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Nucleic acid-induced antiviral immunity in shrimp

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ABSTRACT

Vertebrates detect viral infection predominantly by sensing viral nucleic acids to produce type I interferon (IFN). In invertebrates, it has been believed that the IFN system is absent and RNA interference is a sequence-specific antiviral pathway. In this study, we found that injection of nucleic acid mimics poly(I:C), poly(C:G), CL097, poly C and CpG-DNA, afforded shrimp antiviral immunity, which is similar to the vertebrate IFN system. Using suppression subtractive hybridization (SSH) method, 480 expression sequence tags were identified to be involved in the poly(I:C)-induced antiviral immunity of the model crustacean *Litopenaeus vannamei*, and 41% of them were new genes. In the SSH libraries, several IFN system-related genes such as dsRNA-dependent protein kinase PKR, Toll-like receptor 3 (TLR3) and IFNγ-inducible protein 30 were identified. *L. vannamei* IKKε, whose vertebrate homologs are central regulators of the IFN-producing pathway, could significantly activate IFN reporter genes in HEK293T cells. In crustacean databases, many genes homologous to genes of the vertebrate IFN response, such as IRFs, PKR, ADAR (adenosine deaminase, RNA-specific) and other interferon-stimulated genes (ISGs) were discovered. These results suggest that shrimp may possess nucleic acid-induced antiviral immunity.

1. Introduction

Viruses contain genetic material composed of either DNA or RNA that encodes viral structural components and synthetic and replication enzymes (Akira et al., 2006). Various structural components, including single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), and unmethylated CpG-DNA, are viral nucleic acids that can be recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (Akira et al., 2006; Kawai and Akira, 2009). Recognitions of viral nucleic acids by PRRs commonly induce productions of proinflammatory cytokines, chemokines and type I interferons (IFNs), triggering inflammation and type I IFN responses which are the hallmarks of host innate immune system in defending against viral infections (Sadler and Williams, 2008; Takeuchi and Akira, 2009).

In vertebrates, ssRNA is detected by TLR7 and TLR8, DNA is detected by TLR9, and dsRNA is detected by TLR3 and RLRs (RIG-I and MDA5), resulting in activations of NF-κB and IRF3/7 pathways (Akira et al., 2006; Kawai and Akira, 2009; Takeuchi and Akira, 2009). NF-κB plays a central role in coordinating expression of proinflammatory cytokines and chemokines for eliminating viral infection by provoking inflammation and recruiting innate and acquired immune cells (Akira et al., 2006; Takeuchi and Akira, 2010). IRF3 and IRF7 are the key regulators of type I IFN expression. Their activation in the cytoplasm occurs directly through C-terminal phosphorylation by IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKKε. C-terminal phosphorylation promotes IRF3 and IRF7 homodimerization and subsequent nuclear translocation, resulting in type I IFN gene expression (Takeuchi and Akira, 2009). Type I IFNs activate JAK/STAT pathways via a type I IFN receptor leading to production of interferon-stimulated genes (ISGs) (de Veer et al., 2001; Sadler and Williams, 2008). ISGs, such as IRFs, dsRNA-dependent protein kinase PKR, ADAR (adenosine deaminase, RNA-specific) and 2′5′-oligoadenylate synthase (OAS), mediate inhibition of viral replication and clearance of

Abbreviations: IKK, IκB kinase; PRRs, pattern-recognition receptors; IRF, interferon regulatory factor; RNAi, RNA interference; OAS, 2′5′-oligoadenylate synthase; ADAR, adenosine deaminase, RNA-specific; PRRs, pattern-recognition receptors; RLR, retinoic acid-inducible gene (RIG)-I-like receptor; NLR, nucleotide-binding oligomerization domain (NOD)-like receptor; PAMP, pathogen-associated molecular pattern; SSH, suppression subtractive hybridization; ssRNA, single-stranded RNA; TBK1, TANK-binding kinase 1; ISG, interferon stimulated gene; AMP, antimicrobial peptide genes; RISC, RNA-induced silencing complex; EST, expression sequence tag; WSSV, white spot syndrome virus; ORF, open reading frame; NLS, nuclear localization signal; Mx, Myxovirus resistance.

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virus-infected cells, and induction of non-specific antiviral responses (de Veer et al., 2001; Sadler and Williams, 2008). In invertebrates, the receptors for viral nucleic acids are still unknown and whether nucleic acids could induce an antiviral response is elusive. It has been generally accepted that nucleic acid-induced antiviral immunity of vertebrates is absent in insects because of the lacking of genes homologous to IFN system defined genes e.g., IFNs, IRFs, and PKR. Although insect JAK/STAT pathways still contribute to antiviral responses, RNA interference (RNAi), which represents a sequence-specific antiviral mechanism, is believed to be an essential antiviral pathway (Dostert et al., 2005; Kemp and Imler, 2009; Sabin et al., 2010; Souza-Neto et al., 2009). Like other invertebrates, shrimp possess RNAi, a sequence-specific antiviral mechanism (Chen et al., 2011; Robalino et al., 2007). Injection of siRNA can silence sequence-specific genes of white spot syndrome virus (WSSV) and shows anti-WSSV activity in shrimp (Xu et al., 2007; Wu et al., 2007). Intriguingly, injection of dsRNA and siRNA could induce sequence-independent protection against WSSV in shrimp (Robalino et al., 2004; Westenberg et al., 2005). Recently, the siRNA pathway is revealed to be involved in shrimp antiviral immunity by generating an antiviral siRNA (vp28-siRNA) in response to WSSV infection, which represents a novel mechanism of RNAi in immunity (Huang and Zhang, 2013). In this study, we found that nucleic acid mimics poly(I:C), poly(C:G), CL097, poly C and CpG-DNA could induce an antiviral immunity in shrimp, which is similar to the responses of vertebrate IFN system. Using the suppression subtractive hybridization (SSH) method, genes that contributed to poly(I:C)-induced antiviral immunity of shrimp were identified. We also found LvIKKε, whose vertebrate homologs are central regulators of the IFN-producing pathway, could activate human IFN-producing pathway. In NCBI crustacean database, many IFN system-defined genes, such as IRF, PKR, ADAR and other ISGs, were discovered. These results may reveal new paradigms of how eukaryotic cells resist viruses, especially poly(I:C)-induced antiviral immunity in crustaceans, and raise a question whether the nucleic acid-induced antiviral immunity of shrimp is an IFN-like system or a novel antiviral mechanism.

2. Materials and methods

2.1. Experimental shrimp

Litopenaeus vannamei (~8–10 g each) were obtained from Hengxing shrimp farm in Zhanjiang, Guangdong Province, China. The shrimp were cultured in an indoor tank with sand filtering aerated sea water at ~27 °C, fed a commercial diet at 5% of their body weight twice per day. The shrimp were cultured for at least seven days for acclimation before experiments.

