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Effect of immune gene silencing in WSSV infected tiger shrimp *Penaeus monodon*

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ABSTRACT

White spot syndrome virus, continues to cause huge economic loss to aquaculture industry. In the absence of effective therapeutics to control WSSV, it is important to understand the host pathogen interaction at the molecular level. Suppression subtractive hybridization (SSH) cDNA library was constructed which led to identification of several differentially expressed genes in response to WSSV infection in *Penaeus monodon*. The genes expressed in SSH cDNA library of shrimp gill and gut tissues belonged to a wide range of biological functions. The three differentially expressed genes, Single von Willebrand factor type C domain protein (*pm*SVC), P53 protein gene (*pm*P53) and ADP ribosylation factor (*pm*Arf) were up-regulated against WSSV infection and were further characterized by gene silencing to study the role of these shrimp immune genes on WSSV multiplication. The sequence-specific knock down of *pm*SVC, *pm*P53 and *pm*Arf using the dsRNA revealed that in *pm*SVC-dsRNA inoculated shrimps WSSV replication was more with increased viral copy numbers when compared with *pm*P53-dsRNA and *pm*Arf -dsRNA inoculated shrimps. The varied response of immune genes to WSSV infection, indicated that host genes may either inhibit virus replication to some extent or might act as a target to facilitate viral pathogenesis.

1. Introduction

Disease outbreaks mainly due to viral infections in shrimp aquaculture continue to be a major concern for the sustainable development of the shrimp aquaculture industry. Amongst more than 20 viruses which are known to affect penaeid shrimp, white spot syndrome virus (WSSV) is a severe threat with no effective therapeutics to control the disease outbreak. The spread of the disease continues unabated and cause a huge economic loss to the shrimp farmers. In the absence of effective therapeutics to control WSSV, understanding of viral pathogenesis and the shrimp defence mechanism becomes important. At the host level, several studies have identified shrimp miRNAs and their role in shrimp innate immunity against WSSV infection. These differentially expressed miRNAs are conserved in nature [1] and are reported to be involved in immune related pathways such as phagocytosis, apoptosis and pro-phenoloxidase pathways [2], WSSV DNA replication [3]. The host Dicer2 and Ago2 proteins were reported to be essential in vp28-siRNA functional pathway in *Penaeus* (*Marsupenaeus*) *japonicus* [4]. Numerous other shrimp genes, virus binding proteins, receptors, signalling pathways, apoptosis that are potentially involved in the defence mechanism against WSSV infection have been identified [5].

The RNAi studies on shrimp immune gene silencing have shown to result in either increased cumulative shrimp mortality after WSSV infection [6], significant increase in viral loads [7] or inhibition in virus replication [8]. In the present study, suppression subtractive hybridization (SSH) cDNA library was constructed which led to identification of numerous differentially expressed genes in response to WSSV infection in *Penaeus monodon*. The three differentially expressed genes, Single von Willebrand factor type C domain protein (*pm*SVC) belonging to family of single-domain von Willebrand factor type C proteins, P53 protein gene (*pm*P53) which is associated to apoptotic activity and ADP ribosylation factor (*pm*Arf) belonging to Ras superfamily of small GTPases were further characterized by gene silencing to study the role of shrimp immune genes on WSSV multiplication.

In this study, the two differentially expressed genes, *pm*SVC and *pm*P53 were identified from SSH cDNA library constructed from the gills and gut tissues of WSSV infected *P. monodon* whereas, *pm*Arf was previously identified from SSH cDNA library constructed from

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the hepatopancreas of WSSV infected *P. monodon* [9]. The *pm*SVC gene was selected for gene silencing experiments using double stranded RNA (dsRNA) as this gene is reported to be up-regulated against WSSV infection and it is also involved in RNAi mechanism and gets induced by Dicer 2 in shrimps [10] Similarly, the other two genes *pm*P53 and *pm*Arf are also reported to be up-regulated against WSSV infection in shrimps. The P53 gene is specifically involved in apoptotic activity in response to WSSV infection and P53 gene silencing affect viral replication in WSSV infected shrimps [11]. The knockdown of Arf genes is reported to result in inhibiting expression of WSSV vp28 envelope protein in WSSV infected shrimps [12].

2. Material and methods

2.1. WSSV infection and construction of SSH cDNA library

WSSV challenge experiment was conducted in *P.monodon* shrimps as described previously [9]. Briefly, shrimps were challenged with virus dilution (10^{-7}) made from virus stock (2.62×10^6/μl viral copies) by intramuscular route (0.1 ml). The tissue samples (gills, gut and hepatopancreas) were collected at 6 h, 24 h, 48 h and moribund stage (48 h upto 72 h) of infection and stored in RNA*later* (Qiagen GmbH, Hilden, Germany) for further analysis. The WSSV infection was confirmed in the infected shrimp tissues by nested PCR using the reported outer primers (F:GACAGAGATATGCACGCCAA R: ACCAGTGTTTCGTCATGGAG) and inner primers.

(F: GGTAGATTCTGGTATTAGG R: ACCTGGCGTAGTTCTTGC) for detection of WSSV [13].

