Identification and Functional Study of Long Non-coding RNA Involved in White Spot Syndrome Virus Infection in White Shrimp, *Litopenaeus vannamei*

Ifwa Wirasit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Molecular Biotechnology and Bioinformatics (International Program)

Prince of Songkla University

2023

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ชื่อวิทยานิพนธ์ การระบุและศึกษาหน้าที่ของอาร์เอ็นเอสายยาวที่ไม่แปลรหัสต่อการติดเชื้อตัวแดงดวงขาวของกุ้งขาว

ผู้เขียน นางสาวอิฟวา วิระสิทธิ์

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ปีการศึกษา 2566

บทคัดย่อ

อาร์เอ็นเอสายยาวที่ไม่แปลรหัส (long non-coding RNA, lncRNA) เป็นหนึ่งในชีวโมเลกุลที่มีศักยภาพเชิงควบคุมในกระบวนการชีวภาพ เช่น การควบคุมระบบภูมิคุ้มกัน การสืบพันธุ์ และการเจริญเติบโต ในปัจจุบันการศึกษาเกี่ยวกับทรานสคริปโตม (transcriptomic study) ได้มีการพุ่งเป้าไปที่การติดเชื้อไวรัสในสิ่งมีชีวิตต่างๆ ทว่า บทบาทของ lncRNAs ที่ตอบสนองต่อไวรัสในกุ้งยังไม่เป็นที่ทราบแน่ชัด จึงนำมาสู่การศึกษาในครั้งนี้โดยมีวัตถุประสงค์เพื่อระบุ lncRNAs จากทรานสคริปโตมของกุ้งขาว และศึกษาบทบาทของ lncRNA ในการตอบสนองต่อการติดเชื้อไวรัสตัวแดงดวงขาว (white spot syndrome virus, WSSV) จากผลการวิเคราะห์ข้อมูลทรานสคริปโตมจากตับของกุ้งระหว่างกลุ่มติดเชื้อ WSSV และกลุ่มควบคุมโดยใช้เครื่องมือชีวสารสนเทศนั้น สามารถระบุ lncRNAs เป็นจำนวนทั้งสิ้น 44,539 เส้น จากทรานสคริปทั้งหมด 221,347 เส้น และพบว่ามี lncRNA เพียง 32 ตัวที่ตอบสนองต่อการติดเชื้อ WSSV อย่างมีนัยสำคัญ lncRNA จำนวน 5 ตัวถูกนำมาทดสอบการแสดงออกในเนื้อเยื่อต่างๆของกุ้ง และต่อการติดเชื้อ WSSV และได้เลือก *lnc164* มาศึกษาหน้าที่ต่อการติดเชื้อ WSSV โดยใช้เทคนิกอาร์เอ็นเออินเตอร์เฟเรนซ์ (RNA interference) ทั้งนี้ การลดระดับของ *lnc164* ช่วยชะลอการตายของกุ้งเมื่อติดเชื้อ WSSV ซึ่งทำให้เห็นความสำคัญของ *lnc164* ต่อระบบคุ้มกันของกุ้ง อนึ่ง การลดระดับของ *lnc164* ไม่ได้มีความสัมพันธ์ต่อจำนวนเม็ดเลือดของกุ้ง รวมไปถึงจำนวนไวรัสในเลือดเมื่อกุ้งติดเชื้อ WSSV ต่อมาในการศึกษากลุ่มของยีนที่ควบคุมโดย *lnc164* นั้น เราได้ทำ RNA sequencing พบว่ายีน 251 ตัวมีการตอบสนองต่อการลดระดับของ *lnc164* ในตับของกุ้ง จากนั้นยีน 6 ตัวที่เกี่ยวข้องกับระบบภูมิคุ้มกันได้ถูกนำมาศึกษารูปแบบการแสดงออกในตับกุ้งเมื่อมีการลดระดับของ *lnc164* โดยเทคนิกเรียลไทม์พีซีอาร์ (realtime-PCR) ทั้งนี้ การศึกษาข้างต้นได้นำมาซึ่งความเข้าใจในบทบาทของ lncRNA ในกุ้งขาวมากยิ่งขึ้น โดยเฉพาะอย่างยิ่งในแง่ของการตอบสนองต่อการติดเชื้อไวรัส ทั้งยังแสดงให้เห็นหน้าที่เชิง

ควบคุมของ *lnc164* ในกระบวนการป้องกันไวรัสตัวแดงดวงขาวของกุ้งขาว ซึ่งเป็นแนวทางแก่การศึกษาหน้าที่เชิงลึกของ *lncRNA* ที่เกี่ยวข้องกับการติดเชื้อไวรัสในอนาคต

คำสำคัญ: lncRNA, ไวรัสตัวแดงดวงขาว, กุ้งขาวแวนนาไม, ทรานสคริปโตม

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ABSTRACT

Long non-coding RNA (lncRNA) is a promising class of regulators that plays a significant role in various biological processes, such as immune regulation, reproduction, and development. Despite extensive viral transcriptome studies across various organisms, the role of lncRNAs in shrimp's viral responses remains uncertain. This study aims to uncover potential lncRNAs and their role in white shrimp's response to white spot syndrome virus (WSSV) infection. The WSSV-infected hepatopancreas transcriptome revealed 44,539 putative lncRNAs from 221,347 unigenes. Among them, 32 displayed differential expression between WSSV-infected and control shrimp. To validate the findings, five candidate lncRNAs were selected and assessed their expression levels in response to WSSV infection, and in tissues of the shrimp. We investigated the role of a specific lncRNA, *lnc164*, in the response of *L. vannamei* to WSSV infection. Knockdown of *lnc164* led to increased survival of infected shrimp, suggesting its involvement in shrimp immunity. However, *lnc164* did not directly influence total hemocyte count or viral loads in the shrimp's hemolymph. To explore a set of *lnc164*-regulated genes, RNA sequencing was perform and revealed 251 differentially expressed transcripts, then 6 immune-related genes were validated. The study provided valuable information about lncRNA profiles in response to WSSV infection and paved the way for understanding *lnc164*'s role in the shrimp's antiviral response.

Keywords: Long non-coding RNA, white spot syndrome virus, white shrimp, transcriptome

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This acknowledgment is a tribute to the collective efforts and unwavering support of all those who have played a role, big or small, in making this journey a reality.

With deepest appreciation,

IFWA WIRASIT

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List of Proceeding:

Proceeding I Wirasit, I., Udomkit, A., and Sathapondecha, P., 2023. Identification of Long Noncoding RNAs Associated to White Spot Syndrome Virus Infection in White Shrimp, *Litopenaeus vannamei*. The 8th RSU International Research Conference on Sciences and Technology 2023, April, 451–457

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1. Introduction

Over the years, the Pacific white shrimp, known as *Litopenaeus vannamei*, has become one of the most widely commercialized species in the global marine aquaculture industry (Gucic et al., 2013). However, despite its economic importance and trading value, shrimp farmers often face significant losses due to disease outbreaks, particularly the devastating white spot disease caused by the white spot syndrome virus (WSSV). This virus can rapidly spread in saline waters and cause mortality rates as high as 100% (Santos et al., 2018). To combat WSSV, various strategies have been employed, including stress reduction through improved pond and feed management, as well as enhancing the shrimp's immune defenses by incorporating immune enhancers in their diets. Furthermore, advances in WSSV characterization have enabled farmers to selectively stock WSSV-free post-larvae while also preventing the introduction of the virus and potential vectors into ponds (Verbruggen et al., 2016). Despite these efforts, effective methods or strategies to prevent WSSV outbreaks in the field are still lacking, emphasizing the need for a deeper understanding of the shrimp's immune response to WSSV infection.

Similar to other invertebrates, shrimp rely primarily on their innate immune system, as they lack an adaptive immune system, to defend against invading pathogens (Roy et al., 2020; Zheng et al., 2020). Several signaling pathways, including Toll, IMD, and JAK/STAT, have been extensively studied as part of the shrimp's immune defense system (Roy et al., 2020; Tassanakajon et al., 2010). Additionally, WSSV infection induces changes in metabolic pathways related to mitochondria, similar to the Warburg effect observed in cancer cells (Chen et al., 2011). Consequently, shrimp experience alterations in their transcriptomic profiles, leading to the up- or down-regulation of numerous genes upon WSSV infection. Currently, RNA sequencing has proven to be a valuable tool for analyzing gene expression profiles in the transcriptome, and previous studies have investigated gene profiling in shrimp during pathogen infections (Cao et al., 2023; Miao et al., 2023; Z. Wu & Chu, 2023). Recent research has also focused on identifying differentially expressed genes (DEGs) between WSSV-infected and non-

infected *L. vannamei*, with some of these DEGs implicated in the shrimp's immune response (Millard et al., 2021; Peruzza et al., 2020; F. Wang et al., 2019).

Apart from immune-responsive genes, non-coding RNAs (ncRNAs) constitute a diverse group of genes that lack functional open reading frames (ORFs) and, therefore, do not encode proteins (Aliperti et al., 2021). These ncRNAs can be classified into structural and regulatory ncRNAs, with regulatory ncRNAs, especially miRNAs, playing significant roles in various biological processes (Aliperti et al., 2021). Among regulatory ncRNAs, long non-coding RNAs (lncRNAs) are of particular interest, with lengths exceeding 200 nucleotides (Aliperti et al., 2021; Bridges et al., 2021). Research has shown that lncRNAs play critical roles in regulating both the innate and adaptive immune systems, including antiviral responses (Zhang et al., 2022). Several studies have reported differential expression and regulation of lncRNAs in response to DNA or RNA viral infections (Liu & Ding, 2017), such as human lncRNAs Morrbid and lnc-DC (Kotzin et al., 2016; Zhuang et al., 2018), as well as lncRNA MAVS in teleost fish (Chu et al., 2020). While previous studies identified a set of differentially expressed lncRNAs in *Penaeus japonicas* between WSSV-infected and non-infected groups, their functional roles remain to be elucidated (Zhang et al., 2022). Additionally, in *L. vannamei* hemocytes, lncRNAs are co-expressed with immune-related genes, suggesting their involvement in the regulation of *L. vannamei*'s immune defense (Ren et al., 2020). As part of this study, we aim to employ RNA interference and RNA sequencing to *in silico* identify, characterize, and explore the function of lncRNAs involved in WSSV infection in *L. vannamei*.

2. Objectives

2.1 To identify and characterize lncRNAs that are differentially expressed in *L. vannamei* against WSSV infection.

2.2 To investigate the function of lncRNAs involved in WSSV infection in *L. vannamei*.

3. Results

3.1 Identification of long non-coding RNA related to WSSV infection in *L. vannamei* hepatopancreas

RNA sequencing data from *L. vannamei* hepatopancreas (BioProject No. PRJNA554075) (Peruzza et al., 2020) were analyzed between healthy and WSSV-infected shrimp. Briefly, raw RNA-seq data were cleaned by Trimmomatic (Bolger et al., 2014) and assembled using Trinity package (version 2.9.1) with default parameters (Grabherr et al., 2011). Clean reads were aligned using Bowtie2 (Langmead et al., 2012), quantified by RSEM (Li & Dewey 2011), and gene expression differences analyzed with edgeR (FDR < 0.01, p-value < 0.05, |log2(fold-change)| > 1) (Robinson et al., 2010). Candidate lncRNAs were identified by filtering against databases using BLAST and coding potential tools (CPAT).

The *de novo* assembled transcriptome provided 221,347 unigenes with specific characteristics (GC content of 43.05%, N50 of 893 bp, and an assembly completeness of 97%; Table 1). Among these, 44,539 transcripts were identified as putative lncRNAs. After analyzing the expression profiles between control and WSSV-infected groups, 277 unigenes were significantly induced by WSSV infection, including 145 up-regulated and 132 down-regulated transcripts. Among these differentially expressed genes (DEGs), 32 transcripts were identified as differentially expressed lncRNAs (21 up-regulated and 11 down-regulated, Figure 1). Five candidate lncRNAs were selected from the most significantly up- or down-regulated in WSSV infected group, as specified in Table 2.

Table 1 General characteristics of the *de novo* assembled transcriptome

General information	
Total assembled bases	129,966,101
Number of transcripts	221,347
GC (%)	43.05

Average contig length (bp)	587.16
Contig N50 (bp)	893
Assemble completeness (%)	97.0
No. of protein coding transcript	176,808
No. of total lncRNA in transcriptome	44,539
Differential expression of genes	
No. of differentially expressed transcripts	277
No. of differentially expressed protein coding gene	245
No. of differentially expressed lncRNA	32
Up-regulation upon WSSV infection	21
Down-regulation upon WSSV infection	11

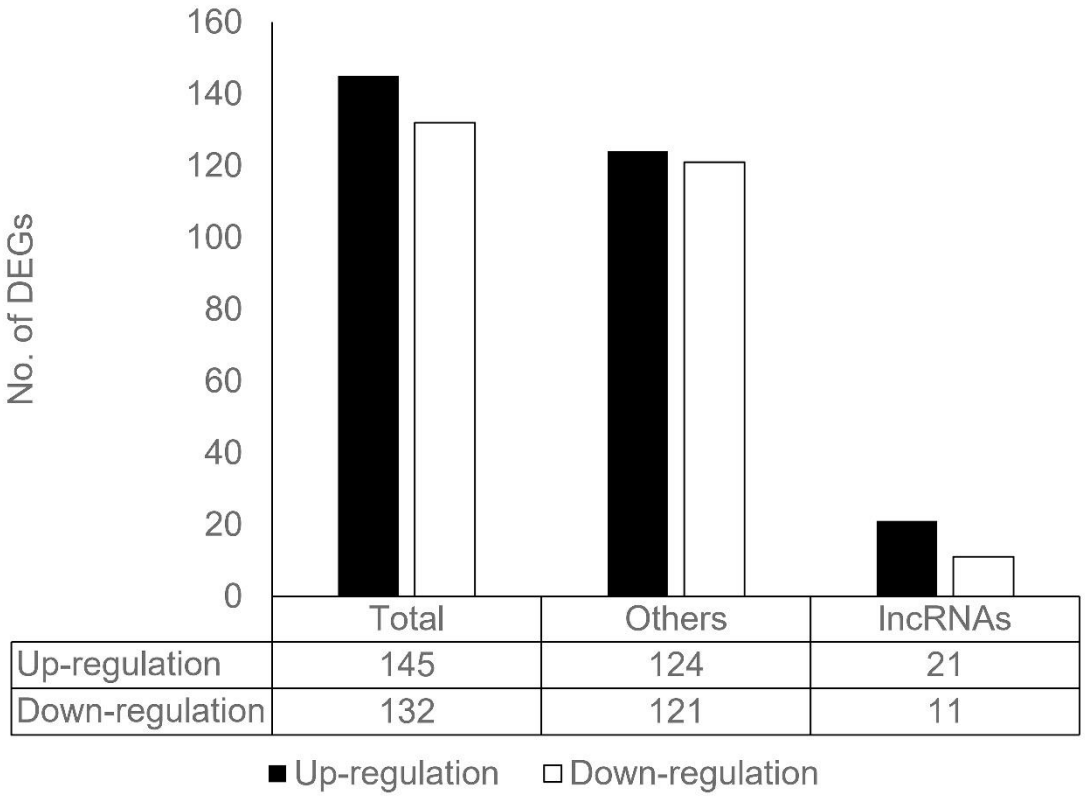

Figure 1 *In silico* analysis of differentially expressed lncRNAs. *De novo* assembled transcriptome was generated and differential expressions of transcripts were analyzed. Number of DEGs composing of total transcripts, mRNA, and putative lncRNAs were shown.

Table 2 Information of the candidate lncRNAs in this study

Candidate lncRNAs	Size (bp)	GenBank Accession No.	DE Patterns	NCBI Hit	Species
lnc164	1,802	OR067088	Down-regulated	XR_003477688	*P. vannamei*
lnc6913	683	OR067089	Down-regulated	-	-
lnc140	1,445	OR067090	Down-regulated	XR_003475561	*P. vannamei*
lnc7225	575	OR067091	Up-regulated	XR_003477627	*P. vannamei*
lnc3615	1,084	OR067092	Up-regulated	-	-

3.2 Validation of lncRNA expressions in response to WSSV infection

Shrimp were injected with either WSSV or 1X PBS pH 7.4, after 12, 24, and 48 hpi, their hepatopancreas was used to extract total RNA to examine candidate lncRNA expressions through qRT-PCR. In brief, total RNA were used for first-strand cDNA synthesis, which RNA sample was added to a mixture of 1X reverse transcriptase buffer, 0.2 mM dNTP, 0.2 µM oligo-dT primer, and 100 U of M-MuLV reverse transcriptase (Vivantis). Given that common lncRNAs are transcribed by RNA polymerase II, and they contain normal 5'-caps and 3' poly-A tails, thus lncRNAs can be detected in RT-PCR using oligo-dT primer. Then, 1/10 diluted cDNA was mixed with SYBR Green qPCR Master Mix and primers, PCR was set with a thermal cycler

of 95 °C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60 °C for 30 sec. A melting curve was subsequently generated by heating at 95°C for 1 min, 60°C for 1 min, and 95°C for 1 min. Beta-actin served as the internal control. Relative lncRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. Primer list was shown in Appendix 1. In this experiment, five shrimp served as biological replicates.

