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Short communication

Characterization of two novel ADP ribosylation factors from giant freshwater prawn *Macrobrachium rosenbergii* and their responses to WSSV challenge

Zheng-Feng Ding ^{a,c,1}, Jie Ren ^{a,1}, Jing-Min Tan ^a, Zheng Wang ^a, Shao-Wu Yin ^a, Ying Huang ^a, Xin Huang ^a, Wen Wang ^a, Jiang-Feng Lan ^{b,**}, Qian Ren ^{a,*}

^a Jiangsu Key Laboratory for Biodiversity & Biotechnology and Jiangsu Key Laboratory for Aquatic Crustacean Diseases, College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210046, China

^b College of Fisheries, Huazhong Agricultural University, Wuhan, Hubei 430070, China

^c Freshwater Fisheries Research Institute of Jiangsu Province, 79 Chating East Street, Nanjing 210017, China

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ABSTRACT

ADP-ribosylation factors (Arfs) are small GTP-binding proteins that have an essential function in intracellular trafficking and organelle structure. To date, little information is available on the Arfs in the economically important giant freshwater prawn *Macrobrachium rosenbergii* and their relationship to viral infection. Here we identified two Arf genes from *M. rosenbergii* (*MrArf1* and *MrArf2*) for the first time. Phylogenetic analysis showed that *MrArf1*, together with *MjArf1* from shrimp *Marsupenaeus japonicus* belonged to Class I Arfs. By contrast, *MrArf2* didn't match any of the Arfs classes of I/II/III, although it could be clustered with an Arf protein from *M. japonicus* called *MjArfn*, which may represent an analog of the Arf. *MrArf1* was ubiquitously expressed in all the examined tissues, with the highest transcription level in the hepatopancreas, whereas *MrArf2* was only highly expressed in the hepatopancreas and exhibited very low levels in the heart, stomach, gills and intestine. The expression level of *MrArf1* in the gills was down-regulated post 24 h WSSV challenge, and reached the maximal level at 48 h. *MrArf1* in the hepatopancreas went up from 24 to 48 h WSSV challenge. *MrArf2* transcript in the gill also went down at 24 h and then was upregulated at 48 h WSSV challenge. *MrArf2* increased significantly in the hepatopancreas 24 h after infection and then went down at 48 h WSSV challenge. RNAi results showed that knockdown of *MrArf1* or *MrArf2* could inhibit the expression of the envelope protein gene *vp28* of the WSSV. So, it could be speculated that *MrArf1* and *MrArf2* might play important roles in the innate immune system against WSSV infection.

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1. Introduction

Within years after its first discovery in Asia in the early 1990s, the white spot syndrome virus (WSSV) developed into an epizootic disease. WSSV is currently found in most crustacean farming areas in the world, where it causes large economic losses to the aquaculture industry. Aside from its economic effect, the disease also poses a threat to natural marine and freshwater ecology

(Jiravanichpaisal et al., 2004; Nambi et al., 2012). As such, understanding the molecular mechanism of the innate immune defense against virus infection is imperative. Recent studies have demonstrated that the small GTPases found in shrimp participate in antiviral immunity by regulating phagocytosis (Liu et al., 2009; Wu et al., 2007). In *Drosophila* S2 cells (Schneider line 2), Ran protein, a small Ras-like GTPase, was involved in the regulation of phagocytosis against *Drosophila* C virus (DCV). Some members of the ADP ribosylation factors (Arfs) found in mammals (D'Souza-Schorey and Chavrier, 2006; Nie et al., 2003) and the shrimp *Marsupenaeus japonicus* (Ma et al., 2010; Wang et al., 2009; Zhang et al., 2010), which are classified with small GTP-binding proteins (~21 kDa), are involved in virus infection. The relationship between various Arfs and virus invasion is thus worth investigating.

Arfs are small, ubiquitously expressed GTP-binding proteins best known for their role in membrane trafficking (Beck et al., 2011; D'Souza-Schorey and Chavrier, 2006; Popoff et al., 2011; Sannerud et al., 2011) and in regulating actin cytoskeleton assembly (Myers

* Corresponding author. Jiangsu Key Laboratory for Biodiversity & Biotechnology and Jiangsu Key Laboratory for Aquatic Crustacean Diseases, College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210046, China. Tel.: +86 25 85891955; fax: +86 25 85891526.