2.2. Nucleic acid mimics injection and WSSV infection

A total of 250 shrimp (~8–10 g each) were divided into seven groups. Groups 1–5 were injected with 50 μl poly(I:C) (2 μg/g) diluted in PBS, 50 μl poly(C:G) (2 μg/g) diluted in PBS, 50 μl CL097 (2 μg/g) diluted in PBS, 50 μl poly C (2 μg/g) diluted in PBS and 50 μl ODN2006 (2 μg/g) diluted in PBS, respectively. Groups 6 and 7 were both injected with 50 μl PBS (Fig. S1). After 6 h, groups 1–6 were intramuscularly challenged with WSSV (10^4 copies/g, the minimum does that caused 100% mortality) at the third abdominal segment, and the untreated group 7 was used as a control (Fig. S1). Mortality was recorded daily, and water exchange and feeding regimes were as described above.

2.3. Construction of the suppression subtractive hybridization (SSH) library

According to the results of the nucleic acid injection experiments above, dsRNA mimic poly(I:C) was chosen as the best inducer in SSH construction. SSH was performed using a PCR-select cDNA subtraction kit (Clontech, USA) according to the manufacturer's protocol described in our previous study (Zhao et al., 2007). For the construction of the poly(I:C)-PBS SSH libraries, hepatopancreas mRNA from poly(I:C)-injected shrimp at 48 h was used as the tester, and hepatopancreas mRNA from PBS-injected shrimp at 48 h was used as the driver to construct the forward SSH library and vice-versa for the reverse SSH library. poly(I:C)-injected shrimp and PBS-injected shrimp were prepared as described in Section 2.2. At 48 h, hepatopancreas were collected for mRNA isolation using PolyATtract® mRNA Isolation Systems (Promega, USA). Likewise, for the construction of the poly(I:C)+WSSV-WSSV SSH libraries, hepatopancreas mRNA from poly(I:C)-injected shrimp at 48 h was used as a tester, and hepatopancreas mRNA from WSSV-injected shrimp at 48 h as the driver to construct the forward SSH library and vice-versa for the reverse SSH library. Shrimp (~8–10 g each) were injected with 50 μl poly(I:C) (2 μg/g) diluted in PBS or 50 μl PBS. At 6 h post-injection, these shrimp were intramuscularly challenged with WSSV (10^4 copies/g) at the third abdominal segment. At 48 h post-poly(I:C) injection, hepatopancreas were collected for mRNA isolation. The efficiency of the subtraction was evaluated by comparing the abundance of the constitutively expressed *β-actin* in the subtracted and unsubtracted populations using PCR with the following conditions: 94 °C for 2 min, 28 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and 72 °C for 10 min. The subtracted PCR products were cloned separately into the pGEM-T easy vector (Promega, USA) for sequencing. Real-time quantitative RT-PCR was performed to confirm the differential expressions of 10 genes selected from all the genes identified in the SSH libraries the same as described in our previous study (Wang et al, 2013b).

2.4. Plasmid construction and mutations

For protein expression in HEK293T cells, the complete ORFs of *LvIKKε* (GenBank Accession No. JN180644) and *Homo sapiens IKKε* (*HsIKKε*) were inserted into pCMV-C-MYC (Beyotime, China) to construct pCMV-LvIKKε and pCMV-HsIKKε, respectively. The point-mutations of pCMV-LvIKKε(K41A) and pCMV-HsIKKε(K38A) were prepared using pCMV-LvIKKε and pCMV-HsIKKε as templates by primer extension and fusion PCR as described in our previous study (Wang et al., 2009). The luciferase reporter vectors were constructed as described previously (Wang et al., 2009, 2012b). In brief, the gene promoter region of human IFNα and IFNβ (~1000 bp upstream of the transcription start sites) was inserted into pGL3-Basic luciferase reporter vectors (Promega, USA) at *Kpn* I and *Xho* I sites by digesting, purifying, ligating, and transforming into DH5α competent cells. After confirmed by sequencing, pGL3-IFNα and pGL3-IFNβ were successfully constructed. The pRL-TK luciferase reporter vector was chosen as an internal standard.

2.5. Cell culture, transfection and luciferase assays

HEK293T cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 incubator. In luciferase reporter assays, the expression plasmid, reporter gene plasmid, and pRL-TK *Renilla* luciferase plasmid were co-transfected into HEK293T cells seeded in a 96-well plate 24 h before transfection as described previously (Wang et al., 2013a). HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen, USA). Cells were harvested 36 h later and lysed for the examination of protein expression and

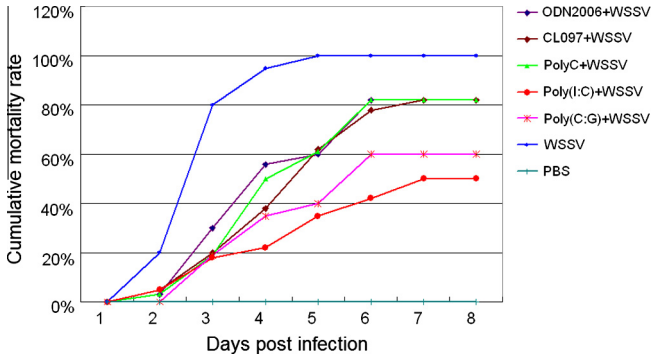

Fig. 1. Nucleic acid-induced antiviral immunity in shrimp. *L. vannamei* (~8–10 g each) were injected intramuscularly with nucleic acid mimics poly(I:C), poly(C:G), CL097, poly C and CpG-DNA. At 6 h after injection, *L. vannamei* (n = 30–35) were infected by intramuscular injection with WSSV. Mortality was recorded daily. The chi-square statistic was performed to assess the significance of the observed antiviral protection by comparing the mortality in nucleic acids infection groups and the positive control group: dsRNA mimics poly(I:C) (χ^2 = 17.953, p = 0.00002) and poly(C:G) (χ^2 = 12,856, p = 0.0003), ssRNA mimics CL097 (χ^2 = 6384, p = 0.012) and poly C (χ^2 = 4781, p = 0.029), and DNA mimics CpG-DNA ODN2006 (χ^2 = 4276, p = 0.039). Differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

luciferase activities using the dual luciferase reporter assay system (Promega, USA), as described previously (Wang et al., 2011a,b, 2012a).

2.6. Statistical analysis

The chi-square statistic was performed to assess the significance of the observed antiviral protection by comparing the mortalities in the nucleic acids injection groups and the control groups using SPSS 13.0. Student's *t*-test was used to compare means between two samples using Microsoft Excel. The data are presented as mean ± standard error (standard error of the mean, SEM). In all cases, differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results

3.1. Protection against WSSV by nucleic acids pre-injection

To investigate whether nucleic acids could induce antiviral immunity in shrimp, we performed nucleic acids-injection experiments. Injection of shrimp with nucleic acids, including dsRNA mimics poly(I:C) and poly(C:G), ssRNA mimics CL097 and poly C, and DNA mimics CpG-DNA ODN2006, afforded antiviral protection for these shrimps after they were challenged with WSSV ($p < 0.05$) (Fig. 1). We observed that dsRNA mimics protected shrimp from WSSV infection more effectively than ssRNA mimics and DNA mimics, reducing cumulative mortality to 50% and 60% for poly(I:C) and poly(C:G), respectively (Fig. 1). Injection of poly(I:C) was most effective in protecting shrimp from WSSV infection, suggesting that dsRNA can strongly induce an antiviral state in shrimp similar to vertebrates.

