**Characterization of Four Novel Caspases from *Litopenaeus vannamei* (Lvcaspase2-5) and Their Role in WSSV Infection through dsRNA-Mediated Gene Silencing**

Pei-Hui Wang1*, Ding-Hui Wan1, Yong-Gui Chen2, Shao-Ping Weng1, Xiao-Qiang Yu3, Jian-Guo He1,2*

1 MOE Key Laboratory of Aquatic Product Safety/State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-Sen University, Guangzhou, People’s Republic of China, 2 School of Marine Sciences, Sun Yat-Sen University, Guangzhou, People’s Republic of China, 3 Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, Missouri, United States of America

**Abstract**

Apoptosis plays an important role in white spot syndrome virus (WSSV) pathogenesis, and caspases are central players in apoptosis. Here, we cloned four novel caspases (Lvcaspase2-5) from the Pacific white shrimp *Litopenaeus vannamei*, and investigated their potential roles in WSSV replication using dsRNA-mediated gene silencing. Lvcaspase2-5 have the typical domain structure of caspase family proteins, with the conserved consensus motifs p20 and p10. Lvcaspase2 and Lvcaspase5 were highly expressed in muscle, while Lvcaspase3 was highly expressed in hemocytes and Lvcaspase4 was mainly expressed in intestine. Lvcaspase2-5 could also be upregulated by WSSV infection, and they showed different patterns in various tissues. When overexpressed in *Drosophila* S2 cells, Lvcaspase2-5 showed different cellular localizations. Using dsRNA-mediated gene silencing, the expression of Lvcaspase2, Lvcaspase3, and Lvcaspase5 were effectively knocked down. In Lvcaspase2-, Lvcaspase3-, or Lvcaspase5-silenced *L. vannamei*, expression of WSSV VP28 gene was significantly enhanced, suggesting protective roles for Lvcaspase2, Lvcaspase3 and Lvcaspase5 in the host defense against WSSV infection.

**Introduction**

Apoptosis plays a protective role in eliminating harmful cells and in the host response to viral infections [1,2]. When virus-infected cells undergo apoptosis, the viruses already replicated in these cells are unable to diffuse and infect other cells [1,2]. Viruses have developed distinct strategies to escape or retard apoptosis triggered by various apoptotic pathways [1-3]. For instance, viruses can block apoptosis to prevent premature death of a host cell, thereby maximizing the viral progeny from a lytic infection or facilitating a persistent infection; in contrast, viruses can also actively promote apoptosis to spread viral progeny to neighboring cells [1-3]. Viruses may perform both pro- and anti-apoptotic functions to facilitate different stages of infection.

Interference with apoptosis by inhibiting the proteolytic activity of cysteine aspartic acid proteases (caspases) prolongs the life of virus-infected cells, resulting in enhanced viral replication and viral persistence [4]. Caspases are a family of structurally related cysteine proteases, and they play a central role in apoptosis. Caspases contain three main domains, namely a prodomain, a large (p20, 20 kDa) catalytic subunit, and a small (p10, 10 kDa) catalytic subunit [5-7]. Based on their roles in apoptosis, the caspase family proteins are divided into two subgroups, initiator caspases and effector caspases [2,6]. The initiator caspases have a long prodomain (> 90 amino acids) containing specific protein-protein interaction motifs that are necessary for their activation, whereas the effector caspases usually have a short prodomain of only 20-30 residues [8]. Initiator caspases such as caspases 2, 8, 9, and 10 can be activated by autocatalysis in response to apoptotic...
Materials and Methods

2.1: Microorganisms and experimental shrimp

Gram-negative *Vibrio alginolyticus* and WSSV inocula were prepared as described previously [29-31]. Pacific white shrimp, *L. vannamei* (~8-10 g each for gene expression analysis; ~1-2 g each for dsRNA-mediated gene silencing), were purchased from a shrimp farm in Zhuhai, Guangdong Province, China. The shrimp were cultured in a recirculating water tank system filled with air-pumped seawater (2.5% salinity) at 24-26°C and were fed a commercial diet at 5% of their body weight twice daily. The shrimp were cultured for at least seven days to acclimate before beginning experiments.

2.2: Rapid amplification of cDNA ends

Total RNA (0.5 μg) was isolated from shrimp gills using an RNeasy Mini Kit (Qiagen, Germany) and reverse transcribed into cDNA using a SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) for cloning the 5' and 3' cDNA ends of genes. Based on the expression sequence tag (EST) of *L. vannamei* in the NCBI database, the full-length cDNA sequences of *Lvcaspase2-5* were obtained using a RACE-PCR approach as described previously [29-32]. All conditions were as described except for the primer sequences (listed in Table 1).

2.3: Bioinformatic analysis

Using the NCBI database, nucleotide blast searches were conducted to retrieve potential caspase-like ESTs. Multiple sequence alignments were performed using the ClustalX 2.0 program (http://www.ebi.ac.uk/tools/clustalw2). The simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de) was used to analyze the domain structure of *Lvcaspase2-5*. Neighbor Joining (NJ) phylogenetic trees were constructed using MEGA 4.0 software (http://www.megasoftware.net/index.html) based on protein sequences of caspase family proteins in typical species. Bootstrap sampling was reiterated 1,000 times.