The gills and gut tissues of shrimp collected at moribund stage of WSSV infection were used for constructing two separate SSH cDNA libraries using PCR-Select cDNA subtraction kit (Clontech, California, USA), following the procedure described previously [9]. The pooled gill and gut tissues collected from six infected and control shrimps were used as tester and driver respectively for the SSH cDNA library construction. Briefly, the protocol included extraction of the total RNA using NucleoSpin RNA II kit (Macherey-Nagel GmBH & Co, Duren, Germany), cDNA synthesis by using Super SMART-PCR cDNA Synthesis Kit (Clontech, California, USA), long distance PCR amplification using Advantage 2 PCR kit (Clontech, California, USA) and cDNA purification by using CHROMA SPIN-1000 DEPC-H2O columns (Clontech, California, USA). *Rsa*I restriction enzyme was used to digest the tester and driver cDNAs and the tester cDNA was ligated with the adapter. Following two steps of hybridization and PCR amplification, the PCR products were ligated into pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). The clones were sequenced (SciGenom Labs Pvt. Ltd, Cochin, Kerala, India) and the sequences of the clones obtained were analysed by BLASTX, BLASTN and TBLASTX (www.ncbi.nlm.nih.gov/blast).

The tissue samples (gills, gut and hepatopancreas) collected at 6 h, 24 h, 48 h and moribund stage of infection were also used for gene expression of *pm*SVC, *pm*P53 and *pm*Arf.

2.2. Amplification of pmSVC and pmP53 genes

The cDNA was synthesized using ProtoScript M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs Inc, Ipswich, Masachusettes, USA), from the total RNA extracted from gill tissues of shrimp using NucleoSpin RNA II kit (Macherey-Nagel GmBH & Co, Duren, Germany). This cDNA was used as a template to amplify the *pm*SVC gene using gene specific primers based on the reported ORF of *P. vannamei* single VWC domain protein 1 (GenBank accession HQ541158). The *pm*P53 gene was amplified by primers designed based on reported sequence of the gene isolated from *P. vannamei* (GenBank accession KC422442). The PCR products of *pm*SVC gene and *pm*P53 were purified by QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and cloned into pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) before sequencing. Amplification of 5′ end of *pm*SVC gene by Rapid Amplification of cDNA ends (RACE) was carried out using SMARTer RACE cDNA Amplification Kit (Clontech, California, USA). The sequences of gene specific and 5′ RACE primers are shown in Table 1.

2.3. Sequence analysis

The sequence of *pm*SVC and *pm*P53 genes were analysed for sequence homology using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein domains were analysed using the SMART software (http://smart.embl-heidelberg.de/). Signal peptide was predicted using SignalP 4.0 software (www.cbs.dtu.dk/services/SignalP).

2.4. Expression of dsRNA of pmSVC, pmP53 and pmArf shrimp genes, WSSV vp28 and gfp gene

The dsRNA was expressed for *pm*SVC, *pm*P53 and *pm*Arf shrimp genes, WSSV vp28 gene and green fluorescent protein (gfp) gene in LITMUS28i transcription vector (New England Biolabs, Inc, Ipswich, Masachusettes,USA). The primers used for expression of dsRNA *pm*SVC gene contained restriction sites *Stu*I and *Bgl*II. Primers sequences for expression of dsRNA sequences for *pm*P53, *pm*Arf, WSSV vp28 and gfp genes contained restriction sites *Xba*I and *Sac*I as shown in Table 1. The PCR products of genes after restriction digestion with respective restriction enzymes were ligated into the polylinker region of LITMUS28i transcription vector and transformed into *E. coli* HT115 (DE3) strain. The cloned recombinant plasmids were confirmed by releasing of the insert DNA using the respective restriction enzymes. The expression of *pm*SVC-dsRNA, *pm*P53-dsRNA, *pm*Arf-dsRNA, vp28-dsRNA and gfp-dsRNA was induced with 0.4 mM/ml of IPTG. The bacterial culture (50 ml) was incubated for 4 h and cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. The ds RNA was isolated by resuspending the bacterial pellet in 4 ml of TRIzol solution (TRI Reagent, Sigma-Aldrich, USA) followed by incubation at room temperature for 5 min. Chloroform (1 ml) was added to the resuspended cell pellet and incubated for 2–3 min. The resuspended cell pellet was centrifuged at 12000 rpm for 15 min at 4 °C and the aqueous phase was transferred into new tube. Two ml of Isopropanol was added and vortex briefly. The solution mix was incubated for 20 min at −80 °C and centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was decanted and the pellet was washed with 70% ethanol and air dried. Finally the dsRNA pellet was resuspended in 100 μl nuclease free water and dsRNA concentration was estimated by Nano drop 2000c spectrophotometer (Thermo scientific, USA). The dsRNA was stored in −80 °C for further use.

2.5. Gene silencing by dsRNA inoculation in shrimps

Shrimps with mean body weight of 5.07 ± 1.19 g were divided into seven groups, with 24 numbers in each group. The *pm*SVC-dsRNA, *pm*P53-dsRNA, *pm*Arf-dsRNA, vp28-dsRNA and gfp-dsRNA (6 μg/g body weight) was injected separately in five groups of shrimps. After 24 h the shrimps were given a second dose of dsRNA with the same concentration of dsRNA. The dsRNA injected shrimps were challenged with WSSV 10^{-4} dilution of virus stock (3.44×10^5/μl viral copies) by intramuscular injection at 24 h post second dose of dsRNA injection. Gills, gut and hepatopancreas from six shrimps each were collected at 6 h, 24 h, 48 h and 96 h post

Table 1
Primers used for gene amplification, RT-qPCR and expression of dsRNA.