The results indicated that the expression of *lnc164* was notably down-regulated at 24 and 48 h post-WSSV infection (Figure 2A). Similarly, *lnc6913* and *lnc140* expression levels significantly decreased in WSSV-infected shrimp at 24 h post-infection (Figure 2B and C). The expression level of *lnc7225* significantly dropped in WSSV-infected shrimp at 12 h post-infection and then increased at 24-48 h post-infection compared to the control shrimp (Figure 2D). Conversely, the *lnc3615* expression was significantly up-regulated in WSSV-infected shrimp at 24-48 h post-infection (Figure 2E).

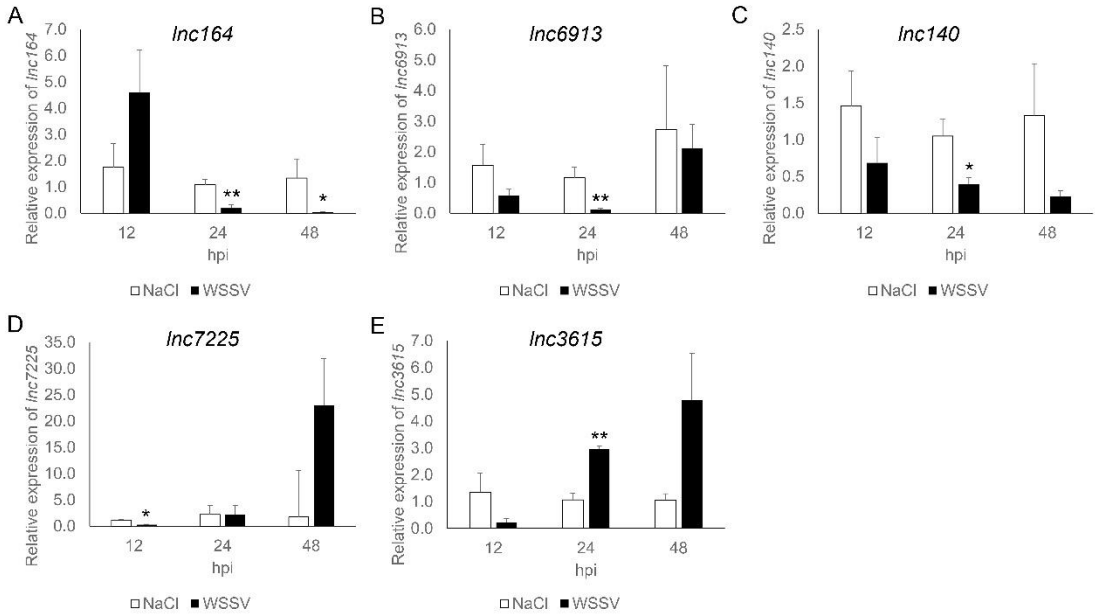

Figure 2 Relative expression of candidate lncRNAs in hepatopancreas during WSSV infection. Hepatopancreas were isolated from control and WSSV-infected shrimp at 12, 24, 48 hpi, and were determined for their expression by qRT-PCR. Bars and error bars represent means and SEMs, respectively. Asterisks, "*" and "**" represent the

significant difference ($p<0.05$ and $p<0.01$, respectively) of infected group when compare to control group.

3.3 Detemination of lncRNA expressions in *L. vannamei* tissues

The expressions of the candidate lncRNAs were assessed in various shrimp tissues, including muscle, brain, gills, epidermis, eyestalk, and hepatopancreas, from healthy shrimp by using qRT-PCR. The results revealed that *lnc164* was predominantly expressed in hepatopancreas and showed minimal expression in other tissues (Figure 3A). On the other hand, the expressions of *lnc6913*, *lnc140*, and *lnc7225* were found ubiquitously across all tested tissues (Figure 3B-D). In contrast, the expression of *lnc3615* was primarily detected in muscle and epidermis, with very low levels observed in other tissues (Figure 3E).

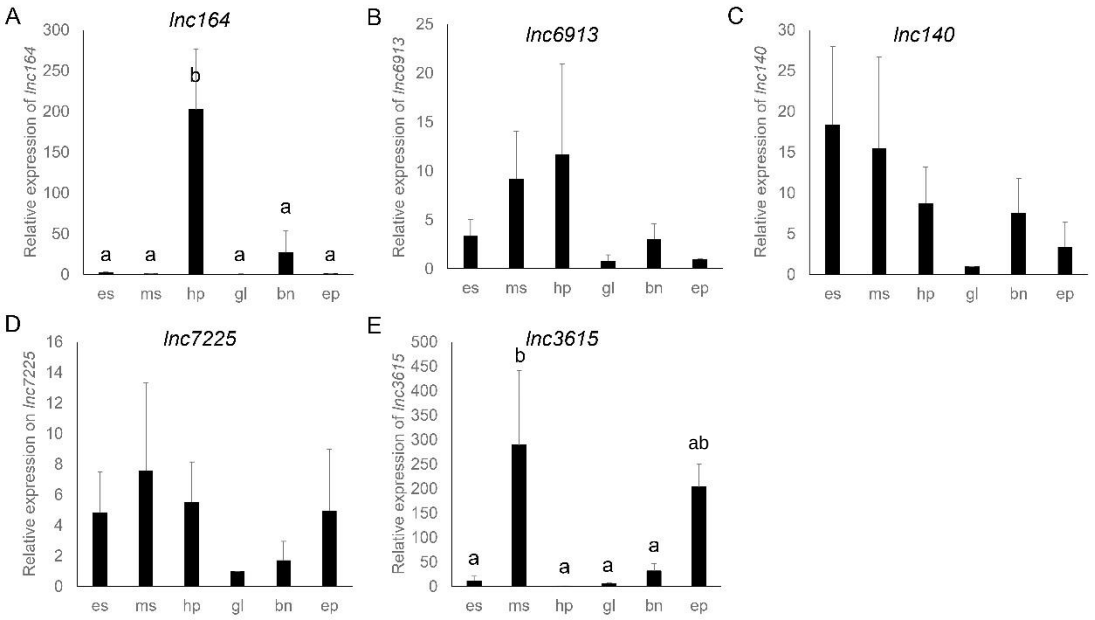

Figure 3 Constitutive expressions of candidate lncRNAs throughout *L. vannamei's* body. Various shrimp tissues including eyestalk (es), muscle (ms), hepatopancreas (hp), gills (gl), brain (bn), and epidermis (ep) were isolated from three healthy *L. vannamei*, and were used to detect the expression of candidate lncRNAs by qRT-PCR. Bars and error bars represent means and SEMs, respectively. Letters indicate significant

difference ($p<0.05$) among groups analyzed by one-way ANOVA and pairwise comparison by Duncan's test.

3.4 Effects of *lnc164* suppression upon WSSV infection in shrimp

To investigate the functional role of lncRNA in WSSV infection, we selected *lnc164* due to its substantial down-regulation upon WSSV infection (Figure 2A) and its predominant expression in hepatopancreas (Figure 3A). To assess gene silencing activity, we successfully synthesized double-stranded RNA specific to *lnc164* (ds164) and administered it through injection into shrimp. In short, an inverted repeat sequence mimicking a stem and its corresponding stem-loop from the *lnc164* sequence was PCR-synthesized. This recombinant was cloned into the pET28 plasmid. Following transformation into *Escherichia coli* HT115 and expression using our modified protocol, dsRNA products were extracted using the ethanol method (Posiri et al., 2013). The results of the experiment demonstrated a significant reduction in the expression level of *lnc164* in hepatopancreas of shrimp injected with ds164. Approximately 78% and 93% decrease in expression was observed at 24-48 hours post-injection, respectively, in comparison to shrimp injected with dsGFP (Figure 4A).

To further investigate the role of *lnc164* in response to the WSSV, we examined the effect of *lnc164* knockdown on shrimp mortality following WSSV infection. Shrimp were first injected with ds164 for 24 h before being subsequently injected with the WSSV. The results revealed significant differences in mortality rates among the different experimental groups. Shrimp injected with NaCl followed by WSSV (NaCl/WSSV) exhibited 10% mortality at 30 h post-infection (hpi), which progressively increased to reach 50% at 69 hpi and eventually reached 100% by 102 hpi (Figure 4B). In contrast, shrimp injected with dsGFP before WSSV infection (dsGFP/WSSV) displayed initial mortality record of 6.25 at 30 hpi. The mortality then increased, reaching 50% and 100% at 78 and 114 hpi, respectively. Interestingly, all *lnc164*-silenced shrimp injected with the WSSV (ds164/WSSV) survived up to 95% at 30 hpi, with only 5% mortality was observed. The mortality rate continued to increase consistently, reaching 50% at 87.5 hpi and 100% at 120 hpi (Figure 4B). Notably, no

shrimp dead was found in the control groups until approximately 60 hpi, and only 10% mortality were found including shrimp injected with NaCl, dsGFP, or ds164 alone without WSSV injection.

Furthermore, we investigated the impact of *lnc164* knockdown on total hemocyte count (THC) and viral loads following WSSV infection. Shrimp were injected with ds164 for 1 day before being subsequently injected with WSSV. To determine amount of hemocytes, shrimp were injected with ds164, dsGFP, or NaCl for 24 h followed by injection of WSSV or NaCl for 24 h. For THC, hemolymph samples (10 µl) were mixed with 10 µl trypan blue and loaded only 10 µl into a hematocytometer. THC was determined using Mani's procedure by counting live and dead cells under a light microscope (Mani et al., 2021).

At 24 hpi, THC levels were dramatically decreased in all WSSV-infected shrimp, whether they received dsRNA injection or NaCl injection, when compared to their respective controls (Figure 4C). THC levels remained unchanged among shrimp injected with WSSV and also among shrimp without WSSV infection (Figure 4C). Similarly, we examined viral loads in gills of individual shrimp using qRT-PCR. The results revealed a significant reduction in viral loads, approximately 88% and 85% in shrimp injected with dsGFP and ds164, respectively, when compared with NaCl group (Figure 4D).

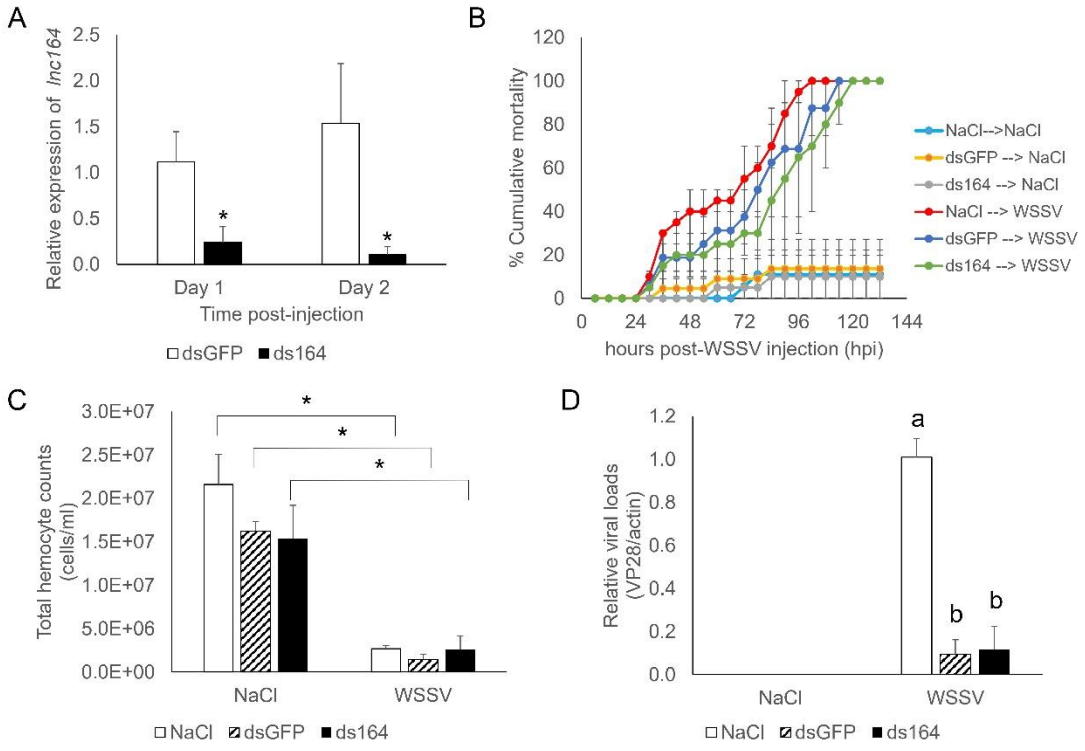

Figure 4 Determination of silencing activity of ds164 and its effect on shrimp mortality, hemocytes, and viral loads. (A) Shrimp were injected with ds164 and dsGFP, and hepatopancreas were isolated on day 1 and day 2 post injection to detect *lnc164* expression level by qRT-PCR. Shrimp were injected with ds164, dsGFP, or NaCl 24 h prior to WSSV infection, (B) cumulative mortality of shrimp when challenged with WSSV were recorded within a 6 h interval, (C) hemolymph were used for total hemocyte count measured by hemocytometry. (D) Relative viral loads in gills of shrimp after 24 h post-WSSV infection. Bars and error bars represent means and SEMs, respectively, an asterisk indicates the significant different ($p<0.05$) between target group compare to control.

3.5 Transcriptomic profiles upon *lnc164* depletion in *L. vannamei*'s hepatopancreas

To investigate a group of genes regulated by *lnc164*, we conducted RNA sequencing on shrimp with *lnc164* knockdown. A *de novo* assembled transcriptome was generated with a total of 166,906 non-redundant transcripts, exhibiting a GC

content of 42.92%, an N50 value of 1,727 base pairs, 98% assembly completeness, and 96.2% representation of the reads in the transcriptome (Table 3). Following this, we analyzed the differential expression in the *lnc164*-depleted shrimp and identified 251 differentially expressed transcripts across the various groups. The expression patterns of these differentially expressed genes (DEGs) were visually represented through a heatmap (Figure 5A). Among the DEGs, only 9 transcripts were significantly up-regulated, while 12 transcripts were significantly down-regulated upon *lnc164* knockdown (Figure 5B).

The functional annotation of the DEGs was performed using GO, KEGG, and COG analyses. The GO analysis revealed that the majority of DEGs were found within cell, cell part, and organelle, respectively, and were primarily associated with binding and catalytic activities. Concerning biological processes, the DEGs were mainly annotated for cellular process, metabolic process, biological regulation, and regulation of biological process (Figure 5C). Regarding KEGG-enriched functional processes, the majority of DEGs were associated with metabolic pathways, with a smaller representation in processes related to nucleocytoplasmic transport and endocytosis (Figure 5D). In the COG categories, many DEGs had unknown functions, followed by the next significant categories involving processes related to post-translational modification, protein turnover, chaperone functions, transcription, and signal transduction (Figure 5E).

