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First characterization of three cyclophilin family proteins in the oyster, *Crassostrea ariakensis* Gould

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ABSTRACT

Cyclophilins (CyPs) are a family of proteins that bind the immunosuppressive agent cyclosporin A (CsA) with high-affinity and belong to one of the three superfamilies of peptidyl-prolyl *cis-trans* isomerases (PPIase). In this report, three cyclophilin genes (Ca-CyPs), including Ca-CyPA, Ca-CyPB and Ca-PPIL3, were identified from oyster, *Crassostrea ariakensis* Gould in which Ca-CyPA encodes a protein with 165 amino acid sequences, Ca-CyPB encodes a protein with 217 amino acid sequences and Ca-PPIL3 encodes a protein with 162 amino acid sequences. All of the three Ca-CyPs genes contain a typical CyP-PPIase domain with its signature sequences and Ca-CyPB contains an N-signal peptide sequences. Tissue distribution study revealed that Ca-CyPs were ubiquitously expressed in all examined tissues and the highest levels were observed in hemocytes. RLO incubation upregulated the mRNA expression levels of Ca-CyPs, indicating that three Ca-CyPs might be involved in oyster immune response against RLO infection.

1. Introduction

Cyclophilins (CyPs) are a family of proteins that contain a single highly conserved peptidyl-prolyl *cis-trans* isomerases (PPIase) domain and belong to one of the three PPIase superfamilies [1–3]. Under physiological conditions, CyPs possess PPIase activity that mediates protein folding and assembly, involving in several biological processes [4–7]. CyPs are also well known as immunophilin family proteins that bind the immunosuppressive agent cyclosporin A (CsA) with high affinity [8]. And recent studies have shown that CsA mediates its immunosuppressive activity through the formation of a CsA-Cyp complex which inhibits the function of calcineurin, subsequently dephosphorylates nuclear factor of activated T-cells (NF-AT), results in the activation of T cells and secretion of cytokines [9–11].

Moreover, later studies have revealed that some CyPs function as proinflammatory secretory product in response to inflammatory stimuli and secreted CyPs serve as a potent chemoattractant for monocytes, neutrophils, eosinophils, and T cells [12–14]. Lee et al. demonstrated that some CyPs possess antioxidant activity via interacts with peroxiredoxins [15]. Furthermore, CyPs has been implicated in diverse pathological conditions and microbial pathogenesis, such as HIV [16], hepatitis B and C viral infection [17]. And in some aquatic animals, studies have suggested that some CyPs played an important role in the innate immune system. When stimulated with bacterial or LPS, the expression of some CyPs was identified to be upregulated in the shellfish *Chlamys farreri* [18], *Venerupis philippinarum* [19], shrimp *Penaeus monodon* [20] and fish *Pelteobagrus fulvidraco* [21], suggesting its involvement in the defense response against the bacterial infections.

CyPs are ubiquitously expressed protein and until now various CyPs have been identified in diverse organisms ranging from bacteria to humans [2,22–24]. In human, 16 CyPs have been identified and among which, the functions of five classic Cyp isoforms (CyPA, B, C, D and 40) are mostly studied [1,25–27]. Here we reported three novel Cyp isoforms (Ca-CyPs) identified from the oyster *Crassostrea ariakensis* Gould, which we named Ca-CyPA, Ca-CyPB and Ca-PPIL3.

Crassostrea ariakensis is one of the most economically important oysters cultivated in southeastern China, which suffered severe mortality caused by the pathogen rickettsia-like organism (RLO) in recent years [28,29]. Rickettsias are Gram-negative bacteria,

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generally described as obligate intracellular pathogens that multiply only within host cells [30]. This prokaryote has been reported as a new species of pathogen in many aquatic animals including fishes [31,32], crustaceans [33,34] and mollusks [35,36]. Now prevention and control of this disease is becoming the priority for the development of oyster aquaculture. In the past years, we have made significant progress in collecting data regarding the oyster defense system [37–45]. In this paper, we report the sequence analysis and tissue distributions of three novel CyPs in the *C. ariakensis*. The temporal expression profiles of three Ca-CyPs under RLO challenge were also studied.

2. Material and method

2.1. Oyster experimentation and tissue isolation

Healthy oysters aged 2–3 years old were obtained from Qinzhou bay (Guangxi, China) and maintained in artificial seawater with a cycling system at 19 ± 1 °C for one week before experiments were carried out. The individual size was about 8.1 × 5.3 × 11.6 (length × width × height, cm) on average. Three untreated oysters were randomly sampled for hemocytes, gill, mantle, digestive gland, gonads and adductor muscle tissue in order to characterize tissue-specific Ca-CyPs expression. Total RNA extraction was performed immediately after dissection using the $RNA_{fast1000}$ purification kit (Feijie, China) according to kit procedures. Total RNA isolated from each organ was reverse transcribed into cDNA with M-MLV RTase cDNA Synthesis Kit (Takara, Japan) following the kit instructions and stored at −20 °C.

2.2. RLO preparation

RLOs were prepared as reported previously [41]. Moribund oysters sampled from Qinzhou bay (Guangxi, China) were washed with PBS (phosphate-buffered saline, pH 7.4), and the isolation and purification of RLOs were carried out following the 'differential speed centrifugation and renografin density gradient centrifugation' method established by Li and Wu [46]. After that, purified RLOs were cultured using the 'chick embryo culture' method (unpublished data), then were collected and centrifuged at 12,000*g* for 20 min, and finally resuspended in sterile seawater (OD_{600} = 1.151) and stored at −80 °C.

