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Molecular characterization and expression analysis of an insulin-like gene from the androgenic gland of the oriental river prawn, *Macrobrachium nipponense*

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ABSTRACT

The androgenic gland (AG), a male-specific endocrine organ in crustacean, is responsible for the maintenance of male characteristics and gender differentiation. In this study, an AG-specific gene, the *Macrobrachium nipponesne* insulin-like androgenic gland factor (*MnIAG*) was isolated from a transcriptome library of *M. nipponesne* and its full-length cDNA sequences were obtained by RACE method. The cDNA was 1,547 bp in length and encoded a precursor protein of 175 amino acids. The deduced precursor protein consisted of a signal peptide, B chain, C peptide and an A chain, which exhibited the same structural organization as that of previously identified insulin-like androgenic gland in crustacean. The mature peptide of the *MnIAG* owned two additional conserved cysteine residues, which were also found in the Palaemonidae species reported. Results of the tissue distribution and *in situ* hybridization showed the *MnIAG* expressed exclusively in androgenic gland. The quantitative RT-PCR results demonstrated that the *MnIAG* transcript was present at blastula stage and later developmental stages with low levels, which suggested that the primordial cells of the AG might form at these stages.

1. Introduction

Decapod crustaceans contain many commercially important species, including penaeid shrimps, freshwater prawns, crayfishes, lobsters and crabs (Jung et al., 2011; Ma et al., 2012; Wang et al., 2006). The oriental river prawn *Macrobrachium nipponense*, a member of the Palaemonidae family of decapod crustaceans, is naturally distributed throughout almost freshwaters in China, able to overwinter outdoors by itself, and can survive in freshwater for its entire life cycle (Fu et al., 2012; Ma et al., 2012; Ventura et al., 2009). In 2009, the culture yields of this prawn exceeded 200,000 tons, and the price of *M. nipponense* has exhibited a continuing rise during the past decade (Fu et al., 2012). Owing to its excellent adaptability and high benefit, *M. nipponense* has been an important species for freshwater aquaculture in China (Feng and Li, 2008; Ma et al., 2011; Zhang et al., 2011) and other Asian countries or regions (Cai and Shokita, 2006; S. De Grave et al., 1849; Yu and Miyake, 1972). However, due to gonochoristic and sex dimorphism, the males grow much faster than females and reach a larger size at harvest (Ma et al., 2012). Hence monosex culture of an all-male stock could result in a significantly higher yield for this prawn.

In crustaceans, as an endocrine organ unique to males, the androgenic gland (AG) plays a crucial role in the development of the male gonad and secondary sexual characteristics, while inhibiting female secondary characteristics (Sagi et al., 1990). Although the AG was discovered in 1947, its function of controlling male sexual characteristics in crustaceans was first described in the amphipod crustacean, *Orchestia gammarella* 15 years later (Charniaux-Cotton, 1954; Cronin, 1947). Thereafter, it was also discovered in other crustacean species, including isopod and decapod species (Banzai et al., 2011; Charniaux-Cotton, 1954; Chung et al., 2011; Manor et al., 2007; Mareddy et al., 2011; Sagi and Khalaila, 2001). Further studies showed that ablation or implantation of the AG at a certain stage of development could result in sex reversal to female or male, respectively. In intersex individuals, *Cherax quadricarinatus*, ablation of the AG leaded to the loss of male-like behavior, demasculinization and physiological shifts (Barki et al., 2006). In gonochoristic *Macrobrachium rosenbergii*, ablation of the

Abbreviations: AG, androgenic gland; IAG, insulin-like androgenic gland factor; AGH, androgenic gland hormone; ORF, open reading frame; DIG, digoxigenin; qRT-PCR, quantitative real-time PCR.

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AG at early developmental stage of normal males also resulted in sexual reversal (Aflalo et al., 2006), whilst the AG implantation into normal young females caused the appearance of male secondary sexual characteristics (Nagamine et al., 1980). Until now, it is widely accepted that sexual differentiation in crustaceans is mediated by the AG.

Studies have indicated that the AG secretes a kind of hormone, called androgenic gland hormone (AGH). In 1999, the AGH was isolated from the terrestrial isopod *Armadillidium vulgare* and the active component was verified to be a glycosylated protein, which was composed of two peptide chains connecting by two disulfide bridges (Martin et al., 1999). Subsequently, the ultrastructure of AG cells showed cytoplasm consisting of numerous rough endoplasmic reticulum, Golgi complex and secretory granules, which also supported the AGH was a protein hormone (Negishi et al., 2001). However, in decapods, the AGH has not yet been purified until now. Instead, a AG-specifically expressed gene (IAG) was identified from the AG of several decapods species (Manor et al., 2007). The factor owned the similarity linear and the typical three-dimensional structure with the isopod AGH (Ventura et al., 2011). Silence of the IAG gene expression by RNAi can inhibit development of male secondary sex characteristics in the *M. rosenbergii*, indicated that the IAG plays a key role on maintenance of male sexual differentiation in decapods similar to isopod AGH (Ventura et al., 2009).