Table 3 General characteristics of *lnc164*-knockdown transcriptome

Statistics of *de novo* assembled transcriptome	
Total assembled bases	182,436,258
Number of transcripts	166,906
GC (%)	42.92
Average contig length (bp)	734.11
Contig N50 (bp)	1,727

Assemble completeness (%)	98.0
No. of differentially expressed transcripts	251

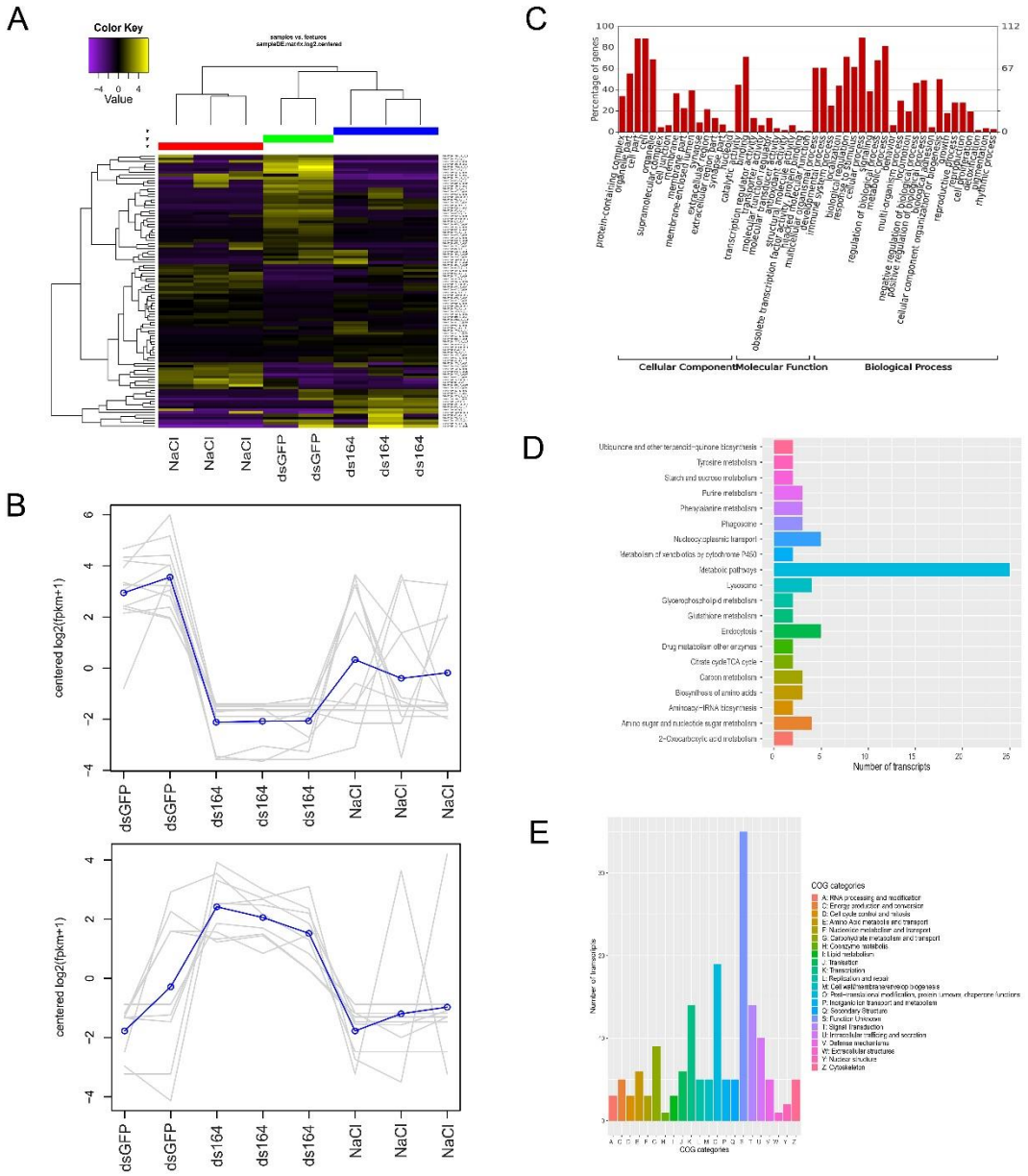

Figure 5 Functional annotation of differentially expressed genes upon *lnc164* depletion. DEGs were analyzed from *de novo* assembled transcriptome from hepatopancreas of *lnc164*-kncokdown shrimp, and were visualized their patterns as (A)

heatmap and (B) graph. Functional annotation were demonstrated by (C) GO, (D) KEGG, and (E) COG.

3.6 Determination of gene expressions in response to *lnc164* depletion

Based on the transcription profiles and functional annotation of the DEGs, we selected the metabolic and immune-related genes, specifically insulin-like growth factor-binding protein-related protein 1 (*IGFBP-rP1*), brain protein I3 (*BRI3*), caspase-3-like (*Casp3*), sprout-related EVH1 domain-containing protein 2-like (*SPRED2*), nose resistant to fluoxetine protein 6-like (*NRF-6*), and cofilin/actin-depolymerizing factor (*Cofilin*), to examine their expression levels in *lnc164* knockdown shrimp using qRT-PCR method (Table 4). The experimental results demonstrated that the expression of *IGFBP-rP1* was significantly increased in both dsGFP- and ds164-injected shrimp compared to the control shrimp (Figure 6A). On the other hand, the expressions of *BRI3* and *Casp3* were significantly up-regulated only in the *lnc164* knockdown shrimp (Figure 6B and 6C). Regarding genes *NRF-6* and *SPRED2*, their expressions were significantly down-regulated in ds164-injected shrimp compared to the other control groups (Figure 6D and 6E). However, there was no significant change observed in the expression of *Cofilin* among the experimental groups (Figure 6F).

Table 4 Information of the candidate genes in response to *lnc164* depletion

Genes	**Gene names**	**GenBank Accession No.**	**E-value**	**Expression patterns**
Cofilin	Cofilin/actin-depolymerizing factor homolog	XP_027225677.1	5.09E-43	Down-regulated
BRI3	Brain protein I3-like	XP_047497091.1	3.92E-31	Down-regulated

IGFBP-rP1	Insulin-like growth factor-binding protein-related protein 1	XP_027221413.1	2.02E-46	Up-regulated
Casp3	Caspase-3-like	XP_027234111.1	0	Up-regulated
SPRED2	Sprouty-related, EVH1 domain-containing protein 2-like	XP_027238969.1	0	Up-regulated
NRF-6	Nose resistant to fluoxetine protein 6-like	XP_042891509.1	0	Down-regulated

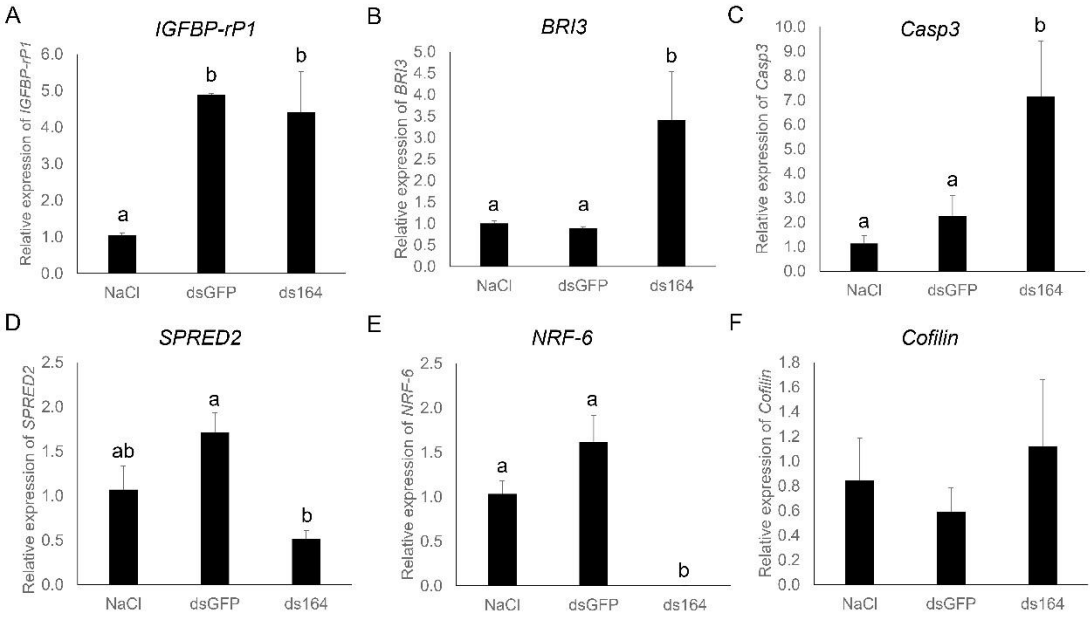

Figure 6 Relative expressions of immune-related genes in *lnc164*-depleted shrimp. Candidate DEGs were validated for their expressions in hepatopancreas of shrimp injected with ds164, dsGFP, or NaCl. Five shrimp were used in each group. Bars and error bars represent means and SEMs, respectively. Letters indicate significant difference ($p<0.05$) among groups analyzed by one-way ANOVA and pairwise comparison by Duncan's test.

3.7 Determination of gene expression in hepatopancreas upon WSSV infection

Five genes related metabolism and immunity were demonstrated for their responsiveness to the knockdown of *lnc164*. Following this, their responses to WSSV infection were further studied (Figure 7). The results demonstrated that the expressions of *IGFBP-rP1*, *Casp3*, and *NRF-6* were notably reduced after 24 h of WSSV infection (Figure 7A-C). Conversely, *SPRED2* exhibited a significant increase in expression upon infection (Figure 7D). However, no significant difference in expression was observed for *BRI3* when comparing the examined groups (Figure 7E).

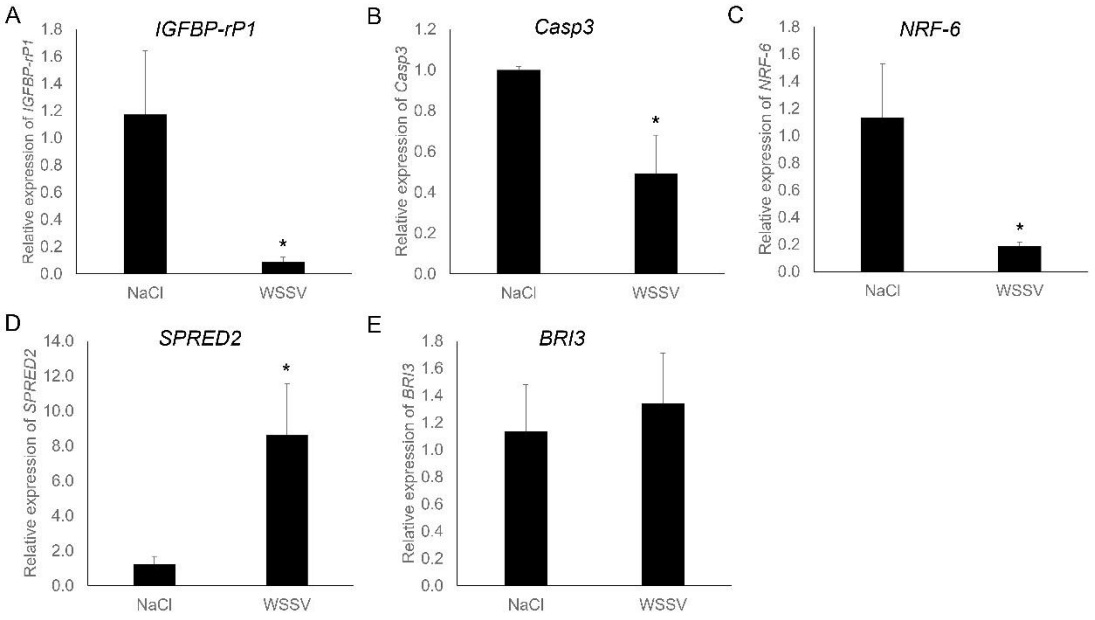

Figure 7 Relative expressions of immune-related genes in shrimp when challenged with WSSV. Candidate DEGs were validated for their expressions in hepatopancreas of shrimp injected with WSSV or NaCl for 24 h, five shrimp were used in each group. Bars and error bars represent means and SEMs, respectively, an asterisk indicates the significant different ($p<0.05$) between target group compare to control.

4. Discussion

4.1 Identification and differential expression of lncRNAs in shrimp antiviral defense against WSSV Infection

To facilitate modifications across numerous pathways implicated in anti-viral activities, shrimp undergo changes in their transcriptomic profiles, impacting a multitude of responsible genes. Besides coding genes and regulatory miRNAs, long non-coding RNAs (lncRNAs) constitute the most substantial faction of non-coding RNAs, exerting regulatory functions across various cellular and physiological processes (Oo et al., 2022). Recent investigations into the transcriptome have unearthed and examined lncRNAs as pivotal regulators in viral infections, primarily due to their distinctive expressions in response to viral invasion and their control over the development and progression of infectious diseases (Liu & Ding, 2017; Peng et al., 2010). Within the scope of this inquiry, we identified 44,539 potential lncRNAs from the *de nov*o assembled transcriptome sourced from the hepatopancreas of both healthy and WSSV-infected shrimp, with only 32 lncRNAs undergoing differential induction post WSSV infection (Table 2). This parallels analogous investigations; for instance, *L. vannamei* hemocytes housed 8,077 potential lncRNAs, among which 1,168 were subject to differential expression upon *Spiroplasma eriocheiris* infection (Ren et al., 2020). In a recent study, 6,544 lncRNAs were pinpointed, of which 457 exhibited differential expression in the hepatopancreas of *P. japonicus* following WSSV infection (Zhang et al., 2022). Moreover, another investigation resulted in the discovery of a total of 12,165 potential lncRNAs, with 709 of these lncRNAs experiencing significant induction due to spring viraemia of carp virus (SVCV) infection in zebrafish (Valenzuela-muñoz et al., 2019).

4.2 Candidate lncRNA expression changes in response to WSSV infection

In our research, we selected five potential lncRNAs for investigating their activity during WSSV infection. The findings showed that the expressions of *lnc164*, *lnc6913*, and *lnc140* decreased, while the expressions of *lnc3615* and *lnc7225* increased during WSSV infection (Figure 2). In shrimp, certain lncRNAs responded differently

when faced with infectious pathogens. For instance, when *P. japonicus* was infected with WSSV (Zhang et al., 2022) or *L. vannamei* was infected with *S. eriocheiris* (Ren et al., 2020), specific lncRNAs displayed varied levels of expression. Similar studies have pointed out the role of lncRNAs in the immune defense against viral infections. For instance, in human, the lncRNA *Morrbid* is induced by T cell receptor (TCR) and type I IFN stimulation in the early stages of both acute and chronic lymphocytic choroidal meningitis virus (LCMV) infection (Kotzin et al., 2016). In lower vertebrates, the lncRNA MARL works alongside miR-122 to influence a regulatory network known as a competing endogenous RNA (ceRNA), which impacts the mitochondrial antiviral signaling protein (MAVS)-mediated RIG-I-like receptors (RLRs) pathway in teleost fish (Chu et al., 2020). These findings suggest that lncRNAs might play a role in the response to harmful pathogens, especially WSSV infection in shrimp.

4.3 Constitutive expression of candidate lncRNAs in L. vannamei tissues

LncRNAs are known to be found in particular body parts (Bridges et al., 2021), but they are also present in various organs (Jiang et al., 2016). In our research, *lnc164* was mainly found in the hepatopancreas (Figure 3A), while other candidate lncRNAs including *lnc6913*, *lnc140*, *lnc7225*, and *lnc3615* were found throughout all the tissues examined (Figure 3B-E). Unlike genes that encode proteins, lncRNAs are usually only active in certain cell types. For instance, in rhesus macaques infected with Ebola virus, lncRNAs are produced in specific cells, especially monocytes, which are the main target cells of the virus (Santus et al., 2023). Herein, the *lnc164* was investigated for its roles in the WSSV infection in shrimp due to the significance of its down-regulation upon the WSSV infection and its specific expression in hepatopancreas.

4.4 *lnc164* depletion and prolonged survival in WSSV-infected shrimp

Investigating *lnc164*'s role in shrimp's response to WSSV infection, we employed RNA interference via ds164 injection. In Figure 4B, the mortality patterns between three groups of WSSV-positive shrimp were not clearly separated because we used the different doses of WSSV in each replicate. However, the trend of delayed pattern can be seen in shrimp with lnc164-knockdown, suggesting a negative regulation

of lnc164 in antiviral response. Additionally, we assessed total hemocyte count (THC) in WSSV-infected shrimp and those with *lnc164* knockdown. THC reduction upon WSSV infection (Figure 4C) was consistent with Koiwai et al.'s observations. WSSV infection disturbs immune-related gene levels within hemocytes, potentially contributing to shrimp mortality (Cui et al., 2020; F. Wang et al., 2019). Notably, *lnc164* knockdown did not affect THC, implying an independent interaction between *lnc164*-modulated immune response and hemocyte apoptosis. Moreover, injecting dsRNA, such as dsGFP and ds164, notably diminished the relative viral loads (Figure 4D). In the study of the impact of non-specific RNA in shrimp during WSSV infection, the injection of dsGFP triggered the up-regulation of various genes encoding immune-related proteins. Consequently, shrimp encountered a disruption in viral accumulation, effectively prevented significant mortalities (Maralit et al., 2015).

4.5 Transcriptome profiling reveals impact of *lnc164* depletion in *L. vannamei* hepatopancreas

We conducted an analysis of the transcriptomic profiles of *L. vannamei* hepatopancreas following the depletion of *lnc164*. This analysis yielded a total of 251 differentially expressed genes (DEGs) in the *de novo* assembled transcriptome. Pathway assessment using KEGG and GO indicated that these genes primarily participated in metabolic pathways and processes. Some of them were specifically linked to sugar metabolism and the TCA cycle (Figure 5C and D). In the context of WSSV pathogenicity, a well-studied aspect is the alteration of metabolic pathways, which is crucial for the virus to infiltrate the host. Since viruses lack their own metabolic machinery, they depend on host mechanisms for replication (Munger et al., 2006). Metabolic changes driven by WSSV influence glycolysis, the TCA cycle, glutaminolysis, and lipid metabolism (Kumar et al., 2022). Consequently, it is plausible that the impact of *lnc164* on WSSV infection might occur through interactions with one or multiple metabolic pathways. This investigation also identified COG categories of DEGs related to lipid metabolism, amino acid metabolism, and nucleotide metabolism (Figure 5E). These categories align with the distinctive metabolic reprogramming associated with WSSV (Kumar et al., 2022). Additionally, the depletion of *lnc164* led

to perturbations in genes associated with immune system processes and defense mechanisms, as indicated by enriched GO and COG analyses (Figure 5A and C). This underscores the significant roles of *lnc164* in the realm of immune defense.