3.2. Suppression subtractive hybridization

SSH was performed to determine genes that are involved in poly(I:C)-induced antiviral immunity. The success of SHH was evaluated by comparing *β-actin* gene abundance in the subtracted and unsubtracted populations. The results showed that the *β-actin* transcript was dramatically decreased after subtraction compared with the unsubtracted sample (Fig. S2), implying that the SSHs were successful. The recombinant percentage of four SSH libraries was approximately 80–90%, as determined by calculating the ratio of white colonies to total colonies. Approximately 150 randomly selected positive white clones from each forward SSH library and 100 clones from each reverse SSH library were sequenced. After deleting vector sequences and poor-quality sequences, a total of 480 qualified ESTs from the four libraries were grouped into 317 consensus sequences (Fig. 2A). Differential expressions of 10 genes

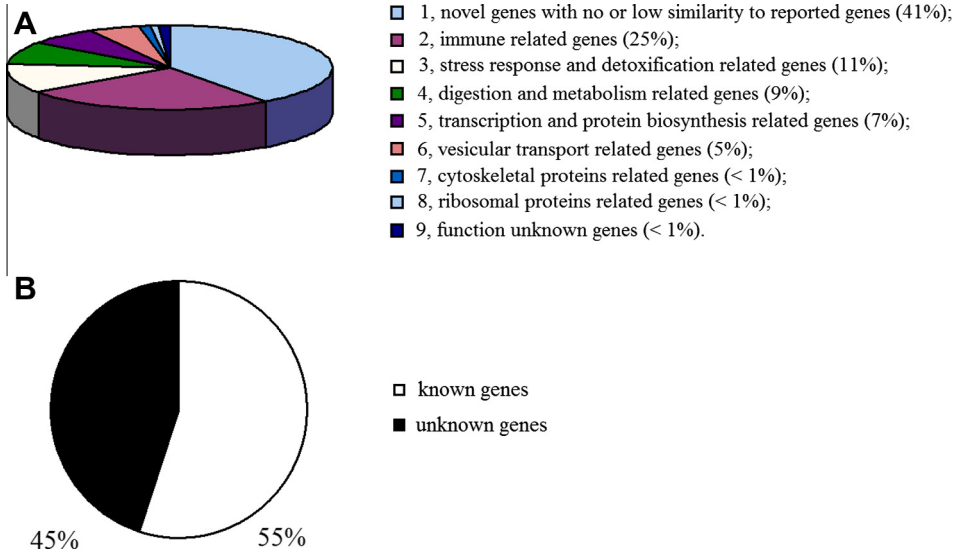

Fig. 2. Construction of SSH libraries and identificaiton of genes involved in poly(I:C)-induced antiviral immunity. According to the results of the nucleic acids injection experiments, dsRNA mimic poly (I:C) was chosen as the best inducer in SSH construction. (A) In SSH libraries, a total of 480 qualified ESTs from the four libraries were identified and grouped into 317 consensus sequences. The 317 genes involved in shrimp poly(I:C)-induced antiviral immunity were categorized according to the major functions of their encoded proteins. 1, novel genes with no or low similarity to reported genes (41%); 2, immune related genes (25%); 3, stress response and detoxification related genes (11%); 4, digestion and metabolism related genes (9%); 5, transcription and protein biosynthesis related genes (7%); 6, vesicular transport related genes (5%); 7, cytoskeletal proteins related genes (<1%); 8, ribosomal proteins related genes (<1%); 9, function unknown genes (<1%). (B) In SSH libraries, 55% of the 480 qualified ESTs showed significant homology (*e*-values <10^{-4}) to known proteins in the GenBank database, while approximately 45% of ESTs had no similarity to any known proteins.

Table 1
Putative functions of ESTs in the library subtracted between poly(I:C) and PBS.