2.3. Sequence characterization and phylogenetic analysis of Ca-AIF1

Three Ca-CyPs clones that contains full open reading frame (ORF) were identified from the cDNA library we constructed before [41]. Homology search was carried out using the BLAST program against the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST). ORF was acquired with ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/). Domains/motifs were identified using the PROSITE profile data base (http://cn.expasy.org/prosite). Multiple sequence alignment was performed using ClustalW program version 1.8. A phylogenetic tree was constructed using the neighbor-joining method with MEGA 3.1 package and the reliability of the tree was estimated via bootstrap analysis with 1000 replicates.

2.4. Hemocyte monolayer preparation and immune challenge

Hemocyte monolayers were prepared as described previously [40,41] and according to Lacoste et al. [47] and Canesi et al. [48]. Briefly, hemolymph was extracted from the pericardial cavity of ten oysters for each experiment, pooled to about 15 ml volume and adjusted to 10^6 cells/ml by addition of Hank's balanced salt solution (HBSS, adjusted to ambient seawater salinity). Hemolymph serum was obtained by centrifugation at 800*g* for 5 min and supernatant was filtered through a 0.22 μm filter. Hemolymph (1 ml) was dispensed into sterile Petri dishes and incubated at 15 °C for 30 min. Non-adherent hemocytes were carefully rinsed with HBSS, and 1 ml of above filtered hemolymph serum containing penicillin G (50 units/ml) and streptomycin (50 μg/ml) was added to the hemocyte monolayer and kept at 15 °C before use.

Twenty-four hours after the hemocyte monolayers were seeded, the hemolymph serum was replaced with fresh serum also containing penicillin G and streptomycin. Then the hemocytes were incubated with RLOs (OD_{600} = 1.15, final concentration, 1 μl RLO/10^6 hemocytes) for different periods of time (0, 1.5, 3, 6, 9. 12 h).

Total RNA from each set of treated hemocytes was then extracted and reverse transcribed as described above.

2.5. Quantitative RT-PCR

Quantitative RT-PCR was performed to analyze the following: (1) tissue-specific expression of Ca-CyPs in untreated oysters; (2) Ca-CyPs mRNA expression profile with RLO incubation.

Gene-specific primers were designed using Primer 5.0 software based on the obtained sequence and reported sequences while the 28s rDNA gene (GenBank No: AF137052) was used as an expression standard (Table 1). The real-time RT-PCR was performed in a 25 μl volume (10 μM of each primer, 2 μl of cDNA and 12.5 μl SYBR Premix Ex Taq™) with a SYBR Premix Ex Taq™ Kit (Takara, Japan) in an iCycler iQ™ thermocycler (Bio-Rad) using the following procedure: initial denaturation at 95 °C for 3 min; followed by 40 cycles of amplification (95 °C for 20 s and 53 °C for 40 s).

The relative expression level of each gene was determined by the Livak $2^{-\triangle\triangle CT}$ method [49,50] and the values were presented as mean ± SEM of independent experiments done in triplicates. Before this method used, we have verified that PCR efficiency of reference and target genes were approximately equal and 28s rDNA gene was used as endogenous control. And the data were analyzed by Student's *t*-test. Differences were considered statistically significant when p values were less than 0.05.

3. Results

3.1. Sequence analysis of Ca-AIF1

By screening the oyster cDNA library we constructed previously [40], three clones were putatively identified as novel oyster cyclophilins for their encoding proteins containing the conserved PPIase

Table 1
Sequences of primer pairs used in this study.

Putative gene	Primer sequence(5′–3′)
Ca-CyPA ORF primer F	5′-GTGAATTCATGGGGAAACCACAGGTC -3′
Ca- CyPA ORF primer R	5′- GGCTCGAGTTAAAGTTGACCAGAGT -3′
Ca-CyPB ORF primer F	5′-TTGAATTCATGGCGTGGGAGGGGACT -3′
Ca-CyPB ORF primer R	5′ CTAAGCTTTCACTCTGGGCTGGGTTT -3′
Ca-PPIL3 ORF primer F	5′-TCCGAATTCATGGCAGTAACGCTACAC-3′
Ca- PPIL3 ORF primer R	5′- TTCTCGAGTCATCCTGCCAATGGAT -3′
Ca-CyPA real-time RT-PCR F	5′-CACAATGGCACTGGAGGA-3
Ca-CyPA real-time RT-PCR R	5′-CTGGCTTCCGTTGGTGTT-3
Ca-CyPB real-time RT-PCR F	5′-GGACACCAACTCTTCACAA-3′
Ca-CyPB real-time RT-PCR R	5′-CACCTGGCGACGATTATCTC-3′
Ca-PPIL3 real-time RT-PCR F	5′-TTGTGCCAGCGATTACTAC-3′
Ca- PPIL3 real-time RT-PCR R	5′-GCCGTTGTTAGCCATAGAT-3′
28s real-time RT-PCR F	5′-GAATCCCTCATCCTAGCGA-3′
28s real-time RT-PCR R	5′-CACGTACTCTTGAACTCTCTC-3′

Restriction enzyme sequences was underlined.