4.6 Knockdown of *lnc164* amplifies immune responsive genes and shapes WSSV interaction in shrimp

4.6.1 Gene interactions and immune response

In the context of immune response, our study successfully validated six immune-responsive genes upon the knockdown of *lnc164*. Notably, among these genes, five demonstrated a significant induction in expression following *lnc164* depletion: *IGFBP-rP1*, *BRI3*, *Casp3*, *SPRED2*, and *NRF-6* (Figure 6). An intriguing observation was made regarding *IGFBP-rP1*, which showed a decrease in expression upon WSSV infection. However, this down-regulation was effectively countered by RNA interference through dsGFP and ds164 injection, revealing the intricate interplay between *lnc164* and this gene. Notably, *IGFBP-rP1*'s established role as a tumor suppressor in human cancer biology via the insulin/IGF-1 signaling pathway adds a compelling layer to its involvement (Y. Ma et al., 2008; Zhu et al., 2014). Furthermore, *NRF-6*, a gene inhibited by both *lnc164* knockdown and WSSV infection (Figure 6E and 7C), presents an enigmatic role in arthropods, but its function in *C. elegans* is linked to molecule uptake and lipid transport (Choy et al., 2006), implying potential effects on WSSV transport and replication upon its down-regulation following *lnc164* suppression.

4.6.2 Apoptosis regulation and shrimp survival

Apoptosis induction is a pivotal facet of the host's antiviral response, yet viruses often subvert this mechanism to bolster their replication. Caspases, central players in apoptosis, have been recognized for their protective roles against WSSV infection (L. Wang et al., 2008; P. Wang et al., 2013). Remarkably, our study noted the down-regulation of *Casp3* upon WSSV infection (Figure 7B), possibly reflecting the virus's anti-apoptotic strategy. The subsequent up-regulation of Caspase-3 at later infection stages could facilitate WSSV distribution (P. Wang et al., 2013). Additionally, the up-

regulation of *SPRED2* in response to WSSV infection is associated with the activation of apoptosis (X. N. Ma et al., 2011). A hypothesis emerges that the heightened levels of *SPRED2* at 24 hpi could trigger *Casp3* activation, peaking at 48 hpi, thus accelerating apoptosis and contributing to the decline of infected shrimp.

Our investigation yielded a significant extension in shrimp survival during WSSV infection upon *lnc164* knockdown (Figure 4B). This effect appears to be correlated with elevated *Casp3* levels and concurrent down-regulation of *SPRED2* expression (Figure 6C and 6B). This orchestrated manipulation potentially initiates early-stage apoptosis, limiting WSSV propagation and leading to prolonged shrimp survival. Moreover, the reduction in *SPRED2* expression disrupts *Casp3* function in later infection stages, inducing a delay in extensive apoptosis and ultimately supporting the sustained survival of infected shrimp.

Furthermore, an unexpected discovery was the up-regulation of the *BRI3* gene upon *lnc164* depletion. *BRI3*'s established role in facilitating tumor necrosis factor (TNF)-induced cell death, particularly in L929 cells, adds intriguing dimensions to its involvement (H. Wu et al., 2003). Interestingly, our results did not reveal a significant disparity in *BRI3* expression between infected shrimp and the control group. Collectively, these findings enhance our comprehension of the intricate *lnc164*-WSSV relationship, shedding light on the complex interactions between *lnc164* and immune-related genes within the framework of shrimp innate immunity.

5. Conclusion

In this research, we identified 44,539 putative long non-coding RNAs (lncRNAs) from the *de novo* assembled transcriptome of healthy and WSSV-infected shrimp's hepatopancreas. After WSSV infection, only 32 lncRNAs showed differential induction. Among these, five candidate lncRNAs were selected for validation of their expressions during WSSV infection and in various shrimp tissues. The lncRNA, named *lnc164*, exhibited predominant expression in the hepatopancreas and demonstrated a down-regulation pattern during WSSV infection. To investigate *lnc164*'s function, we conducted a functional study using RNA interference through ds164 injection. The

knockdown of *lnc164* resulted in a delay in shrimp mortality when infected with WSSV. Despite WSSV infection inducing total hemocyte counts (THC), no significant differences were observed between control and *lnc164*-depleted shrimp. While injecting dsRNA, including dsGFP and ds164, notably diminished the relative viral loads. To explore *lnc164*-regulated genes, we performed RNA sequencing, which revealed 251 differentially expressed transcripts (DEGs) in the *lnc164*-depleted samples. Most of these DEGs were associated with metabolic processes, while some were related to the immune response. Six of the DEGs were validated for their expression upon *lnc164* knockdown. This study not only provided valuable insights into the lncRNA profile of *L. vannamei* but also shed light on the interactions between *lnc164* and WSSV in the innate immunity of shrimp.

REFERENCES

Aliperti, V., Skonieczna, J., & Cerase, A. (2021). Long non-coding RNA (lncRNA) roles in cell biology, neurodevelopment and neurological disorders. *Non-coding RNA*, 7(2), 36. https://doi.org/10.3390/ncrna7020036

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114-2120. https://doi.org/10.1093/bioinformatics/btu170

Bridges, M. C., Daulagala, A. C., & Kourtidis, A. (2021). LNCcation: lncRNA localization and function. *Journal of cell biology*, *220*(2), 1-17. https://doi.org/10.1083/JCB.202009045

Cao, Z., Gao, Y., Xu, J., Yang, N., Li, T., Chang, L., Si, L., & Yan, D. (2023). Transcriptome analysis of the hepatopancreas in *Penaeus vannamei* under experimental infection with *Enterocytozoon hepatopenaei* (EHP). *Fish & shellfish immunology*, *134*(February), 108605. https://doi.org/10.1016/j.fsi.2023.108605

Chen, I. T., Aoki, T., Huang, Y. T., Hirono, I., Chen, T. C., Huang, J. Y., Chang, G. D., Lo, C. F., & Wang, H. C. (2011). White spot syndrome virus induces metabolic changes resembling the warburg effect in shrimp hemocytes in the early stage of infection. *Journal of virology*, *85*(24), 12919-12928. https://doi.org/10.1128/jvi.05385-11

Choy, R. K., Kemner, J. M., & Thomas, J. H. (2006). Fluoxetine-resistance genes in *Caenorhabditis elegans* function in the intestine and may act in drug transport. *Genetics*, *172*, 885-892. https://doi.org/10.1534/genetics.103.024869

Chu, Q., Xu, T., Zheng, W., Chang, R., & Zhang, L. (2020). Long noncoding RNA MARL regulates antiviral responses through suppression miR-122-dependent MAVS downregulation in lower vertebrates. *PLoS pathogens*, *16*(7), 1-27. https://doi.org/10.1371/journal.ppat.1008670

Cui, C., Liang, Q., Tang, X., Xing, J., Sheng, X., & Zhan, W. (2020). Differential apoptotic responses of hemocyte subpopulations to white spot syndrome virus infection in *Fenneropenaeus chinensis*. *Frontiers in immunology*, *11*(December),

1-13. https://doi.org/10.3389/fimmu.2020.594390

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., Di Palma, F., Birren, B. W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., & Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature biotechnology*, *29*(7), 644-652. https://doi.org/10.1038/nbt.1883

Gucic, M., Cortés-Jacinto, E., Civera-Cerecedo, R., Ricque-Marie, D., & Martínez-Córdova, L. R. (2013). Apparent carbohydrate and lipid digestibility of feeds for whiteleg shrimp, *Litopenaeus vannamei* (Decapoda: Penaeidae), cultivated at different salinities. *Revista de Biología Tropical*, *61*(3), 1201-1213. https://doi.org/10.15517/rbt.v61i3.11935

Jiang, C., Li, Y., Zhao, Z., Lu, J., Chen, H., Ding, N., Wang, G., Xu, J., & Li, X. (2016). Identifying and functionally characterizing tissue-specific and ubiquitously expressed human lncRNAs. *Oncotarget*, *7*(6), 7120-7133. https://doi.org/10.18632/oncotarget.6859

Koiwai, K., Kondo, H., & Hirono, I. (2023). scRNA-seq analysis of hemocytes of penaeid shrimp under virus infection. *Marine biotechnology*, *25*(June). 488-502 https://doi.org/10.1007/s10126-023-10221-8

Kotzin, J. J., Spencer, S. P., McCright, S. J., Kumar, D. B. U., Collet, M. A., Mowel, W. K., Elliott, E. N., Uyar, A., Makiya, M. A., Dunagin, M. C., Harman, C. C. D., Virtue, A. T., Zhu, S., Bailis, W., Stein, J., Hughes, C., Raj, A., Wherry, E. J., Goff, L. A., Klion, A. D., Rinn, J. L., Williams, A., Flavell, R. A., & Henao-Mejia, J. (2016). The long non-coding RNA Morrbid regulates Bim and short-lived myeloid cell lifespan. *Nature*, *537*(7619), 239-243. https://doi.org/10.1038/nature19346

Kumar, R., Huang, J. Y., Ng, Y. S., Chen, C. Y., & Wang, H. C. (2022). The regulation of shrimp metabolism by the white spot syndrome virus (WSSV). *Reviews in aquaculture*, *14*(3), 1150-1169. https://doi.org/10.1111/raq.12643

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods*, *9*(4), 357-359. https://doi.org/10.1038/nmeth.1923

Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC bioinformatics*, *12*, 323. https://doi.org/10.1186/1471-2105-12-323

Liu, W., & Ding, C. (2017). Roles of LncRNAs in viral infections. *Frontiers in cellular and infection microbiology*, *7*(May), 205. https://doi.org/10.3389/fcimb.2017.00205

Ma, X. N., Liu, X. Y., Yang, Y. F., Xiao, F. J., Li, Q. F., Yan, J., Zhang, Q. W., Wang, L. S., Li, X. Y., & Wang, H. (2011). Regulation of human hepatocellular carcinoma cells by Spred2 and correlative studies on its mechanism. *Biochemical and biophysical research communications*, *410*(4), 803-808. https://doi.org/10.1016/j.bbrc.2011.06.068

Ma, Y., Lu, B., Ruan, W., Wang, H., Lin, J., Hu, H., Deng, H., Huang, Q., & Lai, M. (2008). Tumor suppressor gene insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) induces senescence-like growth arrest in colorectal cancer cells. *Experimental and molecular pathology*, *85*(2), 141-145. https://doi.org/10.1016/j.yexmp.2008.04.005

Mani, S., Mullaivanam Ramasamy, S., Chakrapani, S., Krishna, A., PS, S. A., Lalramchhani, C., Antony, J., & Panigrahi, A. (2021). The effect of natural and artificial periphytic substrates with biofloc system on shrimp *Penaeus vannamei* (Boone 1931) culture: growth and immune response. *Aquaculture international*, *29*(April), 651-668. https://doi.org/10.1007/s10499-021-00646-0

Maralit, B.A., Komatsu, M., Hipolito, S.G., Hirono I., Kondo H. (2015). Microarray analysis of immunity against WSSV in response to injection of non-specific long dsRNA in kuruma shrimp, *Marsupenaeus japonicus*. *Marine biotechnology*, *17*(August), 493–501. https://doi.org/10.1007/s10126-015-9637-9

Miao, M., Li, S., Liu, Y., Yu, Y., & Li, F. (2023). Transcriptome analysis on hepatopancreas reveals the metabolic dysregulation caused by *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei*. *Biology*, *12*(3), 417. https://doi.org/10.3390/biology12030417

Millard, R. S., Bickley, L. K., Bateman, K. S., Farbos, A., Minardi, D., Moore, K., Ross, S. H., Stentiford, G. D., Tyler, C. R., van Aerle, R., & Santos, E. M. (2021).

Global mRNA and miRNA analysis reveal key processes in the initial response to infection with WSSV in the pacific whiteleg shrimp. *Viruses*, *13*(6), 1140. https://doi.org/10.3390/v13061140

Munger, J., Bajad, S. U., Coller, H. A., Shenk, T., & Rabinowitz, J. D. (2006). Dynamics of the cellular metabolome during human cytomegalovirus infection. *PLoS pathogens*, *2*(12), e132. https://doi.org/10.1371/journal.ppat.0020132

Oo, J. A., Brandes, R. P., & Leisegang, M. S. (2022). Long non-coding RNAs: novel regulators of cellular physiology and function. *Pflugers Archiv: European journal of physiology*, *474*(2), 191-204. https://doi.org/10.1007/s00424-021-02641-z

Peng, X., Gralinski, L., Armour, C. D., Ferris, M. T., Thomas, M. J., Proll, S., Bradel-Tretheway, B. G., Korth, M. J., Castle, J. C., Biery, M. C., Bouzek, H. K., Haynor, D. R., Frieman, M. B., Heise, M., Raymond, C. K., Baric, R. S., & Katze, M. G. (2010). Unique signatures of long noncoding RNA expression in response to virus infection and altered innate immune signaling. *mBio*, *1*(5), e00206-10. https://doi.org/10.1128/mBio.00206-10

Peruzza, L., Thamizhvanan, S., Vimal, S., Vinaya Kumar, K., Shekhar, M. S., Smith, V. J., Hauton, C., Vijayan, K. K., & Sahul Hameed, A. S. (2020). A comparative synthesis of transcriptomic analyses reveals major differences between WSSV-susceptible *Litopenaeus vannamei* and WSSV-refractory *Macrobrachium rosenbergii*. *Developmental and comparative immunology*, *104*, 103564. https://doi.org/10.1016/j.dci.2019.103564

Posiri, P., Ongvarrasopone, C., & Panyim, S. (2013). A simple one-step method for producing dsRNA from *E. coli* to inhibit shrimp virus replication. *Journal of virological methods*, *188*(1-2), 64-69. https://doi.org/10.1016/j.jviromet.2012.11.033

Ren, Y., Li, J., Guo, L., Liu, J. N., Wan, H., Meng, Q., Wang, H., Wang, Z., Lv, L., Dong, X., Zhao, W., Zeng, Q., & Ou, J. (2020). Full-length transcriptome and long non-coding RNA profiling of whiteleg shrimp *Penaeus vannamei* hemocytes in response to *Spiroplasma eriocheiris* infection. *Fish & shellfish immunology*, *106*, 876-886. https://doi.org/10.1016/j.fsi.2020.06.057

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)*, *26*(1), 139-140. https://doi.org/10.1093/bioinformatics/btp616

Roy, S., Bossier, P., Norouzitallab, P., & Vanrompay, D. (2020). Trained immunity and perspectives for shrimp aquaculture. *Reviews in aquaculture*, *12*(4), 2351-2370. https://doi.org/10.1111/raq.12438

Santos, C. A., Andrade, S. C. S., Teixeira, A. K., Farias, F., Kurkjian, K., Guerrelhas, A. C., Rocha, J. L., Galetti, P. M., Jr, & Freitas, P. D. (2018). *Litopenaeus vannamei* transcriptome profile of populations evaluated for growth performance and exposed to white spot syndrome virus (WSSV). *Frontiers in genetics*, *9*(April), 120. https://doi.org/10.3389/fgene.2018.00120

Santus, L., Sopena-Rios, M., García-Pérez, R., Lin, A. E., Adams, G. C., Barnes, K. G., Siddle, K. J., Wohl, S., Reverter, F., Rinn, J. L., Bennett, R. S., Hensley, L. E., Sabeti, P. C., & Melé, M. (2023). Single-cell profiling of lncRNA expression during Ebola virus infection in rhesus macaques. *Nature communications*, *14*(1), 3866. https://doi.org/10.1038/s41467-023-39627-7

Tassanakajon, A., Amparyup, P., Somboonwiwat, K., & Supungul, P. (2010). Cationic antimicrobial peptides in penaeid shrimp. *Marine biotechnology (New York, N.Y.)*, *12*(5), 487-505. https://doi.org/10.1007/s10126-010-9288-9

Valenzuela-Muñoz, V., Pereiro, P., Álvarez-Rodríguez, M., Gallardo-Escárate, C., Figueras, A., & Novoa, B. (2019). Comparative modulation of lncRNAs in wild-type and rag1-heterozygous mutant zebrafish exposed to immune challenge with spring viraemia of carp virus (SVCV). *Scientific reports*, *9*(1), 14174. https://doi.org/10.1038/s41598-019-50766-0

