Fish & Shellfish Immunology 32 (2012) 373–380

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short Communication

Isolation and characterization of cDNAs encoding Ars2 and Pasha homologues, two components of the RNA interference pathway in *Litopenaeus vannamei*

Yi-Hong Chen [a], Li Zhao [a], Xiao-Ting Jia [a], Xiao-Yun Li [a], Chao Zheng Li [a], Hui Yan [a], Shao-Ping Weng [a], Jian-Guo He [a,b,*]

[a] *MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, PR China*
[b] *School of Marine Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, PR China*

ARTICLE INFO

Article history:
Received 13 January 2011
Received in revised form
22 November 2011
Accepted 25 November 2011
Available online 6 December 2011

Keywords:
Arsenite resistance gene 2
Partner of drosha
RNAi
Dicer2

ABSTRACT

The RNA interference (RNAi) is an evolutionarily conserved protective mechanism in eukaryotes against parasitic foreign nucleic acids. Previous studies demonstrated that the RNAi mechanism is important for shrimp antiviral immunity. Here, we report the identification and functional analysis of two key components of the shrimp RNAi activity: *Litopenaeus vannamei* arsenite resistance gene 2 (LvArs2) and partner of drosha (LvPasha). The full-length cDNA of *LvArs2* was 3470 bp, including a 5′ untranslated region (UTR) of 167 bp, a 3′ UTR of 639 bp, and an open reading frame (ORF) of 2664 bp that encoded 887 amino acid residues with an estimated molecular mass of 102.5 kDa. The full-length cDNA of *LvPasha* was 2654 bp, including a 5′ UTR of 99 bp, a 3′ UTR of 560 bp, and an ORF of 1995 bp that encoded 664 amino acid residues with an estimated molecular mass of 74.2 kDa. Co-immunoprecipitation demonstrated that LvArs2 interacted with *L. vannamei* Dicer2 (LvDcr2) and LvPasha in *Drosophila* Schneider 2 (S2) cells, suggesting that LvArs2 may be involved in regulation of the miRNA/siRNA pathways in *L.vannamei*. Subcellular localization assays demonstrated both LvArs2 and LvPasha proteins mainly presented in the nucleus. After Poly(C–G) stimulation, the expression of *LvArs2* was suppressed and expression of *LvPasha* was enhanced in shrimp gills. These results suggest that *LvArs2* and *LvPasha* may participate in the defense against RNA viruses in crustacea.

1. Introduction

The RNA interference (RNAi) mechanism defends the host from invasion of foreign nucleic acids and participates in transcriptional regulation in all eukaryotes [1]. The antiviral function of RNAi has been most intensively investigated in plants, arthropods, and worms [2–7]. Dicers (Dcrs) are key proteins of the RNAi pathway. They interact with other proteins, such as R2D2 in *Drosophila melanogaster* or the transactivating response RNA-binding protein (TRBP) in *Homo sapiens*, to recruit Argonaute proteins (Ago) and form the RNA-induced silencing complex (RISC)/RISC-loading complex (RLC) [8–10]. Dcrs can cut long double-stranded RNAs (dsRNAs) into small interfering RNAs (siRNAs) or microRNAs (miRNAs) [11,12]. Recent studies also demonstrated that Dcrs participated in fragmenting chromosomal DNA during apoptosis and antiviral gene induction in a RNAi pathway-independent manner [13,14]. As reported, insects have two Dcr proteins with various functions, and Dcr2 (but not Dcr1) workes in the siRNA pathway and mediates the post-transcriptional silencing of exogenous dsRNAs and viruses [2].

In contrast to most siRNAs, miRNAs are exogenous dsRNAs that are first transcribed as primary miRNAs (pri-miRNAs) [15]. In the miRNA pathway, processing of pri-miRNAs into precursor miRNAs (pre-miRNAs) within the nucleus required an RNase III family protein, the Drosha [16]. Drosha interactes with Partner of Drosha (Pasha) to form the Drosha–Pasha/DGCR8 (homologous protein of Pasha) complex. Pasha/DGCR8 was proposed to bind to the double-stranded stem near the loop, orienting Drosha in position to bind closer to the base [17]. In *D. melanogaster*, Pasha (DmPasha) is a member of the Microprocessor which is shown to interact physically with Ars2 (DmArs2). Depletion of Ars2 or Pasha/DGCR8 leads to alterations in pri-miRNAs processing in the nucleus [18,19].