Accession number	Genes and putative functions	Species with closed similarity	Library redundancyF R	Except value
Immunity and putative immunity				
emb|CAA57880.1|	hemocyanin	*Litopenaeus vannamei*	39	0.0E + 00
gb|AAL36892	crustin	*Litopenaeus vannamei*	1	2.00E-29
gb|AAZ22828.1|	lymphoid organ expressed yellow head virus receptor protein	*Penaeus monodon*	1	2.00E-39
gb|AAT94175.1|	cathepsin B	*Paralichthys olivaceus*	1	1.00E-46
gb|ABD65298.1|	destabilase I/lysozyme		3	2.00E-42
emb|CAM36311.1|	hypothetical protein	*Thermobia domestica*	2	6.00E-62
gb|ABS45569.1|	QM protein	*Marsupenaeus japonicus*	1	2.00E-26
emb|CAO98765.1|	double-stranded RNA activated protein kinase 2	*Xenopus tropicalis*	2	
gb|ABI97374.1|	C-type lectin	*Litopenaeus vannamei*	5	4.00E-19
gb|ABU92557.1|	lipopolysaccharide and beta-1,3-glucan binding protein	*Litopenaeus vannamei*	2	4.00E-62
gb|ABY70643.1|	chitinase precursor	*Litopenaeus vannamei*	4	4 5.00E-63
ref|XP_800780.2|	similar to tetraspanin family protein	*Strongylocentrotus purpuratus*	1	4.0E-28
Stress response and detoxification				
gb|ABR66910.1|	heat shock protein 90	*Metapenaeus ensis*	1	
gb|AAP21806.1|	cytochrome c oxidase subunit I	*Litopenaeus vannamei*	2	1.0E-88
ref|XP_001202234.1|	reductase, DHCR24	*Strongylocentrotus purpuratus*	1	1.00E-04
ref|XP_001651717.1|	subunit B14.5b	*Danio rerio*	1	1.00E-13
ref|YP_001315044.1|	NADH dehydrogenase subunit 1	*Litopenaeus vannamei*	1	4.00E-100
gb|ABS19964.1|	ubiquitin/ribosomal L40 fusion protein	*Artemia franciscana*	1	5.00E-15
ref|XP_001568232.1|	ubiquitin-conjugating enzyme E2, putative	*Leishmania braziliensis*	1	6.00E-24
gb|EAT46434.1|	proteasome subunit beta type	*Aedes aegypti*	1	1.0E-41
ref|XP_623784.1|	similar to Rpt4 CG3455-PA	*Apis mellifera*	1	1.00E-59
ref|XP_001652004.1|	sodium-dependent phosphate transporter	*Danio rerio*	1	4.00E-39
gb|ABD65301.1|	zinc proteinase Mpc1	*Litopenaeus vannamei*	12	6.0E-31
emb|CAC42504.1|	metallothionein	*Homarus americanus*	1	1.0E-03
ref|NP_001103586.1|	hypothetical protein LOC571365	*Danio rerio*	1	9.00E-36
ref|XP_974620.1|	CG14235-PA, isoform A	*Tribolium castaneum*	1	3.00E-19
Digestion and metabolism				
ref|XP_001622502.1|	predicted protein	*Nematostella vectensis*	14	3.00E-07
gb|AAY55575.1|	IP10822p	*Drosophila melanogaster*	2	2.00E-12
gb|AAH67599.1|	GTP binding protein 4	*Danio rerio*	1	6.00E-05
gb|ABI52804.1|	Mitochondrialassociated endoribonuclease MAR1-isochorismatase superfamily	*Argas monolakensis*	1	1.00E-33
ref|NP_957153.1|	solute carrier family 25	*Danio rerio*	1	3.00E-13
ref|XP_395289.3|	similar to Aldehyde dehydrogenase type III CG11140-PI,isoform I	*Apis mellifera*	1	4.00E-23
ref|XP_001662127.1|	sterol carrier protein-2, putative	*Aedes aegypti*	1	3.00E-56
ref|XP_784116.2|	PREDICTED: hypothetical protein	*Strongylocentrotus purpuratus*	1	6.00E-62
ref|XP_972926.1|	similar to hyaluronidase 1	*Tribolium castaneum*	1	1.00E-17
gb|ABE01157.2|	carboxylesterase	*Spodoptera litura*	1	8.00E-07
gb|ABD65300.1|	carboxypeptidase B	*Litopenaeus vannamei*	1	1.00E-46
ref|XP_969000.1|	similar to Glycine cleavage system H protein, mitochondrial precursor	*Tribolium castaneum*	1	1.00E-11
gb|AAN75002.1|	late trypsin	*Ochlerotatus triseriatus*	1	2.00E-18
Transcription and protein biosynthesis				
ref|NP_501804.1|	Seryl tRNA Synthetase family member	*Caenorhabditis elegans*	1	1.00E-38
ref|XP_001661064.1|	translation elongation factor g	*Danio rerio*	1	1.00E-09
ref|XP_001190200.1|	polyprotein	*Strongylocentrotus purpuratus*	21	2.0E-19
gb|ABU41071.1|	receptor for activated protein kinase C-like protein	*Lepeophtheirus salmonis*	1	6.00E-128
ref|NP_001037556.1|	elongation factor 1 beta	*Bombyx mori*	1	5.00E-06
Vesicular transport proteins				
ref|XP_975476.1|	similar to CG6056-PA	*Tribolium castaneum*	1	3.00E-50
ref|XP_001600708.1|	similar to ENSANGP00000021999	*Nasonia vitripennis*	1	3.00E-37
Cytoskeletal proteins				
gb|AAR82846.1|	actin E	*Litopenaeus vannamei*	1	4.00E-49
ref|XP_001499689.1|	similar to FKSG18	*Equus caballus*	1	1.00E-46
Ribosomal proteins				
gb|AAX62406.1|	ribosomal protein P2 isoform B	*Lysiphlebus testaceipes*	1	9.00E-12
emb|CAH04310.1|	acidic p0 ribosomal protein	*Dascillus cervinus*	2	4.0E-61
gb|ABI52692.1|	ribosomal protein LP1	*Argas monolakensis*	1	5.00E-21
ref|XP_001653874.1|	mitochondrial ribosomal protein, L36	*Aedes aegypti*	1	4.00E-19
gb|AAK92187	ribosomal protein S18	*Spodoptera frugiperda*	1	6.00E-65
gb|AAB46716.1|	40S ribosomal protein S27E	*Homarus americanus*	1	6.00E-10
gb|ABI52808.1|	40S ribosomal protein S23	*Argas monolakensis*	1	6.00E-75
ref|NP_476631.1|	Ribosomal protein L19 CG2746-PA, isoform A	*Drosophila melanogaster*	1	1.00E-36
ref|XP_532112.2|	similar to 40S ribosomal protein S10	*Canis familiaris*	1	1.00E-28
ref|NP_649887.1|	RpL34b CG9354-PA, isoform A	*Drosophila melanogaster*	1	2.00E-31

(*continued on next page*)

Table 1 (*continued*)

Accession number	Genes and putative functions	Species with closed similarity	Library redundancyF R	Except value
gb|ABV44714.1|	60S ribosomal protein L35A-like protein	*Phlebotomus papatasi*	1	8.00E-36
gb|ABW23163.1|	ribosomal protein rpl7a	*Arenicola marina*	1	4.00E-13
gb|AAN05591.1|	ribosomal protein L7	*Argopecten irradians*	1	1.00E-32

selected from a total of 317 genes were confirmed by real-time quantitative PCR the same as described in our previous study (Wang et al, 2013b). The results showed that genes from forward libraries are significantly up-regulated and genes from reverse libraries are significantly down-regulated (Fig. S3), confirming the efficiency of the SSH again. After searching for sequence homology in the NCBI GenBank database using the BLASTN and BLASTX programs, 55% of the 480 ESTs showed significant homology (*e*-values $<10^{-4}$) to known protein sequences in the GenBank database, while approximately 45% had no similarity to any known protein (Fig. 2B). Known genes of the 317 consensus sequences were categorized according to the major function of their encoded proteins (Tables 1 and 2). Genes involved in immune responses were most abundant, accounting for 25% of all ESTs (Fig. 2A). Some genes that may have roles in nucleic acid-induced antiviral immunity were discovered, such as the complete ORF of IFNγ-inducible protein 30 and partial sequences of the PKR and TLR3-like protein (Tables 1 and 2). Compared with poly(I:C)+WSSV-WSSV SSH libraries, dsRNA activated protein kinase 2, lymphoid organ expressed yellow head virus receptor protein, chitinase precursor, lipopolysaccharide and beta-1,3-glucan binding protein, and polyprotein were specifically found in poly(I:C)-PBS SSH libraries, suggesting that these genes may be specific to dsRNA (poly(I:C)) stimulation (Tables 1 and 2).

3.3. The activation of IFN promoter by LvIKKε

In mammals, IKKε is the central regulator of IFN-producing pathways. IKKε can phosphorylate IRF3 and IRF7 directly in the cytoplasm and promotes IRF3 and IRF7 homodimerization and subsequent nuclear translocation, resulting in type I IFN gene expression (Hacker and Karin, 2006; Takeuchi and Akira, 2009). In this study, we obtained the full-length cDNA of shrimp IKKε (LvIKKε, GenBank Accession No. JN180644) using degenerated primer-PCR approach as described in our previous studies (Wang et al., 2009, 2012a,b). When transfected into HEK293T cells, LvIKKε activated the IFNα and IFNβ reporters ~123.4 and ~24.0 folds, respectively, comparing with the control group (Fig. 3A). Activity of IFNα reporter activated by LvIKKε was lower than that activated by human IKKε (HsIKKε) (Fig. 3B). The inactive mutant form of HsIKKε (HsIKKε(K38A)) did not activate IFNα reporter (Fig. 3B), a result consistent with previous report (Sharma et al., 2003). After sequence alignment, we identified the K38 of HsIKKε is evolutionary conserved in shrimp (Fig. 3C). We generated a mutant form of LvIKKε (LvIKKε(K41A)) and found that, similar to HsIKKε(K38A), LvIKKε(K41A) did not activate IFNα reporter (Fig. 3B).