Verbruggen, B., Bickley, L. K., van Aerle, R., Bateman, K. S., Stentiford, G. D., Santos, E. M., & Tyler, C. R. (2016). Molecular mechanisms of white spot syndrome virus infection and perspectives on treatments. *Viruses*, *8*(1), 23. https://doi.org/10.3390/v8010023

Wang, F., Li, S., Xiang, J., & Li, F. (2019). Transcriptome analysis reveals the activation of neuroendocrine-immune system in shrimp hemocytes at the early

stage of WSSV infection. *BMC genomics*, *20*(1), 247. https://doi.org/10.1186/s12864-019-5614-4

Wang, L., Zhi, B., Wu, W., & Zhang, X. (2008). Requirement for shrimp caspase in apoptosis against virus infection. *Developmental and comparative immunology*, *32*(6), 706-715. https://doi.org/10.1016/j.dci.2007.10.010

Wang, P. H., Wan, D. H., Chen, Y. G., Weng, S. P., Yu, X. Q., & He, J. G. (2013). Characterization of four novel caspases from *Litopenaeus vannamei* (Lvcaspase2-5) and their role in WSSV infection through dsRNA-mediated gene silencing. *PloS one*, *8*(12), e80418. https://doi.org/10.1371/journal.pone.0080418

Wu, H., Liu, G., Li, C., & Zhao, S. (2003). bri3, a novel gene, participates in tumor necrosis factor-alpha-induced cell death. *Biochemical and biophysical research communications*, *311*(2), 518-524. https://doi.org/10.1016/j.bbrc.2003.10.038

Wu, Z., Chu, K. H., & Ma, K. Y. (2023). Transcriptome analysis of multiple tissues in the shrimp *Penaeus vannamei* reveals the typical physiological response to three pathogens. *Journal of marine science and engineering*, *11*(2), 389. https://doi.org/10.3390/jmse11020389

Zhang, Y., Yao, N., Zhang, C., Sun, X., Huang, J., Zhao, B., & Li, H. (2022). LncRNA-mRNA integrated profiling analysis in response to white spot syndrome virus in hepatopancreas in *Penaeus japonicus*. *Fish & shellfish immunology*, *129*, 251-262. https://doi.org/10.1016/j.fsi.2022.08.061

Zheng, J., Wang, P., Mao, Y., Su, Y., & Wang, J. (2020). Full-length transcriptome analysis provides new insights into the innate immune system of *Marsupenaeus japonicus*. *Fish & shellfish immunology*, *106*(August), 283-295. https://doi.org/10.1016/j.fsi.2020.07.018

Zhu, S., Xu, F., Zhang, J., Ruan, W., & Lai, M. (2014). Insulin-like growth factor binding protein-related protein 1 and cancer. *Clinica chimica acta*, *431*, 23-32. https://doi.org/10.1016/j.cca.2014.01.037

Zhuang, L., Tian, J., Zhang, X., Wang, H., & Huang, C. (2018). Lnc-DC regulates cellular turnover and the HBV-induced immune response by TLR9/STAT3 signaling in dendritic cells. *Cellular & molecular biology letters*, *23*(1), 43. https://doi.org/10.1186/s11658-018-0108-y

APPENDICES

Appendix 1 Oligonucleotides used in this study

Name	Forward Sequence (5´ to 3´)	Reverse Sequence (5´ to 3´)	Purposes
Actin	GCGACGTGGACATCCGTAAG	GAGCGAGGGCAGTGATTTCCT	Expression analysis
lnc164	TTCCACACCCAAAGCCATGA	GCTGTAACCACTCACGAGTCT	Expression analysis
lnc6913	TGTGGGAACGGACCATGTTT	ACGGAGCCTTGCTTGAAAGT	Expression analysis
lnc140	TCCATGTCGGAGCAGAGGTA	GGGAGTGTAGCAACCCTGTG	Expression analysis
lnc7225	GGAGGGTCGTTCCTTGTGTC	CGTCGGTCCACAAACAAACG	Expression analysis
lnc3615	GGGCGGTTCCTAGACACATC	CTGGCTTTTGAGAGCGGGTA	Expression analysis
St_164	CATGAACAAGGCTCCATGACG	TCGTGCATTGTCCTGTCAGT	ds164 production
StL_164	CATGAACAAGGCTCCATGACG	GTCCTTTCTGGGTGAGAATCGAAC	ds164 production
LvCofilin	CCGGAGTACAAGTAGCAGATG	ATATAGACCATAGCGGCACTG	Expression analysis
LvBRI3	GTAGGGTCGGTCGGTTACAA	AGAGAGCTTTGCAGCCATCA	Expression analysis
LvIGFBP-rP1	GCCTCGTCAAGAATGTGTGC	TTACCATAGGAGACGGGGTG	Expression analysis

Appendix 1 Oligonucleotides used in this study (cont.)

Name	**Forward Sequence (5´ to 3´)**	**Reverse Sequence (5´ to 3´)**	**Purposes**
LvCasp3	TGCGAGTACCAGTAACACCAG	TGAGGTCAGGGCGATCCTTA	Expression analysis
LvSPRED2	GTTCATCAAAGCCCGCTGTC	TCACATGGACGGTGAGCAAA	Expression analysis
LvNRF-6	CATTAGTGCCCAGCGAAACG	ATTGCCGTCAAGAATCCCGT	Expression analysis
Vp28	AGGTGTGGAACAACACATCAAG	TGCCAACTTCATCCTCATCA	WSSV detection

Paper I

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Long noncoding RNA profiling in hepatopancreas of white shrimp and its role in response to white spot syndrome virus infection

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Abstract:	Long noncoding RNA (lncRNA) is a potential regulator of biological processes, including immunity, reproduction, and development. Although several transcriptome studies have focused on responses of viral infections in several organisms, the role of lncRNAs in viral responses in shrimp is still unclear. Therefore, this work aimed to identify putative lncRNAs and study their role in white spot syndrome virus (WSSV) infection in white shrimp. The hepatopancreas transcriptome from WSSV infected shrimp was analyzed in silico to identify putative lncRNAs. Among 221,347 unigenes of the de novo assembled transcriptome, 44,539 putative lncRNAs were identified, 32 of which were differentially expressed between WSSV-infected and control shrimp. Five candidate lncRNAs were validated for their expressions in shrimp tissues and in response to WSSV infection. Lnc164 was chosen for further investigation of its role in WSSV infection. Knockdown of lnc164 prolonged survival of shrimp when challenged with WSSV, suggesting a role in shrimp immunity. In addition, lnc164 was not directly involved in the control of total hemocytes and viral loads in hemolymph of WSSV-infected shrimp. A set of lnc164-regulated genes was obtained by RNA sequencing among which 251 transcripts were differentially expressed between lnc164 knockdown and control shrimp. Six immune-related genes were validated for their expression profiles. Our work sheds light on lncRNA profiles in L. vannamei in response to WSSV infection and paves the way to a functional study of lnc164 in host antiviral response.
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Long noncoding RNA profiling in hepatopancreas of white shrimp and its role in response to white spot syndrome virus infection

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Abstract

Long noncoding RNA (lncRNA) is a potential regulator of biological processes, including immunity, reproduction, and development. Although several transcriptome studies have focused on responses of viral infections in several organisms, the role of lncRNAs in viral responses in shrimp is still unclear. Therefore, this work aimed to identify putative lncRNAs and study their role in white spot syndrome virus (WSSV) infection in white shrimp. The hepatopancreas transcriptome from WSSV infected shrimp was analyzed *in silico* to identify putative lncRNAs. Among 221,347 unigenes of the *de novo* assembled transcriptome, 44,539 putative lncRNAs were identified, 32 of which were differentially expressed between WSSV-infected and control shrimp. Five candidate lncRNAs were validated for their expressions in shrimp tissues and in response to WSSV infection. Lnc164 was chosen for further investigation of its role in WSSV infection. Knockdown of *lnc164* prolonged survival of shrimp when challenged with WSSV, suggesting a role in shrimp immunity. In addition, *lnc164* was not directly involved in the control of total hemocytes and viral loads in hemolymph of WSSV-infected shrimp. A set of *lnc164*-regulated genes was obtained by RNA sequencing among which 251 transcripts were differentially expressed between *lnc164* knockdown and control shrimp. Six immune-related genes were validated for their expression profiles. Our work sheds light on lncRNA profiles in *L. vannamei* in response to WSSV infection and paves the way to a functional study of *lnc164* in host antiviral response.

Keywords: Long noncoding RNA, white spot syndrome virus, shrimp, transcriptome

1. Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, has long been one of the most commercialized species in marine aquaculture worldwide [1]. Because of its importance and

value to the aquaculture industry, a disease outbreak among *L. vannamei* results in significant losses to farmers. White spot disease caused by the white spot syndrome virus (WSSV), a double stranded DNA virus, can spread very quickly in saline waters and may lead to 100% mortality [2]. Many strategies have been initiated to control WSSV in farms. Pond and feed management have been adjusted to minimize stress to shrimp and the immune defenses of d shrimp have been boosted by supplementing shrimp diets with immunity enhancers. At the same time, considerable progress has been made in the characterization of WSSV [3]. This helps farmers selectively stock only WSSV-free post-larvae and exclude the virus and potential vectors from ponds. However, there are still no effective methods or strategies to prevent WSSV outbreaks in the field. Therefore, more understanding of the shrimp immune response to WSSV infection is necessary.

Like invertebrates, shrimp lack an adaptive immune system relying instead on their innate immune system to combat invading pathogens [4-5]. Signaling pathways involved in shrimp immunity have been identified and studied, including Toll, IMD and JAK/STAT pathways studied [4, 6]. In addition, WSSV infection induces changes in metabolic pathways related to mitochondria, similar to the Warburg effect that occurs in cancer cells [7]. To support changes in these various pathways, shrimp undergo alterations in their transcriptomic profiles that cause certain genes to be up- or down-regulated in response to WSSV infection. Transcriptome analysis of expression profiles has been applied in several studies of pathogen infections in shrimp [8-10], and RNA sequencing is a powerful tool in the analysis of gene expression profiles. Recent investigations of differentially expressed genes (DEGs) in WSSV-infected and control *L. vannamei* implicated some of the DEGs in shrimp immunity [11-13].

Apart from immune-responsive genes, noncoding RNA (ncRNA) comprises a heterogenous group of genes that lack functional open reading frames (ORFs) and do not

encode protein [14]. NcRNAs can be divided into two main classes: structural ncRNAs and regulatory ncRNAs. Regulatory ncRNAs especially miRNA, have been reported for their significance in many biological processes [14]. Indeed, a large and diverse class among regulatory ncRNAs are those longer than 200 nucleotides, which are classified as long noncoding RNA (lncRNA) [14-16]. LncRNAs were recently reported to play a vital role in the regulation of innate and adaptive immune systems, including antiviral response [17]. Differentially expressed lncRNAs regulated by DNA or RNA viral infection [18] include the human lncRNAs *Morrbid* and *lnc-DC* [19-20] and the lncRNA MAVS in teleost fish [21]. In *Penaeus japonicas*, a set of lncRNAs was identified from hepatopancreas transcriptome that was differentially expressed between WSSV-infected and uninfected groups. However, the functional roles of those lncRNAs have not been elucidated [17]. In *L. vannamei* hemocytes, lncRNAs were co-expressed with immune related genes, including participation in the regulation of *L. vannamei* immune defenses [22]. In this *in silico* study, we implement RNA interference and RNA sequencing to identify, characterize, and study the function of lncRNAs involved in WSSV infection in *L. vannamei*.

2. Materials and Methods

2.1 Animal sample

Approximately 5 g of *L. vannamei* were purchased from a local shrimp farm in Songkhla, Thailand. They were acclimatized in 10 ppt seawater for two days and were given commercial feed three times a day. The standards of animal use in this study were passed by the Institutional Animal Care and Use Committee, PSU (Reference No. 73/2021). The animal protocol was in accordance with the Animals for Scientific Purposes Act, B.E. 2558 (A.D. 2015), Thailand.

2.2 Identification of WSSV infection-related lncRNAs in *L. vannamei* hepatopancreas

To identify lncRNAs potentially involved in the regulation of WSSV infection, RNA seq data from hepatopancreas of healthy and WSSV-infected *L. vannamei* at 24 h post-infection (hpi) were retrieved from the NCBI database (BioProject No. PRJNA554075) [12]. The raw reads were checked for their quality with the FastQC program, and adaptors and low-quality sequences were removed using the Trimmomatic program [23]. Using Trinity package (version 2.9.1) with default parameters, the clean reads were used to generate a *de novo* assembled transcriptome [24]. The transcripts were removed and clustered for their redundant sequences with CD-HIT-EST program [25]. The quality of the assembled transcriptome was determined by BUSCO and read representation. To identify putative lncRNAs, the transcriptome data was in-house analyzed by BLASTx and BLASTn against the arthropod database from NCBI, *L. vannamei* genome and RNA (RefSeq Accession No. GCF_003789085.1). Matched transcripts were removed. The filtered transcripts were checked against the rfam and miRBase databases, and non-related non-coding RNAs were removed. Finally, putative lncRNAs in the remaining transcripts were predicted by the Coding Potential Assessment Tools (CPAT). The predicted putative lncRNAs were then *in silico* analyzed for differential expression in response to WSSV infection. The clean reads were aligned to the *de novo* assembled transcriptome by Bowtie2 [26], mapped reads were counted by RSEM [27]. DEGs were analyzed by edgeR with FDR < 0.01, p-value < 0.05, and |log2(fold-change)| > 1 [28], and then identified using the method described above for putative lncRNAs.

2.3 White spot syndrome virus challenge

Shrimp were intramuscularly injected with 50 μl of a diluted WSSV that killed 50% of shrimp on day 2-3, or with 150 mM NaCl. At 12, 24, and 48 h post injection, hepatopancreas and gills were collected for RNA and DNA extraction, respectively. Five shrimp were used in each group. Total RNA was extracted using TRIzol® reagent (Ambion)

following the manufacturer's instructions. Quantity and quality of nucleic acids were determined by Nanodrop (Thermo Scientific) and agarose gel electrophoresis. To confirm WSSV infection, gill DNA was extracted by a conventional phenol/chloroform method. The gill DNA was used as a template in a PCR reaction with the VP28 gene specific primers [29]. Fifty nanograms of DNA were added to a PCR mixture of 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM primer pair, and 1 U of *Taq* DNA polymerase (Vivantis). The initial PCR temperature was 95°C for 2 min, which was followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. Beta-actin was used as an internal control. The primer sequences were shown in Table 1.

2.4 First-strand cDNA synthesis

Total RNA was extracted from muscle, brain, gill, epidermis, eyestalk, and hepatopancreas isolated from three *L. vannamei* individuals. One microgram of total RNA was treated with DNase I in a mixture of 1X DNase I buffer and 1 U of DNase I (Thermo Scientific). The mixture was incubated at 37°C for 30 min, and then 0.5 µl of 50 mM EDTA was added. The sample was heated at 65°C for 5 min and then placed on ice. The RNA sample was then added to a mixture of 1X reverse transcriptase buffer, 0.2 mM dNTP, 0.2 µM oligo-dT primer, and 100 U of M-MuLV reverse transcriptase (Vivantis). The temperature profile was 25°C for 5 min, 42°C for 60 min, and 70°C for 10 min.

2.5 Determination of gene expression by quantitative real-time PCR (qRT-PCR)

The candidate lncRNA expressions were determined by qRT-PCR using a 1/10 dilution of the first-strand cDNA as a template. The template was mixed with 1X Maxima SYBR Green qPCR Master Mix (Thermo Scientific) and 0.2 µM primer pair. The mixture was heated at 95 °C for 10 min, followed by 40 cycles of 95°C for 10 s and 60 °C for 30 s (CFX96, Biorad). After the qRT-PCR step, a melting curve was generated by heating the product at 95°C for 1 min, 60°C for 1 min, and 95°C for 1 min. Beta-actin was used as an

internal control The relative expressions of lncRNA were calculated using the $2^{-\Delta\Delta CT}$ method [30]. The list of primers used was shown in Table 1.

2.6 Cloning and expression of dsRNA specific to lncRNA

DNA fragments of a selected lncRNA, lnc164, were amplified to generate stem and stem-loop fragments using the respective primer pairs st164-F/st164-R and stL164-F/stL164-R tagged with specific restriction enzyme sites (Table 1). Hepatopancreas cDNA was used as a template in a conventional PCR reaction. The DNA fragments were cloned into an expression vector, pET28 plasmid, to obtain an inverted repeat sequence of lnc164. After confirming the nucleotide sequence by DNA sequencing, the recombinant plasmid was then transformed in *Escherichia coli* strain HT115.