E-mail address: renqian0402@126.com (Q. Ren).

** Corresponding author. College of Fisheries, Huazhong Agricultural University, Wuhan, Hubei 430070, China. Tel: +86 2787282113; Fax: +86 2787282113.

E-mail address: sjs15@163.com (J.-F. Lan).

¹ Zheng-Feng Ding and Jie Ren equally contributed to this paper.

and Casanova, 2008). The six mammalian Arfs are divided into three classes based on their amino-acid identity. Class I contains Arf1, Arf2, and Arf3; class II contains Arf4 and Arf5; and class III contains only Arf6. Class I and class II Arfs are mainly concentrated in the Golgi apparatus, although they also function in endosomal compartments. By contrast, Arf6 is primarily localized in the plasma membrane and is a subset of the endosomes. A large body of evidence indicates that Arfs promote the biogenesis of carrier vesicles by nucleating the assembly of coat protein complexes in the sites of vesicle formation (Cheung et al., 2014; Myers and Casanova, 2008). However, different Arfs seem to possess various intracellular localizations and functions, which depend on the specific membrane they bind according to their recruitment of diverse groups of proteins. Such specificities can be defined by determining which regulators of Arfs, GEFs (guanine nucleotide-exchange factors), and GAPs (GTPase-activating proteins) function with which Arf proteins (Donaldson and Honda, 2005; Sun et al., 2011). The binding of Arfs to membranes appears to be essential for viral replication (Belov et al., 2007a), and they cycle between GTP- and GDP-bound states. In the former cycle, the activated form Arf-GTP is bound to the membrane; in the latter cycle, Arf-GDP is released by the cellular GTPase activity into the cytoplasm.

During the evolutionary interplay between virus infection and host defense, the Arf-dependent secretion pathway may be involved in anti-virus action through secretion of interferons and presentation of viral antigens by MHC I (Ma et al., 2010; Molla et al., 1991). The pathway of secretion or endocytic can easily be made to cause viral immune evasion and virus propagation, and Arfs can be recruited to the specific target membrane to induce disintegration of intracellular membranes and formation of specific vesicles by viral proteins mediating their interactors (Belov et al., 2007b). Arf1 was reported to have important roles in the regulation of viral RNA replication and the production of infectious hepatitis C virus (HCV), a kind of plus-strand RNA virus (Matto et al., 2011). GBF1, a kind of Arf GEFs, was crucial for the RNA replication of coxsackievirus B3 (CVB3) (Lanke et al., 2009). In addition to the function in the viral RNA replication, class II Arf proteins (Arf4 and Arf5) played important roles in the secretion of dengue virus (DENV) (Kudelko et al., 2012). Mouse hepatitis coronavirus (MHV) replication was dependent on the GBF1-mediated Arf1 activation (Verheije et al., 2008). The replication of plant RNA virus also needed the participation of Arf1 (Hyodo et al., 2013). The previous research showed that Arfs were related to RNA virus replication and secretion; however, the relationship between Arfs and DNA virus infection was rarely reported. WSSV is a double stranded DNA virus and *MjArf4* from *M. japonicus* was significantly up-regulated with the propagation of WSSV (Zhang et al., 2010). The relatively low expression level of *MjArf1* in WSSV-resistant shrimp may suggest its roles in WSSV invasion (Ma et al., 2010).

Compared with the extensive studies on shrimp Rab proteins (Attasart et al., 2009; Ongvarrasopone et al., 2011; Ye et al., 2012), Arfs were rarely studied. In this study, two Arf genes (*MrArf1* and *MrArf2*) were identified for the first time from *M. rosenbergii*, an economically important crustacean in the aquaculture industry (Mohanakumaran Nair et al., 2006). The expression level of *MrArfs* were up-regulated with the WSSV propagation, which might preliminarily indicate the roles of *MrArfs* in WSSV infection.

