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Identification and expression pattern analysis of Piwi genes during the spermiogenesis of *Portunus trituberculatus*

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ABSTRACT

The Piwi genes have an important role in stem cell development, gametogenesis and RNA interference in diverse organisms. So far, most of the studies have focused on the function of Piwis in vertebrates, but their function during spermiogenesis in invertebrates still remains largely unclear. In order to investigate the function of Piwis during spermiogenesis in the crab *Portunus trituberculatus*, we use RT-PCR and RACE to identify three Piwi complete cDNA sequences from the total RNA of the testis in *P. trituberculatus*. The deduced amino acid sequences of *P. trituberculatus* Piwi-1, Piwi-2 and Piwi-3 showed that each contains a well-conserved PAZ domain and PIWI domain. RT-PCR analyzed the tissue expression pattern of *P. trituberculatus* Piwi-1, Piwi-2 and Piwi-3 in the testis, heart, muscle, hepatopancreas and gill. All of the Piwis are found in germ cells of adult testis in *P. trituberculatus* by in situ hybridization, suggesting that these genes may play function during spermiogenesis in this species.

1. Introduction

The Argonaute superfamily is a large family of proteins and characterized by the presence of conserved PAZ and PIWI domains (Cerutti et al., 2000). The N-terminal domain PAZ, is the acronym for Piwi and has their homologs in *Arabidopsis*, Argonaute, and Zwille, which together with the mid (middle) domain to bind a small RNA (Song et al., 2004). The C-terminal domain PIWI, is often capable of cleaving target RNAs and resembles RNase H (Gunawardane et al., 2007; Okamura et al., 2004). Based on the sequence similarity with *Arabidopsis* Argonaute protein or *Drosophila* Piwi protein, the Argonaute superfamily is divided into two branches: the Ago subfamily that is related to the *Arabidopsis* Argonaute protein and Piwi subfamily proteins that is homologous with the *Drosophila* Piwi protein (Farazi et al., 2008; Hock and Meister, 2008; Hutvagner and Simard, 2008).

The Ago subfamily protein expression is ubiquitously in biological tissues. Ago proteins bind microRNAs (miRNA) and small-interfering RNAs (siRNA), both of them by Dicer into 20–22-nucleotide length from double-stranded precursors (Kim et al., 2009; Wu and Belasco, 2008). However, Piwi protein expression is mostly restricted to germ cells and stem cells (Thomson and Lin, 2009). Piwi proteins bind to Piwi-interacting RNAs (piRNA, 26–31-nucleotide length in general), which are processed in a Dicer-independent pattern from long single-stranded precursors (Aravin et al., 2006; Grivna et al., 2006a,b; Houwing et al., 2007).

Homologues of Piwi have been identified in a number of diverse organisms, especially in vertebrates. The three types of Piwi proteins identified in *Drosophila* are Piwi, Aubergine and Ago3, Piwi and Aubergine are germ cell-specific in *Drosophila*, Piwi is necessary for the self-renewal of germline stem cells, and mutation of Aubergine causes male sterility and maternal lethality (Cox et al., 1998, 2000; Lin and Spradling, 1997). Three have been identified in mouse (*Mus musculus*): MIWI, MILI and MIWI2. MIWI and MILI are restricted to germ lineages and knockout of MIWI or MILI leads to male sterility by blocking the process of spermatogenesis (Deng and Lin, 2002; Grivna et al., 2006a,b; Kuramochi-Miyagawa et al., 2001, 2004). Mutation of MIWI2 causes a loss of germ cells (Carmell et al., 2007). Two Piwi homologues Ziwi and Zili were identified in zebrafish (*Danio rerio*), and both of them are crucial for germ cells. Ziwi, Piwi like 1 of zebrafish, is co-expressed with Vasa in germ cells during embryogenesis. Reducing Ziwi causes a loss of germ cells during larval development and abnormal apoptosis of germ cells in adults. Zili, Piwi like 2 of zebrafish, is required

