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The nuclear receptor E75 from the swimming crab, *Portunus trituberculatus*: cDNA cloning, transcriptional analysis, and putative roles on expression of ecdysteroid-related genes

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ABSTRACT

The nuclear receptor E75 is an early-responsive gene in 20-hydroxyecdysone (20E) signaling pathway, and is found to play essential roles in many aspects of arthropods development. In this study, a cDNA encoding the E75 nuclear receptor of the swimming crab, *Portunus trituberculatus* was cloned using RT-PCR and RACE. The *PtE75* cDNA was 3211 bp in length, and encodes a protein of 795 amino acids. The DBD region of the predicted amino acid sequence for PtE75 was highly conserved with other arthropoda E75s, while its LBD region was more similar to decapod E75s. Tissue distribution analysis showed that *PtE75* transcript was widespread among tissues and relatively abundant in Y-organ, epidermis, eyestalk, and muscles. *PtE75* exhibited tissue-specific expression patterns in these four tissues, which may depend on the distinct action of 20E on different tissues. The expression of *PtE75* in Y-organ and epidermis were induced by eyestalk ablation (ESA), indicating its responsiveness to the increasing hemolymph 20E titer. To identify potential targets for ecdysteroid control the in Y-organ, epidermis, and eyestalk, the expression change of an ecdysteroid synthesis gene PtSad in Y-organ, a chitin degradation gene PtChi1 in epidermis, and the molt-inhibiting hormone gene PtMIH in eyestalk were investigated after silencing of PtE75 mRNA. The double stranded RNA (dsRNA) of PtE75 caused a loss in *PtChi* and *PtMIH* expression, while an increase in *PtSad* expression. The results suggested that the ecdysteroids may act through E75, and play a stimulatory role on chitin degradation in epidermis and MIH synthesis in eyestalk, and an negative feedback effect on ecdysteroid synthesis in Y-organ.

1. Introduction

The ecdysteroids are polyhydroxylated C27 steroids that play vital roles in arthropod growth, development, metamorphosis, diapause and reproduction (Laufer et al., 2002; Qu et al., 2015; Riddiford et al., 2003; Rotllant et al., 2000; Schwedes and Carney, 2012). A pair of molting glands, Y-organ in crustaceans and the prothoracic gland in insects, synthesized and secreted the inactive ecdysteroids into the hemolymph which are then converted by peripheral tissues into more active forms of the hormone, primarily 20-hydroxyecdysone (20E) (Mykles, 2011).

Most effects of 20E are mediated through the transcriptional regulatory functions of a heterodimer formed by two members of the nuclear receptor (NR) superfamily, the ecdysone receptor (EcR) and retinoid-X-receptor (RXR, also known as USP in insects). This hormone/receptor complex directly regulates expression of ecdysone-responsive genes by binding to specific promoter sequences called ecdysone response elements (EcREs), and these genes, in turn regulate further downstream gene expression cascades directing appropriate biological responses to the ecdysteroid pulse during development (King-Jones and Thummel, 2005; Schwedes and Carney, 2012).

The molecular mechanism of the 20E-induced gene cascades has been described mostly in insects, especially in *Drosophila melanogaster*. Central to this hierarchical model was the genes responsible for early 20E-induced puffs in the giant larval salivary gland polytene chromosomes (Ashburner et al., 1974). One well-characterized gene is E75 at the 75B puff, which is also the member of the nuclear receptor superfamily and its sequence is most similar to the vertebrate nuclear receptor Rev.-erbα (Reinking et al., 2005). E75 is required for molting and metamorphosis in *D. melanogaster* (Bialecki et al., 2002) and *Blattella germanica* (Mane-Padros et al., 2008). It also serves an essential role in oogenesis and vitellogenesis in several insect species (Bialecki et al., 2002; Bryant et al., 1999; Raikhel et al., 2002; Swevers et al., 2002).

In crustaceans, the putative orthologs of E75 were identified in the shrimp *Metapenaeus ensis* (Chan, 1998), *Fenneropenaeus chinensis* (Priya et al., 2010), *Litopenaeus vannamei* (Qian et al., 2014), and the land crab *Gecarcinus lateralis* (Kim et al., 2005), and the water flea *Daphnia magna* (Hannas and LeBlanc, 2010) and *Daphnia pulex* (Litoff

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et al., 2014). Injection with E75 double-stranded RNA (dsRNA) completely arrested the molting process in *F. chinensis* attributable to the defective epidermal retraction and the poor development of setae and new cuticle (Priya et al., 2010). E75, together with the EcR and RXR, were reported to be crucial for the time- and tissue-specific functions of ecdysteroids in L. *vannamei* (Qian et al., 2014).

The swimming crab, *Portunus trituberculatus*, is a commercially important crustacean species and is one of the most common edible crabs in Chinese waters. A comprehensive understanding on the 20E-induced hierarchical model of this species may be essential for controlling its growth and development. In this study, the cDNA of nuclear receptor E75 from the swimming crab, *P. trituberculatus* was identified and its expression changes during the molt cycle and after eyestalk ablation (ESA) were determined. In addition, the roles of PtE75 on expression of ecdysteroid-related genes in different tissues were investigated using the dsRNA-mediated RNA interference (RNAi) technique.