2.4: Sample preparation and real-time quantitative PCR

For tissue distribution studies, the hemocyte, eyestalk, gill, heart, hepatopancreas, stomach, intestine, nerve, muscle, pyloric cecum, and epithelium samples were collected from healthy *L. vannamei* to extract total RNA for first-strand cDNA preparation. For immune challenges, healthy *L. vannamei* were injected intramuscularly at the third abdominal segment with 2.4×10⁶ V. *alginolyticus* or 100 μl of WSSV inoculum (approximately 10² copies/shrimp). PBS-injected shrimp were used as controls. At 0, 3, 6, 12, 24, 36, 48 and 72 hours post-injection (hpi), five shrimp from each group were randomly selected for the gill, hemocyte, hepatopancreas, intestine, and muscle sample collection. Shrimp total RNA isolation and preparation of cDNA templates for PCR were conducted as previously described [29-32]. Five-fold dilutions of cDNA templates were prepared, and 1 μl was used to detect the expression of *Lvcaspase2-5* in healthy and immune-challenged shrimp using the Master SYBR Green I system and a LightCycler (Roche) with the following program: 1 cycle of 95°C for 30 s and 40 cycles of 95°C for 5 s, 57°C for 20 s, and 78°C for 1 s. Three qPCR replicates were performed per sample, and three shrimp were analyzed for each sample. The expression of *L. vannamei* elongation factor 1α (*LvEF-1α*) was used as an internal control. Standard curves for *Lvcaspase2-5* and *LvEF-1α* were generated by running triplicate reactions of a 10-fold dilution series (10 different cDNA concentrations). The primer amplification efficiencies for *Lvcaspase2*, *Lvcaspase3*, *Lvcaspase4*, *Lvcaspase5* and *LvEF-1α* were 1.943, 1.958, 2.019, 1.851 and 1.953, respectively. The relative standard curve method was used for calculation of the fold changes in gene expression [33-35].
### Table 1. PCR primers used in this study.

| Primer Location   | Primer sequence (5’-3’)                                              |
|-------------------|-----------------------------------------------------------------------|
| cDNA cloning      |                                                                        |
| 5’ Lvcasp2-RACE1  | TTGGAAATCCCGAGTTAGCTGAAG                                              |
| 5’ Lvcasp2-RACE2  | ACCGTTGACAGTTTCTCCATT                                                 |
| 3’ Lvcasp2-RACE1  | TCTTTCAACCCTTCGGTCTT                                                  |
| 3’ Lvcasp2-RACE2  | TGGCTACCAGGCCTTACAGATT                                                |
| 5’ Lvcasp3-RACE1  | CACCCCGACCCTTCGGTCTT                                                  |
| 5’ Lvcasp3-RACE2  | CACCATGAGCAGTATGGAAGC                                                |
| 3’ Lvcasp3-RACE1  | ACCGACCTCATCCAACTCACC                                                 |
| 3’ Lvcasp3-RACE2  | ACCGAAAGAGGTTTCGTCACCAC                                               |
| 5’ Lvcasp4-RACE1  | GGTGGCTTCCTGCGTCTT                                                   |
| 5’ Lvcasp4-RACE2  | GGAAGGAGAGGTTTCGTCACCAC                                               |
| 3’ Lvcasp4-RACE1  | TTCTCCCTGTAATGGGAGATT                                                |
| 3’ Lvcasp4-RACE2  | TTATACAGGGAGGTTTCGACCAG                                              |
| qPCR analysis     |                                                                        |
| qPCR-Lvcasp2-F    | ATGGCTCTGGTGAATTCATTCAAG                                              |
| qPCR-Lvcasp2-R    | CATCAGGGTGGAGACAATAACAG                                               |
| qPCR-Lvcasp3-F    | AGTTATGACAAACACAGATTGGAGG                                             |
| qPCR-Lvcasp3-R    | TCTTGGACAGACAGTATGGAAGC                                              |
| qPCR-Lvcasp4-F    | CATGCTCTGACTACCCAGTGAT                                                |
| qPCR-Lvcasp4-R    | TGTCCTCCAAATCTGTGCTT                                                 |
| dsRNA preparation*|                                                                        |
| dsGFP-F           | AGTGCTTCCAGCGCTCAGCC                                              |
| dsGFP-R           | GCCGTTCTGCTGGGGTC                                                   |
| dsGFP(T7)-F       | TAAATACGACTCACTATAGAGGAGTGCTTCCAGCCGCTACCC                             |
| dsGFP(T7)-R       | TAAATACGACTCACTATAGAGGAGTGCTTCCAGCCGCTACCC                             |
| dslvcasp2-F       | ACCATGAGCGCTGATTGTC                                                  |
| dslvcasp2-R       | AGTCACCGCGTGGTGGGAAT                                                |
| dslvcasp3(T7)-F   | TAAATACGACTCACTATAGAGGAGTGCTTCCAGCCGCTACCC                             |
| dslvcasp3(T7)-R   | TAAATACGACTCACTATAGAGGAGTGCTTCCAGCCGCTACCC                             |
| dslvcasp3-F       | GCCCTGCTTCACTATAGGCG                                                 |
| dslvcasp3-R       | ACCATGAGCGCTGATTGTC                                                  |
| dslvcasp5(T7)-F   | TAAATACGACTCACTATAGAGGAGTGCTTCCAGCCGCTACCC                             |
| dslvcasp5(T7)-R   | TAAATACGACTCACTATAGAGGAGTGCTTCCAGCCGCTACCC                             |
| Cellular localization |                                                              |
| pA5.1Lvcasp2-F   | CGGGGTAACCATGAGGAAACTGTCACACG                                       |
| pA5.1Lvcasp2-R   | GCTCTGAAATACCTGCTTGGCGTAAGTACCCCTT                                  |
| pA5.1Lvcasp3-F   | AAGAGAAAAGCAGGCGCCGCAGCCACATGAGACATCANAAT-CAGGC                      |
| pA5.1Lvcasp3-R   | GCTCTGAGCCCTCCTGCTTCC                                                  |
| pA5.1Lvcasp4-F   | CGGAATTCGCCGCCACCATGTTGAGAGGAAACAGTCC                                |
| pA5.1Lvcasp4-R   | GCTCTGAGTTCCACCCGCCGCCGCCG                                           |
| pA5.1Lvcasp5-F   | CGGGGTAACCATGAGGAAACTGTCACACG                                       |
| pA5.1Lvcasp5-R   | GCTCTGAGTTCCACCCGCCGCCGCCG                                           |
2.5: Plasmid construction

