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Calcium signaling-related genes in *Penaeus monodon* respond to abiotic stress and pathogenic bacteria common in aquaculture

Virak Visudtiphole*, Sasithorn Petkon, Kanchana Sittikankaew, Jariya Sakayaroj, Sirawut Klinbunga

National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Paholyothin Rd., Khlong 1, Khlong Luang, Pathum Thani 12120, Thailand

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ABSTRACT

Stress of cultured species is a common problem, affecting the host health, in aquatic farming. It occurs when interactions among the three elements – host, environment and pathogens, are not in balance, particularly under intensive culturing conditions (de la Vega et al., 2006). Hosts respond to stress by triggering cellular Ca^{2+} signaling to activate many downstream pathways for their functions in order to mitigate the stress effect. Calreticulin (CRT), Calnexin (CNX) and Endoplasmic reticulum protein 57 (ERp57) are involved in Ca^{2+} signaling in stress response mechanisms and are essential for survival and viability of organisms under stress conditions. In this study, the full length cDNA of *CNX* and *ERp57* in the giant tiger shrimp (*Penaeus monodon*) were identified. Amino acid sequences deduced from both cDNA showed significantly high similarity with those present in other crustaceans. One and two forms of *PmCNX* and *PmERp57* transcripts were found, respectively. For *PmERp57*, its genomic sequence was additionally identified. It spanned 8257 base pairs (bp), composing of 10 exons (84–226 bp long) and 9 introns (93–2787 bp long). Both genes were expressed in all examined tissues of both juvenile and broodstock *P. monodon*. Effects of *Vibrio harveyi* and abotic stress commonly found in aquaculture on expression of *PmCRT*, *PmCNX* and *PmERp57* were examined in hemocytes by quantitative real-time PCR. High temperature, hypo- and hyper-osmosis and *V. harveyi* infection induced expression all of the three genes ($P < 0.05$). In contrast, analysis of hypoxia effect on expression of the Ca^{2+} signaling-related genes, which was firstly studied in aquatic species, showed to suppress their expression ($P < 0.05$). Results from this study suggested that *PmCRT*, *PmCNX* and *PmERp57* are related to stress response mechanisms and immune system of *P. monodon*. Their expression is therefore a potential candidate for markers to detect or monitor the early sign of shrimp's stress and immunity changes in farming.

1. Introduction

As the wild aquatic species are declining, aquaculture becomes increasingly important to serve the rising world population. With the limited available land area, especially for marine cultures, productivity per land size is a concern and high-density stocking is desired to be attained without undermining host's health and growth (Schock et al., 2013). Giant tiger shrimp (*Penaeus monodon*) is one of the commercially-cultured crustacean species. However, its production has declined for the past several years owing to disease and growth problems (Limsuwan, 2004; Preechaphol et al., 2007). Under intensive farming conditions, shrimp is prone to be exposed to suboptimal environmental conditions e.g., increased water temperature, low dissolved oxygen, outgrowing opportunistic pathogens. These conditions cause stress to internal physiology of shrimp and consequently affect their health and immunity (de la Vega et al., 2006; de la Vega et al., 2007; Huang et al., 2008; Wickins et al., 2002). In addition, stress could be induced by exterior environmental variations - seasons and changing climate. Heavy rain leads to salinity and pH changes of the water, or hot and strong sun light during a long summer day can cause evaporation of water in the pond, resulting in higher salinity.

Stress occurs when the homeostasis of interactions among the environment, pathogens and host in the culture is disrupted. To attenuate the effect from stress, host cells employ Ca^{2+} signaling system to trigger several cascade pathways e.g., the unfolded protein response (UPR), DNA repair, immune defending mechanisms, phagocytosis and ER stress-induced apoptosis (Chichiarelli et al., 2007; Grillo et al., 2006; Guerin et al., 2008; Muller-Taubenberger et al., 2001; Wang et al., 2012; Zuppini et al., 2002). Two major proteins regulating Ca^{2+} signaling are Calreticulin (CRT) and Calnexin (CNX). They bind to Ca^{2+}

* Corresponding author.
E-mail address: virak.vis@biotec.or.th (V. Visudtiphole).

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and are involved in the cellular signaling by controlling storage and release of Ca^{2+} in the Endoplasmic Reticulum (ER) (Ellgaard and Helenius, 2003).

The UPR is one of the main mechanisms that are triggered to tackle the cellular harm from stress. This mechanism involves in activation of protein-folding chaperones to correct the mis- and un-folded proteins caused by stress (Schroder and Kaufman, 2005). Complexing with ERp57, a member of the protein disulfide isomerase (PDI) family, CRT and CNX themselves also act as chaperones by associating with glycoproteins to facilitate the folding while ERp57 is responsible specifically for disulfide bond formation of the folded protein (Frickel et al., 2004; Oliver et al., 1999), some of which e.g., integrin are important for the immune system (Rudd et al., 2001). They are, therefore, vital for organisms to maintain growth and viability under stress conditions (Coe et al., 2008; Elagoz et al., 1999; Lee et al., 2005; Solda et al., 2006). Activation of *CRT*, *CNX* and *ERp57* expression by biotic (pathogens) and abiotic (temperature, Ca^{2+} deprivation) stress has been found in many species e.g., human, *Caenorhabditis elegans*, *Fenneropenaeus chinensis*, *Litopenaeus vannamei* (Boden and Merali, 2011; Lee et al., 2005; Luana et al., 2007; Vargas-Albores et al., 2009). In *P. monodon*, *CRT (PmCRT)* was previously identified and its expression was activated by heat stress (Visudtiphole et al., 2010).