2. Materials and methods

2.1. Animals, tissue collection and immune challenge by WSSV

M. rosenbergii (about 15 g each) were purchased from an aquaculture market in Zhenjiang, Jiangsu Province, China. The prawns were acclimatized in freshwater tanks under laboratory conditions at room temperature (25 °C). The prawns were divided into

a control group and an experimental group designated as WSSV-challenged group. The WSSV inoculum was prepared following the previous paper (Wu et al., 2007). In brief, it was extracted from virus-infected *M. japonicus* by homogenizing muscle in TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4). Then the tissue homogenates were centrifuged at 2000×g for 10 min. The supernatant was diluted 100 times with 0.9% NaCl and was subsequently filtered through 0.45 µm filter. About 100 µL of filtrate (10⁵ WSSV copies/mL) was injected into the lateral area of the fourth abdominal segment of the prawns using a syringe with 29-gauge needle. The hepatopancreas and gills of the prawns challenged with WSSV at 0, 24, and 48 h (3 prawns at each time) were collected for RNA extraction. Tissues (i.e., heart, hepatopancreas, gills, stomach, and intestine) were also collected for RNA extraction.

2.2. Total RNA isolation, cDNA synthesis

The total RNA was extracted from the above-mentioned tissues using an EasyPure RNA Kit (TransGen, Beijing, China) according to the manufacturer's protocols respectively. The concentration of RNA were measured using Nanodrop (Thermo). For the qRT-PCR analysis, the first-strand cDNA was synthesized using a PrimeScript™ RT Reagent Kit (Perfect Real Time) (Takara, Dalian, China) with the primer oligo dT primer. For cloning the 3' fragments of *MrArfs*, the first strand cDNA was synthesized using 3'-Full RACE Core Set Ver. 2.0 (Takara, Dalian, China) according to the manufacturer's protocol using 3' RACE Adaptor primer. For cloning the 5' fragments of *MrArfs*, 5'-RACE-Ready cDNA was produced using a Clontech SMARTer™ RACE cDNA amplification kit from TAKARA (Dalian, China). The detailed methods were according to the manufacturer's instructions with 5'-CDS Primer A (T25VN, N = A, C, G, or T; V = A, G, or C), SMARTer IIA oligo (AAGCAGTGGTATCAACGCA GAGTACXXXX, X = undisclosed base in the proprietary SMARTer oligo sequence).

2.3. Cloning of the full-length cDNA of *MrArf1* and *MrArf2*

Two EST fragments (*MrArf1* and *MrArf2*) similar to Arf genes were found from the hepatopancreas transcriptome data. The EST sequences of the two genes were obtained from the hepatopancreas from *M. rosenbergii* using Illumina's Solexa Sequencing Technology performed by the Chinese National Human Genome Center at Shanghai. In order to obtain the full length of *MrArf1* and *MrArf2*, the 3' and 5' RACE were performed. The 3' ends of *MrArf1* and *MrArf2* were all cloned from the hepatopancreas using gene-specific primers (*MrArf1*-F: 5'- CAGTGTGGGATGTAGGTGGTC -3' and *MrArf2*-F: 5'-TCAACGTCGAGACGGTGGAGC-3') and 3' RACE Outer Primer (5'-TACCCTCGTTCACCTAGTATT-3'). The 5' ends of *MrArf1* and *MrArf2* were amplified using gene-specific primers (*MrArf1*-R: 5'-ATCACCAAAATCCCTCGGAAAACGC-3' and *MrArf2*-R: 5'-AGGAC CCTTGATTAGTGACAGGATGCCG-3') and a Universal Primer A Mix (UPM: long, CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAA CGCAGAGT; Short, CTAATACGACTCACTATAGGGC) according to the following procedures: 5 cycles at 94 °C for 30 s and 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; and 25 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The reaction was prepared in a Bioteke machine using a Clontech Advantage 2 PCR kit from Takara (Dalian, China). The full length of the *MrArf1* and *MrArf2* cDNAs were obtained by overlapping the 5', middle, and 3' fragments.

2.4. Sequence and phylogenetic analyses

Nucleotide sequence homology and comparisons of the deduced amino acid sequence were accomplished using the online program BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). cDNA translation and

deduced protein prediction were obtained using Expasy (<http://www.au.expasy.org/>). Signal sequence and domain prediction were performed using SMART (<http://www.smart.embl-heidelberg.de/>). MEGA 4.0 and GENDOC were used for the multiple alignments of *MrArf1* and *MrArf2* from *M. rosenbergii*. MEGA 4.0 was also used for constructing a phylogenetic tree and NJ (Neighbor-Joining) method was selected for the phylogenetic analysis (Kumar et al., 2008).