The pAc5.1-N-GFP vector constructed in our previous study expressed sufficient green fluorescent protein (GFP) in Drosophila S2 cells [20,30,31,36]. For cellular localization of Lvcaspase2-5, PCR products containing the complete open reading frames (ORFs) of Lvcaspase2-5 were inserted into pAc5.1-N-GFP using standard molecular cloning methods to construct the expression vectors pAc5.1-Lvcaspase2-5-GFP.

2.6: Cell culture

Drosophila S2 cells were maintained at 28°C without CO₂ in Schneider’s Drosophila medium (SDM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA). When the culture density reached approximately 6-20 × 10⁶ viable cells ml⁻¹, the Drosophila S2 cells were passaged onto a new plate at a density of approximately 5 × 10⁶ viable cells ml⁻¹.

2.7: Confocal microscopy analysis

Drosophila S2 cells were seeded onto poly-L-lysine-coated cover slips in 24-well plates. Approximately 24 hours later, cells were transfected with pAC5.1-Lvcaspase2-5-GFP. At 36 hours post-transfection, the cells on the cover slips were washed twice with PBS, fixed using Immunol Staining Fix Solution (Beyotime, China) and stained with Hoechst 33258 Solution (Beyotime, China). The cells on the cover slips were observed using a Leica laser scanning confocal microscope as previously described [30,31,36].

2.8: dsRNA preparation and dsRNA mediated gene silencing in vivo

Double-stranded RNA (dsRNA) sequences corresponding to Lvcaspase2-5 and GFP (dsLvcaspase2, dsLvcaspase3, dsLvcaspase4, dsLvcaspase5, and dsGFP, respectively) were prepared using the T7 RibomAX Express Kit (Promega, USA) as previously described [37]. In dsRNA-mediated gene silencing experiments, the experimental group (1.2 g per shrimp) was injected with dsLvcaspase2, dsLvcaspase3, dsLvcaspase4 or dsLvcaspase5 (1 µg/g shrimp) by intramuscular injection, while the control groups were injected with dsGFP or PBS. To evaluate silencing, gill samples from at least 3 shrimp in each treatment group were collected at 0, 24, 72, 120 and 144 hours post-dsRNA injection (hpi) for total RNA extraction. The first-strand cDNA prepared from the gill total RNA was used to detect gene silencing efficiency using qPCR as described in Section 2.5.

2.9: WSSV infection experiments in dsRNA-injected L. vannamei

The gene silencing efficiency of Lvcaspase2, Lvcaspase3, and Lvcaspase5 was significant compared with the control groups (> 80%) at all the examined time points. In the WSSV infection experiments, L. vannamei were infected intramuscularly with 100 µl WSSV inoculum (approximately 10⁷ copies/shrimp) at 48 hours post dsRNA injection (hpi), and gill samples were collected at 0, 3, 6, 12, 24, 36 and 48 hours post WSSV infection for detection of WSSV VP28 expression.

2.10: Statistical analysis

Student’s t-test was used to compare means between pairs of samples using Microsoft Excel. In all cases, differences were considered significant at p < 0.05 and highly significant at p < 0.01. The data are presented as the means ± standard error (standard error of the mean, SEM).