Elevated water temperature, salinity change, lower dissolved oxygen and pathogenicity are common problems in shrimp farming, especially under intensive culture conditions (Kungvankij et al., 1986). The present study hence examined how these abiotic stress and a common opportunistic pathogen, *Vibrio harveyi*, affected molecular biology of the shrimp host through expression of three important Ca^{2+} signaling-related genes: *calreticulin*, *calnexin* and *ERp57*. cDNAs from two new Ca^{2+} signaling and UPR-related genes, *PmCNX* and *PmERp57*, were also identified.

2. Methods and materials

2.1. Experimental animals

P. monodon juveniles (15 ± 3 g body weight, 4 months old) were supplied from a commercial farm in the eastern region of Thailand and acclimated under the laboratory condition (15 ppt salinity and 28–30 °C temperature) for 7 days before subjected to the challenge experiments or tissue dissection for the tissue distribution study.

To compare gene expression between juveniles and broodstock, wild broodstock were additionally included in the tissue distribution study. They were live-caught from the Andaman Sea (west of the Thai Peninsula) and acclimated for 5 days before tissue dissection. Haemolymph was individually collected from the ventral sinus using 10% sodium citrate as an anticoagulant (1:1 *v*/v) and immediately centrifuged at 3000*g* at 4 °C for 5 min to collect hemocytes. All tissues dissected from the animals were immediately placed in liquid N_2 and stored at − 80 °C until required.

2.2. Stress challenge experiments

Stress challenges were performed as followed. Hemocytes were collected by centrifugation of hemolymph withdrawn from the experimental animals with 10% sodium citrate (1:1 *v*/v). The collected samples were quickly frozen in liquid N_2 and stored at − 80 °C until used for the quantitative real-time PCR analysis.

2.2.1. Heat stress

Experimental juvenile shrimp were transferred from the acclimating condition (ambient temperature, 28 °C) to 35 ± 1 °C for 6 h and then returned to the acclimating temperature. Hemocyte samples were time-interval collected at 1, 3, 6 h during treatment (hdt) and at 1, 3, 6, 12, 24, 48 h post treatment (hpt) ($N = 5$ for each group). Control was the shrimp collected at time 0 h before the challenge.

2.2.2. Hypo- and hyper-salinity

Experimental juvenile shrimp were transferred from the acclimating condition (15 ppt) to 5 or 30 ppt for 1 week. Hemocyte samples were time-interval collected at 1, 3, 6, 12, 24, 48 h and 1 week during treatment ($N = 5$ for each group). Control was the shrimp collected at time 0 h before the challenge.

2.2.3. Hypoxia

Low-oxygen environment was created by bubbling nitrogen gas into the water to suppress the dissolved oxygen (DO) level to 1 ppm. The condition was maintained throughout the entire experiment by a DO controller (Hanna). Experimental juvenile shrimp were transferred from the acclimating condition (ambient DO 5.2 ppm) to the 1-ppm DO environment for 6 h. Hemocyte samples were time-interval collected at 1, 3, 6 hdt and 1, 3, 6, 12, 24, 48 hpt ($N = 5$ for each group). Control was the shrimp collected at time 0 h before the challenge.

2.2.4. Vibrio harveyi infection

To determine the maximum challenge pressure of 0% mortality during the experimental period (48 h), juvenile shrimp were initially injected with 100 µl of various *V. harveyi* dilutions (10^5–10^9 cfu/ml) in normal saline. The injection was carried out at the third abdominal segment. Consequently, 10^7 cfu/ml was the effective working concentration for the infection experiment. For the control, shrimp were injected with normal saline. Both sample groups were maintained separately at the normal acclimating condition. Hemocyte samples were time-interval collected at 1, 3, 6, 12, 24, 48 h post injection (hpi) ($N = 5$ for each group).

2.3. Isolation of total RNA and preparation of first-strand cDNA

Total RNA was extracted from the tissue dissected from both juveniles and wild broodstock using TRI-Reagent (Molecular Research Center). Total RNA samples were treated with DNase I (0.5 U/µg of RNA) at 37 °C for 30 min to remove contaminated genomic DNA and then subjected to phenol-chloroform extraction. One and a half micrograms of the DNase I-treated total RNA were reverse-transcribed to first-strand cDNA using an Improm-II™ Reverse Transcription System (Promega), following the manufacturer's instruction.

2.4. RACE-PCR

mRNA was purified from the extracted RNA using a QuickPrep micro mRNA Purification Kit (GE Healthcare). One µg of purified mRNA was reverse-transcribed to the 5′ and 3′ RACE-PCR templates using a SMART RACE cDNA Amplification Kit (BD Clontech) following the protocol recommended by the manufacturer. Gene-specific primers (GSP) were designed from an expressed short sequence tag (EST) derived from *P. monodon* (Tassanakajon et al., 2006).

For *PmCNX*, both 5′ and 3′ RACE-PCRs were carried out in two steps. GSPs for the primary 5′ and 3′ RACE-PCRs were CNX-5′RACE and CNX-3′RACE, respectively (Table 1). The primary PCR products were then used as the templates for nested 5′ and 3′ RACE-PCRs, using CNX-N5′RACE and CNX-N3′RACE as GSPs, respectively. For *PmERp57*, only one step of each 5′ and 3′ RACE-PCR was performed, using ERp57-5′RACE and ERp57-3′RACE, as GSPs, respectively. The final product bands were gel-eluted, cloned and sequenced.

Sequences of the RACE-PCR products and EST were assembled and searched against the GenBank database using BLAST*X* ((Altschul et al., 1990); available at http://ncbi.nlm.nih.gov). Protein domains, p*I* value and molecular weight of the deduced PmCNX protein were determined using SMART ((Letunic et al., 2009); available at http://smart.embl-heidelberg.de/) and ProtParam ((Gasteiger et al., 2005); available at (http://www.expasy.org/tools/protparam.html)).