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Characterization of ADP ribosylation factor 1 gene from *Exopalaemon carinicauda* and its immune response to pathogens challenge and ammonia-N stress

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ABSTRACT

ADP ribosylation factors (Arf), as highly conserved small guanosine triphosphate (GTP)-binding proteins, participates in intracellular trafficking and organelle structure. In this study, a full-length cDNA of Arf1 (designated EcArf1) was cloned from *Exopalaemon carinicauda* by using rapid amplification of cDNA ends (RACE) approaches. The full-length cDNA of EcArf1 was 1428 bp, which contains an open reading frame (ORF) of 549 bp, encoding a 182 amino-acid polypeptide with the predicted molecular weight of 20.69 kDa and estimated isoelectric point was 7.24. Sequence analysis revealed that the conserved Arf protein family signatures were identified in EcArf1. The deduced amino acid sequence of EcArf1 shared high identity (95%–98%) with that of other species and clustered together with Arf1 of other shrimp in the NJ phylogenetic tree, indicating that EcArf1 should be a member of the Arf1 family. Quantitative real-time RT-qPCR analysis indicated that EcArf1 was expressed in hemocytes, hepatopancreas, gills, muscle, ovary, intestine, stomach and heart, and the most abundant level was in hemocytes and gills, which were also the two main target tissues of pathogen infection and environmental stress. After *Vibrio parahaemolyticus* challenge, EcArf1 transcripts level significantly increased in hemocytes and hepatopancreas at 3 h and 6 h, respectively. The expression of EcArf1 in hemocytes and hepatopancreas significantly up-regulated at 12 h and 6 h respectively, and down-regulated at 72 h and 48 h, respectively. EcArf1 expression in hepatopancreas and gills both significantly increased at 6 h and decreased at 24 h under ammonia-N stress. The results suggested that EcArf1 might be involved in immune responses to pathogens (*V. parahaemolyticus* and WSSV) challenge and ammonia-N stress in *E. carinicauda*.

1. Introduction

The ridgetail white prawn *Exopalaemon carinicauda* is an important economical shrimp species naturally distributed in the coasts of the Yellow Sea and the Bohai Sea, China [1]. *E. carinicauda* possesses multiple merits, such as rapid growth, wide environmental adaptability and good reproductive performance, which makes its culture areas expanding rapidly and contributes to one third of the gross output of the polyculture ponds in eastern China [2,3]. However, with the development of intensive culture and the ecologic environmental deterioration, frequent outbreaks of various diseases caused by pathogens and environmental stresses have caused large scale economic losses to commercial shrimp aquaculture [4,5]. The suboptimal environmental conditions have been demonstrated that could affect the immunity of *E. carinicauda*, for example, pH and ammonia stress could cause affect the immune response of HSP90 [6], pathogens challenge could induce the immune-related genes such as selenium dependent glutathione peroxidase (GPx) [4], non-metastatic oncoprotein 23 (NM23) [1] and calreticulin (CRT) [5]. Therefore, better understanding of the

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innate immune abilities and immune defense mechanisms of *E. carinicauda* will be beneficial to the selective breeding and health management in shrimp aquaculture.

ADP ribosylation factors (Arf) are highly conserved small guanosine triphosphate (GTP)-binding proteins, which belonged to Ras-like GTPase family and involved in several cellular functions, such as membrane transport, maintenance of organelle structures and remodeling of the cytoskeleton [7–9]. The Arf proteins family can be separated into three categories based on their sequence homology, such as class I (Arf1, Arf2, Arf3), class II (Arf4, Arf5) and class III (Arf6) [10,11]. It was reported that different Arf forms have different intracellular localizations and functions, relying on the specific membrane it binds according to their recruitment of diverse groups of proteins [12–14]. Arf1 is mainly associated with the Golgi complex and participates in vesiculation, cellular material transportation, secretory membrane transport, modulate actin cytoskeleton assembly [11,14,15].

To date, Arf1 gene have been isolated from some crustaceans and has documented functions in virus invasion, for example, Arf1 transcripts were up-regulated significantly in shrimp upon WSSV challenge [9–11]. In addition, the knockdown of Arf1 gene could inhibit the expression of the WSSV envelope protein gene vp28 [9]. However, none is known about the potential role of EcArf1 in *E. carinicauda* against pathogen bacteria challenge (such as *Vibrio parahaemolyticus*) and ammonia-N stress. Ammonia nitrogen (ammonia-N) stress have been demonstrated that could affect the immunity [16–18], growth and molting [19], oxygen consumption and ammonia excretion of crustaceans [20]. *V. parahaemolyticus* is the mainly causative agents of acute hepatopancreatic necrosis disease (AHPND) and/or early mortality syndrome (EMS), which has caused large scale losses in farmed shrimp production [21,22], and so far there are still no effective preventive and therapeutic measures. In *E. carinicauda* ponds, “milky shrimp” disease caused by *Hematodinium* resulted in up to 100% mortality in Zhoushan, China [23]. *E. carinicauda* is one of the hosts of WSSV and WSSV could cause severe economic loss to *E. carinicauda* in Jiangsu province, China since 2010 and 2011 [24,25]. In addition, ammonia-N could affect the antioxidant system [26], increased the lipid peroxidation [26], and induced the HSP90 [6] and NM23 [1] expression in *E. carinicauda*.