Results

3.1: Cloning and sequence analysis of four novel caspases from L. vannamei

Based on the ESTs of L. vannamei in the NCBI database, the full-length cDNA sequences of four novel caspases were identified and named Lvcaspase2, Lvcaspase3, Lvcaspase4 and Lvcaspase5 after the reported Lvcaspase1 (called Penaeus vannamei cas-3 in the original report). The Lvcaspase2 cDNA was 1,490 bp and contained a 924-bp ORF encoding a putative 307-amino acid protein, a 5′ untranslated region of 96 bp, and a 3′ untranslated region of 470 bp (Figure S1A). The Lvcaspase3 cDNA was 2,083 bp and contained a 1,482-bp ORF encoding a putative 494-amino acid protein, a 5′ untranslated region of 47 bp, and a 3′ untranslated region of 545 bp (Figure S1D). The Lvcaspase4 cDNA was 1,634 bp and contained a 1,176-bp ORF encoding a putative 496-amino acid protein, a 5′ untranslated region of 59 bp, and a 3′ untranslated region of 399 bp (Figure S1B). The Lvcaspase5 cDNA was 1,161 bp and contained an 873-bp ORF encoding a putative 290-amino acid protein, a 5′ untranslated region of 246 bp, and a 3′ untranslated region of 42 bp (Figure S1C). Based on the sequence identities and domain structures, we identified Lvcaspase2 and Lvcaspase5 as effector caspases, while Lvcaspase3 and Lvcaspase4 were initiator caspases (Figure S2).

3.2: Phylogenetic tree construction

Phylogenetic analysis of caspase family proteins showed that Lvcaspase1 (Penaeus vannamei cas-3), Pmcaspase1 (PmCasp) and Fmcaspase1 clustered in a group (Figure S3). Lvcaspase2, Pmcaspase2 (Pm caspase) and Lvcaspase5 clustered in another group; Lvcaspase3, Mjcaspase3 (PjCasp) and DmNedd2 clustered in a third group; and Lvcaspase4 and Dmdream clustered in a group (Figure S3). These results also revealed that Lvcaspase4 is a completely novel type of shrimp caspase.
3.3: Tissue distribution of Lvcaspase2-5

In healthy shrimp, when normalized to the mRNA expression level in the eyestalk (1.00-fold), Lvcaspase2 was expressed at a higher level in epithelium (1.15-fold), hepatopancreas (1.81-fold increase), nerve (2.03-fold), gill (2.06-fold), pyloric cecum (4.09-fold), heart (4.17-fold), hemocytes (4.17-fold), stomach (7.24-fold), intestine (8.21-fold), and muscle (18.81-fold) (Figure 1A); Lvcaspase3 was highly expressed in eyestalk (1.53-fold), epithelium (1.79-fold), intestine (1.86-fold), pyloric cecum (2.48-fold), nerve (3.51-fold), muscle (4.18-fold), gill (6.39-fold), hepatopancreas (8.40-fold), heart (20.53-fold), and hemocytes (41.92-fold) when normalized to the mRNA expression level in the stomach (1.00-fold) (Figure 1B); Lvcaspase4 was highly expressed in gill (3.43-fold), epithelium (4.41-fold), nerve (8.82-fold), eyestalk (9.94-fold), heart (14.86-fold), pyloric cecum (237.18-fold), muscle (366.40-fold), hepatopancreas (654.03-fold increase), stomach (706.68-fold), and intestine (2843.09-fold) when normalized to the mRNA expression level in hemocytes (1.00-fold) (Figure 1C); and Lvcaspase5 was highly expressed in hemocytes (1.88-fold), stomach (1.90-fold), epithelium (2.03-fold), intestine (2.06-fold), eyestalk (2.92-fold), gill (3.90-fold), nerve (4.41-fold), pyloric cecum (5.55-fold), heart (8.51-fold), and muscle (32.51-fold) when normalized to the mRNA expression level in the hepatopancreas (1.00-fold) (Figure 1D).

3.4: Expression profiles of Lvcaspase2-5 after WSSV challenges

After WSSV infection, Lvcaspase2 expression in the gill and hemocytes were increased compared with the PBS injection group, but no significant changes in Lvcaspase2 transcript level occurred in the hepatopancreas or intestine (Figure 2). Lvcaspase3 was upregulated in the gill, hemocytes, hepatopancreas and intestine after WSSV infection (Figure 3). Lvcaspase4 was upregulated in the hemocytes but downregulated in the intestine after WSSV infection (Figure 4). Lvcaspase5 was upregulated in the gill and hemocytes after WSSV infection (Figure 5). In the muscle, Lvcaspase2, Lvcaspase3, and Lvcaspase5 were all upregulated after WSSV infection (Figure 6).

3.5: Subcellular localization of Lvcaspase2-5 in Drosophila S2 cells

The subcellular localization of Lvcaspase2-5 proteins may provide clues about their functions or positions in the caspase cascades. Fluorescent imaging of Lvcaspase2-GFP in Drosophila S2 cells showed that Lvcaspase2 was localized to the cytoplasm as speck-like aggregates near the membrane, while Lvcaspase3-5-GFP proteins localized in distinct patterns to the nucleus and cytoplasm of Drosophila S2 cells (Figure 7).