Gene	Primer/probe sequence (5′-**3**′)	Amplification/analysis	GenBank accession no.
β-actin	F: CCCTGTTCCAGCCCTCATT	RT-qPCR	JN808449
	R: GGATGTCCACGTCGCACTT		
	[6FAM]CCACCTACAACTCCATCAT [TAM]	probe	JN808449
*pm*SVC	F: ATGAAGTTCTTGCTGATTGCT	gene specific	HQ541158
	R: TTAAGCAGTTCCTGGTGCTGC		
	F:CGGTCCTCAGCCCAAGAA	RT-qPCR	KU342002
	R:CACGCTGGGTATTCGAGGAT		
	[6FAM] ACGATTTCCAACCTAGATGA [TAM]	probe	KU342002
	AAGAGTCGGAAGTTTGTTGA	5′RACE	KU342002
	F:CGGAGATCTATGAAGTTCTTGCTGATTGCT	dsRNA	KU342002
	R:CGGAGGCCTTTAAGCAGTTCCTGGTGCTGC		
*pm*P53	F: ATGCAGCGGTCGGACTCCGA	gene specific	KC422442
	R: TTAGTTACTCTCCTCTTCAGGA		
	F:CGCCCATTCTGCATTGC	RT-qPCR	KU342003
	R:TGCAGCACTTAATGTCCAAGATC		
	[6FAM]ACTCTTAGAAACTCTGTCACTG [TAM]	probe	KU342003
	F:CGGTCTAGAATGCAGGTCGGACTCGCCGA	dsRNA	KU342003
	R:CGGGAGCTCTTAGTTACTCTCCTCTTCAGGA		
*pm*Arf	F:GGTCTTGATGCTGCTGGTAAAA	RT-qPCR	KM210090
	R:TGATGTTCTTGTACTCGACGGTTT		
	[6FAM]TTAGGAGAGATTGTCACCACC [TAM]	probe	KM210090
	F: CGGTCTAGAATGGGACTTACGCTCTCGA	dsRNA	KM210090
	R:CGGGAGCTCTTACTTCTTTGGCAATTCATTG		
WSSV vp28	F: ATGTGTCCTTTGACAGCGAC	RT-qPCR	DQ681069
	R: GCCCTCCACGGGAGTGATGA		
	[6FAM]AAGTCTGATGCACAGATGA [TAM]	probe	DQ681069
	F:CGGTCTAGAATGGATCTTTCTTTCACT	dsRNA	DQ681069
	R:CGGGAGCTCTTACTCGGTCTCAGTGCCAG		
gfp	F:CGGTCTAGAATGGGCGTGATCAAGCCCGA	dsRNA	AY218848
	R:CGGGAGCTCTTAGCCGGCCTGGCGGGGT		

The restriction enzyme sites are underlined in primer sequences used for dsRNA cloning and expression.

WSSV challenge for further analysis. The sixth group of shrimps was challenged with same dose of WSSV (3.44×10^5/μl viral copies) and this shrimp group was used as positive control for WSSV infection. Gill tissues from six shrimps each were collected at 6 h, 24 h, 48 h and 96 h post WSSV challenge to estimate viral copy numbers. The PBS inoculated seventh group of shrimps was maintained as uninfected negative control group. The tissues were stored in RNA*later* (Qiagen GmbH, Hilden, Germany) for further analysis.

2.6. Expression analysis of gene transcripts

Gene expression analysis of *pm*SVC and *pm*P53 genes was carried out by RT-qPCR in WSSV infected gills, gut and hepatopancreas tissues collected at 6 h, 24 h, 48 h and moribund stage of infection. RT-qPCR was carried out as described previously [9]. Briefly, the cDNA was synthesized using Protoscript M-MuLV first strand cDNA synthesis kit (New England Biolabs, Inc, Ipswich, Masachusettes,USA) from the total RNA extracted from tissues of WSSV infected shrimps and uninfected control shrimps by NucleoSpin RNA II kit (Macherey-Nagel, GmBH & Co, Duren, Germany). The cDNAs of *pm*SVC and *pm*P53 were analysed for their relative expression using the TaqMan Universal master mix II (Applied Biosystems, Foster city, California, USA). Shrimp β-actin gene was used as an endogenous control in RT-qPCR. The gene specific primers and probes used in RT-qPCR are shown in Table 1. The fold change values were obtained by comparative ΔC_T method. The expression values were $2^{-\Delta\Delta CT}$ without applying negative inverse which gives actual number of folds decrease in expression of target gene in treated groups compared to control. The error bars were estimated using $2^{-\Delta CT}$ values of individual shrimp. One-way ANOVA was used in the statistical analysis of the data. The significance of observations ($p < 0.05$) in treated group at each data point were tested against observations of untreated negative control at that data point.

2.7. Validation of the gene silencing effect of expressed dsRNA of pmSVC, pmP53 and pmArf genes and WSSV vp28 gene by RT-qPCR

To validate the gene silencing effect of *pm*SVC, *pm*P53 and *pm*Arf genes, the gills, gut and hepatopancreas respectively were collected from shrimp inoculated with respective dsRNA of *pm*SVC, *pm*P53 and *pm*Arf genes. The respective shrimp tissues were collected at 6 h, 24 h, 48 h and 96 h post WSSV challenge. The gene silencing effect of *pm*SVC, *pm*P53 and *pm*Arf genes in comparison with control group of shrimps were analysed by RT-qPCR. The expression values in control group (shrimp without infection) were normalized to unity while estimating relative expression values in treated groups. The expression values were $2^{-\Delta\Delta CT}$ without applying negative inverse which gives actual number of folds decrease in expression of target gene in treated groups compared to control. The $2^{-\Delta CT}$ values of individual shrimp were used to conduct statistical tests between control and treatment groups at $p < 0.05$ by one-way ANOVA and to estimate the error bars.