To express dsRNA, the bacteria was cultured in LB broth containing 50 µg/ml kanamycin at 37 °C for 14-16 h. The bacterial starter was then inoculated in 2XYT medium at 37 °C, 250 rpm, until OD_{600} reached 0.4-0.8. The expression of dsRNA was induced by adding IPTG to a final concentration of 0.2 mM and shaking continued for 2-4 h. The bacterial cells were then harvested, and the dsRNA products were extracted by the ethanol method [31].

2.7 *In vivo* silencing of *lnc164* with dsRNA

To determine the effect of ds164 on *lnc164* expression, approximately 3-4 g shrimp were injected with 5 µg/g body weight of either ds164 or dsGFP. On days 1, 2 and 3 post-injection, hepatopancreas was used to determine *lnc164* expression by qRT-PCR. Three shrimp were used in each group.

2.8 Effect of *lnc164* knockdown on shrimp mortality after WSSV infection

Shrimp were divided into three groups and injected with 5 µg/g body weight of ds164, dsGFP, or 150 mM NaCl. After 24 h, shrimp from each group were injected with WSSV or

150 mM NaCl. Ten shrimp were used in each subgroup. The number of dead shrimp was recorded daily for seven days. Two independent experiments were carried out.

2.9 RNA sequencing and data analysis

Hepatopancreas from shrimp injected with ds164, dsGFP, and NaCl were extracted for their total RNA two days post-injection. Expression of *lnc164* in each group were verified by qRT-PCR. After treating total RNA with DNase I, samples were analyzed by RNA sequencing. Using a stranded specific mRNA library kit, libraries were prepared from samples in each group and then sequenced on a DNBseq platform (BGI Genomics, Hongkong). The clean reads were used to generate a *de novo* assembled transcriptome with the Trinity program, and DEG was analyzed as described above. The DEGs were identified by BLAST and eggNOG mapper [32], and were functionally annotated by KEGG, GO, and COG.

2.10 Validation of DEG in response to *lnc164* knockdown and DEG expressions in WSSV infection

DEGs were investigated for their roles in immune response and metabolic processes. The selected candidate DEGs were those with the highest differential expression between *lnc164* knockdown and control samples. Shrimp were divided into three groups: 5 µg/g ds164, 5 µg/g dsGFP, or 150 mM NaCl. On day 2, hepatopancreas was isolated and used to determine expressions of *lnc164*-regulated genes by qRT-PCR. Five shrimp were used in each group.

2.11 Total hemocyte count

Shrimp were injected with ds164, dsGFP, or NaCl. After 24 h, shrimp were injected with WSSV or 150 mM NaCl. Hemolymph was collected by a syringe and deposited in an anticoagulant solution (10 mM Tris pH 8.0, 250 mM sucrose, 50 mM EDTA, and 100 mM sodium citrate). Ten microliters of hemolymph were mixed with 10 µl of trypan blue (Gibco),

and 10 µl of the mixture were loaded into a hematocytometer. Live and death cells were counted under a light microscope, and the total hemocytes count (THC) was calculated based on Mani's procedure [33].

2.12 Viral loads

Gill DNA sample was extracted by a conventional phenol/chloroform method and 50 ng of DNA were used as the template in a realtime PCR reaction as described in Section 2.5. The purified Vp28 DNA fragment generated by PCR using the specific primer pair (Table 1) was analyzed for its copy number and used to generate a standard curve. Viral loads were determined using qRT-PCR. Four shrimp were used in each group.

2.13 Statistical analysis

All data were statistically analyzed by t-test or one-way ANOVA and pairwise comparison by Duncan's test.

3. Results

3.1 Identification of WSSV infection-related lncRNA to in *L. vannamei* hepatopancreas

RNA seq data from hepatopancreas of healthy and WSSV-infection shrimp [12] generated a total of 221,347 unigenes from the *de novo* assembled transcriptome. The GC content of the transcriptome was 43.05%, N50 was 893 bp, and assembly completeness 97% (Table 2). Among the unigenes, 44,539 transcripts were identified as putative lncRNAs. The DEG analysis between control and WSSV-infected groups revealed that expressions of 277 unigenes were significantly altered of which 145 were upregulated and 132 were downregulated after WSSV infection. Among the 277 DEGs, only 32 transcripts were differentially expressed lncRNAs: 21 upregulated and 11 downregulated (Fig. 1). Five candidates were selected from the most significantly altered lncRNAs in the WSSV-infected group (Table 3). Among these, lnc164, lnc140, and lnc7225 were matched to intergenic non-

coding transcripts in the *L. vannamei* genome, whereas lnc3615 and lnc6913 were not found in the databases.

3.2 Validation of candidate lncRNA expressions in response to WSSV infection

Shrimp were injected with WSSV or 1X PBS pH 7.4, and their hepatopancreas were used to determine candidate lncRNA expressions by qRT-PCR. The results showed that the expression of *lnc164* downregulated at 24 and 48 h post-WSSV infection (hpi) (Fig. 2A). The expression levels of *lnc6913* and *lnc140* were also significantly lower in WSSV-infected shrimp at 24 hpi (Fig. 2B and C). The expression of *lnc7225* was significantly lower in WSSV-infected shrimp at 12 hpi but elevated at 24 and 48 hpi compared with the control shrimp (Fig. 2D). In contrast, the expression of *lnc3615* was significantly upregulated in WSSV-infected shrimp at 24 and 48 hpi (Fig. 2E).

3.3 Determination of lncRNA expressions in *L. vannamei* tissues

The expressions of the five candidate lncRNAs were also determined in muscle, brain, gills, epidermis, eyestalk, and hepatopancreas from the healthy shrimp. The results indicated that *lnc164* was expressed strongly only in hepatopancreas and less in other tissues (Fig. 3A). The expressions of *lnc6913*, *lnc140*, and *lnc7225* were found in all tested tissues (Fig. 3B-D). In contrast, the expression of *lnc3615* was mainly found only in muscle and epidermis, and at very low levels in other tissues (Fig. 3E).

3.4 Determination of ds164 efficiency to *lnc164* knockdown in shrimp

The functional role of lncRNAs in WSSV infection was investigated by studying the effects of *lnc164* knockdown because expression of the gene was significantly downregulated in response to WSSV infection (Fig. 2A) and dominant in the hepatopancreas (Fig. 3A). Double-stranded RNA specific to *lnc164* (ds164) was successfully produced in *E. coli* and was injected into shrimp to examine gene silencing activity. The expression level of *lnc164* in

hepatopancreas of shrimp injected with ds164 was approximately 78% lower at 24 hpi and 93% lower at 48 hpi, compared with dsGFP-injected shrimp (Fig. 4A).

3.5 Suppression of *lnc164* prolonged the survival of shrimp infected with WSSV

Shrimp were injected with ds164 24 h before being injected with WSSV. The results showed that 10% of shrimp injected with NaCl followed by WSSV (NaCl/WSSV) were dead at 18 hpi. Their mortality reached 50% at 69 hpi and 100% and 102 hpi (Fig. 4B). All shrimp injected with dsGFP prior to WSSV challenge (dsGFP/WSSV) survived to 42 hpi. Then, 5% of shrimp were dead at 30 hpi, 50% at 78 hpi, and 100% at 114 hpi. Interestingly, all *lnc164* silenced shrimp injected with WSSV (ds164/WSSV) survived to 30 hpi. Mortality was 50% at 87.5 hpi, and 100% at 120 hpi (Fig. 4B). While no shrimp dead was found in the control groups until approximately 60 hpi, and only 10% mortality were found including shrimp injected with NaCl, dsGFP, or ds164 without WSSV injection.

3.6 Effect of *lnc164* knockdown on total hemocyte counts and viral loads after WSSV infection

To investigate the effect of *lnc164* knockdown on THC and viral loads after WSSV infection, shrimp were injected with ds164 or 150 mM NaCl 24 h before being injected with WSSV. At 24 hpi, THCs were decreased in all ds164- and NaCl-injected shrimp compared to their controls (Fig. 4C). There was no different between shrimp injected with WSSV and not injected with WSSV (Fig. 4C). In contrast, viral loads were significantly reduced by approximately 88% and 85% in dsGFP- and ds164-injected shrimp, respectively, compared with control. In non-infected groups, viral loads were undetected (Fig. 4D).

3.7 Transcriptomic profiles upon *lnc164* depletion in *L. vannamei*'s hepatopancreas

To explore the set of genes regulated by *lnc164*, hepatopancreas of the *lnc164* knockdown shrimp was analyzed by RNA sequencing. The generated transcriptome comprised 166,906 non-redundant transcripts with a GC content of 42.92%, N50 of 1,727 bp,

an assembly completeness of 98%, and 96.20% read representation (Table 4). Analysis of DEG in the *lnc164* depleted shrimp found 251 differentially expressed transcripts among groups (Supplementary material 1). The patterns of DEGs were visualized in the form of a heatmap (Fig. 5A). Among the DEG patterns of *lnc164* knockdown shrimp, nine transcripts were significantly upregulated and 12 were downregulated compared to NaCl and dsGFP groups (Fig. 5B).

Functional annotation of DEGs was analyzed by GO, KEGG, and COG. The GO result revealed that most DEGs located within cell, cell part, and organelle and were mostly involved with binding and catalytic activity. In the biological processes, cellular process, metabolic process, biological regulation, and regulation of biological process were most annotated (Fig. 5C). In the KEGG-enriched functional processes, most DEGs were shown to be involved in metabolic pathways, fewer in nucleocytoplasmic transport and endocytosis. (Fig. 5D). Among COG categories, most DEGs exhibited unknown functions, while the next biggest categories were post-translational modification, protein turnover, chaperone functions, transcription, and signal transduction (Fig. 5E).

3.8 Determination of gene expressions in response to *lnc164* depletion

We selected the metabolic and immune-related genes to determine their expressions in *lnc164* knockdown shrimp. Based on the transcription profiles and functional annotation of the DEGs, insulin-like growth factor-binding protein-related protein 1 (*IGFBP-rP1*), brain protein I3 (*BRI3*), caspase-3-like (*Casp3*), sprout-related EVH1 domain-containing protein 2-like (*SPRED2*), nose resistant to fluoxetine protein 6-like (*NRF-6*), and cofilin/actin-depolymerizing factor (*Cofilin*) were chosen (Table 5). The results showed that expression of *IGFBP-rP1* was significantly increased compared to control in both dsGFP- and ds164-injected shrimp (Fig. 6A), while *BRI3* and *Casp3* expression levels were significantly increased only in *lnc164* knockdown shrimp (Fig. 6B and C). In contrast, expressions of

297 *SPRED2* and *NRF-6* were significantly down-regulated in ds164-injected shrimp compared
298 with controls (Fig. 6D and E). However, there was no significant change in the expression of
299 *Cofilin* among the groups (Fig. 6F).

300 **3.9 Determination of gene expression in hepatopancreas upon WSSV infection**

301 Five immune-related genes that were responsive to knockdown of *lnc164* were further
302 examined for their response to WSSV infection. The results indicated that expressions of
303 *IGFBP-rP1*, *Casp3*, and *NRF-6* were significantly decreased at 24 hpi (Fig. 7A-C), whereas
304 the expression of *SPRED2* was significantly elevated upon infection (Fig. 7D). However, no
305 differential expression of *BRI3* was observed between groups (Fig. 7E).

306 **4. Discussion**

307 To support changes in several pathways involved in anti-viral activities, shrimp
308 undergo alteration in their transcriptomic profiles that affect many genes. Apart from coding
309 genes and regulatory miRNAs, lncRNAs are the largest group of ncRNAs and play regulatory
310 roles in cellular and physiological processes [34]. Recent transcriptome studies have
311 identified lncRNAs as important regulators that respond to viral infections and regulate the
312 development and progression of infectious diseases [18,36]. In this study, 44,539 putative
313 lncRNAs were identified from a *de novo* assembled transcriptome derived from
314 hepatopancreas of healthy and WSSV-infected shrimp. Only 32 lncRNAs were differentially
315 induced after WSSV infection (Table 2). Other studies have produced similar findings. For
316 instance, 8,077 putative lncRNAs were found in *L. vannamei* hemocytes, and 1,168 lncRNAs
317 were differentially expressed after infection with *Spiroplasma eriocheiris* [22]. Recently,
318 6,544 lncRNAs were identified in *P. japonicus* hepatopancreas, and 457 were differentially
319 expressed after WSSV infection [17]. In a study of zebrafish, 12,165 putative lncRNAs were
320 identified and 709 lncRNAs were significantly induced by infection with the spring viraemia
321 of carp virus [36].

In our study, the expressions of five candidate lncRNAs during WSSV infection were investigated. The results revealed that the expressions of *lnc164*, *lnc6913,* and *lnc140* were downregulated, while those of *lnc3615* and *lnc7225* were upregulated (Fig. 2). In previous studies, putative lncRNAs were differentially expressed in *P. japonicus* infected with WSSV [17] and *L. vannamei* infected with *S. eriocheiris* [22]. Several studies of other organisms reported the post-viral infection modulation of lncRNAs in both innate and adaptive immune systems. For example, during the early stages of acute and chronic infection with the lymphocytic choroidal meningitis virus, the human lncRNA *Morrbid* was induced by stimulation of the T cell receptor and type I IFN [19]. In teleost fish, the lncRNA MARL interacted with miR-122 to regulate the mitochondrial antiviral signaling protein (MAVS)-mediated retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) pathway [21]. These studies indicated the involvement of lncRNA in response to infectious pathogens, especially in shrimp infected with WSSV.

The expression of lncRNAs has been reported to be tissue-specific [15] but many lncRNAs found in various organs [37]. In this study, *lnc164* was mainly expressed only in hepatopancreas (Fig. 3A), whereas other candidate lncRNAs (*lnc6913*, *lnc140*, *lnc7225*, and *lnc3615*) were expressed in all tested tissues (Fig. 3B-E). However, compared to protein-coding genes, the expression of lncRNA is more restricted to specific cell types. For instance, in rhesus macaques, the expression of lncRNA induced by Ebola virus infection was dynamic and mostly presented in specific cell type, especially monocytes, which are the main target of the Ebola virus [38]. LncRNA *PCGEM1* expression is found only in the human prostate [39], and some lncRNAs involved in the molt process have been found in gills and epidermis of white shrimp [40]. In contrast, the broad expression of lncRNAs in multiple tissues simultaneously is thought to play roles in chromosome compaction and transcriptional regulation [41]. Herein, the *lnc164* was investigated for its roles in the WSSV infection of

shrimp due to the significance of its downregulation after WSSV infection and its specific expression in hepatopancreas.

The role of *lnc164* in response to WSSV infection in shrimp was investigated by RNA interference using a double-stranded RNA. The effect of *lnc164* depletion on shrimp mortality upon WSSV infection was investigated. The result indicated that knockdown of *lnc164* prolonged the survival of WSSV-infected shrimp (Fig. 4B). Thus, the *lnc164* somehow facilitated the WSSV infection, perhaps by blocking pathways involved in shrimp antiviral activity. Indeed, since transcript levels of *lnc164* were diminished after WSSV infection, the negative effect of *lnc164* on shrimp immune defense against WSSV might be inhibited by certain immune-related pathways (Fig. 2A). Our investigation of THC and viral loads after *lnc164* knockdown and challenged with WSSV showed that THC was lower in infected shrimp but that there was no difference among tested groups (Fig. 4C). Similarly, circulating hemocytes in *P. japonicus* and *Fenneropenaeus chinensis* were reduced following WSSV infection [42,43]. Our results therefore indicated that lnc164 was not involved in the control of THC. In contrast, viral loads were significantly reduced in both dsGFP- and ds164-injected shrimp under WSSV challenge (Fig. 5D). A prior study of *L. vannamei* observed that copies of WSSV were lower in gill, hepatopancreas, and muscle of shrimp injected with dsGFP compared with shrimp injected with PBS [44]. Our results indicated that the lower viral loads in ds164-injected shrimp were due to dsRNA molecules.

