C and fed the diet with 30% fish meal and 30% soybean meal.

The ovary tissues from three individuals in each group were pooled to provide sufficient RNA for cDNA library construction. Total RNA was isolated using the E.Z.N.A.® Total RNA Kit II (OMEGA) following the manufacturer's instructions. Agarose gel and A260/A280 ratio were used to determine the quality of the total RNA. Equal amounts of the RNAs from the two groups were mixed to generate a pooled sample.

2.2. Full-length normalization subtractive hybridization (FNSH) for generating differentially expressed cDNAs

First-strand cDNA was synthesized using tTScap-P and AP-polyT by an improved template-switching method as reported by Dai et al. (2007), except that a modified TdT (Terminal deoxynucleoitidyl transferase) tailing step (Xia et al., 2008) was added for generating poly(dC) tail to the 3′ end of first-strand cDNA before replicating the tTScap-P sequence into the cDNA in case of the incomplete 5′cap structure of mRNA.

The double-stranded cDNA synthesis of tester and driver was performed and the FNSH libraries were generated according to Dai et al. (2009). Briefly, the first-strand cDNA of 3′ terminal ampullae was amplified by PCR using two sets of primers: one set with PH5'CP and 3′ AP (amplified as tester 1) and the other set with 5'CP and PH3'AP (amplified as tester 2). The double-strand (ds) driver cDNA was generated from the first-strand cDNA by using primers 5'BK and 3′BK. For tester cDNA ligation, 200 ng of purified tester 1 was mixed with 1 μl ADP I, and the same amount of tester 2 was mixed with ADP II. Ligation was performed at 16 °C for 16 h. For the preparation of single-strand (ss) cDNA-enriched drivers, purified ds driver cDNA (2 μg) was used as a template. The template amplified by primer 3'BK was designated as driver 1 and other part amplified by primer 5′BK as driver 2. The single primer extension was performed in a 25 μl reaction mixture containing 0.4 μM of primer (3′BK or 5′BK), 1.25 U of Taq & Pfu DNA polymerase (Sangon) and other PCR components.

A mixture of 1.25 μl of 4× Hybridization buffer and 0.25 μl of TScap & AP-polyT (5 μM each) was added into two tubes, respectively. The first tube contained 1.5 μl of ligated tester 1 and 2 μl of driver 1; and the

Table 1
oligonucleotides (primers) and adaptors.

Oligos	Sequences
tTScap-P[a]	5′-**TGGTTGCCATAAGCGGATCAT***CCATAGAATT(G)*10-p-3′
TScap[a]	5′-**TGGTTGCCATAAGCGGATCAT***CCATAGAATTGGG*-3′
AP-polyT[a]	5′-**TGGTTGGACTCGGTTTGGACG***CCATAGAATTGG*(T)15VN-3′
5′CP[a]	5′-**TGGTTGCCATAAGCGGATCATC**-3′
PH5′CP[a]	5′-p**TGGTTGCCATAAGCGGATCATC**-3′
3′AP[a]	5′-**TGGTTGGACTCGGTTTGGACG**-3′
PH3'AP	5′-pTGGTTGGACTCGGTTTGGACG-3′
ADP I	5′-GTAATACGACTCACTATAGGGCTGTAGCGTGAAGACGACAGAAT
	TCTTAAGGTAGCT-3′
	3′-AGAATTCCATCG-5′
ADP II[a]	5′-GTAATACGACTCACTATAGGGCTGCAGGGAACCAATCCTC
	TCTTAAGGTAGCT-3′
	3′-AGAATTCCATCG-5′
5′BK[a]	5′-AATTAACCCTCACTAAAGGG**TGGTTGCCATAAGCGGATCATC**-3′
3′BK[a]	5′-AATTAACCCTCACTAAAGGG**TGGTTGGACTCGGTTTGGACG**-3′
P1	5′-GTAATACGACTCACTATAGGGC-3′
P2	5′-TGTAGCGTGAAGACGACAGAA-3′
AD2P	5′-CTGCAGGGAACCAATCCTCT-3′
3′F	5′-TGGTTGCCATAAGCGGATCATCCATAGAATT-3′
β-actin F	5′-GGCGTGATGGTTGGTATGGG-3′
β-actin R	5′-TCCGTCAGCAGGACTGGGTG-3′
PcC1q-like F	5′-CCGTGGAAGCGAGACAAC-3′
PcC1q-like R	5′-TGAGCGAAGGCGTAGAGG-3′
18s rRNA F	5′-TGAGAAACGGCTACCACAT -3′
18s rRNA R	5′-GCTTTTTAACCGCAACAAC-3′
PcC1q-like – Fi[b]	5′-*taatacgactcactatagggaga*TACCGTGGAAGCGAGACA-3′
PcC1q-like – Ri[b]	5′-*taatacgactcactatagggaga*AGGAAAGAGCAGAAAACCC-3′
PcC1q-like – Fh	5′-CTCTACGCCTTCGCTCATCT-3′
PcC1q-like –Rh	5′-CCTTGTGCCATAAGCAGTCA-3′
GFP-Fi[b]	5′-*taatacgactcactatagggaga*AGGGCGAGGGCGATGCCACC-3′
GFP-Ri[b]	5′-*taatacgactcactatagggaga*TGTACTCCAGCTTGTGCCCC-3′
Vg F	5′-CCACAAGGTAGGAGGCAATG-3′
Vg R	5′-CGCACAAGCCTTCAGACAA-3′
VgR F	5′-TGTCCTCCTCACAAGTTCACC-3′
VgR R	5′-CATCGGCACAATCTGGGTCA-3′

[a] The BstX I recognition site is given in *italics*. The I-Ppo I recognition site is underlined. The sequence of 5'CP is given in bold. The sequence of 3'AP is given in bold and underlined.
[b] The T7 promoter sequence is given in *italics*.