The gene silencing effect of expressed dsRNA of WSSV vp28-dsRNA was validated by estimating WSSV viral copy numbers in gill tissues of WSSV challenged shrimps.

2.8. Estimation of WSSV viral copy numbers

Estimation of WSSV viral copy numbers was carried out in gill tissues of dsRNA inoculated shrimps with *pm*SVC-dsRNA, *pm*P53-dsRNA, *pm*Arf-dsRNA, vp28-dsRNA and gfp-dsRNA and

subsequently challenged with WSSV. A standard curve was plotted using 10 fold dilution of cloned pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) containing the WSSV vp28 insert. The C_T value of the target was compared with the standard curve. The gill tissues were collected at 6 h, 24 h, 48 h and 96 h for estimation of viral copy numbers by RT-qPCR.

3. Results

3.1. Functional classification of differentially expressed genes in SSH cDNA library of shrimp gill and gut tissues

The insert size of the clones obtained in the SSH cDNA library generated from the shrimp tissues of the WSSV infected *P.monodon* ranged from 200 bp to 1.5 kb. The putative functions of the SSH clones on sequencing were identified by BLAST analysis and were classified into different functional categories. The putative functions of the genes expressed in SSH cDNA library of shrimp gill and gut tissues related to a wide range of biological roles (Supplementary Table 1).

3.2. Isolation and sequence analysis of pmSVC gene

The *pm*SVC gene was one of the differentially expressed gene from SSH cDNA library prepared from WSSV infected shrimp gill tissues. The *pm*SVC for the first time was identified from *P. monodon* in the present study and its sequence was submitted to the GenBank (GenBank accession KU342002). The *pm*SVC sequence showed homology with single VWC domain protein 1 of *P. vannamei* (*Lv*SVC1) having 168 amino acids (AEB54791). The *Pm*SVC protein sequence revealed deletion of 3 amino acids in its sequence and 89% identity when compared with the SVC of *P. vannamei*. These amino acid deletions were represented with 18Gly-Pro-Ala20 in the SVC sequence of *P. vannamei*. The analysis of *Pm*SVC protein for signal peptide prediction using SignalP 4.0 server, showed it to be a secreted protein with a signal peptide region. The domain analysis of the amino acid sequence showed SVWC domain spanning from 48 to 117 region with E-value of 0.0564 (Supplementary Fig. S1).

3.3. Isolation and sequence analysis of pmP53 gene

The sequence of P53 cDNA (1353 bp) identified for the first time from *P. monodon* was submitted to the GenBank (GenBank accession KU342003) (Supplementary Fig. S2). The sequence analysis showed high similarity to the P53 gene sequence isolated from *P. vannamei* (KC422442).

3.4. Expression analysis of pmSVC and pmP53 genes after WSSV challenge

The RT-qPCR analysis revealed that the WSSV challenge resulted in peak expression of *pm*SVC in the gill tissue at 48 h (13.28 folds) which reduced to 1.91 folds at moribund stage of WSSV infection. Similar peak expression profile was recorded at 48 h (14.79 folds) with reduced expression levels of 1.93 folds at moribund stage of WSSV infection in the gut tissue (Fig. 1).

The *pm*P53 gene expression significantly increased in the gut tissues from 24 h (3.44 folds) to moribund stage (4.25 folds) of WSSV infection as compared to gills and hepatopancreas (Fig. 2).

3.5. Validation of the gene silencing effect of expressed dsRNA of pmSVC, pmP53 and pmArf genes and WSSV vp28 gene by RT-qPCR

To validate the gene silencing using expressed dsRNA, RT-qPCR

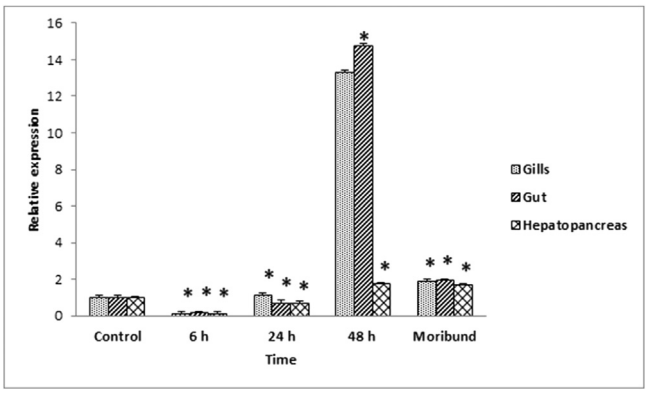

Fig. 1. Gene expression analysis of *pm*SVC in gills, gut tissues and hepatopancreas of WSSV challenged *P. monodon* by RT-qPCR at 6 h, 24 h, 48 h and moribund stage of post WSSV infection. Significant difference is indicated by asterisk (*) as compared to untreated negative control.