The transcriptomic profiles of *lnc164*-depleted *L. vannamei* hepatopancreas returned a total of 251 DEGs in the *de novo* assembled transcriptome. Functional annotation analysis of DEGs by KEGG and GO revealed that those genes were mostly involved with metabolic processes and some with sugar metabolism and the TCA cycle (Fig. 5C and D). In viral pathogenicity, one of the most studied aspects is metabolic reprogramming, which is a critical virus-host interaction [45]. The metabolic changes modulated by WSSV affects glycolysis,

372 the TCA cycle, glutaminolysis and lipid metabolism in the host [46]. Hence, the modulation
373 of *lnc164* under WSSV challenge could occur via interactions with one or more metabolic
374 pathways. In this study, COG annotated DEGs to lipid, amino acid, and nucleotide
375 metabolisms (Fig. 5E), which were identified as characteristics of the metabolic
376 reprogramming modulated by WSSV [46]. GO and COG enrichment analyses showed that
377 depletion of *lnc164* affected genes associated with immune system processes and defense
378 mechanisms, respectively (Fig. 5C and E), highlighting the immune defense roles of lnc164.
379 From the total DEGs, six immune responsive genes were validated for their
380 expressions upon knockdown of *lnc164*. The expressions of five candidate genes were
381 significantly altered by *lnc164* depletion (Fig. 6). We found that the level of *IGFBP-rP1* was
382 significantly down-regulated after WSSV infection but was stimulated when *lnc164* was
383 depleted, regardless of the *lnc164* function. In humans, *IGFBP-rP1* plays a role as a tumor
384 suppressor, mediated by the insulin/IGF-1 signaling pathway [47-48], but the involvement of
385 *IGFBP-rP1* in viral infection is rarely seen in any organisms. It remains to be seen whether
386 the upregulation of *IGFBP-rP1* by *lnc164* knockdown controls metabolic processes that
387 inhibit WSSV infection. The expression of *NRF-6* was inhibited by the knockdown of *lnc164*
388 and WSSV challenge in this study (Fig. 6E and 7C). Although there is no evidence of *NRF-6*
389 function in arthropods, it has been reported that *NRF-6* in *C. elegans* plays a role in the up
390 take from the intestine to surrounding tissues of a range of molecules that includes lipids and
391 xenobiotic compounds, and in the transport of lipids from the intestine to the reproductive
392 tract [49]. Therefore, the downregulation of *NRF-6* by suppression of *lnc164* might also
393 interrupt the transport of WSSV through shrimp tissues and modulate WSSV replication.
394 In hos-virus competition, the host induces apoptosis to eliminate infected cells and to
395 stop the diffusion of viral progeny toward neighboring cells, while viruses adapt to evade or
396 interrupt apoptosis [50-52]. Caspases are widely studied in various species due to their role as

central regulators in apoptosis [53-54]. In WSSV-infected shrimp, the silencing of *L. vannamei* caspase-3 [55] and *Marsupenaeus japonicus* caspase-3 [56] resulted in increased WSSV loads, suggesting protective roles for caspase-3 in the defense against WSSV infection. In this study, *Casp3* was stimulated by the suppression of *lnc164* expression (Fig. 6C) but significantly downregulated 24 hpi with WSSV (Fig. 7B). This result suggested that an increase in apoptotic processes via caspase-3 activity might be involved in the prolonged survival of *lnc164* depleted shrimp. In this study, the expression of *SPRED2* was upregulated upon *lnc164* knockdown and WSSV infection (Fig. 6D and 7D), suggesting that lnc164 inhibits SPRED2. *SPRED2* plays a role in cancer regulation in human hepatocellular carcinoma cells [57] and the overexpression of *SPRED2* induced the activation of caspase-3 and apoptosis in both human hepatocellular carcinoma cells [57] and retinal endothelial cells [58]. The findings of these studies might imply that the downregulation of *lnc164* expression under WSSV challenge (Fig. 2A) leads to the upregulation of *SPRED2* expression and accelerates the activation of caspase and apoptosis. In L929 cells, BRI3 has been reported for its function in promoting tumor necrosis factor (TNF)-induced cell death [59]. In addition, BRI3 is one of targets in the Wnt/beta-catenin signaling pathway [60], which controls several physiological processes, including development and immune responses [61]. The silencing of *lnc164* increased *BRI3* expression (Fig. 6B), suggesting the negative regulation of BRI3. Whether or not the regulation of BRI3 by lnc164 is involved in the stimulation of Wnt/beta-catenin signaling and TNF-induced cell death in shrimp is matter for further study. The findings of this study elucidated lncRNA expression profiles involved in response to WSSV infection. They also revealed the relationship between *lnc164* expression and WSSV infection and advance our understanding of the interactions between *lnc164* and immune-related genes in the innate immunity of shrimp.

5. Conclusions

In this study, 44,539 putative lncRNAs were identified from a *de novo* assembled transcriptome derived from hepatopancreas of healthy and WSSV-infected shrimp. Only 32 lncRNAs were differentially induced after WSSV infection. Five candidate lncRNAs were chosen for validation of their expressions in various shrimp tissues and during WSSV infection. The gene lnc164, that was predominantly expressed in hepatopancreas, was downregulated during WSSV infection. A functional study of lnc164 was carried out using RNA interference by ds164 injection. The knockdown of *lnc164* delayed the mortality of shrimp infected with WSSV. Both viral loads and total hemocyte counts were changed after WSSV infection but only between control and *lnc164*-depleted shrimp were no significant differences observed. RNA sequencing was performed to explore *lnc164* responsive genes. In *lnc164*-depleted samples, we found 251 differentially expressed transcripts, most of which were involved in metabolic processes and immune response. Six of the DEGs were validated for their expression upon *lnc164* knockdown. This study provided lncRNA profiles of *L. vannamei* and shed light on the interactions between *lnc164* and WSSV in the innate immune response of shrimp.

Data Availability Statement

The RNA sequencing data analyzed for lncRNAs identification (BioProject No. PRJNA554075) can be found at https://www.ncbi.nlm.nih.gov/bioproject/ PRJNA554075. The raw reads from RNA sequencing data (PRJNA995044) generated from the *lnc164* knockdown experiment were deposited at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA995044.

Declaration of competing interest

None

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References

[1] M. Gucic, E. Cortés-Jacinto, R. Civera-Cerecedo, D. Ricque-Marie, L. R. Martínez-Córdova, Apparent carbohydrate lipid digestibility of feeds for whiteleg shrimp, *Litopenaeus vannamei* (Decapoda: Penaeidae), cultivated at different salinities, Rev. Biol. Trop. 61 (2013) 1201-1213. https://doi.org/10.15517/rbt.v61i3.11935.

[2] C. A. Santos, S. C. S. Andrade, A. K. Teixeira, F. Farias, K. Kurkjian, A. C. Guerrelhas, J. L. Rocha, P. M. Galetti, Jr, P. D. Freitas, *Litopenaeus vannamei* transcriptome profile of populations evaluated for growth performance exposed to white spot syndrome virus (WSSV), Front. Genet. 9 (2018) 120. https://doi.org/10.3389/fgene.2018.00120.

[3] B. Verbruggen, L. K. Bickley, R. van Aerle, K. S. Bateman, G. D. Stentiford, E. M. Santos, C. R. Tyler, Molecular mechanisms of white spot syndrome virus infection perspectives on treatments, Viruses 8 (2016) 23. https://doi.org/10.3390/v8010023.

[4] S. Roy, P. Bossier, P. Norouzitallab, D. Vanrompay, Trained immunity perspectives for shrimp aquaculture, Rev. Aquac. 12 (2020) 2351-2370. https://doi.org/10.1111/raq.12438.

[5] J. Zheng, P. Wang, Y. Mao, Y. Su, J. Wang, Full-length transcriptome analysis provides new insights into the innate immune system of *Marsupenaeus japonicus*, Fish Shellfish Immunol. 106 (2020) 283-295. https://doi.org/10.1016/j.fsi.2020.07.018.

[6] A. Tassanakajon, P. Amparyup, K. Somboonwiwat, P. Supungul, Cationic

antimicrobial peptides in penaeid shrimp, Mar. Biotechnol. (NY). 12 (2010) 487-505. https://doi.org/10.1007/s10126-010-9288-9.

[7] I. T. Chen, T. Aoki, Y. T. Huang, I. Hirono, T. C. Chen, J. Y. Huang, G. D. Chang, C. F. Lo, H. C. Wang, White spot syndrome virus induces metabolic changes resembling the warburg effect in shrimp hemocytes in the early stage of infection, J. Virol. 85 (2011) 12919-12928. https://doi.org/10.1128/jvi.05385-11.

[8] Z. Cao, Y. Gao, J. Xu, N. Yang, T. Li, L. Chang, L. Si, D. Yan, Transcriptome analysis of the hepatopancreas in *Penaeus vannamei* under experimental infection with *Enterocytozoon hepatopenaei* (EHP), Fish Shellfish Immunol. 134 (2023) 108605. https://doi.org/10.1016/j.fsi.2023.108605.

[9] M. Miao, S. Li, Y. Liu, Y. Yu, F. Li, Transcriptome analysis on hepatopancreas reveals the metabolic dysregulation caused by *Vibrio parahaemolyticus* infection in *Litopenaeus vannamei*, Biology 12 (2023) 417. https://doi.org/10.3390/biology12030417.

[10] Z. Wu, K. H. Chu, K. Y. Ma, Transcriptome analysis of multiple tissues in the shrimp *Penaeus vannamei* reveals the typical physiological response to three pathogens, J. Mar. Sci. Eng. 11 (2023) 389. https://doi.org/10.3390/jmse11020389.

[11] R. S. Millard, L. K. Bickley, K. S. Bateman, A. Farbos, D. Minardi, K. Moore, S. H. Ross, G. D. Stentiford, C. R. Tyler, R. van Aerle, E. M. Santos, Global mRNA miRNA analysis reveal key processes in the initial response to infection with WSSV in the pacific whiteleg shrimp, Viruses 13 (2021) 1140. https://doi.org/10.3390/v13061140.

[12] L. Peruzza, S. Thamizhvanan, S. Vimal, S., K. Vinaya Kumar, M. S. Shekhar, V. J. Smith, C. Hauton, K. K. Vijayan, A. S. Sahul Hameed, A comparative synthesis of transcriptomic analyses reveals major differences between WSSV-susceptible *Litopenaeus vannamei* WSSV-refractory *Macrobrachium rosenbergii*, Dev. Comp.

Immunol. 104 (2020) 103564. https://doi.org/10.1016/j.dci.2019.103564.

[13] F. Wang, S. Li, J. Xiang, F. Li, Transcriptome analysis reveals the activation of neuroendocrine-immune system in shrimp hemocytes at the early stage of WSSV infection, BMC Genomics 20 (2019) 247. https://doi.org/10.1186/s12864-019-5614-4.

[14] V. Aliperti, J. Skonieczna, A. Cerase, Long non-coding RNA (lncRNA) roles in cell biology, neurodevelopment neurological disorders, Non-coding RNA 7 (2021) 36. https://doi.org/10.3390/ncrna7020036.

[15] M. C. Bridges, A. C. Daulagala, A. Kourtidis, 2021. LNCcation: lncRNA localization function. J. Cell Biol.. 220, e202009045. https://doi.org/10.1083/JCB.202009045.

[16] M. Kazimierczyk, M. K. Kasprowicz, M. E. Kasprzyk, J. Wrzesinski, Human long noncoding RNA interactome: detection, characterization function, Int. J. Mol. Sci. 21 (2020) 1027. https://doi.org/10.3390/ijms21031027.

[17] Y. Zhang, N. Yao, C. Zhang, X. Sun, J. Huang, B. Zhao, H. Li, LncRNA-mRNA integrated profiling analysis in response to white spot syndrome virus in hepatopancreas in *Penaeus japonicus*, Fish Shellfish Immunol. 129 (2022) 251-262. https://doi.org/10.1016/j.fsi.2022.08.061.

[18] W. Liu C. Ding, Roles of lncRNAs in viral infections, Front. Cell. Infect. Microbiol. 7 (2017) 205. https://doi.org/10.3389/fcimb.2017.00205.

[19] J. J. Kotzin, S. P. Spencer, S. J. McCright, D. B. U. Kumar, M. A. Collet, W. K. Mowel, E. N. Elliott, A. Uyar, M. A. Makiya, M. C. Dunagin, C. C. D. Harman, A. T. Virtue, S. Zhu, W. Bailis, J. Stein, C. Hughes, A. Raj, E. J. Wherry, L. A. Goff, A. D. Klion, J. L. Rinn, A. Williams, R. A. Flavell, J. Henao-Mejia, The long non-coding RNA Morrbid regulates Bim short-lived myeloid cell lifespan. Nature 537 (2016) 239-243. https://doi.org/10.1038/nature19346.

[20] L. Zhuang, J. Tian, X. Zhang, H. Wang, C. Huang, Lnc-DC regulates cellular turnover

the HBV-induced immune response by TLR9/STAT3 signaling in dendritic cells, Cell. Mol. Biol. Lett. 23 (2018) 43. https://doi.org/10.1186/s11658-018-0108-y.

[21] Q. Chu, T. Xu, W. Zheng, R. Chang, L. Zhang, 2020. Long noncoding RNA MARL regulates antiviral responses through suppression miR-122-dependent MAVS downregulation in lower vertebrates. PLoS Pathog. 16, e1008670. https://doi.org/10.1371/journal.ppat.1008670.

[22] Y. Ren, J. Li, L. Guo, J. N. Liu, H. Wan, Q. Meng, H. Wang, Z. Wang, L. Lv, X. Dong, W. Zhao, Q. Zeng, J. Ou, Full-length transcriptome long non-coding RNA profiling of whiteleg shrimp *Penaeus vannamei* hemocytes in response to *Spiroplasma eriocheiris* infection, Fish Shellfish Immunol. 106 (2020) 876-886. https://doi.org/10.1016/j.fsi.2020.06.057.

[23] A. M. Bolger, M. Lohse, B. Usadel, Trimmomatic: A flexible trimmer for Illumina sequence data, Bioinformatics 30 (2014) 2114-2120. https://doi.org/10.1093/bioinformatics/btu170.

[24] M. G. Grabherr, B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. Di Palma, B. W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol. 29 (2011) 644-652. https://doi.org/10.1038/nbt.1883.

[25] L. Fu, B. Niu, Z. Zhu, S. Wu, W. Li, CD-HIT: Accelerated for clustering the next-generation sequencing data, Bioinformatics 28 (2012) 3150-3152. https://doi.org/10.1093/bioinformatics/bts565.

[26] B. Langmead S. L. Salzberg, Fast gapped-read alignment with Bowtie 2, Nat. Methods 9 (2012) 357-359. https://doi.org/10.1038/nmeth.1923.

546 [27] B. Li C. N. Dewey, RSEM: Accurate transcript quantification from RNA-seq data with
547 or without a reference genome, BMC Bioinf. 12 (2011) 323.
548 https://doi.org/10.1186/1471-2105-12-323.
549 [28] M. D. Robinson, D. J. McCarthy, G. K. Smyth, edgeR: A Bioconductor package for
550 differential expression analysis of digital gene expression data, Bioinformatics 26
551 (2009) 139-140. https://doi.org/10.1093/bioinformatics/btp616.
552 [29] F. Mendoza-Cano A. Sánchez-Paz, Development validation of a quantitative real-time
553 polymerase chain assay for universal detection of the white spot syndrome virus in
554 marine crustaceans, Virol. J. 10 (2013) 186. https://doi.org/10.1186/1743-422X-10-
555 186.
556 [30] T. D. Schmittgen K. J. Livak, Analyzing real-time PCR data by the comparative C(T)
557 method, Nat. Protoc. 3 (2008) 1101-1108. https://doi.org/10.1038/nprot.2008.73.
558 [31] P. Posiri, C. Ongvarrasopone, S. Panyim, A simple one-step method for producing
559 dsRNA from *E. coli* to inhibit shrimp virus replication, J. Virol. Methods 188 (2013)
560 64-69. https://doi.org/10.1016/j.jviromet.2012.11.033.
561 [32] C. P. Cantalapiedra, A. Herņandez-Plaza, I. Letunic, P. Bork, J. Huerta-Cepas,
562 eggNOG-mapper v2: Functional annotation, orthology assignments, domain prediction
563 at the metagenomic scale, Mol. Biol. Evol. 38 (2021) 5825-5829.
564 https://doi.org/10.1093/molbev/msab293.
565 [33] S. Mani, S. Mullaivanam Ramasamy, S. Chakrapani, A. Krishna, S. A. PS, C.
566 Lalramchhani, J. Antony, A. Panigrahi, The effect of natural artificial periphytic
567 substrates with biofloc system on shrimp *Penaeus vannamei* (Boone 1931) culture:
568 growth immune response, Aquac. Int. 29 (2021) 651-668.
569 https://doi.org/10.1007/s10499-021-00646-0.
570 [34] J. A. Oo, R. P. Brandes, M. S. Leisegang, Long non‐coding RNAs: Novel regulators

of cellular physiology function, Pflug. Arch. Eur. J. Physiol. 474 (2022) 191-204. https://doi.org/10.1007/s00424-021-02641-z.

[35] X. Peng, L. Gralinski, C. D. Armour, M. T. Ferris, M. J. Thomas, S. Proll, B. G. Bradel-Tretheway, M. J. Korth, J. C. Castle, M. C. Biery, H. K. Bouzek, D. R. Haynor, M. B. Frieman, M. Heise, C. K. Raymond, R. S. Baric, M. G. Katze, 2010. Unique signatures of long noncoding RNA expression in response to virus infection altered innate immune signaling. Mbio. 1, e00206-10. https://doi.org/10.1128/mBio.00206-10.