3.4. The discovery of IFN system-defined genes

Searching for IFN system-defined genes of crustaceans was performed using IFN system-defined genes as seeds. After comparison with the sequences in NCBI GenBank using the tBLAST program, more than 15 genes were hypothesized to be IFN system-relevant, including IRF, PKR, ADAR, OAS, PKR activator, PKR inhibitor, IFNα-inducible protein 27 (IFI27), IFNγ-inducible protein 30, IFI35, IFI44, IFN-inducible and antiviral protein viperin, IFN-inducible protein Gig2, IFN regulatory factor 2-binding protein 1, IFN-related developmental regulator 1, and IFN-induced guanylate-binding protein 1 (Table S1). We did not find similar sequences in the crustacean EST database for the other two important components of the IFN system, Myxovirus resistance (Mx) and RNASE L. However, several Mx sequences have been reported in disk abalone, and RNASE L has also not been found even in fish genomes based on sequence comparison (De Zoysa et al., 2007). Some homologs involved in the IFN-producing pathways (TLR and RLR pathways) and IFN response signaling (JAK/STAT) were also found in the database (data not shown). Two ESTs similar to mouse TLR3 were also found to be induced by dsRNA mimic poly(I:C) using SSH. Recently, it has been reported that *Drosophila* Dicer proteins and mammalian RLRs belong to the same family of helicases that are essential in sensing viral infection in multicellular organisms (Deddouche et al., 2008). RIG-I-like helicases are absent from insect genomes, instead they have Dicer-2. It has been proposed that Dicer-2 is the functional equivalent of mammalian RLRs (Deddouche et al., 2008). Shrimp RIG-I-like helicases Dicer-1 (GenBank Accession No. ACF96960) and Dicer-2 (GenBank Accession No. HQ541163) have been found and they may be candidates for dsRNA recognition (Chen et al., 2011). Homologous components of the RLR pathways, such as Eya4, Traf3, MITA/STING, IKKε/TBK1, and IRF3, have also been found in crustaceans. Although we did not find IPS-1 homolog in the crustacean EST database, several IPS-1 like genes have been found in the ancient invertebrate *Nematostella vectensis*. These results suggest that RLR-mediated signal pathways may exist in invertebrates, especially in crustaceans. Therefore, the main components of both IFN-producing and IFN-responsive pathways have been identified in crustacean EST databases. See Fig. 4.

4. Discussion

In vertebrates, after recognition of viral nucleic acids by TLRs and RLRs, innate immunity is activated to trigger the release of inflammatory cytokines and type I IFNs through activation of NF-κB and IRF3/7 (Akira et al., 2006; Kumar et al., 2009). The IFN system is the most prominent non-specific innate antiviral response in vertebrates (Sadler and Williams, 2008). Until now, IFNs have only been found in vertebrates, and the nucleic acid-induced antiviral immunity had been believed to be a privilege of the vertebrate immune system (Krause and Pestka, 2005; Robalino et al., 2004, 2007). In invertebrates, RNAi mediated sequence-specific antiviral responses is believed to be a replacement of the vertebrate IFN system (Robalino et al., 2004; Sabin et al., 2010). Here, we investigate whether shrimp innate immune system can be provoked by nucleic acids and its potential mechanism.

Nucleic acids injection experiments indicated that poly(I:C), poly(C:G), CL097, poly C and CpG-DNA all could protect shrimp from WSSV infections (Fig. 1). Injection of dsRNA mimics reduced cumulative mortality to 50% and 60% for poly(I:C) and poly(C:G), respectively (Fig. 1) ($p < 0.0001$). ssRNA mimics and DNA mimics could also induce antiviral responses in shrimp, reducing cumulative mortality to ~82% for CL097, poly C and CpG-DNA (Fig. 1) ($p < 0.05$). So shrimp probably have receptors for dsRNA, ssRNA and DNA recognitions and possess nucleic acid induced-antiviral immunity like vertebrates. The mechanisms of nucleic acid induced-antiviral immunity are of great interests, especially dsRNA

Table 2
Putative functions of ESTs in the library subtracted between poly(I:C)+WSSV and WSSV.