2.5. Real-time PCR

The tissue distribution of *MrArf1* and *MrArf2* at mRNA level in the heart, hepatopancreas, gills, stomach, and intestine was analyzed using Taqman probe qRT-PCR methods. The primers were (*MrArf1*-RT-F: 5'-CCAATCTCTTTAAGGGCCTTTTG-3', *MrArf1*-RT-R: 5'-TTTACACCAATTTGAGCTTGTAAG-3', TaqMan fluorogenic probe: 5'-FAM-ACCAGCAGCATCTAACCCACCA-Eclipse-3'; *MrArf2*-RT-F: 5'-CCCCTGTGGAGGCATTATTTTC-3', *MrArf2*-RT-R: 5'-CATTACGAGCAGAGGGCAGTAG-3', TaqMan fluorogenic probe: 5'-FAM-CGTCCGCCCATCTTCATCGT-Eclipse-3'). The GAPDH from the prawns were also amplified as the reference gene using primers as follows (GAPDH-F: 5'-AGGTCTTCAACGAGATGAAG-3'; GAPDH-R: 5'-GGAGTACTTCTCCAAGTTC-3'; TaqMan fluorogenic probe: 5'-FAM-CAAGGCCGAGCCGAGTACATC-Eclipse-3'). The time-course analysis of *MrArf1* and *MrArf2* in the hepatopancreas and gills of *M. rosenbergii* challenged with WSSV at 0, 24, and 48 h was performed using qRT-PCR methods with primers identical to those used during the tissue distribution analysis. A 2× Premix Ex Taq™ (Probe qPCR) (Takara, Dalian, China) was used in the qRT-PCR experiment following the manufacturer's instructions. The methods were undertaken as described by a previously published paper (Liu et al., 2009). All samples were repeated in triplicates in the qRT-PCR analysis. The data were calculated using 2^{-ΔΔCt} methods (Livak and Schmittgen, 2001) and were subjected to statistical analysis. An unpaired sample *t*-test was conducted, and the difference was considered significant if *P* < 0.05.

2.6. RNA interference assay

DsRNA preparation: Gene-specific primers for *MrArf1*, *MrArf2* and green fluorescent protein (GFP) were incorporated with the T7 promoter at their 5'-ends (RNAi-Arf1-F, 5'-GCG TAA TAC GAC TCA CTA TAG GAT ACT GGA GCC CAG TGT TC-3'; RNAi-Arf1-R, 5'-GCG TAA TAC GAC TCA CTA TAG GCT AAT ATA CAA GCT CCA CA-3'; RNAi-Arf2-F, 5'-GCG TAA TAC GAC TCA CTA TAG GGC ACT GTG ATC ACT TAA C-3'; RNAi-Arf2-R, 5'-GCG TAA TAC GAC TCA CTA TAG GAT CCT ATA AAT ACT TTC-3'; RNAi-GFP-F, 5'-GCG TAA TAC GAC TCA CTA TAG AGG TGG TCC CAA TTC TCG TGG AAC-3'; RNAi-GFP-R, 5'-GCG TAA TAC GAC TCA CTA TAG GCT TGA AGT TGA CCT TGA TGC C-3'). These primer sets were used to amplify the corresponding PCR products as templates for dsRNA synthesis. The dsRNA was synthesized using T7 polymerase (Fermentas, USA) following the previous method (Wang et al., 2009).

The Giant Freshwater Prawn was randomly divided into four groups. All Giant Freshwater Prawn in this assay were kept in tanks at about 22 °C. The first group was injected with 80 μg of *dsMrArf1* RNA. The second group was injected with 80 μg of *dsMrArf2* RNA. The third group was injected with 80 μg of dsGFP RNA. The untreated fourth group was used as control. Finally, all four groups were injected with WSSV (1 × 10⁵ virions) at 48 h after dsRNA were injected. Total RNA were extracted from gills at 36 h after the WSSV injection. qRT-PCR analysis was used to detect the efficiency of the RNAi and WSSV levels. The primers of *MrArf1* and *MrArf2* were the same as part 2.5. *Vp28* gene expression was detected using primers as follows (*vp28*-F: 5'-CACAGACAATATCGAGAC-3'; *vp28*-R: 5'-GCATCTTCTCCTTCATC-3'; TaqMan fluorogenic probe: 5'-FAM-

AACCTCCGATTCCTGTGACT-Eclipse-3'). The experiments were repeated three times.