3.6: In vivo knock-down of Lvcaspase2-5 by dsRNA-mediated gene silencing

Using dsRNA-mediated gene silencing, we successfully suppressed the expression of Lvcaspase2, Lvcaspase3 and Lvcaspase5, but not Lvcaspase4, in the gill, as previously described [37]. Using qPCR, we observed that Lvcaspase2, Lvcaspase3, and Lvcaspase5 transcript levels were significantly reduced, while Lvcaspase4 transcript level was not affected. These results suggest that Lvcaspase2, Lvcaspase3, and Lvcaspase5 may play important roles in the immune response against WSSV infection in shrimp.
Figure 2. Temporal expression patterns of Lvcaspase2 in the gill (A), hepatopancreas (B), hemocyte (C) and intestine (D) after PBS, WSSV and V. alginolyticus injection. Healthy L. vannamei were injected intramuscularly at the third abdominal segment with PBS, V. alginolyticus or WSSV inocula. Gill, hemocyte, hepatopancreas, and intestine samples were collected at the indicated time points. The expression levels of Lvcaspase2 in the tissues of immune-challenged shrimp were determined by qPCR analysis. The expression of Lvcaspase2 in the untreated shrimp (0 hpi) was set as 1.0. The mRNA expression levels of Lvcaspase2 were normalized to those of LvEF-1α using the relative standard curve method. qPCR was performed on three replicates per sample. Data are expressed as the means ± S.E. (n =3).

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Figure 3. Temporal expression patterns of Lvcaspase3 in the gill (A), hepatopancreas (B), hemocyte (C) and intestine (D) after PBS, WSSV and V. alginolyticus injection. Healthy L. vannamei were injected intramuscularly at the third abdominal segment with PBS, V. alginolyticus or WSSV inocula. Gill, hemocyte, hepatopancreas, and intestine samples were collected at the indicated time points. The expression levels of Lvcaspase3 in the tissues of immune-challenged shrimp were determined by qPCR analysis. The expression of Lvcaspase3 in the untreated shrimp (0 hpi) was set as 1.0. The mRNA expression levels of Lvcaspase3 were normalized to those of LvEF-1α using the relative standard curve method. qPCR was performed on three replicates per sample. Data are expressed as the means ± S.E. (n =3).

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Healthy *L. vannamei* were injected intramuscularly at the third abdominal segment with PBS, *V. alginolyticus* or WSSV inocula. Gill, hemocyte, hepatopancreas, and intestine samples were collected at the indicated time points. The expression levels of *Lvcaspase4* in the tissues of immune-challenged shrimp were determined by qPCR analysis. The expression of *Lvcaspase4* in the untreated shrimp (0 hpi) was set as 1.0. The mRNA expression levels of *Lvcaspase4* were normalized to those of *LvEF-1α* using the relative standard curve method. qPCR was performed on three replicates per sample. Data are expressed as the means ± S.E. (n =3).

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Healthy *L. vannamei* were injected intramuscularly at the third abdominal segment with PBS, *V. alginolyticus* or WSSV inocula. Gill, hemocyte, hepatopancreas, and intestine samples were collected at the indicated time points. The expression levels of *Lvcaspase5* in the tissues of immune-challenged shrimp were determined by qPCR analysis. The expression of *Lvcaspase5* in the untreated shrimp (0 hpi) was set as 1.0. The mRNA expression levels of *Lvcaspase5* were normalized to those of *LvEF-1α* using the relative standard curve method. qPCR was performed on three replicates per sample. Data are expressed as the means ± S.E. (n =3).

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**Lvcaspase3** and **Lvcaspase5** transcripts in the gill were significantly reduced at 24, 72, 120 and 144 hpi compared with the dsGFP control group (Figure 8).

Figure 6. **Temporal expression patterns of **Lvcaspase2** (A), Lvcaspase3 (B) and Lvcaspase5 (C) in the muscle after PBS and WSSV injection.** Healthy *L. vannamei* were injected intramuscularly at the third abdominal segment with 100 µL of PBS (control group) or 100 µL of WSSV inoculum (10⁷ copies). At 0, 3, 6, 12, 24, 36, 48, and 72 hours post-injection (hpi), five shrimp from each group were randomly selected to take muscle samples for qPCR analysis. The expression levels in untreated shrimp (0 hpi) were set as 1.0. The mRNA expression levels of **Lvcaspase2**, **Lvcaspase3**, and **Lvcaspase5** were normalized to those of **LvEF-1α** using the relative standard curve method. qPCR was performed on three replicates per sample. Data are expressed as the means ± S.E. (n =3).

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*Lvcaspase3* and *Lvcaspase5* transcripts in the gill were significantly reduced at 24, 72, 120 and 144 hpi compared with the dsGFP control group (Figure 8).
Figure 7. Determination of the subcellular localization patterns of Lvcaspase2 (A), Lvcaspase3 (B), Lvcaspase4 (C) and Lvcaspase5 (D) using confocal microscopy. *Drosophila* S2 cells were transfected with pAC5.1-Lvcaspase2-5-GFP. At 36 hours post-transfection, cells on the cover slips were washed twice with PBS, fixed using Immunol Staining Fix Solution (Beyotime, China) and stained with Hoechst 33258 Solution (Beyotime, China). The cells were observed using a Leica laser scanning confocal microscope as previously described [30,31,36].

