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Molecular cloning, characterization and effects of catechol-*o*-methyltransferase (comt) mrna and protein on aggressive behavior in the swimming crab *portunus trituberculatus*

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ABSTRACT

In the present study, a cDNA that encodes catechol-*O*-methyltransferase (*COMT*) was cloned from the muscle of the swimming crab *Portunus trituberculatus* and named *PtCOMT*. *PtCOMT* was proven to be a conserved gene that encodes a protein of 222 amino acid residues with a molecular mass 25.4 kDa that constitutes only the signature motif "SAM_OMT_I." The RT-PCR results showed that *PtCOMT* was expressed in all five larval stages (zoea stage I, Z_1; zoea stage II, Z_2; zoea stage III, Z_3; zoea stage IV, Z_4; and megalopa, M) of *P. trituberculatus*. The *PtCOMT* mRNA levels were higher ($P < 0.05$) in the Z_4 and M stages than in the Z_1, Z_2, and Z_3 stages. In addition, *PtCOMT* was expressed in the tissues of healthy crabs, namely, ganglion, muscle, and hepatopancreas. On the basis of the RT-PCR and immunohistochemistry analyses, the level of expression in the ganglion and muscle was significantly higher than that in the hepatopancreas ($P < 0.05$). Aggressiveness of DA injected crabs was tested through a behavioral study where dopamine-treated crabs walked through the "middle" of the pool more frequently than control crabs. When compared with the control group, *PtCOMT* expression level was significantly upregulated ($P < 0.05$) at 2 h and significantly downregulated at 4 h in the muscles after dopamine (DA) injection ($P < 0.05$), whereas it was significantly downregulated ($P < 0.05$) at 2 h and upregulated after 4 h ($P < 0.05$) in the ganglia. These results show that DA promotes the release of *PtCOMT*. Our findings indicate that the expression level of *PtCOMT* may be directly related to aggressiveness and affect the central nervous system of *P. trituberculatus* to act on its aggressive behavior. Our results can help further studies on the aggressive behavior mechanism of *P. trituberculatus*.

1. Introduction

The swimming crab *Portunus trituberculatus* is one of the most economically important marine crabs (Hamasaki et al., 2006), and it is widely distributed along the coast of China. With the development of artificial breeding techniques, the *P. trituberculatus* aquaculture industry has developed rapidly: The output in 2016 was about 125,000 tons (China Fisheries Statistical Yearbook, 2017). However, *P. trituberculatus* is a very aggressive animal species who shows strong territoriality and cannibalism just like other crustaceans. Previous studies have indicated that 30% crabs may loss their limbs or be injured when aggressive behavior happen in aquaculture (Smith and Hines, 1991; He et al., 2016). Consequently, feeding, growth, regeneration, reproduction, competitive ability, predator avoidance, and survival ability of crabs in aquaculture will be affected by the loss of limb (Juanes and Smith, 1995; He et al., 2016). Moreover, a large number of *P. trituberculatus* crabs die or are damaged during cultivation and breeding because of aggressive behavior, resulting in a poor survival rate in pond culture and low output (He et al., 2016). To a certain extent, the aggression of *P. trituberculatus* hinders the healthy and sustainable development of its breeding industry.

Among invertebrates, crustaceans have large amine neurons and clear modular nervous system structures (Kravitz and Huber, 2003). Besides, amniotic-neuron changes in behavioral-related neural circuits have been confirmed to be readily observable (Mulloney et al., 2003) and can act as a template for analyzing aggressive behavioral mechanisms (Panksepp et al., 2003). Currently, the main response to crustacean attack in behavioral observation and physiological studies is physical medium isolation, turbid culture, and reduction of exposure to increased culture area (Zhao et al., 2017; He et al., 2017). In

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Table 1
Oligonucleotide primers used in the study.

Primer	Sequence (5′-3′)	Sequence information
AOLP	GGCCACGCGTCGACTAGTACT(16)	Oligo dT primer
3′ outer primer	TCAAACCCTCCAGCGTCTCATC	*COMT*-specific primer (for sequencing)
3′ inner primer	CTAATCTTGTTCAGTAGCCTTTGGTG	*COMT*-specific primer (for sequencing)
5′ outer primer	CACCAAAGGCTACTGAACAAGATTA	*COMT*-specific primer (for sequencing)
5′ inner primer	CGTCCAGCACCTTCTTCCCACC	*COMT*-specific primer (for sequencing)
M13–47	CGCCAGGGTTTTCCCAGTCACGAC	Vector primer (for sequencing)
RV-M	GAGCGGATAACAATTTCACACAGG	Vector primer (for sequencing)
COMT-F	CTGGGCGCTCCTGAAGTGCT	Specific primer (for real-time PCR)
COMT-R	GGCTTGTCGGCGTCGATAAA	Specific primer (for real-time PCR)
Actin-F	CGAAACCTTCAACACTCCCG	Primers for β-actin
Actin-R	GGGACAGTGTGTGAAACGCC	Primers for β-actin
Pt-F	CTCGAGTTAGTGGTGGTGGTGGTGCTTGATGAAGCACAGCGTGAGGCCG	Recombinant primer (for expression)
Pt-R	CATATGGCGACGACAGTCAAGAGCTACA	Recombinant primer (for expression)

crustaceans, aggression has been widely reported in crayfish (Vorburger and Ribi, 1999), lobster (Panksepp and Huber, 2002; Panksepp et al., 2003), Chinese mitten crab (Yu et al., 2012; He et al., 2016), and many other economic species.