Abbreviations: A, acrosome; aa, amino acids; AC, apical cap; Ago3, Argonaute3; APES, 3-Aminopropyl-triethoxysilane; AT, acrosomal tubule; BCIP, 5-bromo-4-chloro-3-indolylphosphate; DEPC, diethylpyrocarbonate; DIG, Digoxigenin; EV, endoplasmic reticulum vesicle; GSP, gene specific primer; IL, inner layer; MC, membrane complex; ML, middle layer; NBT, nitroblue tetrazolium chloride; NJ, neighbor-joining; NUP, Nested Universal Primer; OL, outer layer; ORF, open reading frame; PAZ, Piwi–Argonaute–Zwille; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PG, proacrosomal granule; piRNA, piwi-interacting RNA; Piwi, P-element induced wimpy testis; PM, plasma membrane; PV, proacrosomal vesicle; RA, radial arms; RACE, rapid-amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SSC, standard saline citrate; TD-PCR, touchdown PCR; UPM, Universal Primer Mix; UTR, untranslated region.

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for germ cell differentiation and meiosis (Houwing et al., 2007, 2008; Tan et al., 2002). These researches showed that Piwi may play an important role in germ cell development in vertebrate.

While the research of Piwi in invertebrate is extremely rare, the invertebrate *Portunus trituberculatus* is an important aquaculture and export economical crabs of China, the functions of Piwis in this species have not been reported. In the present study, we hypothesize that Piwis also exist in invertebrates and have a role in spermiogenesis. For the first time, we use the methods of RT-PCR, 3′ RACE, 5′ RACE, in situ hybridization and relevant software to research the expression of them in *P. trituberculatus* and to testify our hypothesis. Here, we obtained three full length cDNA sequences coding for peptides that belongs to the Piwi family from the testis in *P. trituberculatus*, we named it Piwi-1, Piwi-2 and Piwi-3. We found that all of Piwis were expressed in spermiogenesis of *P. trituberculatus* and their expression was a little different.

2. Materials and methods

2.1. Animals

We purchased mature *P. trituberculatus* from the aquatic products market at Luojia Village (Hangzhou, China) and each individual weight is 100 g–200 g. The testis, heart, hepatopancreas, gill, and muscle were dissected from mature individuals, sharp-frozen in liquid nitrogen and then stored at −80 °C for further study.

2.2. Molecular cloning of cDNA ends

We used Trizol reagent (TIANGEN, Beijing, China) to extract total RNA from the testis of *P. trituberculatus* and then about 1 μg of total RNA employed to synthetize first-strand cDNAs according to the manufacturer's instructions (Takara, Dalian, China). Followed by the Touchdown PCR (TD-PCR) to clone the middle fragment of Piwis cDNA, we designed several degenerate primers (Table 1). Afterwards, PCR amplifier was made in Mygene Series Peltier Thermal Cycler (Mygene, Hangzhou, China) with the following program: 94 °C for 5 min, 14 cycles in a TD program (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a 0.5 °C decrease of the annealing temperature per cycle), then 30 cycles were running (30 s at 94 °C, 30 s at 48 °C, and 30 s at 72 °C) with 10 min at 72 °C for the final extension. The PCR products were isolated by agarose electrophoresis, purified, cloned in the pMD18-T vector (Takara, Dalian, China), transformed into DH5α competent cells (Takara, Dalian, China), coated plates, select monoclonal colony PCR and then sequenced by BGI Company, Hangzhou, China.

According to the obtained Piwi cDNA fragment, we designed specific primers (Table 1) to amplify the complete Piwi cDNAs. Subsequently, we used 3′ Full RACE Amplification Kit (Takara, Dalian, China) and Smart RACE cDNA Amplification kit (Clonetech, USA) to get the 3′ and 5′ cDNA ends, respectively.

For 3′ RACE of different Piwi genes, we adopted Nest PCR and TD PCR. The first round PCR was performed using a 3′ outer primer as the reverse primer that included in the kit, the designed specific primers piwi1-3GSP1, piwi1-3GSP3, piwi2-3GSP1, and piwi3-3GSP1 as the forward primer (Table 1), respectively. Subsequently, the second round PCR was performed using 3′ inner primer as the reverse primer that was included in the kit, and the designed specific primers piwi1-3GSP2, piwi1-3GSP4, piwi2-3GSP2, and piwi3-3GSP2 as a forward primer (Table 1) to get three Piwi genes, respectively.