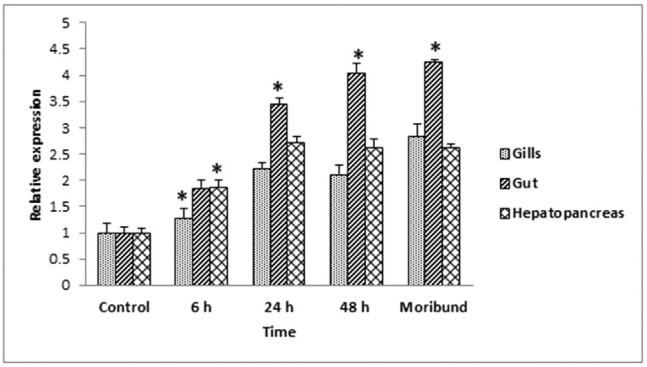

Fig. 2. Gene expression analysis of *pm*P53 in gills, gut tissues and hepatopancreas of WSSV challenged *P. monodon* by RT-qPCR at 6 h, 24 h, 48 h and moribund stage of post WSSV infection. Significant difference is indicated by asterisk (*) as compared to untreated negative control.

was carried out for gene expression analysis of *pm*SVC, *pm*P53 and *pm*Arf in gill, gut tissues and hepatopancreas respectively of dsRNA inoculated shrimps. The fold change values obtained by comparative ΔC_T method revealed down-regulation for *pm*SVC, *pm*P53 and *pm*Arf genes at all time points, indicating gene silencing of respective genes in dsRNA inoculated shrimps (Fig. 3). The gene silencing effect of expressed dsRNA of WSSV vp28-dsRNA was validated by estimating WSSV viral copy numbers in gill tissues of WSSV challenged shrimps which revealed significant reduction till 48 h in the viral copy numbers estimated at 6 h (6.0×10^2/μl), 24 h (2.4×10^3/μl), 48 h (4.4×10^3/μl) as compared to viral copy numbers estimated in WSSV positive shrimp group which showed high viral copy numbers at 6 h (2.2×10^4/μl), 24 h (3.0×10^4/μl), and 48 h (1.9×10^6/μl). However, at late stage of infection at 96 h the viral copy numbers estimated in vp28-dsRNA inoculated shrimp group (1.0×10^8/μl) did not vary much with WSSV positive shrimp group (8.9×10^7/μl). (Fig. 4).

3.6. Analysis of WSSV replication in dsRNA inoculated shrimps

To evaluate the role of *pm*SVC, *pm*P53, *pm*Arf and gfp-dsRNA genes in the shrimp defense against WSSV infection, WSSV challenge was carried out in *P. monodon* injected with dsRNA. Estimation of WSSV viral copy numbers was carried out in gill tissues of dsRNA inoculated shrimps with *pm*SVC-dsRNA, *pm*P53-dsRNA, *pm*Arf-dsRNA and gfp-dsRNA and subsequently challenged with

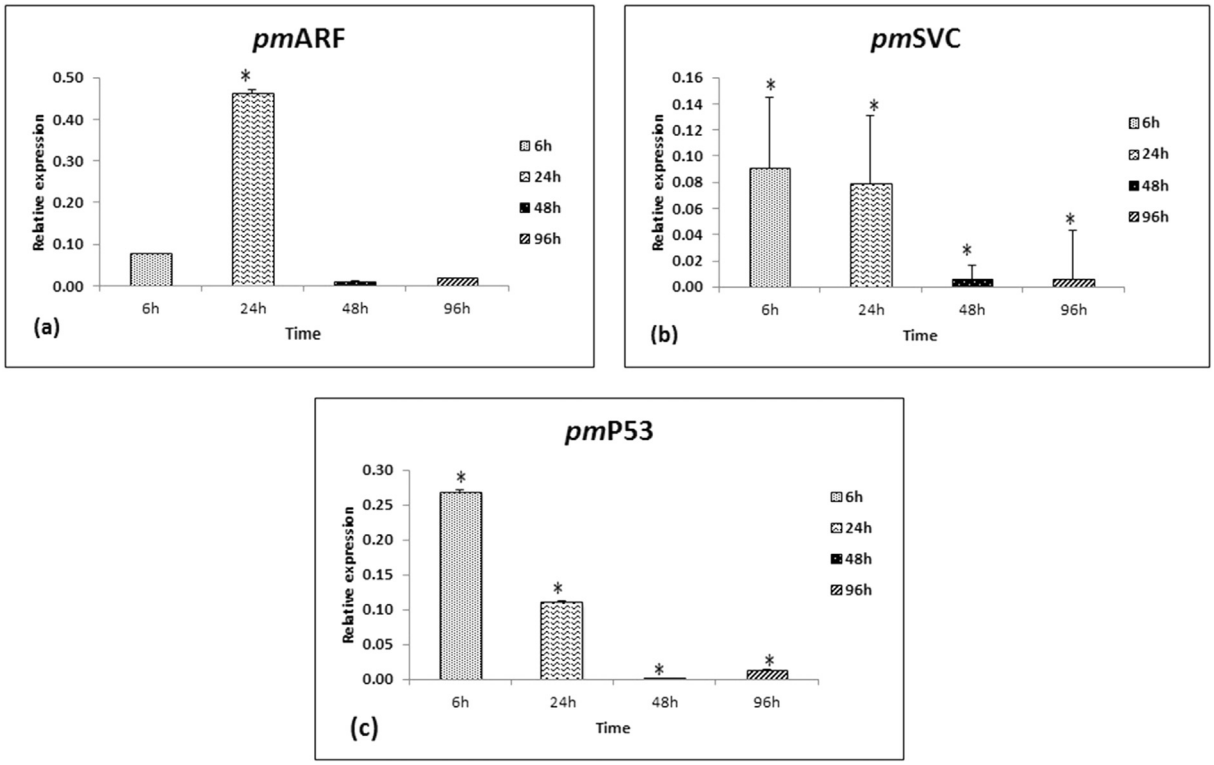

Fig. 3. Gene expression analysis of (a) *pm*Arf in hepatopancreas, (b) *pm*SVC in gills and (c) *pm*P53 in gut tissues of shrimps inoculated by dsRNA of respective genes by RT-qPCR at 6 h, 24 h, 48 h and 96 h post WSSV infection. Significant difference is indicated by asterisk (*) as compared to untreated negative control.