Accession number	Genes and putative functions	Species with closed similarity	Library redundancy	Except value
Immunity and putative immunity				
emb|CAA57880.1|	hemocyanin	*Litopenaeus vannamei*	13	0.0E+00
gb|DQ858900.1|	clone c002 C-type lectin 1-like	*Litopenaeus vannamei*	6	0.00E+00
gb|ABI97374.1|	C-type lectin	*Litopenaeus vannamei*	1	8.00E-07
gb|ABD65298.1|	destabilase I/lysozyme	*Litopenaeus vannamei*	6	2.00E-42
gb|AAZ39947.1|	chitinase	*Aedes aegypti*	2	5.00E-06
ref|XP_533981.2|	cathepsin C	*Canis familiari*	1	1.0E-12
gb|AAH87708.1|	Legumain	*Rattus norvegicus*	1	3.00E-57
ref|NP_001040347.1|	salivary secreted ribonuclease	*Bombyx mori*	1	4.00E-19
ref|NP_956482.1|	hypothetical protein LOC393157	*Danio rerio*	1	2.00E-12
gb|AAY56093.1|	death-associated protein-like	*Penaeus monodon*	1	3.00E-51
ref|XP_001177412.1|	similar to interferon gamma-inducible protein 30	*Strongylocentrotus purpuratus*	2	8.00E-15
gb|AAO92933.1|	High density lipoprotein/1,3-beta-D-glucan-bindig protein precursor	*Litopenaeus vannamei*	1	5.00E-137
gb|EDL35548.1|	toll-like receptor 3, isoform CRA_a	*Mus musculus*	2	2e-04
gb|DQ858899.1|	clone c001 C-type lectin 2-like mRNA	*Litopenaeus vannamei*	16	0.0E+00
gb|DQ871243.1|	C-type lectin mRNA	*Penaeus semisulcatus*	2	8.00E-31
gb|AAQ75589.1|	PMAV Penaeus monodon	*purpuratus*	1	2.00E-17
gb|ABP73289.1|	anti-LPS factor isoform 3	*Penaeus monodon*	1	7.00E-08
gb|ABD65299.1|	ENSANGP00000021035-like	*Litopenaeus vannamei*	1	6.00E-13
gb|ABC87809.1|	leucine-rich repeat protein	*Penaeus monodon*	1	9.00E-91
emb|CAM36311.1|	hypothetical protein	*Thermobia domestica*	1	6.00E-62
Stress reponse and detoxification				
gb|ABD65301.1|	zinc proteinase Mpc1	*Litopenaeus vannamei*	6	1.0E-16
dbj|BAB13775.1|	oxygenase	*Oplophorus gracilorostris*	2	8.00E-15
ref|XP_001183266.1|	similar to apolipoprotein D; apoD	*Strongylocentrotus purpuratus*	1	2.00E-20
ref|XP_320183.2|	AGAP012374-PA	*Anopheles gambiae*	1	9.00E-17
gb|AAT76663.2|	glutathione S-transferase	*Litopenaeus vannamei*	1	2.00E-41
ref|XP_001636741.1|	predicted protein	*Nematostella ectensis*	1	2.00E-04
gb|ABC59528.1|	cytosolic manganese superoxide dismutase	*Litopenaeus vannamei*	1	3.00E-12
ref|YP_001315034.1|	cytochrome c oxidase subunit II	*Aedes aegypti*	1	4.00E-21
ref|XP_968146.1|	similar to CG14028-PA	*Tribolium castaneum*	1	1.00E-06
ref|XP_001607066.1|	similar to ubiquitin-activating enzyme E1	*Nasonia vitripennis*	2	6.00E-92
gb|ABM74399.1|	ubiquitin	*Portunus pelagicus*	1	4.00E-12
ref|NP_001040456.1|	NADH-ubiquinone oxidoreductase	*Litopenaeus vannamei*	1	6.00E-32
ref|XP_001674422.1|	Hypothetical protein CBG19031	*Caenorhabditis briggsae*	1	4.00E-22
ref|YP_001315037.1|	cytochrome c oxidase subunit III	*Litopenaeus vannamei*	1	8.00E-127
gb|AAQ93009.1|	cytochrome P450 CYP330A1	*Carcinus maenas*	1	6.00E-39
ref|XP_001608297.1|	similar to prefoldin subunit	*Nasonia vitripennis*	1	1.00E-1
ref|XP_798646.2|	similar to testis-enriched protein tyrosine phosphatase	*Strongylocentrotus purpuratus*	1	2.0E-38
gb|AAL68262.1|	RE09301p	*Drosophila melanogaster*	1	1.0E-14
Digestion and metabolism				
ref|XP_001648219.1|	cyclohex-1-ene-1-carboxyl-CoA hydratase, putative	*Aedes aegypti*	1	5.00E-68
ref|NP_998296.1|	hypothetical protein LOC406405	*Danio rerio*	1	2.00E-14
ref|NP_001018532.1|	asparagine-linked glycosylation 3 homolog	*Danio rerio*	1	5.00E-42
ref|XP_001662127.1|	sterol carrier protein-2	*Aedes aegypti*	1	3.00E-56
gb|ABU41107.1|	S-adenosylhomocysteine hydrolase	*Lepeophtheirus salmonis*	1	3.00E-81
gb|ABB76924.1|	beta-N-acetylglucoasminidase 1	*Spodoptera frugiperda*	1	8.00E-15
gb|EAX08769.1|	esterase D	*Homo sapiens*	1	8.00E-85
ref|XP_001633389.1|	predicted protein	*Nematostella vectensis*	1	9.00E-15
ref|XP_001660815.1|	succinate dehydrogenase	*Aedes aegypti*	1	3.00E-50
gb|ABE01157.2|	carboxylesterase	*Spodoptera litura*	1	8.00E-07
gb|ABI52804.1|	mitochondrial associated endoribonuclease MAR1-isochorismatase superfamily 1	*Argas monolakensis* 1.00E-33		
gb|AAN75002.1|	late trypsin triseriatus	*Ochlerotatus triseriatus*	1	2.00E-18

(*continued on next page*)

Table 2 (*continued*)

Accession number	Genes and putative functions	Species with closed similarity	Library redundancy	Except value
ref|NP_001006854.1|	high density lipoprotein binding protein (vigilin)	*Xenopus tropicalis*	1	4.00E-65
ref|YP_238259.1|	ATP synthase F0 subunit 6	*Marsupenaeus japonicus*	1	2.0E-71
Transcription and protein biosythesis				
gb|AAF26416	20S proteasome beta5 subunit	*Drosophila melanogaster*	1	4.0E-61
ref|XP_001663131.1|	26S protease regulatory subunit	*Aedes aegypti*	1	4.00E-21
dbj|BAF63671.1|	protein disulfide isomerase-2	*Haemaphysalis longicornis*	2	9.00E-56
gb|ABN04118.1|	ATP/ADP translocase	*Marsupenaeus japonicus*	1	1.00E-04
gb|AAB46716.1|	ref|XP_001190200.1| polyprotein	*Strongylocentrotus purpuratus*	1	2.0E-19
ref|XP_313678.3|	AGAP004394-PA	*Anopheles gambiae*	1	3.00E-19
ref|XP_001607323.1|	similar to putative beta-NAC-like protein	*Spodoptera frugiperda*	1	8.00E-15
ref|NP_001037094.1|	kiser	*Bombyx mori*	1	4.00E-32
ref|XP_396057.3|	similar to poly A binding protein, cytoplasmic 1 isoform 1	*Apis mellifera*	1	4.00E-131
Vesicular transport proteins				
ref|XP_966498.1|	similar to Vesicle-associated membrane protein-associated protein B/C (VAMP-associated protein B/C)	*Tribolium castaneum*	1	8.00E-42
ref|XP_623495.1|	similar to Vacuolar ATP synthase catalytic subunit A, osteoclast isoform (V-ATPase subunit A 2)	*Apis mellifera*	1	4.00E-53
gb|AAF08281	vacuolar ATP synthase subunit B	*Carcinus maenas*	1	2.00E-24
Cytoskeletal proteins				
gb|AAG16253	beta-actin	*Litopenaeus vannamei*	1	6.00E-76
Ribosomal proteins				
emb|CAJ17232.1|	ribosomal protein L6e	*Carabus granulatus*	1	2.00E-18
ref|NP_001037263.1|	ribosomal protein S8	*Bombyx mori*	1	2.00E-32
emb|CAF89492.1|	unnamed protein product	*Tetraodon nigroviridis*	1	3.00E-58
gb|AAR10084.1|	similar to Drosophila melanogaster CG12775	*Drosophila yakuba*	1	2.00E-14
ref|NP_001013473.1|	hypothetical protein LOC541327	*Danio rerio*	1	2.00E-43
ref|NP_001037269.1|	ribosomal protein S18	*Bombyx mori*	1	8.00E-65
ref|XP_971759.1|	PREDICTED: similar to CG7726-PA	*Tribolium castaneum*	1	2.00E-18
4gb|ABI52808.1|	40S ribosomal protein S23	*Argas monolakensis*	1	6.00E-75
Others				
ref|XP_001600606.1|	similar to ENSANGP00000020083	*Nasonia vitripennis*	1	8.00E-07
ref|XP_001086604.1|	similar to CG9119-PA isoform 1	*Macaca mulatta*	1	3.00E-12
ref|XP_001624581.1|	predicted protein	*Nematostella vectensis*	1	2.00E-04
ref|XP_969512.1|	similar to CG33170-PA	*Tribolium castaneum*	1	4.00E-05
gb|AAL49119.1|	RE55745p	*Drosophila melanogaster*	1	7.00E-04
ref|XP_001655215.1|	conserved hypothetical protein	*Aedes aegypti*	1	5.00E-05