To accumulate sequence information of additional IAGs, better known the evolution of IAGs in decapods, and further investigate gene expression of IAGs during early development, here we cloned and reported for the first time the complete *MnIAG* transcript sequence, encoding the insulin-like androgenic gland factor in the commercially valuable freshwater prawn *M. nipponense*. Meanwhile we investigated the AG tissue-specific gene expression patterns of *MnIAG* in various tissues by RT-PCR and *in situ* hybridization, respectively. The cloning of the *MnIAG* gene provides a useful tool for the studies of male sex differentiation and monosex culture of an all-male stock in *M. nipponense*.

2. Materials and methods

2.1. Animal treatment and tissue sampling

Adult *M. nipponense* prawns used in this study were purchased from a local fish market in Shanghai and transported to our laboratory. The prawns were fed once daily and maintained at 28 ± 2 °C in 120 L aerated aquaria for three days before tissues and embryos were collected. A variety of tissues, including heart, eyestalk, hepatopancreas, gill, muscle, ovary, androgenic gland and testis, were collected from each female/male prawn.

The developmental stages of embryos were determined following the criteria by Chen et al. Chen et al. (2012). These stages included: unfertilized eggs; eggs at 0 h post-fertilization; embryos at the 2–4-cell stage, embryos at the 8–16-cell stage, embryos at the 32–128-cell stage, blastula stage, gastrula stage, nauplius stage, protozoea stage and zoea stage. All of the samples were immediately frozen in liquid nitrogen and stored at −80 °C until total RNA extraction. Androgenic gland was also fixed in Bouin's fixative or 4% paraformaldehyde solution overnight at 4 °C for histological observation or *in situ* hybridization (ISH).

2.2. Cloning of the full-length MnIAG complementary DNA (cDNA)

Total RNA was extracted from the tissues and embryos stated above using RNAiso Plus reagent (TaKaRa, Japan) and stored at −80 °C. A 1,519 bp fragment of *MnIAG* gene was obtained from a *de novo* transcriptomic library (Ma et al., 2012) of *M. nipponense* and used to clone the full-length cDNA of *MnIAG* by Rapid Amplification of cDNA Ends (RACE) method using the RNA PCR Kit (AMV) ver. 3.0 (TaKaRa, Japan) according to the user's manual provided by the manufacturer.

The full-length cDNA of *MnIAG* was obtained by 3′-RACE with a gene-specific primer (Table 1). The 3′-RACE reaction was performed in a 50 μl volume containing 10 μl of 5 × PCR buffer, 36.75 μl of PCR-grade water, 0.25 μl of TaKaRa Ex Taq® HS (TaKaRa, Japan), 0.5 μl gene-specific primer (10 μM), 0.5 μl M13 Primer M4 (10 μM) and 2 μl of RACE-Ready cDNA under the following cycle profile: 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min. The PCR product was ligated into pGEM®-T easy vector (Promega, USA), transformed into the competent *Escherichia coli* DH5α cells, plated on the LB-agar Petri dish and incubated overnight at 37 °C. Positive clones containing the insert with expected size were identified by colony PCR. Six of the positive clones were picked up and sequenced on an ABI PRISM 3730 Automated Sequencer using BigDye terminator v3.1 (Applied Biosystems, USA).

2.3. Bioinformatics analysis

Nucleotide and amino acid sequence identity were performed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame (ORF) of *MnIAG* cDNA was determined using the ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/); the presence of signal peptide and potential endoproteinase arginine cleavage sites for removal of C peptide were processed with ProP 1.0 server (http://www.cbs.dtu.dk/services/ProP/).

The known sequences (Fig. S1) of IAG homologs, including 10 decapods and seven isopods, were obtained from the GenBank database for multiple sequence alignment and phylogenetic analysis of *MnIAG*. The sequences were aligned using the ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). A phylogenetic tree was constructed based on the deduced full-length amino acid sequence alignments by the Neighbor-Joining (NJ) algorithm embedded method in Mega 5.0 program (Tamura et al., 2011). The bootstrap test was employed based on 10,000 pseudo-replications to assess the reliability of the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset.

2.4. Tissue-specific gene expression of MnIAG

In order to examine the expression of the *MnIAG* mRNA, tissues were collected from four adult *M. nipponense*. Total RNA treated with DNase I (Promega, USA) from the tissues was submitted to reverse transcription (RT) using RNAiso plus reagent (TaKaRa, Japan). The sequences of *MnIAG* cDNA specific primers used in RT-PCR were showed in Table 1. The reaction conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, and finally, 72 °C for 5 min. Meanwhile, β-actin was amplified as a positive control using a pair of primers (Table 1). The reaction conditions were the same as those stated above except for annealing at 58 °C for 30 s. The RT-PCR products were separated by electrophoresis on a 1.5% agarose gel and verified by sequencing.

2.5. Preparation of digoxigenin (DIG)-labeled RNA probe and ISH

The cDNA fragments corresponding to nucleotides 745–1305 of *MnIAG* transcript was amplified and sub-cloned into pGEM®-T Easy vector (Promega, USA), and then the recombinant plasmid was linearized either with SacII (Promega, USA) or with SpeI (Promega, USA).