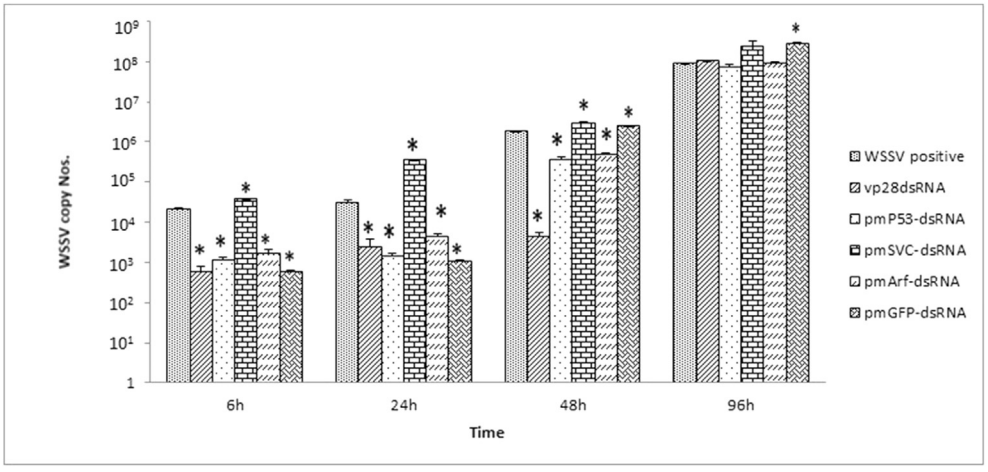

Fig. 4. RT-qPCR estimation of WSSV viral copy numbers carried out in gill tissues of dsRNA inoculated shrimps with *pm*SVC-dsRNA, *pm*P53-dsRNA, *pm*Arf-dsRNA, WSSV vp28-dsRNA and gfp-dsRNA at (a) 6 h, (b) 24 h, (c) 48 h and (d) 96 h by RT-qPCR. The copy number of virus in treated groups is tested for significant deviation ($p < 0.05$) from WSSV infected control group using one-way ANOVA.

WSSV. The WSSV copy numbers were higher in *pm*SVC-dsRNA inoculated shrimps at 6 h (3.7×10^4/μl), 24 h (3.6×10^5/μl) and 48 h (2.9×10^6/μl). The WSSV copy numbers was less in *pm*Arf-dsRNA shrimps at 6 h (1.7×10^3/μl), 24 h (4.3×10^3/μl) and 48 h (4.7×10^5/μl) and in the shrimp group inoculated with *pm*P53-dsRNA at 6 h (1.1×10^3/μl), 24 h (1.4×10^3/μl) and 48 h (3.5×10^5/μl) when compared with *pm*SVC-dsRNA inoculated shrimps and WSSV infected positive group. The shrimp group inoculated with gfp-dsRNA showed less viral copy numbers at 6 h (5.8×10^2/μl) and 24 h (1.0×10^3/μl) with increase in viral copies at 48 h (2.4×10^6/μl) when compared to WSSV infected positive group (Fig. 4).

At late stage of infection at 96 h the WSSV copies was in the same range in all the dsRNA inoculated shrimp groups of *pm*SVC-dsRNA (2.4×10^8/μl), *pm*Arf-dsRNA (8.8×10^7/μl), *pm*P53-dsRNA (7.2×10^7/μl) when compared with WSSV infected positive group (8.9×10^7/μl) and gfp-dsRNA control group (2.7×10^8/μl).

4. Discussion

The differentially expressed genes identified from SSH cDNA library from the shrimp gill tissues were penaeidin, glutathione peroxidase, dicer2, single von Willebrand factor type C domain

protein (*pm*SVC) and lymphoid organ expressed yellow head virus receptor protein (Supplementary Table 1). *pm*SVC was selected for further characterization as this gene is reported to respond to viral infections [10,14]. To date, this gene has not yet been isolated and identified from *P. monodon* and no study has been carried out on WSSV pathogenesis after *pm*SVC gene silencing in shrimp. Single domain von Willebrand factor type C (SVWC) belongs to family of single-domain von Willebrand factor type C proteins. Thirteen short *Drosophila melanogaster* proteins showing similarity to a single von Willebrand factor C-domain (VWC) and belonging to family of single VWC domain proteins have been identified. The members of this protein family are named as single VWC domain proteins (SVC) [15]. These proteins respond to bacterial and viral infections and to nutritional status of the organism. For example, the Vago gene from *Drosophila* having the VWC domain responded to infection with *Drosophila* C virus and its induction was dependent on Dicer2 [14]. A recent study revealed that *Penaeus vannamei* interferon regulatory factor (IRF) binds to the Vago promoter to regulate its transcription. Vago is therefore suggested as an interferon (IFN) -like molecule having a functional role in IRF-Vago-JAK/STAT pathway [16]. In shrimps, five VWC domain proteins having motif similar to *Drosophila melanogaster* SVC proteins have been identified from *P. vannamei*. These shrimp SVCs which ranged between 102 and 190 amino acids in length were activated by Dicer2, a component of RNA interference (RNAi) pathway and responded against WSSV infection [10].