3. Results and discussion

3.1. Cloning of full-length cDNA of *MrArf1* and *MrArf2*

Ran, Rab, and Rho proteins, members of small GTPases found in crustaceans, are involved in innate defense response (He et al., 2004; Liu et al., 2009; Pan et al., 2005; Wu et al., 2007). *Arfs* in mammalian cells participate in virus assembly, replication, and release (Belov et al., 2007a). Some of the *Arfs* of the shrimp *M. japonicus* are engaged in virus–host interaction events (Ma et al., 2010; Wang et al., 2009; Zhang et al., 2010). These findings suggest that investigating whether or not Arf GTPases in the giant freshwater prawn *M. rosenbergii* are involved in innate immunity against WSSV is noteworthy.

The sequencing of the transcriptome of the hepatopancreas and intestine of the freshwater prawns using the Solexa methods resulted in two different EST sequences similar to the *Arf* gene, which were designated as *MrArf1* and *MrArf2*. The full-length cDNA of *MrArf1* was 1819 bp, including a 132-bp 5' untranslated region (UTR), a 549-bp open reading frame (ORF) encoding 182 amino acid proteins and a 1138-bp 3' UTR. *MrArf2* was 1364 bp in length with a 537-bp ORF encoding a protein with 178 amino acids. The 5' and 3' UTR of *MrArf2* were 141 and 686 bp, respectively. The molecular weights of *MrArf1* and *MrArf2* were 20.7 and 19.9 kDa, respectively, and their *pI* values were 6.15 and 5.93, respectively. Each of them had an ARF domain (Supplement 1A and 1B). BLAST P analysis showed that *MrArf1* was 100% identical with the ADP-ribosylation factor 1 from *M. japonicus* (MjArf1). However, the coding region cDNA sequence of *MrArf1* was only 86% identical with that of MjArf1. The 3' UTR of *MrArf1* and MjArf1 were 64.3% identical to each other. In contrast to that of MjArf1, the 3' UTR of *MrArf1* had many extra insertions of nucleotides. Blast P analysis showed that *MrArf2* shared 60% identity with MjArfn. The coding region of *MrArf2* was 64% identical with that of MjArfn.

3.2. Multiple alignment and phylogenetic analysis of *MrArf1* and *MrArf2* with other *Arfs*

The multiple alignment of the *MrArf1* with other *Arf1*s showed that the *Arf1* proteins were highly conserved and had an N-terminal myristoylation site of G. The protein sequences of P loop (GLDAAGK), switch regions (GEIVT/ATIPTIGF, DVGGQDKIRPLWRHY), and interswitch region (NVETVEYKNISFTVW) from different *Arf1*s were nearly the same (Supplement 2A). *MrArf2* also had an N-terminal myristoylation site of G. However, the protein sequences of P loop (GLDGVGKT), switch regions (GKVVQTIPTIGF, DIPSHSKMRPLWRHY), and interswitch region (NVETVEHGNISFTVW) of *MrArf2* (Supplement 2B) were different from those of the MjArfn protein.

Phylogenetic analysis showed that the *Arf* proteins from different species could be divided into 4 different classes. Class I consists of *Arf1*, *Arf2* and *Arf3*. *MrArf1* from giant freshwater prawns and MjArf1 from shrimp *M. japonicus* belong to class I. Class II contains *Arf4* and *Arf5*. MjArf4 and insect *Arfs* belong to class II. *Arf6* proteins constitute class III. *MrArf2* is not clustered into these 3 classes and *MrArf2* and MjArfn belong to a unique class (Supplement 3).

The *Arf* proteins are ubiquitously expressed and well conserved in all eukaryotes with remarkable fidelity (D'Souza-Schorey and Chavrier, 2006; Li et al., 2004). The amino acid conservation among *Arfs* is striking, and the distinct clusters of class-specific residues may indicate unique interactions with regulators and effectors. Investigating these interactions will be challenging, but will reveal

the full range of Arf functions in cells. Our results help lay the foundation for such future undertaking.

3.3. Tissue distribution of MrArf1 and MrArf2

qRT-PCR was used to examine the tissue distribution of *MrArf1* and *MrArf2*. The results showed that *MrArf1* was expressed in all analyzed tissues, including heart, hepatopancreas, gills, stomach, and intestine. The transcription level was significantly higher in the hepatopancreas than in the others (Supplement 4A). *MrArf2* was only highly expressed in the hepatopancreas (Supplement 4B). *MjArf1*, *MjArfn*, and *MjArf4* are all extensively expressed genes (Ma et al., 2010; Zhang et al., 2010). The characterization of the high conservation of protein sequences of shrimp *Arfs* and their constitutive and widespread expression in tissues may indicate that shrimp *Arfs* have important roles.