* Corresponding author. MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory for Biocontrol, School of Life Sciences, Sun Yat-sen University, 135 Xingang Road West, Guangzhou 510275, PR China. Tel.: +86 20 39332988; fax: +86 20 39332849.
E-mail address: lsshjg@mail.sysu.edu.cn (J.-G. He).

1050-4648/$ – see front matter
doi:10.1016/j.fsi.2011.11.032

Increasing evidences suggest that miRNAs have important functions in viral replication as well as roles in host antiviral immunity [20–22].

Arsenite resistance gene 2 (Ars2) is known to confer arsenite resistance in arsenite-sensitive Chinese hamster ovary (CHO) cells 2. In mammals, Ars2 was first shown to be essential for early mammalian development and was also suggested to participate in RNA metabolism [23]. It was subsequently confirmed as a component of the nuclear RNA cap-binding complex (CBC) [18]. In *D. melanogaster*, DmArs2 not only directly interacts with Dcr2 and facilitates the production of siRNAs in the cytoplasm, but it is also shown to participate in Microprocessor association and pri-miRNA stabilization. Thus, DmArs2 participates in both the miRNA pathway and siRNA pathway regulation [24]. Knockdown of DmArs2 increases infection by Drosophila C virus (DCV), Flock House Virus (FHV), and Sindbis virus (SINV) [24]. These studies indicate important roles of Ars2 in RNAi pathway regulation.

Penaeid shrimp culture is a major economic activity throughout the world [25]. *Litopenaeus vannamei* is the primary farmed shrimp species in the world, and is susceptible to a variety of pathogens, including the RNAs viruses such as taura syndrome virus (TSV) and yellow head virus (YHV) [26–28]. A better understanding of immune responses to pathogens would aid in developing novel strategies for disease prevention and control. Evidence is accumulating that RNAi pathway is important for shrimp antiviral immunity [29–32]. Recently, three Dcr1s have been identified. Knockdown of *Penaeus monodon Dcr1* (*PmDcr1*) resulted in more rapid mortality and higher viral loads after gill-associated virus (GAV) infection [33]; *L. vannamei Dcr1* (*LvDcr1*) mRNA was upregulated in hemocytes and gills after TSV injection [34]; *Marsupenaeus japonicus Dcr1* (*MjDcr1*) has not yet been studied in detail. Other components of the shrimp RNAi pathway, such as *Fenneropenaeus chinensis* transactivating response RNA-binding protein 1–3 (FcTRBP1–3), *L. vannamei Argonaute 2* (*LvAgo2*), *L. vannamei* systemic RNA interference-deficient (*LvSID-1*) and *P. monodon Argonaute 1* (*PmAgo1*), were also shown to participate in antiviral immunity [30,35,36].

In this study, cDNAs encoding full-length *L. vannamei* Ars2 (LvArs2) and *L. vannamei* Pasha (LvPasha) were identified. We demonstrated that LvArs2 interacted with LvPasha and LvDcr2 in S2 cells. Expression of *LvArs2* and *LvPasha* responded to Poly(C–G) stimulation. These results suggested that LvArs2 may have similar roles as DmArs2 in RNAi pathway.

2. Materials and methods

2.1. Cloning of LvArs2 and LvPasha cDNAs from L. vannamei

Based on *L. vannamei* expressed sequence tag (EST) sequences homologous to *D. melanogaster* Ars2 and Pasha (available at www.marinegenomics.org), specific primers (see primers in Table 1) were designed to obtain the 3′ and 5′ ends of *LvArs2* and *LvPasha* cDNA sequences by rapid amplification of cDNA ends (RACE). The cDNA template for RACE PCR was prepared using the BD SMART RACE cDNA Amplification kit (Clontech, Japan). The LvArs2–5′ RACE1 and LvArs2-3′ RACE1 primers were used for the first round of 5′-end and 3′-end RACE PCR. Thermocycle program settings were 94 °C for 2 min, followed by 7 cycles of 94 °C for 30 s, 68 °C for 30 s (decreasing by 1 °C each cycle), and 68 °C for 2 min, then 34 cycles of 94 °C for 30 s, 61 °C for 30 s, and 68 °C for 2 min, followed by a single final extension at 68 °C for 5 min. The conditions of the second round 5′-end and 3′-end PCR using LvArs2–5′ RACE2 and LvArs2–3′ RACE2 primers were the same as the first round. The PCR products were cloned into the pMD-19 vector (TaKaRa, Japan) and sequenced (ABI PRISM, Applied Biosystems, USA).