In the SSH cDNA library constructed from the shrimp gut tissues, the tumour suppressor gene P53 gene was represented along with other immune genes such as anti-lipopolysaccharide factor, elongation factor, cyplasin, YLR154W-A-like protein, beta-integrin, innexin 2 and ribosomal protein L30 (Supplementary Table 1). The *pm*P53 was further characterized in this study as very limited information of this gene is available and the tumour suppressor gene P53 is reported to be associated with apoptosis [17] and in inducing the cell cycle arrest against DNA damage [18]. In shrimps such as *P. japonicus*, the miRNA mediated P53 protein has been reported to contribute to shrimp innate immunity by regulating the apoptotic activity in response to WSSV infection. The expression levels of P53 was upregulated in the WSSV infected shrimp and P53 gene silencing resulted in decrease of apoptotic activity with decrease in WSSV infection. The gene silencing of P53 indicated negative effect on WSSV infection in shrimp [11]. The *pm*P53 has a functional role in shrimp survival by regulating antioxidant enzymes in response to environmental stress [19] and in influencing WSSV infection by regulating apoptotic activity in shrimp [11].

In our earlier study, we have reported *pm*Arf expression was induced by WSSV infection indicating that shrimp Arfs are involved in immune response against WSSV [9]. Hence, this gene was selected to study its functional role in immune response and WSSV pathogenesis by gene silencing. The ADP ribosylation factor (Arf), which belongs to Ras superfamily are involved in signal transduction pathways, membrane trafficking, activation of lipid modifying enzymes and membrane surface modifications [20]. The mammalian Arfs are classified into class I (Arf1-3), class II (Arf4-5) and class III (Arf6) [21], however, very limited Arfs have been identified from shrimp. The shrimp Arfs have been isolated from *P. japonicus* [22,23] and *P. monodon* [9] and were shown to be involved in WSSV infection.

There is no clear reasons why the WSSV genes were not represented as differentially expressed genes in the SSH cDNA libraries in the present study. However, there are other several studies which have reported similar results. For example, no viral transcripts could be detected in SSH cDNA library prepared from *Penaeus merguiensis* hepandensovirus infected *P. merguiensis* lymphoid organ [24], parvovirus infected gill tissues of crayfish [25] and from WSSV infected hepatopancreas of *P. vannamei* [26]. The absence of viral transcripts in SSH cDNA libraries could be due several reasons such as low abundance of the target mRNA which should be at least 0.1% of the total mRNA [27] or absence of poly A tail in target transcripts. WSSV is reported to possess only 61.5% of the structural genes with a polyadenylation signal and some structural protein genes mRNA lacks a poly(A) tail [28].

In the present study, *pm*SVC was highly expressed in the gill (13.28 folds) and gut (14.79 folds) tissues at initial stages (48 h) of WSSV infection which later reduced in the moribund stage of infection. The gill tissues of WSSV infected penaeid shrimp is reported to show altered expression profiles of immune genes [29,30]. The gut tissue of shrimp is also closely associated with viral pathogenesis as oral route is also a mode of WSSV transmission [31]. Similar expression profile of this gene has been reported in WSSV infected *P. vannamei*. The SVC1 mRNA from *P. vannamei* was shown to be highly expressed in gill and epidermal tissue of shrimp. The gene was upregulated upon WSSV challenge with peak expression (55.6-fold) at 24 h and the expression levels of gene gradually decreased to about 50% of peak at 30 h, and plateaued for the next 36–72 h in the gill tissues [10]. The present results indicate that expression of *pm*SVC gets stimulated against WSSV infection in *P. monodon* in gill and gut tissues of the shrimp.

The *pm*SVC gene on sequencing showed homology with *P.vannamei* (*Lv*SVC1) (AEB54791), and revealed eight conserved cysteine residues. The SCV isolated from *Drosophila* are known to have the consensus pattern C–(17, 32)–C–(4,5)–C–(10, 11)–C–(6, 10)–C–(12,14)–(FY)–P–X–C–C–(2, 5)–C [15]. The VWC domains which are cysteine rich domain generally consists of ten cysteines [32], however in arthropod proteins eight cysteines have been observed. This study also confirms the presence of eight cysteines in shrimps.

To find the interaction between *pm*P53 and WSSV infection in the present study, the gene expression level of *pm*P53 was determined in WSSV infected shrimp tissues. Stress mediated P53 activation has been reported under hypoxic conditions [33] and in response to viral infections in humans [34] and shrimps [11]. The P53 gene expression in *P. vannamei* is reported to be induced and up-regulated in a time-and concentration-dependent manner in response to acidic pH challenge and cadmium exposure to mediate oxidative cell stress with antioxidant enzymes [19]. The *pm*P53 gene expression significantly increased in the gut tissues from 24 h (3.44 folds) to moribund stage (4.25 folds) of WSSV infection. This increase in the fold change gene expression level is in agreement with the results obtained by others [11], who showed a similar range of increase from about 2.5 folds at 36 h post infection to about 5.0 folds at 60 h post infection in the gene expression levels of P53 in WSSV infected *P. japonicus* haemolymph. The high similarities (91%) in the amino acid sequence of *pm*P53 with that of *P. vannamei* P53 and the conserved P53 DNA binding domain with DNA binding, zinc binding and dimerization sites, indicated similar functional role of the gene isolated from *P. monodon* in the present study. The increased levels in the gene expression of P53 upon WSSV indicates that P53 plays an important role in virus infection in shrimps.