3.4. Transcriptional analysis of MrArf1 and MrArf2 after WSSV infection

Given our hypothesis that *MrArf1* and *MrArf2* are WSSV related, their gene transcriptional differential expressions during WSSV infection were evaluated. The results displayed a distinguished up-regulation of *MrArf1* and *MrArf2* post WSSV challenge, demonstrating that *MrArf1* and *MrArf2* can significantly be triggered by WSSV infection. Specific evidence was also presented to verify our hypothesis that *MrArf1* and *MrArf2* have a pivotal function in WSSV-related prawn innate immunity. However, *MrArf1* and *MrArf2* transcription patterns were distinct from those of *Arf1*, *Arf4*, and *Arfn* in the hepatopancreas of *M. japonicus*, which were up-regulated and quickly reached the highest level only 6 h after infection. In our study, *MrArf1* reached the maximal level 48 h after infection (Fig. 1A and 1C). The *MrArf2* transcript in the gills was significantly down-regulated at

Fig. 1. Analysis of *MrArf1* and *MrArf2* expression in gills (A, B) and hepatopancreas (C, D) from the giant freshwater prawns *M. rosenbergii* challenged with WSSV using qRT-PCR methods.

Fig. 2. *MrArf1* and *MrArf2* knockdown decreases WSSV in giant freshwater prawn. The giant freshwater prawn was subjected to RNA interference with dsGFP as the control. (A, B) Effect of *MrArf1* and *MrArf2* RNAi on the target gene levels in the gills analyzed via qRT-PCR. (C) The giant freshwater prawn was infected with WSSV after *MrArf1* and *MrArf2* knockdown, and the amount of WSSV was detected via qRT-PCR using *vp28* as a marker. Bars represent the mean of three individual measurements $\pm$ SEM. Differences between groups were analyzed using one-way ANOVA followed by a Tukey's multiple comparison test. Different letters indicate significant difference ($P < 0.05$).

first 24 h after infection (Fig. 1B) but up-regulated and reached the highest value at 48 h. In hepatopancreas, the *MrArf2* was significantly up-regulated at 24 h after infection (Fig. 1D). These findings are consistent with the transcriptional tendency of *Mjcent*, the *Arf6* GAP of *M. japonicus* (Wang et al., 2009). In the previous research, it was found that *Arf1*s and *GBF1* were involved in the RNA virus replication and secretion (Kudelko et al., 2012; Matto et al., 2011; Verheije et al., 2008).

3.5. *MrArf1* and *MrArf2* knockdown decreased the replication level of WSSV

In order to further indicate the roles of *MrArf1* and *MrArf2* in WSSV infection, RNAi experiment was performed. The results showed that *MrArf1* and *MrArf2* were knocked down in gills after *MrArf1* and *MrArf2* dsRNA were injected into the prawns (Fig. 2A and 2B) and the envelope protein gene *vp28* of the WSSV was obviously inhibited compared with the normal (untreated) control and the dsGFP injection group (Fig. 2C). Enterovirus 71 RNA replication was dependent on the activity of Class I Arfs and depletion of *Arf1* and *Arf3* inhibited viral replication in cells (Wang et al., 2014). In plants, RNA virus replication also needed the participation of *Arf1* (Hyodo et al., 2013). Both the levels of hepatitis C virus (HCV) RNA and the pro-

duction of infectious viral particles was downregulated when *Arf1* expression was inhibited by small interfering RNA (siRNA) (Matto et al., 2011). Simultaneous depletion of class II Arf (*Arf4* and *Arf5*) blocked dengue flavivirus secretion (Kudelko et al., 2012). The roles of Arfs in DNA virus infection was less reported. Centaurin- α 1 is a shrimp GTPase activating protein (GAP) of *Arf6* and it notably decreased in WSSV-resistant shrimps (Wang et al., 2009). Other member of *Rab* family, such as *Rab27a*, is required for human cytomegalovirus assembly (Fraile-Ramos et al., 2010). So, it could be speculated that *MrArf1* and *MrArf2* (members of *Rab* family) might play important roles in the innate immune system against WSSV infection.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.dci.2014.10.006.

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