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Role of Halloween genes in ecdysteroids biosynthesis of the swimming crab (*Portunus trituberculatus*): Implications from RNA interference and eyestalk ablation

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ABSTRACT

Molting, including metamorphosis molting in arthropods are controlled by the ecdysteroids that are synthesized and secreted by the crustacean Y-organ (YO) or the insect prothoracic gland (PG). The Halloween genes encoding the enzymes mainly involved in the biosynthesis of ecdysteroids are well studied in insects but not in crustaceans. Given the importance of Halloween genes in ecdysteroids biosynthesis, we have previously reported the cDNA cloning of disembodied (Dib) in *P. trituberculatus*. Here, cDNA sequences of another two Halloween genes, Spook (Spo) and Shadow (Sad), were further identified and characterized. The predicted amino acid sequences for these two Halloween genes of *Portunus trituberculatus* were compared to those of several other arthropods, and several typical domains of the cytochrome P450 mono-oxygenase (CYP) were identified. Similar to the tissue distribution of *Dib*, the *Spo* and *Sad* also showed high specificity to the YO. RNA interference (RNAi) of these 3 genes indicated they all play essential role in ecdysteroids biosynthesis. To investigate the relationships of the Halloween genes to the eyestalk neuropeptides such as molt-inhibiting hormone (MIH), effects of eyestalk ablation (ESA) on the expression of *Dib*, *Spo* and *Sad* were detected. Expression of *Dib* and *Sad*, but not *Spo*, was significantly induced by ESA. The result indicated that the inhibition of MIH in ecdysteroids biosynthesis may be partly through the transcriptional regulation of certain Halloween genes, such as Dib and Sad, while the Spo might not be the target for MIH signal.

1. Introduction

Molting, including metamorphosis molting in arthropods are controlled by the ecdysteroids, which function as crucial coordinators of cell proliferation, differentiation, and apoptosis (Schwedes and Carney, 2012). The ecdysteroids are polyhydroxylated C27 steriods that synthesized and secreted by a pair of molting glands: Y-organ (YO) in crustaceans and the prothoracic gland (PG) in insects. 20-hydroxyecdysone (20E) has been characterized as the primary physiologically active form of the ecdysteroids in insects, whereas the crustaceans may possess more than one active form in addition to 20E (Mykles, 2011).

Recent advances in elucidating the biosynthetic pathway for ecdysteroids in insects and crustaceans suggest the origin of this pathway is deeply rooted in the evolutionary history of arthropods (Rewitz and Gilbert, 2008; Sin et al., 2015). Among seven enzymes that are mainly involved in the biosynthesis of ecdysteroids, five of them are cytochrome P450 mono-oxygenases (CYPs). The genes for these seven enzymes, collectively known as Halloween genes, include neverland (nvd), non-molting glossy/shroud (sro), spook (spo, CYP307A1), phantom (phm, CYP306A1), disembodied (dib, CYP302A1), shadow (sad: CYP315A1) and shade (shd: CYP314A1). In early steps of the steroidogenic pathway, the dietary cholesterol is taken up by the molting gland, and converted to 7-dehydrocholesterol (7DC) by 7, 8-dehydrogenase, encoded by the Nvd gene (Yoshiyama et al., 2006). 7DC is then converted to Δ4-Diketol, the central precursor to the multiple derivative steroid products, by a series of reactions involving enzymes encoded by Sro and Spo or its paralogous genes (Ono et al., 2006; Niwa et al., 2010). The last four steps of this pathway are catalyzed by the hydroxylases at C25, C22, C2, and C20, which are encoded by Phm, Dib, Sad and Shd, respectively (Mykles, 2011).

Although the ecdysteroids biosynthesis pathways are similar in insects and crustaceans, their neurohormonal control seemed to be opposite in action. Unlike the PG ecdysteroidogenesis is under stimulatory regulation by prothoracicotropic hormone (PTTH), the primary regulation of YO ecdysteroid synthesis is inhibitory (Covi et al., 2012). The YO ecdysteroidogenesis is negatively regulated by two neuropeptides synthesized and secreted by the X-organ/sinus gland (XO-SG) complex in eyestalk, namely molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH). These neuropeptides are members of

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CHH family which control growth, reproduction, osmoregulation, and metabolism in crustaceans (De Kleijn and Van Herp, 1995; Lacombe et al., 1999; Nakatsuji et al., 2009; Chung et al., 2010; Webster et al., 2012). Both peptides have been proved to suppress the secretion of ecdysteroids by YO in vitro (Nakatsuji and Sonobe, 2004; Lee et al., 2007) and in vivo (Chang et al., 1990; Chung, 2010), although MIH is 10–20 times more effective than CHH (Chung and Webster, 2003). The suppression of MIH may act through the transcriptional or translational regulation (Chang and Mykles, 2011). As in the shrimp, *Marsupenaeus japonicus*, the sharply increase in *Phm* expression during premolt can be blocked by sinus gland extract and recombinant MIH (Asazuma et al., 2009), the Halloween genes may be a target of MIH signaling.