Catechol-*O*-methyltransferase (*COMT*) is a key enzyme in many organisms such as bacteria (Pospiech et al., 1996), fungi (Averbeck et al., 2000), animals (Bonifa'Cio et al., 2000), and plants (Ibrahim et al., 1998), and it is involved in biological growth, development, signal transduction, and disease resistance. *COMT* plays an important role in the catabolism and *O*-methylation of endogenous catecholamines, such as dopamine (DA), in animals (Filipenko et al., 2001). Additionally, the substrates of *COMT* contained xenobiotics with a catechol moiety in the small molecule (Chen et al., 2014), which was used as regulatory substances of catecholamine neurotransmitters (DA, adrenaline, and noradrenaline) to act on human emotion and disease (Odlind et al., 2001; Han et al., 2017). Previous studies have confirmed that fighting, cannibalism, and other attacks are mainly regulated by biogenic amines in the nervous system of crustaceans (Sneddon et al., 2000; Panksepp et al., 2003). In crustaceans, DA is a biogenic amine neurotransmitter that is widely distributed, and it modulates numerous physiological functions (Tierney et al., 2003; Khornchatri et al., 2015). *COMT* is a major metabolic enzyme for DA (Kravitz and Huber, 2003), and it acts on motor, affective, and cognitive behaviors by controlling the metabolism of DA in the synapses; its activity is closely related to aggression (Yu et al., 2014; Cunha-Bang et al., 2016). The release of DA is believed to be a stressful induction of neuroendocrine responses (Ottaviani and Franceschi, 1996) that can promote or inhibit physiological processes in crustaceans (Sarojini et al., 1995; Kuo and Yang, 1999). Few studies have identified aggression-related genes and molecular regulation mechanisms underlying aggressive behavior in crustaceans, especially the mechanism underlying the involvement of the *COMT* gene in the metabolism of biogenic amines, including DA, during the attack behavior of *P. trituberculatus*.

Currently, to the best of our knowledge, there is no information on the characteristics of *COMT* as well as the relationship between *COMT* and aggressive behavior in *P. trituberculatus*. In this study, we first cloned the full-length *COMT* gene of *P. trituberculatus* and obtained the recombinant protein in vitro via a prokaryotic expression system. Then, we prepared the polyclonal antibody. Finally, we analyzed the tissue distribution and larval development characteristics of the *COMT* in *P. trituberculatus* and preliminarily evaluated the relationship between *COMT* and DA. The aim of this study was to provide a basis for understanding the molecular mechanism underlying *P. trituberculatus* attack behavior and effectively reduce the damage and death caused by crab attacks during seed culture and breeding.

2. Materials and methods

2.1. Experimental materials and reagents

RNA iso™ Plus, TRIzol® reagent, pMD18-T vector, restriction enzymes *NdeI* and *Xho*I, and SMARTer® RACE5′/3′ Kit were purchased from TaKaRa Biotechnology Company (Dalian, China). Taq DNA polymerase and M-MLV reverse transcriptase were obtained from Promega (USA). *Escherichia coli* host strain TOP10F' for routine plasmid amplification, *E. coli* host strain BL21 (DE3) pLysS, expression vector pET28a (+), kanamycin, and IPTG were purchased from Suobio (Shanghai, China). Co-NTA His-bind resin was obtained from Qiagen (Germany). Goat-anti-rabbit IgG horseradish peroxidase (HRP) conjugates were purchased from Mai New Biological (Fuzhou, China). Dopamine hydrochloride was obtained from Saint Louis (H8502; Sigma-Aldrich MO, USA). Other reagents were purchased from Sangon (Shanghai, China).

2.2. RNA isolation and cDNA synthesis

P. trituberculatus (100 ± 10 g) specimens and larvae from five zoea developmental stages (Ren et al., 2017) were collected from a commercial farm in Xiangshan, China (28°51′18″–29°39′42″ N, 121°34′03″–122°17′30″E). The crabs were fed with razor fish for a week and adapted to the laboratory conditions. The larvae were sampled and stored at −80 °C. Total RNA was extracted using TRIzol® reagent, followed by reverse transcription of 2 μg of total RNA with M-MLV reverse transcriptase and Oligo dT primer AOLP (shown in Table 1) to synthesize the cDNA.

2.3. Rapid amplification of cDNA ends

A partial sequence of *PtCOMT* was obtained from the transcriptome sequencing data of *P. trituberculatus*. Full-length cDNA was obtained using expressed sequence tag (EST) analysis and rapid amplification of cDNA ends (RACE) technique. The 5′- and 3′-end sequence amplification gene-specific primers are listed in Table 1. PCR was performed using a 50 μL reaction volume, according to the manufacturer's instructions. The PCR product was isolated on 1% agarose gel and purified with a PCR purification kit (BBI, USA). Then, it was cloned into pMD18-T simple vector (TaKaRa) and transformed into competent cells of *E. coli* TOP10F'. The positive recombinants were selected using anti-kanamycin selection before PCR screening with the primers M13–47 and RV-M. Three positive clones were confirmed for sequencing (BGI Company, Shenzhen, China). The full sequence of *PtCOMT* cDNA was assembled using the Vector NTI Suite 7 software after removing the vector sequences.