In dsRNA inoculated shrimps using *pm*SVC-dsRNA, *pm*P53-dsRNA and *pm*Arf-dsRNA, the down-regulation for *pm*SVC in gills, *pm*P53 in gut and *pm*Arf in hepatopancreas respectively was observed. In vp28-dsRNA treated shrimps, significant reduction of WSSV viral copy numbers as compared to control, was observed till 48 h of sample collection. This indicated successful sequence specific gene silencing. In shrimps, gene silencing using dsRNA is highly sequence-specific to trigger a protective RNAi response [8] and the RNAi effect is significantly reduced even with one-nucleotide change in the siRNA [35].

In the present study, two doses of dsRNA (6 μg/g body weight)

after 24 h interval were used for gene silencing. Multiple doses have been used by other workers for successful gene silencing. For example, three injections of the vp28-siRNA, resulted in complete eradication of the virus from WSSV-infected *P. japonicus* and with increase in number of injections of vp28-siRNA the WSSV copies decreased rapidly [35].

The gene silencing results obtained after WSSV challenge of dsRNA injected shrimps at 24 h post second dose of dsRNA injection is in agreement with the observation of others [36] who reported that treatment of shrimps first with vp28-siRNA and later with WSSV challenge instead of simultaneous injection of vp28-siRNA and WSSV give better protection activity of siRNA.

The gene silencing of *pm*P53 and *pm*Arf reduced the WSSV copy numbers when compared to viral copy numbers estimated in WSSV positive infection shrimp group, whereas, the gene silencing of *pm*SVC resulted in increase of WSSV copy numbers. However, at 96 h post WSSV infection, the viral copies increased in all the shrimp groups treated with dsRNA, indicating that at late stage of virus infection, the viral multiplication increases by many folds and the host immune machinery fails to inhibit the virus multiplication.

The shrimp group inoculated with gfp-dsRNA showed decrease in the viral copy numbers in the initial infection period from 6 h to 24 h however late stage of WSSV infection from 48 h to 96 h indicated no significant effect on the inhibition of WSSV replication when compared to WSSV infected positive group of shrimp. The use of long dsRNA including gfp-dsRNA can probably influence in enhancing in shrimp immunity in sequence independent manner as reported by others [37]. Based on use of dsRNA gene silencing experiments, the shrimp immunity is hypothesized to be induced by sequence-independent innate immunity or by RNAi silencing mechanism pathway [38,39]. The exact mechanisms of these genes in WSSV regulation is still unclear and requires further investigation.

The SVWC family proteins responds to environmental stimuli, such as bacterial infection and nutritional status in addition to their anti-viral immunity function. All of these functions are probably linked to SVWC expression being induced by Dicer2 as the promoters of *P. vannamei* SVC are reported to get activated by Dcr2 in S2 cells [10]. It would be interesting to further examine the relationship between various components involved in RNAi mechanism such as dicer, vago gene and the SVC proteins in shrimps. This would offer a new insight into the mechanisms involved in shrimp immunity using RNAi pathway.

The gene silencing of *pm*P53 was observed to reduce the WSSV copy numbers in the present study. The knockdown of P53 expression by P53-siRNA mediated gene silencing is reported to result in decrease in WSSV copies in other shrimps such as *P. japonicus* [11] indicating functional role of P53 in virus infection. The viruses are known to manipulate these P53 mediated pathways as a strategy to survive and facilitate their replication [34,40]. In addition to several innate humoral immune mechanism, shrimps also are reported to use cellular defence mechanism such as phagocytosis and apoptosis, against WSSV infection. The enhancement of caspase dependent apoptosis is shown to result in inhibition of WSSV infection with decrease in shrimp mortality [41]. A caspase gene isolated from WSSV resistant *P. japonicus* shrimp and in particular containing fragment 3 was shown to modulate virus induced apoptosis. The gene silencing of caspase gene by siRNA resulted in increase in WSSV copy numbers [42]. Similarly, shrimp P53 mediated pathways may be involved in host cellular stress response against virus infections and might be regulated by WSSV to facilitate its replication as reported in numerous other viral infections.

ADP ribosylation factor is reported to facilitate viral propagation in other organisms. The siRNA-mediated knockdown of ADP-ribosylation factor-related protein 1 (ARFRP1) was shown to significantly inhibit HCV replication [43]. In shrimps, a recent study in *Macrobrachium rosenbergii*, has shown that knockdown of ADP ribosylation factors MrArf1 or MrArf2 resulted in decrease in the expression levels of the envelope protein gene vp28 of the WSSV [12]. Hence, the gene silencing of *pm*Arf which resulted in reducing the WSSV copy numbers in the present study is in agreement with results obtained by others who showed that gene silencing of ADP ribosylation factor results in inhibition of viral replication. In conclusion, *pm*SVC, *pm*P53 and *pm*Arf genes were observed to respond with varied gene expression levels to WSSV infection. In general, these differentially immune responsive genes can have a two way effect in viral infections, one of which is to respond by inhibiting virus replication or it might act as a target for the viral proteins to facilitate their pathogenesis and replication which needs to be further explored.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.09.019.

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