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3.7: Knock-down of Lvcaspase2, Lvcaspase3, and Lvcaspase5 increases WSSV replication

To further evaluate the role of Lvcaspase2, Lvcaspase3 and Lvcaspase5 in the shrimp defense against WSSV infection, we performed WSSV infection experiments in dsRNA-injected L. vannamei. When L. vannamei were infected with WSSV 48 hours after dsRNA injection, we found that at 48 hours post-infection (hpi), but not at 24 or 36 hpi, the expression of WSSV VP28 in the gill from the dsLvcaspase2-injection group was dramatically higher than in the dsGFP- and PBS-injection groups (Figure 9A). At 36 and 48 hpi (but not 24 hpi), the expression of WSSV VP28 in the gill from the dsLvcaspase3-injection group was dramatically higher than in the dsGFP- and PBS-injection groups (Figure 9B). At 24, 36 and 48 hpi, the expression of WSSV VP28 in the gill from the dsLvcaspase5-injection group was dramatically higher than in the dsGFP- and PBS-injection groups (Figure 9C). We also noticed that at 24 hpi, the expression of VP28 was very low in the PBS-, dsGFP-, dsLvcaspase2- and dsLvcaspase3-injection groups but was very high in the dsLvcaspase5-injection group (Figure 9). This result suggests that silencing Lvcaspase5 might accelerate WSSV infection. Collectively, these data suggest that Lvcaspase2, Lvcaspase3 and Lvcaspase5 are all involved in the host defense against WSSV infection but have different roles.

Discussion

There are two distinct apoptotic pathways in mammals: the extrinsic pathway (or death receptor pathway) and the intrinsic pathway (or mitochondria/cytochrome c pathway) [6,38,39]. In the extrinsic pathway, binding of the death ligand to a death receptor such as TNFα-TNFR leads to death receptor-FADD-procaspase-8 complex formation, thereby resulting in the cleavage and activation of caspase-8 [6,11,40]. The downstream effector caspase-3 is then activated, ultimately resulting in cell death [6,7,11,40]. Intracellular signals such as DNA damage, oxidative stress and viral infection can activate the intrinsic pathway [6,11,40]. All these signals converge on the mitochondria, which then release cytochrome c into the cytoplasm [6,11,39]. The cytochrome c binds to Apaf-1 and forms the apoptosome, which can interact with and activate procaspase-9 [6,7,11,39]. The activated caspase-9 initiates the caspase cascade, allowing the downstream effector caspases to execute the destruction of the cell [6,7,11,39]. The extrinsic and intrinsic pathways converge at the point of activating the effector caspases [6,11,39]. Thus, caspases are central regulators of apoptosis.

In Caenorhabditis elegans, however, the mammalian extrinsic pathway seems not to exist, as this species lacks essential components of this pathway [11]. Although Drosophila encodes homologs of a mammalian death ligand and receptor (called Eiger and Wengen in Drosophila, respectively), the receptor Wengen lacks the death domain to transduce death signaling, suggesting that Drosophila may not have functional extrinsic apoptosis pathway [11,41,42]. In invertebrates, which lack adaptive immunity, programmed cell death (i.e., apoptosis) functions as an important immune response against pathogen infection [43]. In our previous studies, we cloned the TNF superfamily (LvTNFSF) gene and the TNFR superfamily (LvTNFRSF) gene from L. vannamei, and we found that LvTNFRSF, like Drosophila Wengen, lacks the death domain to transduce death signaling [30]. Therefore,
Shrimp may rely mainly on the intrinsic pathway for apoptosis-mediated immune responses. Five caspase genes have been reported in penaeid shrimp that are extremely sensitive to WSSV: Penaeus merguiensis cap-3, Penaeus vannamei cas-3 (called Lvcaspase1 in this study), PjCaspase from P. japonicas (called Mjcaspase3 in this study), PmCasp and Pm caspase from P. monodon (called Pmcaspase1 and Pmcaspase2, respectively, in this study) [11]. These five shrimp caspases fall into 3 different types: caspase-1 type, caspase-2 type, and caspase-3 type (Figure S3). To further investigate function of the caspase family proteins in the host defense against WSSV infection, we cloned four novel caspases from L. vannamei in this study. Lvcaspase2-5 show the typical domain structure of caspase family proteins, with the conserved consensus motifs p20 and p10 (Figure S2). Like Pmcaspase1 (PmCasp) and Mjcaspase3 (PjCaspase), expression of Lvcaspase2-5 mRNA can be induced by WSSV infection but show distinct patterns. Lvcaspase2 mRNA is induced in the gill and in hemocytes (Figure 2); Lvcaspase3 mRNA is induced in all the tissues detected in our study including the gill, hepatopancreas, hemocytes, intestine and muscle (3 and 9); Lvcaspase4 mRNA was mainly induced in the hepatopancreas and hemocytes (Figure 4); and Lvcaspase5 mRNA was induced in the gill, hepatopancreas, hemocytes and muscle (Figures 5 and 9). The different expression patterns observed after WSSV infection may suggest that Lvcaspase2-5 play different roles in host defense.