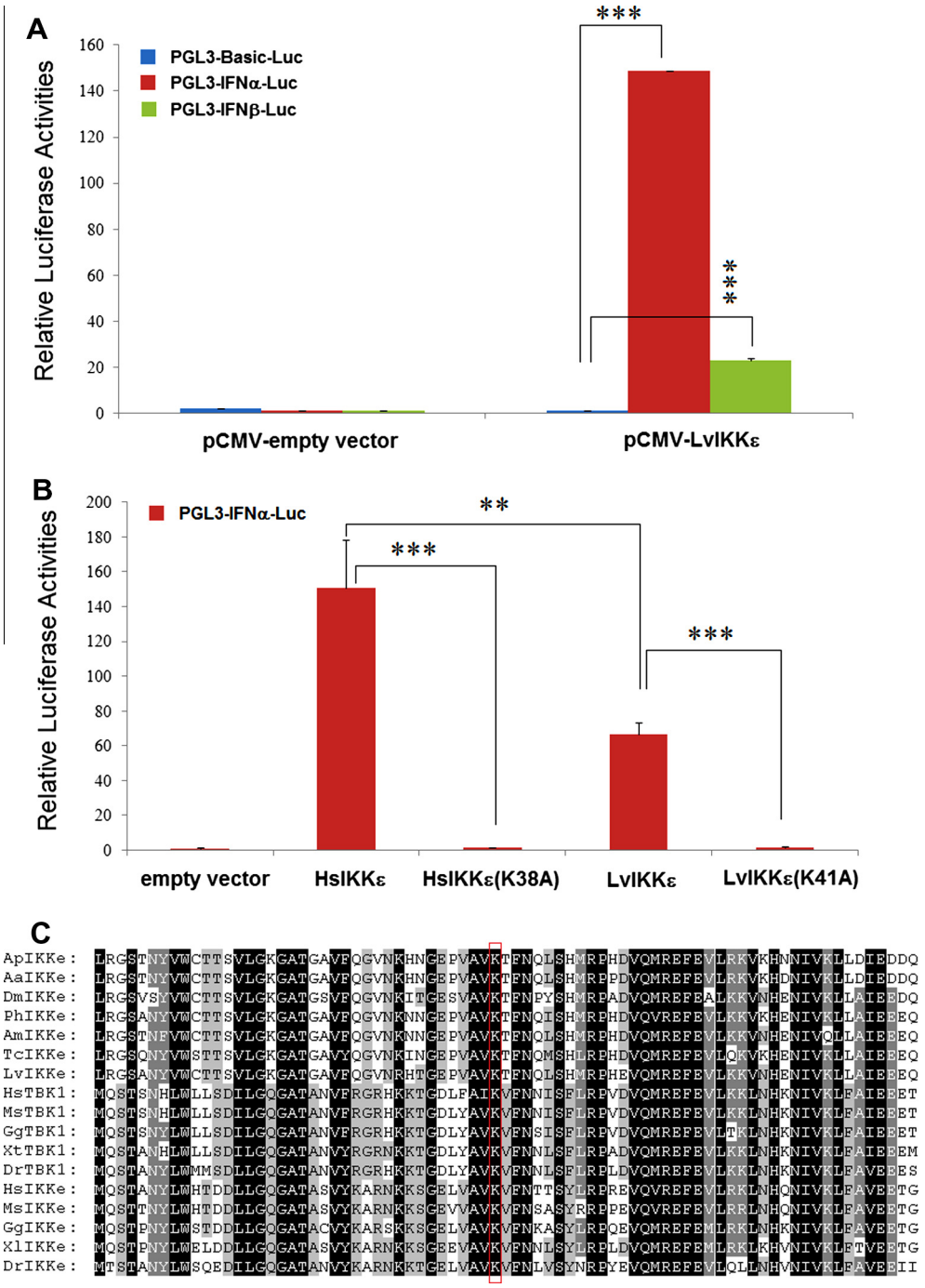

Fig. 3. LvIKKε activated the IFN-producing pathway in HEK293T cells. (A) HEK293T cells were co-transfected with the pCMV empty vector (70 ng) or pCMV-LvIKKε (70 ng) together with the IFNα luciferase reporter vector (30 ng) or IFNβ luciferase reporter vector (30 ng) and the control *Renilla* expression vector (pRL-TK, 3 ng). pCMV empty vector and pGL3-Basic were used as negative controls. (B) The potential inactive mutant form of LvIKKε (LvIKKε(K41A)) was constructed and co-transfected with IFNα luciferase reporter. Like the inactive form of HsIKKε (HsIKKε(K38A)), LvIKKε(K41A) did not activate IFNα reporter. (C) Multiple sequence alignment of N-terminus of IKK-related kinases IKKε and TANK-binding kinase 1 in typical specials. K38 of HsIKKε, which is essential for IKKε in IFN-producing pathway, is evolutionary conserved in shrimp. All data are representative of three independent experiments. $^{**}p < 0.01$.

induced antiviral immunity which is strongest and shows similarity to the vertebrate IFN system.

SSH was performed to identify shrimp genes involved in poly(I:C)-induced antiviral immunity. Approximately 45% of ESTs had no similarity to any known protein (Fig. 2B), suggesting an unknown antiviral program in shrimp. We also identified several genes that have been reported to participate in antiviral responses, including hemocyanin, lymphoid organ-expressed yellow head virus receptor protein, C-type lectin, and anti-lipopolysaccharide factor 3 (Tables 1 and 2) (Assavalapsakul et al., 2006; Liu et al., 2006; Tharntada et al., 2009; Zhang et al., 2004; Zhao et al., 2009). Several IFN system-related genes such as PKR, IFNγ-inducible protein 30 and two TLR3-like ESTs were also identified to be involved in the poly(I:C)-induced antiviral immunity. After IFN secretion, IFNγ-inducible protein 30 can be highly expressed through the JAK/STAT pathway in vertebrates, and the same regulatory mechanism of shrimp IFNγ-inducible protein 30 by the JAK/STAT pathway has been proposed in shrimp (Kongton et al., 2011).