Table 1
Summary of primers used in this study.

Name	Sequence (5′–3′)
For cDNA cloning	
LvPasha–5′RACE1	GAGCATCCTCCAGCTTTAGTTGGTTTATG
LvPasha–5′RACE2	GTGGCATTTGATTGCAGTAAGGTGATTTCT
LvPasha–3′RACE 1	CAAACACCTCCATCATAAACCAACTAAAGC
LvPasha–3′RACE 2	GAAGATTCCTCCCTCCCGAAGATGTTGC
LvArs2–5′RACE 1	TCCGTGCCTCCCTCCAGCTTGATGACC
LvArs2–5′RACE 2	AACAGACACTCTTCTCTTCAAGTTTGACAGC
LvArs2–3′RACE 1	AACAATAGTGAGGAAAAGGCTGAGGGAGA
LvArs2–3′RACE 2	ATTACAATCACTCTGAATACCCCAATGAAGATG
For real-time RT–PCR	
β-actin-F	CCAGAGCAAGCGAGGTATCC
β-actin-R	TCGTCCCAGTTGGTGACGAT
LvPasha-F	CAGCAGTCTTTGGACGAACTTG
LvPasha-R	TCTGGGAGTTGCGGTCTATCTT
LvArs2-F	GCCATTTATTTTGGGAGAGGAC
LvArs2-R	CTGTAACTCCATCTGTTCCTCTGAC
For protein expression[a]	
pACB-LvArs2-Flag-F	CGG**GGTACC**GCCACCATGGCAGACAGTGATGATGAG
pACB-LvArs2-Flag-R	TAG**TCTAGA**TTACTTATCGTCGTCATCCTTGTAATCGT-AGTCTTCCTTGGGTGCATC
pACB-LvArs2-F	CGG**GGTACC**GCCACCATGGCAGACAGTGATGATGAG
pACB-LvArs2-R	CTAG**TCTAGA**CTGTAGTCTTCCTTGGGTGCATC
pACB-LvPasha-F	TAT**GAATTC**GCCACCATGTCCAAGTCAGAGAGTAATTCT
pACB-LvPasha-R	AAG**TCTAGA**CTTAGATCAACATTATTAAGATCGATTC
pACB-eGFP-F	AAT**GGGCCC**GATGGTGAGCAAGGGCGAGGA
pACB-eGFP-R	AAT**GGGCCC**TTACTTGTACAGCTCGTCCATGC

[a] Nucleotides in bold indicate restriction sites introduced for cloning.

The 3′ and 5′ ends of *LvPasha* cDNA sequences were obtained by RACE usingsimilar RACE PCR programs as employed for *LvArs2* RACE PCR. The primers for first round PCR were LvPasha-5′ RACE1 and LvPasha-3′ RACE1. Primers for second round PCR were LvPasha-5′ RACE2 and LvPasha-3′ RACE2.

2.2. Bioinformatics analysis

The Ars and Pasha sequences from other species in the database were obtained and analyzed by BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was performed using the ClusterX v1.83 program. Neighbor-joining (NJ) phylogenic trees were constructed based on the deduced amino acid sequences of LvArs2, LvPasha, and other known Ars or Pasha/DGCR8 proteins using MEGA4.0 software. Bootstrap sampling was evaluated 1000 times. Protein domains were searched against the Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

2.3. Co-immunoprecipitation

Expression vectors pACB-LvArs2-Flag and pACB-LvPasha for the full-length LvArs2 and LvPasha proteins were constructed for a Co-IP assay (primers in Table 1, expression vectors for full-length LvDcr2 were constructed by our laboratory). In brief, S2 cells were co-transfected with LvArs2 (Flag)/LvDcr2 (V5) or LvArs2 (Flag)/LvPasha(V5) expression plasmids and harvested after 48 h for immunoprecipitation. Cells were lysed in NP-40 lysis buffer (Beyotime, China) with protease inhibitor cocktail (Roche Applied Science, Germany) on ice and incubated 3 h at 4 °C with anti-V5 (Invitrogen, USA) or anti-Flag (Sigma–Aldrich, USA) antibody. The proteins were immunoprecipitated with Protein A/G Plus-Agarose (Calbiochem, Germany). After 3 h of incubation, beads were washed three times with NP-40 lysis buffer (Beyotime, China). Proteins were subjected to SDS-PAGE, and then electrotransferred to nitrocellulose membranes for Western blot analyses. The blots