The program of Piwi-1 (primer pairs: piwi1-3GSP1 and 3′ RACE outer, piwi1-3GSP2 and 3′ RACE inner) for PCR amplification as follows: 94 °C 5 min, 10 cycles of touchdown program (94 °C for 30 s, 70 °C for 30 s, 72 °C for 1 min, followed by 1 °C decrease of the annealing temperature per cycle), followed by 29 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 10 min. The program of Piwi-1 (primer pairs: piwi1-3GSP3 and 3′ RACE outer, piwi1-3GSP4 and 3′ RACE inner) for PCR amplification as follows: 94 °C 5 min, 14 cycles of touchdown program (94 °C for 30 s, 68 °C for 30 s, 72 °C for 1 min, followed by 0.5 °C decrease of the annealing temperature per cycle), followed by 29 cycles at 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 10 min.

Table 1
Primers used in the present study.

Primer name	Primer sequence (5′ to 3′)	Usage
DPF1	GCCATCCAGATGAACTGYAARHTNGG	Cloning
DPR1	GCCAGCTTGTGGGCRTAYTGRCA	Cloning
DPR2	TCGCCCACGCCRTCNCKRTA	Cloning
piwi1-3GSP1	TGGTCTAATGGTTTGTGGTGTGGA	3′ RACE for piwi-1
piwi1-3GSP2	TTCTGTGGTGGGGTTTGTAGCCTC	3′ RACE for piwi-1
piwi1-3GSP3	GTTCAGAAGAGACTAAACACAAGG	3′ RACE for piwi-1
piwi1-3GSP4	ATTTCTTCCTTGTATCACAGCACG	3′ RACE for piwi-1
piwi2-3GSP1	CTTTACCCTCGGAGTGAAGAACGC	3′ RACE for piwi-2
piwi2-3GSP2	GCTGGAGACTTACCACAGGGAGAA	3′ RACE for piwi-2
piwi3-3GSP1	ACAATGGTGGTAGGATACGATGCT	3′ RACE for piwi-3
piwi3-3GSP2	TGCTCGGGTCAACGGGTGCCT	3′ RACE for piwi-3
Outer-3GSP (kit)	TACCGTCGTTCCACTAGTGATTT	3′ RACE
Inner-3GSP (kit)	CGCGGATCCTCCACTAGTGATTTCACTATAGG	3′ RACE
piwi1-5GSP	CCACAATGGAGCCAGGAGGAGGGTT	5′ RACE for piwi-1
piwi2-5GSP1	ACCAGCACACGGGAGGGCAGG	5′ RACE for piwi-2
piwi2-5GSP2	GACACCACCGCACCACACGACACATTT	5′ RACE for piwi-2
piwi3-5GSP1	CACCCGTTGACCCGAGCATAG	5′ RACE for piwi-3
piwi3-5GSP2	TGTTCCTGAGTGGCATCAGCACAGACC	5′ RACE for piwi-3
UPM-5GSP (kit)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5′ RACE
NUP-5GSP (kit)	AAGCAGTGGTATCAACGCAGAGT	5′ RACE
piwi1-FH	GCATCACACCTAATGTTCGTCA	RT-PCR & RNA probe for piwi-1
piwi1-RH	CTGCTCGCAATTTCTTTTCTTG	RT-PCR & RNA probe for piwi-1
piwi2-FH	ACTGACGAGATGCGTGCTGAC	RT-PCR & RNA probe for piwi-2
piwi2-RH	AGACGAGGCTTTTTGAAGAGA	RT-PCR & RNA probe for piwi-2
Piwi3-FH	AAGCACTGGAGTTGTGGAAGG	RT-PCR & RNA probe for piwi-3
Piwi3-RH	GCAGCGACGTAGAAGTATGGA	RT-PCR & RNA probe for piwi-3
βActin-F	TGCCCTTCCTCACGCTATCC	RT-PCR for β-Actin
βActin-R	AGGGCGGTGATTTCCTTCTG	RT-PCR for β-Actin
R(A,G) Y(C,T) K(G,T) H(A,C,T) N(A,C,G,T).		