Although shrimp caspases have the signature p20 and p10 domains of the caspase family proteins, their sequence identities with mammalian caspases are not high enough for sequence-based classification into existing caspase classes. In this study, we named shrimp caspases based on their reported orders. According to our analysis, Lvcaspase1 (Penaeus vannamei cas-3), Pmcaspase1 (PmCasp) and Pmcaspase2 (Pm caspase) are effector caspases, and Mjcaspase3 (PjCaspase) is an initiator caspase (Figure S2). The domain structures of Lvcaspase2-5 indicated that Lvcaspase2 and Lvcaspase5 are effector caspases, while Lvcaspase3 and Lvcaspase4 are initiator caspases (Figure S2).

Although five shrimp caspases have been reported, until now their cellular localization has remained unknown. Using confocal microscopy, we found that Lvcaspase2-GFP appeared as speck-like aggregates in the cytoplasm near the membranes of Drosophila S2 cells, while Lvcaspase3-5-GFP localized with distinct patterns to the nucleus and cytoplasm of Drosophila S2 cells (Figure 7). The different cellular localization patterns of GFP-tagged Lvcaspase2-5 may suggest different roles or positions in the caspase cascade.

Silencing Mjcaspase3 (PjCaspase) resulted in increased WSSV virus copy number, indicating a requirement of Mjcaspase3 in apoptotic responses against viral infection [22]. Recently, the same group also found that the sequence diversification of Mjcaspase3 could generate a specifically antiviral defense against WSSV infection [43]. Pmcaspase2 (Pm caspase) from P. monodon can induce apoptosis in SF9 insect cells, and the apoptotic activity can be blocked by AAP-1 (ORF390 or WSSV449) [19]. Further studies confirmed that AAP-1 (ORF390 or WSSV449) can directly bind to and be cleaved by Pmcaspase2 (Pm caspase), thereby inhibiting Pmcaspase2 (Pm caspase) activity [18]. Lvcaspase2 shows high similarity to Pmcaspase2 (Figure S3). To further investigate its function in WSSV infection, we suppressed the expression of Lvcaspase2 using dsRNA-mediated gene silencing. We found that at 24 and 36 hpi, the expression of...
WSSV VP28 in dsLvcaspase2-injected shrimp showed no obvious difference from the dsGFP-injected shrimp, but at 48 hpi, WSSV VP28 expression in the dsLvcaspase2-injected shrimp was dramatically higher than in the dsGFP- and PBS-injected shrimp (Figure 9A). These results suggested that Lvcaspase2 may be required for defending against WSSV infection. In future studies, we will investigate the effect of WSSV449 on the activities of these four novel caspases and will test whether they interact with each other. Lvcaspase3 is a homolog of Mjcaspase3 (PjCaspase). When Lvcaspase3 was silenced, VP28 expression was significantly higher than in the dsGFP control group at 36 and 48 hpi, in accord with results from Mjcaspase3 (Figure 9B) [22]. Lvcaspase4-5 are novel types of shrimp caspases. Unfortunately, we were unable to knock down the expression of Lvcaspase4 using dsRNA-mediated gene silencing. When we suppressed the expression of Lvcaspase5, VP28 expression was dramatically higher than in the dsGFP control group at 24, 36 and 48 hpi (Figure 9C).

We also noticed that Lvcaspase5 was the only caspase when knocked down could cause higher expression of VP28 at the early infection stage of 24 hpi, suggesting a different role or position in the caspase cascade from Lvcaspase2-3 (Figure 9). Pmcaspase2 (Pm caspase) has been targeted by small-molecule drugs to improve the apoptotic activity of shrimp hemocytes and thereby inhibit WSSV infection [11,44]. In future studies, we will investigate the detailed functions of Lvcaspase2-5 at different stages of WSSV infection. Development of drugs targeting caspases and manipulating shrimp apoptosis may provide novel strategies for the prevention and control of WSSV infections.

Supporting Information

Figure S1. Nucleotide and deduced amino acid sequences of Lvcaspase2 (A), Lvcaspase3 (D), Lvcaspase4 (B) and Lvcaspase5 (C) from L. vannamei. The nucleotide (upper row) and deduced amino acid (lower row) sequences of Lvcaspase2-5 are shown. The initiation codon (ATG) and stop codon (TAA, TGA or TAG) are shown in bold. The caspase family p20 and p10 domains in Lvcaspase2-5 are shaded. (TIF)

Figure S2. Domain architectures of shrimp caspases. The full-length protein sequences of shrimp caspases were subjected to the simple modular architecture research tool (SMART; http://smart.embl-heidelberg.de) to generate domain structures. The p20 and p10 domain are indicated as elliptical boxes, and the prodomain upstream of the p20 domain is indicated as a line. The initiator caspases have a long prodomain (> 90 amino acids) containing specific protein-protein interaction motifs that are necessary for their activation, whereas the effector caspases usually have a short prodomain of only 20-30 residues [8]. (TIF)