2. Materials and methods

2.1. Experimental animals

Wild juvenile swimming crabs (*P. trituberculatus*) of 30–50 g body weight were collected and temporarily reared at the Ninghai Deshui Aquaculture Farm. All crab investigations were carried out according to Animal Care and Use of Science and Technology guidelines. Crabs were maintained in large tanks with through-flowing sea water and fed with live razor clams once a day. Intact crabs with no injuries or missing pereiopods were selected for sampling, and the molting stages of experimental animals were determined by observing their morphological characteristics as previously described (Shen et al., 2011). Y-organ, epidermis, eyestalk, and muscle of each staged crab were removed and placed in RNA preservation fluid (Shanghai Sangon), and stored at −20 °C. For the intermolt crabs, tissues including hepatopancreas, brain, gill, mandibular organ, intestines, thoracic ganglia, ovary, testis, and heart were also sampled for tissue distribution analysis. 25 other intermolt crabs were treated with eyestalk ablation (ESA), and the eyestalks of individual crabs were clipped bilaterally using sharp sterile scissors and the wounds were cauterized with flame-heated sharp spatula to minimize hemolymph loss and infection. 5 individuals were randomly selected and sacrificed at 0, 1, 2, 4 and 8 d post ESA. Y-organ and epidermis of each crab were then obtained and stored as described above.

2.2. RNA isolation and cDNA preparation

Total RNA of tissue samples was extracted using Trizol RNA isolation reagent (Sangon) according to the manufacturer's instructions, and genomic DNA was removed by DNase I (Takara). RNA concentrations was determined using a NanoDrop 2000 UV Spectrophotometer (Thermo Fisher), and RNA integrity was determined by visualization of clear bands of the 18S and 28S ribosomal RNA on agarose gels. cDNA was then prepared with 1 μg RNA using Perfect Real Time PrimeScript® RT reagent Kit (Takara), and stored in −80 °C.

2.3. Cloning of the full-length cDNA of PtE75

The cDNA of epidermis was used as templates for validation of the sequence information in our transcriptome database and the primers for RT-PCR were E75-F (5′-ACGCACGCTTCTTAGTGGATT-3′) and E75-R (5′-GGGCTGGTGAT-ACTCGGGAC-3′). To obtain the full-length cDNA, 5′ RACE and 3′ RACE were performed according to the manufacturer's instructions of SMARTer™ RACE cDNA Amplification kit (Clontech, USA). The primer were E75-3F (5′-CGGGAACGGTTACCATCGG-3′) for 3′ RACE and E75-5R (5′-TGCGAAGCCCTGGTCTGTC-3′) for 5′ RACE. The following program was used for each PCR amplification: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 90 s, with a final elongation at 72 °C for 10 min. All PCR products were analyzed by 1% agarose gel electrophoresis, and the PCR products were gel-purified using the PCR purification kit (Sangon), ligated with the pMD19-T vector (Takara), and transformed into competent *Escherichia coli* cells. After transformation, three positive clones were picked for sequencing (Invitrogen).

2.4. Sequence analysis of PtE75

Sequence splicing and open reading frame (ORF) identification were performed using Vector NTI 10.0 software, and the conserved domains were searched from NCBI website. Sequence analysis was performed using the ExPASy Molecular Biology server (http://www.us.expasy.org/). The deduced protein sequence was aligned with known E75 sequences from other crustacean species derived from NCBI database using the Clustal W multiple sequence alignment program. A phylogenetic tree was generated with the neighbor joining option of molecular evolutionary genetics analysis (MEGA) version 5.0 by multiple sequence alignment with 15 known sequences from NCBI database (name and protein id are given in Fig. 2). Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree branch positions.

2.5. Preparation and injection of PtE75 dsRNA

An amplicon of *PtE75* was obtained using the primer E75-dF (5′-TTCATC-TCCACGCTCATCCA-3′) and E75-dR (5′-GACTCATACTGCCGCT GCTACT-3′), while the amplicon of *green fluorescent protein* (*GFP*, *Genbank accession no*. U55762) was obtained using the primer 5′-CGACGTAAACGGCCACAAGT-3′ and 5′-CTTGTACAGCTCGTCCATGC-3′.

Table 1
The qPCR primers used in this study.

Gene	GenBank accession No.	Product Size	Primer (5'-3')
E75	KM016226	118 bp	Forward: CGAGAGCCTAGTGATGTA Reverse: ATGAGTGATGAGCGAGTA
EcR	KC354381	150 bp	Forward: TAAGTGATGACGACTCGGATGC Reverse: ACGAGCAAGCCTTTAGCAGTG
RXR	KF061043	136 bp	Forward: AGCGTCAGAGGACAAAAGGC Reverse: TGGTCCAGTGGCTGCTCAT
Sad	KM880023	110 bp	Forward: CACGGCATTTTCAAGGAGA Reverse: AAGGCGTCATCCAGGCACT
Chi1	AB874469	190 bp	Forward: CCCAGCCGATAGGAAGACC Reverse: CGCTGTCAGTATCATTCCGTTAG
MIH	EU284117	110 bp	Forward: TATCAAGTGCAGGAACTCAG Reverse: GGAACATACAAGCCTAAACA
β-actin	FJ641977	154 bp	Forward: CGAAACCTTCAACACTCCCG Reverse: GATAGCGTGAGGAAGGGCATA