The swimming crab, *Portunus trituberculatus*, is a commercially important crustacean species and is one of the most common edible crabs in Chinese waters. As it has been extensively artificially propagated and cultivated, comprehensive understanding on the ecdysteroids-mediated molting process is essential for controlling its growth and development. Given the importance of Halloween gene in ecdysteroids biosynthesis, we have tried for several years to clone the *P. trituberculatus* Halloween gene orthologs using degenerate primers based on the *Daphnia*, *Drosophila*, and *Bombyx* genes, but only the full-length cDNA of Dib (GenBank accession no. **KM596851**) was obtained (Liu et al., 2015). Here, we cloned the full-length cDNA of another two Halloween genes, Pt-spo and Pt-sad, based on their sequence information in our *P. trituberculatus* transcriptome database. Their tissue distribution and expression patterns during the molt cycle were detected using qPCR. In addition, the roles of these three Halloween genes in ecdysteroids biosynthesis of *P. trituberculatus* were further investigated by RNA interference (RNAi) and eyestalk ablation (ESA).

2. Materials and methods

2.1. Experimental animals

Wild swimming crabs (body weight: 35–50 g) 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. Molting stages of experimental animals were determined by observing their morphological characteristics as previously described (Shen et al., 2011). Intact crabs with no injuries or missing pereiopods were selected for sampling, and YOs of each staged crab were removed, and placed in RNA preservation fluid (Sangon), and stored at −20 °C. For the intermolt crabs, tissues including mandibular organ, eyestalk, thoracic ganglia, hepatopancreas, epidermis, testis, ovary, and heart were also sampled for detecting the tissue distribution of *Pt-spo* and *Pt-sad*.

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 were 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 gel. 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 and sequence analysis of Pt-spo and Pt-sad

All Gene-specific primers in cDNA cloning were designed from the sequence information in our *P. trituberculatus* transcriptome database (Table 1). The cDNA of YO was used as templates for PCR validation of the sequence information. 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 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, 57 °C (58 °C in 3′ RACE) 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).

Sequence splicing and open reading frame (ORF) identification were performed using Vector NTI 10.0 software. The deduced protein sequences were aligned with representative Spo and Sad sequences selected in the NCBI database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).The phylogenetic tree was generated based on the representative protein sequences of Halloween genes using MEGA 5.0.

Table 1
Primers used in this study.

Primer	Sequence (5′–3′)	Purpose
Spo-F	CGCACGGAGACGAAGAAA	RT-PCR
Spo-R	CAAAGCAGGCGTCCACTAAG	RT-PCR
Sad-F	TAACCGCTTTGGGTCGCT	RT-PCR
Sad-R	GGATAACCATCGTTCCACCAG	RT-PCR
Spo-GSPF	GTGGAGGACTTAGTGGACGC	3′-RACE
Spo-GSPR	GCGGTTTCCTCCGAAGTAG	5′-RACE
Sad-GSPF	ACGCCTTCATCCCCTTTG	3′-RACE
Sad-GSPR	TGATAGTGCGGTGTTTTCTCC	5′-RACE
Spo-QF	GGGACGAGCCCAATAAGTT	qPCR
Spo-QR	CTGGTGCTGAAAGGGATGA	qPCR
Dib-QF	TGCGAGTCTGCTTGAGGTG	qPCR
Dib-QR	AGCCATTGTCAGTGGGGAG	qPCR
Sad-QF	CACGGCATTTTCAAGGAGA	qPCR
Sad-QR	AAGGCGTCATCCAGGCACT	qPCR
Actin-QF	CGAAACCTTCAACACTCCCG	qPCR
Actin-QR	GATAGCGTGAGGAAGGGCATA	qPCR
Spo-IF	TAATACGACTCACTATAGGGACTTCCAACAAGCCTCACCG	RNAi
Spo-IR	TAATACGACTCACTATAGGGGGTTGAGGGAGCAGTATGACA	RNAi
Dib-IF	TAATACGACTCACTATAGGGAAGATGAGGCGTGAAGGG	RNAi
Dib-IR	TAATACGACTCACTATAGGGGCAATGAGGTAAAGCCAAGA	RNAi
Sad-IF	TAATACGACTCACTATAGGGCGAAGTGCCTGGATGACG	RNAi
Sad-IR	TAATACGACTCACTATAGGGTGTATTTCGGAGCCAACG	RNAi
GFPIF	TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT	RNAi
GFPIR	TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGC	RNAi

T7 promoter sequence was underlined.

2.4. RNA interference

Amplicons of Pt-spo, Pt-dib, Pt-sad, and green fluorescent protein (GFP) were cloned into pMD19-T vector (Takara) and amplified by PCR using a T7 promoter linked primers (Table 1). The resultant DNA was used as templates to synthesize dsRNA. The dsRNA was synthesized and purified according to the manufacturer's instructions of the MEGAscript RNAi Kit (Ambion). Integrity of the dsRNA was checked on agarose gel, and concentration of the dsRNA was checked with a NanoDrop 2000 UV Spectrophotometer (Thermo Fisher).

The RNAi experiment was conducted in a stage-dependent manner. Pt-spo dsRNA was injected into the intermolt crabs, while Pt-dib and Pt-sad dsRNA were respectively injected into the premolt (D_1 stage) crabs. For each stage, crabs receiving crab saline (Duan and Cooke, 1999) and GFP dsRNA were set as control. 150 μL crab saline or 300 μg (dissolved in 150 μL crab saline) dsRNA was injected via the base of the last walking leg with 1 mL syringe. 5 crabs in each injection group were sacrificed at 0, 1, 2, and 4 d post injection. YO and hemolymph sample of each crab were then obtained for further analysis.