Figure S3. A phylogenetic tree of Lvcaspase2-5 with other caspase family proteins. The full-length amino acid sequences of caspase family proteins from typical organisms were aligned using the ClustalX2.0 program (http://www.ebi.ac.uk/tools/cluster2). The rooted tree was then constructed by the “neighbor-joining” method and was bootstrapped 1,000 times using MEGA 4.0 software (http://www.megasoftware.net/index.html). The numbers at the nodes indicate bootstrap values. Lvcaspase2-5 are boxed in blue lines. Lvcasp1, L. vannamei caspase1 (Accession no. ABK88280); Lvcasp2, L. vannamei caspase2 (Accession no. KC661002); Lvcasp3, L. vannamei caspase3 (Accession no. KC661013); Lvcasp4, L. vannamei caspase4 (Accession no. KC661005); Lvcasp5, L. vannamei caspase5 (Accession no. KC661004); Pmcasp1, Penaeus monodon caspase1 (Accession no. AEW91437); Mjcasp3, Marsupenaeus japonicus caspase3 (Accession no. ABK62771); Pmcasp2, Penaeus monodon caspase2 (Accession no. ABO38430); Hscasp1, Homo sapiens caspase1 (Accession no. NP_001214); Mmcasp1, Mus musculus caspase1 (Accession no. NP_033937); Hscasp2, H. sapiens caspase2 (Accession no. AAH02427); Mmcasp2, M. musculus caspase2 (Accession no. NP_031636); Hscasp3, H. sapiens caspase3 (Accession no. NP_116786); Mmcasp3, M. musculus caspase3 (Accession no. NP_033940); Hscasp4, H. sapiens caspase4 (Accession no. NP_001216); Hscasp5, H. sapiens caspase5 (Accession no. NP_001129584); Hscasp6, H. sapiens caspase6 (Accession no. NP_001217); Hscasp7, H. sapiens caspase7 (Accession no. NP_001253987); Mmcasp7, M. musculus caspase7 (Accession no. NP_031637); Hscasp8, H. sapiens caspase8 (Accession no. NP_001073594); Hscasp9, H. sapiens caspase9 (Accession no. NP_127463); Hscasp10, H. sapiens caspase10 (Accession no. AAD28403); Hscasp14, H. sapiens caspase14 (Accession no. NP_036246); Dmloc, Drosophila melanogaster Ice (Accession no. NP_524551); Dmcasp1, D. melanogaster caspase1 (Accession no. AAB58237); Dmdream, D. melanogaster dream (Accession no. NP_610193); Dmddeath, D. melanogaster death executioner caspase (Accession no. NP_477462); DmNedd2, D. melanogaster Nedd2 (Accession no. NP_524017); Drcasp1, D. rerio caspase1 (Accession no. NP_571580); Drcasp2, D. rerio caspase2 (Accession no. NP_001036160); Drcasp3, D. rerio caspase3 (Accession no. NP_571952); Drcasp6, Danio rerio caspase6 (Accession no. NP_001018333); Drcasp7, D. rerio caspase7 (Accession no. NP_001018443); Drcasp7-2, D. rerio caspase7 like (Accession no. XP_002667104); Drcasp8, D. rerio caspase8 (Accession no. NP_571585); Drcasp9, D. rerio caspase9 (Accession no. NP_001007405); Ggcasp1, Gallus gallus caspase1 (Accession no. NP_003642432); Ggcasp2, G. gallus caspase2 (Accession no. NP_003642432); Ggcasp3, G. gallus caspase3 (Accession no. NP_003642432); Ggcasp4, G. gallus caspase4 (Accession no. NP_003642432); Ggcasp5, G. gallus caspase5 (Accession no. NP_003642432); Ggcasp6, G. gallus caspase6 (Accession no. NP_003642432); Ggcasp7, G. gallus caspase7 (Accession no. XP_421764); Ggcasp8, G. gallus caspase8 (Accession no. NP_989923); Ggcasp9, G. gallus caspase9 (Accession no. XP_424580); Ggcasp10, G. gallus caspase10 (Accession no. XP_421936); Ggcasp18, G. gallus caspase18 (Accession no. NP_001308154); Xlcasp1, X. laevis caspase1 (Accession no. NP_001081223); Xlcasp2, X. laevis caspase2 (Accession no. NP_001081223); Xlcasp3, Xenopus laevis caspase3 (Accession no. NP_001081225); Xlcasp7, X. laevis caspase7 (Accession no. NP_001081408); Xlcasp6, X. laevis caspase6
Shrimp Caspases Defend against WSSV Infection

(TIF)

Author Contributions

Conceived and designed the experiments: PHW JGH.
Performed the experiments: PHW DHW YGC.
Analyzed the data: PHW.
Contributed reagents/materials/analysis tools: SPW.
Wrote the manuscript: PHW.
Revised the draft: QYJ.

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