The aim of this study was to: (1) clone the full-length cDNA of Arf1 from hemocytes of *E. carinicauda*; (2) compare its sequence with other known Arfs; (3) investigate the expression pattern of EcArf1 in various tissues of *E. carinicauda*; (4) evaluate its expression in immune-related tissues of *E. carinicauda* after pathogens (*V. parahaemolyticus* and WSSV) challenge and ammonia-N stress. These results will be essential to understand the role of EcArf1 in immune response against pathogens challenge and ammonia-N stress in *E. carinicauda*.

2. Materials and methods

2.1. Animals materials

Healthy adult *E. carinicauda*, averaging weight 1.33 ± 0.32 g, were collected from a commercial farm in Qingdao, China. They were cultured in 200 L polyvinyl chloride polymer (PVC) tanks with filtered aerated seawater (salinity 30‰, pH 8.2) at 22 ± 0.5 °C for 7 days before processing. There were 100 shrimps in each group. The shrimps were fed daily with a ration of 10% of body weight, and two-thirds of the water in each group was renewed once daily.

2.2. RNA extraction and cDNA synthesis

Hemolymph (400 μL) was extracted from three shrimps with a 1 mL syringe which contained an equal volume (400 μL) of anti-coagulant buffer (1.59 g sodium citrate, 3.92 g sodium chloride, 4.56 g glucose, 0.66 g EDTA-2Na, 200 mL ddH_2O) [27] and placed into a 1.50 mL plastic tube, then centrifuged at 800*g*, 4 °C for 15 min to collect the hemocytes. Total RNA was extracted from hemocytes using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA samples were analyzed in 1.0% agarose electrophoresis and the purity was quantitated at 260 nm, all OD_{260}/OD_{280} were between 1.8 and 2.0. The 3′ and 5′ ends RACE cDNA template were synthesized using SMART™ cDNA Kit (Clontech, USA) following the protocol of the manufacturer.

2.3. Cloning the full-length cDNA of EcArf1

An EST sequence corresponding to Arf1 was obtained from *E. carinicauda* hemocytes cDNA library of our laboratory (GenBank accession no. JK997147), and has been reported by Duan et al. [28]. BLAST analysis showed that it has high identities with Arf1s of other crustaceans. According to the EST sequence, a gene specific primer F1 was designed for 3′ RACE, and primer R1 was designed for 5′ RACE (Table 1) and its 3′ and 5′ ends were obtained using SMART RACE cDNA Amplification Kit (Clontech, USA). For 3′ RACE, the PCR reaction was performed using the primer F1 and the anchor primer UPM (Table 1). The 50 μL PCR reactions contained the cDNA 2.5 μL of template, 10 × Advantage 2 PCR buffer 5 μL, dNTP Mix (10 μmol/L) 1 μL, 50 × Advantage 2 Polymerase Mix 1 μL, primer UPM (10 μmol/L) 5 μL, primer F1 (10 μmol/L) 1 μL, PCR-Grade water 34.5 μL. The PCR reaction conditions were 5 cycles of 94 °C for 30 s, 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, and 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 3 min. For 5′ RACE, the PCR reaction was performed using the primer R1 and the anchor primer UPM (Table 1). The PCR reaction systems and conditions were the same as described above.

The PCR fragments were subjected to electrophoresis on 1.5% agarose gel to determine length differences, and the target band was purified by PCR purification kit (Promega, USA). The purified products were cloned into PMD18-T vector, following the instructions provided by the manufacturer (TaKaRa, Japan). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing.

2.4. Sequence analysis

The nucleotide and deduced amino acid sequences of EcArf1 cDNA were analyzed and compared using the BLAST search programs (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). The signal

Table 1
Primer sequences used in this study.

Primer name	Sequence (5′–3′)
EcArf1	
F1 (forward)	GCCACTGTGGCGCCACT
R1 (reverse)	GCTACAACAATGGAGTA
F2 (forward)	GGAAGTAAGGAAGCAGGTGGT
R2 (reverse)	AGACTGGACAAACAGCAAGGT
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
	CTAATACGACTCACTATAGGGC
18S rRNA	
18S-HF	TATACGCTAGTGGAGCTGGAA
18S-HR	GGGGAGGTAGTGACGAAAAAT
WSSV	
F3 (forward)	ACAATGGTCCCGTCCTCATC
R3 (reverse)	TGCCTTGCCGGAAATTAGTG
Probe (T)	TET-CAGAAGCCATGAAGAATGCCGTCTATCAC-TAMRA