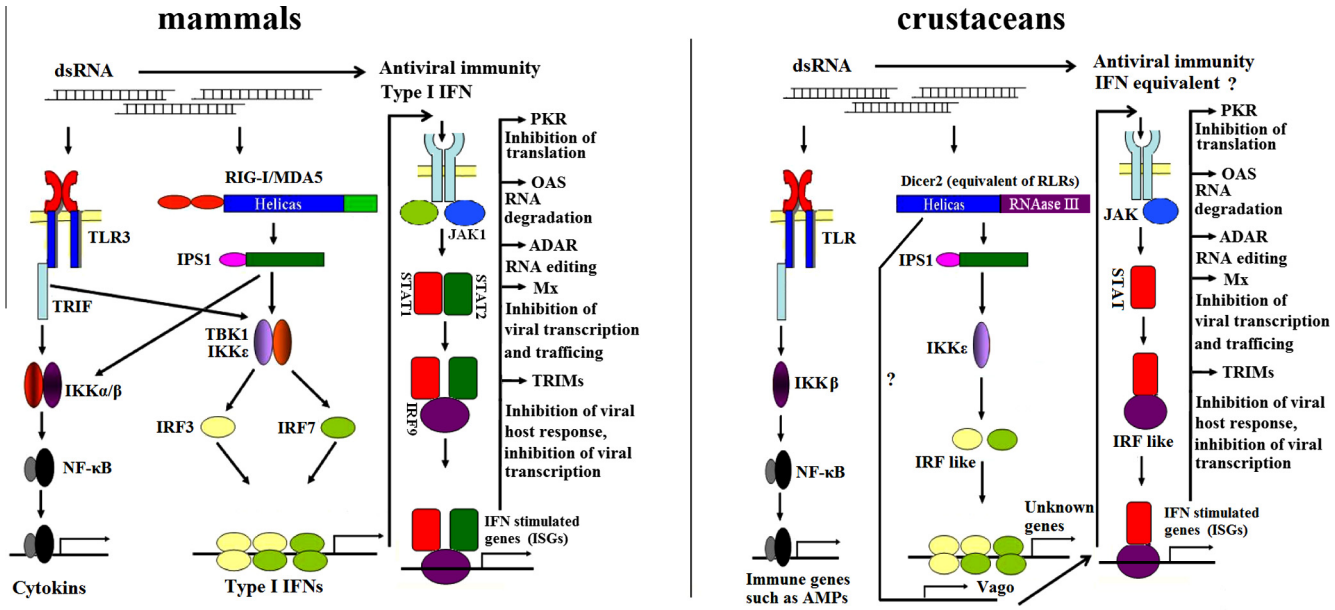

Fig. 4. A comparison of IFN-producing and -responsive pathway genes between mammals (left) and crustaceans (right). The homologous or functionally equivalent genes of mammalian IFN-producing and -responsive pathways were identified in crustaceans, suggesting that crustaceans probably possess an IFN-like system. In mammals, dsRNAs are recognized by endosomal TLR3 and cytosolic RIG-I/MDA5 to trigger the IFN-producing pathway (Kawai et al., 2009). In this study, we found that dsRNA could be recognized in shrimp to induce nonspecific antiviral responses. We identified the homologous or functionally equivalent genes of mammalian IFN-producing and-responsive pathways in crustacean and hypothesize that these genes may contribute to the crustacean nonspecific antiviral immune responses.

In mammals, TLR3 recognizes dsRNA directly and induces IFN expression (Takeuchi and Akira, 2010). In invertebrates, the function and mechanisms of TLR signaling have only been studied in insects, and it is believed that insect TLRs possess antiviral functions (Lemaitre and Hoffmann, 2007; Ramirez and Dimopoulos, 2010; Sabin et al., 2010; Takeuchi and Akira, 2010; Zambon et al., 2005). Further experimental studies are required to better understand the roles of these dsRNA-responsive genes, such as PKR, IFNγ-inducible protein 30 and TLR3-like protein, in shrimp nucleic acid-induced antiviral immunity.

In mammals, IKKε is the central regulator of IFN-producing pathways. In this study, we found that LvIKKε could also activate IFN-producing pathways in HEK293T cells by activating the promoter activities of IFNα and IFNβ to ∼123.4 and ∼24.0 folds, respectively (Fig. 3A). Shrimp NF-κB and STAT signal pathways have been reported to participate in host immune responses to viral infections (Chen et al., 2008; Huang et al., 2010; Liu et al., 2007). In addition to an IRF-like gene found in crustaceans (Table 1), we propose that the three essential immune signal pathways (NF-κB, JAK/STAT and IRFs) might be evolutionarily conserved from some invertebrates to vertebrates (Chen et al., 2008; Dostert et al., 2005; Ghosh et al., 1998; Nehyba et al., 2009; Souza-Neto et al., 2009).

It has been generally accepted that the IFN system is absent from invertebrates because of the lack of genes homologous to IFNs or the major effectors of the IFN response in several fully sequenced insect genomes (Robalino et al., 2004, 2007; Rosa and Barracco, 2008). Until now, shrimp genomes have not been completely sequenced. To explore whether IFN system-defined genes exist in crustaceans, we performed homology searches in crustacean EST databases using vertebrate IFN system-defined genes. We found many genes that showed significant similarity to vertebrate IRF, PKR, OAS, ADAR and other ISGs. In addition, the virally induced *Drosophila* gene CG1667 encodes an ortholog of MITA/STING, which is an essential component of the IFN-producing pathway in mammals (Kemp and Imler, 2009; Takeuchi and Akira, 2009). We also found an ortholog of MITA/STING in crustaceans (GenBank Accession No. FE369720.1). Moreover, the shrimp JAK/STAT pathway has been reported to be activated after WSSV infection (Kemp and Imler, 2009; Takeuchi and Akira, 2009). The Mx protein is one of the most studied antiviral proteins. In this study, we did not find the shrimp *Mx* gene. However, in the invertebrate mollusk abalone, the *Mx* gene has been reported to be present and up-regulated after poly(I:C) challenge in gill and digestive tissues (De Zoysa et al., 2007). OAS has been identified in an invertebrate group (sponges), and it can also be up-regulated in response to poly(I:C) challenge (Schroder et al., 2008). We also found ESTs similar to OAS in shrimp (Table S1). Many IFN system-defined genes, including components of IFN-producing and IFN-responsive pathways, have been found in a crustacean EST database, except *IFNs*. However, even in vertebrates, *IFNs* from different species show low sequence homology; for example, zebrafish IFNα and human IFNα only share 20.4% identity. Using sequence comparison, we only identified IFN-like genes in species that evolved after cartilaginous fish (Fig. S4). Therefore, although invertebrate *IFNs* exist, it is difficult to identify them based on sequence comparison. However, the absence of homologous *IFN* genes does not exclude the existence of invertebrate immune systems analogous to the vertebrate IFN system. It is possible that invertebrates utilize some genes as the functional equivalent of IFNs, similar to the mechanisms of RNAi in budding yeast (Drinnenberg et al., 2009). In *Drosophila*, dsRNA is detected by Dicer2 to trigger an unidentified signal that leads to the inducible expression of the gene *Vago*, which controls the viral load in the fat body (Deddouche et al., 2008). A recently study indicates that secreted Vago can restrict West Nile virus infection in Culex mosquito cells by activating the JAK-STAT pathway (Paradkar et al., 2012). And they further demonstrate that Vago functions as an IFN-like antiviral cytokine in mosquitoes (Paradkar et al., 2012). This antiviral Dicer2-Vago pathway also exists in crustaceans (Chen et al., 2011). And we also found that LvVago promoter could be activated by LvIKKε (Fig. S5). It is possible that Vago may play a role as a cytokine-like *IFN* in nucleic acid-induced antiviral immunity of shrimp. Our results indicate that shrimp possess nucleic acid-induced antiviral immunity similar

to the responses of vertebrate IFN-antiviral system. Our future studies will investigate whether this nucleic acid-induced antiviral immunity of shrimp is an IFN-like system or a novel antiviral mechanism of lower invertebrates.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.05.016.

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