[36] V. Valenzuela-muñoz, P. Pereiro, M. Álvarez-rodríguez, C. Gallardo-escárate, A. Figueras, B. Novoa, Comparative modulation of lncRNAs in wild-type rag1-heterozygous mutant zebrafish exposed to immune challenge with spring viraemia of carp virus (SVCV), Sci. Rep. 9 (2019) 14174. https://doi.org/10.1038/s41598-019-50766-0.

[37] C. Jiang, Y. Li, Z. Zhao, J. Lu, H. Chen, N. Ding, G. Wang, J. Xu, X. Li, Identifying functionally characterizing tissue-specific ubiquitously expressed human lncRNAs, Oncotarget 7 (2016) 7120-7133. https://doi.org/10.18632/oncotarget.6859.

[38] L. Santus, M. Sopena-Rios, R. García-pérez, A. E. Lin, G. C. Adams, K. G. Barnes, K. J. Siddle, S. Wohl, F. Reverter, J. L. Rinn, R. S. Bennett, L. E. Hensley, P. C. Sabeti, M. Melé, Single-cell profiling of lncRNA expression during Ebola virus infection in rhesus macaques, Nat. Commun. 14 (2023) 3866. https://doi.org/10.1038/s41467-023-39627-7.

[39] V. Srikantan, Z. Zou, G. Petrovics, L. Xu, M. Augustus, L. Davis, J. R. Livezey, T. Connell, I. A. Sesterhenn, K. Yoshino, G. S. Buzard, F. K. Mostofi, D. G. McLeod, J. W. Moul, S. Srivastava, PCGEM1, a prostate-specific gene, is overexpressed in prostate cancer, Proc. Nat. Acad. Sci. 97 (2000) 12216–12221. https://doi.org/10.1073/pnas.97.22.12216.

[40] C. Taneerat, P. Olasard, P. Suksri, S. Whankaew, P. Sathapondecha, Identification profiling of long non-coding RNAs during molt cycle: An involvement of lnc1182 in the molt of white shrimp, *Litopenaeus vannamei*, Aquac. Rep. 30 (2023) 101611. https://doi.org/10.1016/j.aqrep.2023.101611.

[41] C. Jiang, Y. Li, Z. Zhao, J. Lu, H. Chen, N. Ding, G. Wang, J. Xu, X. Li, Identifying functionally characterizing tissue-specific ubiquitously expressed human lncRNAs, Oncotarget 7 (2016) 7120-33. https://doi.org/10.18632/oncotarget.6859.

[42] K. Koiwai, H. Kondo, I. Hirono, scRNA-seq analysis of hemocytes of penaeid shrimp under virus infection, Mar. Biotechnol. 25 (2023) 488-502. https://doi.org/10.1007/s10126-023-10221-8.

[43] C. Cui, Q. Liang, X. Tang, J. Xing, X. Sheng, W. Zhan, Differential apoptotic responses of hemocyte subpopulations to white spot syndrome virus infection in *Fenneropenaeus chinensis*, Front. Immunol. 11 (2020) 594390. https://doi.org/10.3389/fimmu.2020.594390.

[44] H. Li, B. Yin, S. Wang, Q. Fu, B. Xiao, K. Lǔ, J. He, C. Li, RNAi screening identifies a new Toll from shrimp *Litopenaeus vannamei* that restricts WSSV infection through activating Dorsal to induce antimicrobial peptides, PLoS Pathog. 14 (2018) e1007109. https://doi.org/10.1371/journal.ppat.1007109.

[45] J. Munger, S. U. Bajad, H. A. Coller, T. Shenk, J. D. Rabinowitz, 2006. Dynamics of the cellular metabolome during human cytomegalovirus infection. PLoS Pathog. 2, e132. https://doi.org/10.1371/journal.ppat.0020132.

[46] R. Kumar, J. Y. Huang, Y. S. Ng, C. Y. Chen, H. C. Wang, The regulation of shrimp metabolism by the white spot syndrome virus (WSSV), Rev. Aquac. 14 (2022) 1150-1169. https://doi.org/10.1371/journal.ppat.0020132.

[47] Y. Ma, B. Lu, W. Ruan, H. Wang, J. Lin, H. Hu, H. Deng, Q. Huang, M. Lai, Tumor

suppressor gene insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) induces senescence-like growth arrest in colorectal cancer cells, Exp. Mol. Pathol. 85 (2008) 141-145. https://doi.org/10.1016/j.yexmp.2008.04.005.

[48] S. Zhu, F. Xu, J. Zhang, W. Ruan, M. Lai, Insulin-like growth factor binding protein-related protein 1 cancer, Clin. Chim. Acta 431 (2014) 23-32. https://doi.org/10.1016/j.cca.2014.01.037.

[49] R. K. Choy, J. M. Kemner, J. H. Thomas, Fluoxetine-resistance genes in *Caenorhabditis elegans* function in the intestine may act in drug transport, Genetics 172 (2006) 885-892. https://doi.org/10.1534/genetics.103.024869.

[50] J. M. Hardwick, Apoptosis in viral pathogenesis, Cell Death Differ. 8 (2001) 109-110. https://doi.org/10.1038/sj.cdd.4400820.

[51] A. Roulston, R. C. Marcellus, P. E. Branton, Viruses apoptosis, Annu. Rev. Microbiol. 53 (1999) 577-628. https://doi.org/10.1146/annurev.micro.53.1.577.

[52] L. Galluzzi, C. Brenner, E. Morselli, Z. Touat, G. Kroemer, 2008. Viral control of mitochondrial apoptosis. PLoS Pathog. 4, e1000018. https://doi.org/10.1371/journal.ppat.1000018.

[53] Q. Bao Y. Shi, Apoptosome: A Platform for the activation of initiator caspases, Cell Death Differ. 14 (2007) 56-65. https://doi.org/10.1038/sj.cdd.4402028.

[54] W. C. Earnshaw, L. M. Martins, S. H. Kaufmann, Mammalian caspases: Structure, activation, substrates, functions during apoptosis, Annu. Rev. Biochem. 68 (1999) 383-424. https://doi.org/10.1146/annurev.biochem.68.1.383.

[55] P. H. Wang, D. H. Wan, Y. G. Chen, S. P. Weng, X. Q. Yu, J. G. He. Characterization of four novel caspases from *Litopenaeus vannamei* (Lvcaspase2-5) their role in WSSV infection through dsRNA-mediated gene silencing. PloS one. 8, e80418. https://doi.org/10.1371/journal.pone.0080418.

[56] L. Wang, B. Zhi, W. Wu, X. Zhang, Requirement for shrimp caspase in apoptosis against virus infection, Dev. Comp. Immunol. 32 (2008) 706-715. https://doi.org/10.1016/j.dci.2007.10.010.

[57] X. N. Ma, X. Y. Liu, Y. F. Yang, F. J. Xiao, Q. F. Li, J. Yan, Q. W. Zhang, L. S. Wang, X. Y. Li, H. Wang, Regulation of human hepatocellular carcinoma cells by Spred2 correlative studies on its mechanism, Biochem. Biophys. Res. Commun. 410 (2011) 803-808. https://doi.org/10.1016/j.bbrc.2011.06.068.

[58] T. Liu, J. Zhao, C. Lin, Sprouty-related proteins with EVH1 domain (SPRED2) prevents high-glucose induced endothelial-mesenchymal transition and endothelial injury by suppressing MAPK activation, Bioengineered 13 (2022) 13882-13892. https://doi.org/10.1080/21655979.2022.2086351.

[59] H. Wu, G. Liu, C. Li, S. Zhao, bri3, a novel gene, participates in tumor necrosis factor-alpha-induced cell death, Biochem. Biophys. Res. Commun. 311 (2003) 518-524. https://doi.org/10.1016/j.bbrc.2003.10.038.

[60] E. Kavak, A. Najafov, N. Ozturk, T. Seker, K. Cavusoglu, T. Aslan, A. D. Duru, T. Saygili, G. Hoxhaj, M. C. Hiz, D. O. Unal, N. Birgül-Iyison, M. Ozturk, A. Koman, Analysis of the Wnt/B-catenin/TCF4 pathway using SAGE, genome-wide microarray and promoter analysis: Identification of BRI3 and HSF2 as novel targets, Cell Signal. 22 (2010) 1523-35. https://doi.org/10.1016/j.cellsig.2010.05.021.

[61] M. Haseeb, R. H. Pirzada, Q. U. Ain, S. Choi, Wnt signaling in the regulation of immune cell and cancer therapeutics, Cells 8 (2019) 1380. https://doi.org/10.3390/cells8111380.

[62] F. Mendoza-Cano, A. Sánchez-Paz, Development validation of a quantitative real-time polymerase chain assay for universal detection of the White Spot Syndrome Virus in marine crustaceans, Virol. J. 10 (2013) 186. https://doi.org/10.1186/1743-422X-10-

186.

Figure captions

Fig. 1 Number of differentially expressed lncRNAs. *De novo* assembled transcriptome was generated and differential expressions of transcripts were analyzed. Number of DEGs shown includes total transcripts, mRNA, and putative lncRNAs.

Fig. 2 Determination of candidate lncRNA expressions in hepatopancreas during WSSV infection. Gene expressions in hepatopancreas isolated from control and WSSV-infected shrimp were determined at 12, 24, 48 hpi by qRT-PCR. Five shrimp were used in each time course. Bars and error bars represent means and SEMs, respectively. The notations "*" and "**" indicate significant differences ($p<0.05$ and $p<0.01$, respectively) between groups analyzed by t-test.

Fig. 3 Determination of candidate lncRNA expressions in shrimp tissues. Shrimp tissues, including eyestalks (es), muscles (ms), hepatopancreas (hp), gills (gl), brains (bn), and epidermis (ep), isolated from three healthy *L. vannamei* were used to determine the candidate lncRNAs by qRT-PCR. Bars and error bars represent means and SEMs, respectively. Letters indicate significant differences between groups analyzed by one-way ANOVA and pairwise comparison by Duncan's test.

Fig. 4 Determination of *lnc164* knockdown and its effect on shrimp mortality, hemocytes, and viral loads. Hepatopancreas from shrimp (n=5) injected with ds164 and dsGFP were isolated on day 1 and 2 post injection to determine *lnc164* expression by qRT-PCR (A). Shrimp were injected with ds164, dsGFP, or 150 mM NaCl 24 h prior to WSSV challenge (n=10), and the percentage of cumulative mortality was recorded for 5 days (B). Total hemocyte count (C) and relative viral loads in gills (D) from shrimp 24 hpi were measured by hemocytometry and qPCR, respectively. Bars and error bars represent means and SEMs,

695 respectively. An asterisk and letter indicate the significant difference (p<0.05) between
696 groups analyzed by t-test and one-way ANOVA, respectively.
697 **Fig. 5** Differentially expressed genes and their functional annotations upon *lnc164* depletion.
698 The analyzed DEGs were from the *de novo* assembled transcriptome from hepatopancreas of
699 *lnc164*-kncokdown shrimp. The DEG patterns were visualized in a heatmap (A) and
700 dendrograms (B) with $\log_2$(FPKM+1). Functional annotations were analyzed with GO (C),
701 KEGG (D), and COG (E).
702 **Fig. 6** Determination of lnc164-responsive gene expression in *lnc164*-depleted shrimp. The
703 candidate DEGs were validated for their expressions in hepatopancreas injected with ds164,
704 dsGFP, or 150 mM NaCl. Five shrimp were used in each group. Bars and error bars represent
705 means and SEMs, respectively. Letters indicate significant difference (p<0.05) among groups
706 analyzed by one-way ANOVA and pairwise comparison by Duncan's test.
707 **Fig. 7** Determination of lnc164-responsive gene expression upon WSSV infection. The
708 candidate DEGs were validated for their expressions in hepatopancreas of shrimp injected
709 with WSSV or NaCl for 24 h. Five shrimp were used in each group. Bars and error bars
710 represent means and SEMs, respectively. An asterisk indicates the significant difference
711 (p<0.05) between target and control groups analyzed by t-test.
712 **Table 1** Oligonucleotides used in this study

Name	**Forward Sequence (5´ to 3´)**	**Reverse Sequence (5´ to 3´)**	**Purposes**
Actin	GCGACGTGGACATCCG TAAG	GAGCGAGGGCAGTGA TTTCCT	qRT-PCR
lnc164	TTCCACACCCAAAGCC ATGA	GCTGTAACCACTCACG AGTCT	qRT-PCR

lnc6913	TGTGGGAACGGACCATGTTT	ACGGAGCCTTGCTTGAAAGT	qRT-PCR
lnc140	TCCATGTCGGAGCAGAGGTA	GGGAGTGTAGCAACCCTGTG	qRT-PCR
lnc7225	GGAGGGTCGTTCCTTGTGTC	CGTCGGTCCACAAACAAACG	qRT-PCR
lnc3615	GGGCGGTTCCTAGACACATC	CTGGCTTTTGAGAGCGGGTA	qRT-PCR
St_164	TCTAGACATGAACAAGGCTCCATGACG	GAACCTTCGTGCATTGTCCTGTCAGT	ds164 production
StL_164	AAGCTTCATGAACAAGGCTCCATGACG	GAACCTGTCCTTTCTGGGTGAGAATCGAAC	ds164 production
LvCofilin	CCGGAGTACAAGTAGCAGATG	ATATAGACCATAGCGGCACTG	qRT-PCR
LvBRI3	GTAGGGTCGGTCGGTTACAA	AGAGAGCTTTGCAGCCATCA	qRT-PCR
LvIGFBP-rP1	GCCTCGTCAAGAATGTGTGC	TTACCATAGGAGACGGGGTG	qRT-PCR
LvCasp3	TGCGAGTACCAGTAACACCAG	TGAGGTCAGGGCGATCCTTA	qRT-PCR
LvSPRED2	GTTCATCAAAGCCCGCTGTC	TCACATGGACGGTGAGCAAA	qRT-PCR
LvNRF-6	CATTAGTGCCCAGCGAAACG	ATTGCCGTCAAGAATCCCGT	qRT-PCR

Vp28 [62]	AGGTGTGGAACAACAC ATCAAG	TGCCAACTTCATCCTC ATCA	WSSV detection

713 **Table 2** General characteristics of the *de novo* assembled transcriptome

General information	
Total assembled bases	129,966,101
Number of transcripts	221,347
GC (%)	43.05
Average contig length (bp)	587.16
Contig N50 (bp)	893
Assembly completeness (%)	97.0
No. of protein coding transcripts	176,808
No. of total lncRNAs in transcriptome	44,539
Differential expression of genes	
No. of differentially expressed transcripts	277
No. of differentially expressed protein coding genes	245
No. of differentially expressed lncRNAs	32
Upregulated upon WSSV infection	21
Downregulated upon WSSV infection	11

714

715 **Table 3** Information of the candidate lncRNAs in this study

Candidate lncRNAs	**Size (bp)**	**GenBank Accession No.**	**DE Patterns**	**NCBI Hit**	**Species**
lnc164	1,802	OR067088	Downregulated	XR_003477688	*P. vannamei*

lnc6913	683	OR067089	Downregulated	-	-
lnc140	1,445	OR067090	Downregulated	XR_003475561	*P. vannamei*
lnc7225	575	OR067091	Upregulated	XR_003477627	*P. vannamei*
lnc3615	1,084	OR067092	Up regulated	-	-

716 **Table 4** General characteristics of *lnc164*-knockdown transcriptome

Statistics of *de novo* assembled transcriptome	
Total assembled bases	182,436,258
Number of transcripts	166,906
GC (%)	42.92
Average contig length (bp)	734.11
Contig N50 (bp)	1,727
Assembly completeness (%)	98.0
No. of differentially expressed transcripts	251

717 **Table 5** Information of the candidate genes in response to *lnc164* depletion

Genes	**Gene names**	**GenBank Accession No.**	**E-value**	**Expression pattern**
Cofilin	Cofilin/actin-depolymerizing factor homolog	XP_027225677.1	5.09E-43	Downregulated
BRI3	Brain protein I3-like	XP_047497091.1	3.92E-31	Downregulated
IGFBP-rP1	Insulin-like growth factor-binding protein-related protein 1	XP_027221413.1	2.02E-46	Upregulated
Casp3	Caspase-3-like	XP_027234111.1	0	Upregulated

SPRED2	Sprouty-related, EVH1 domain-containing protein 2-like	XP_027238969.1	0	Upregulated
NRF-6	Nose resistant to fluoxetine protein 6-like	XP_042891509.1	0	Downregulated

718

Highlights

- LncRNAs were *in silico* identified from gills of healthy and WSSV-infected shrimp.
- Expressions of lncRNA were determined in various tissues and during WSSV infection.
- Knockdown of *lnc164* prolonged shrimp mortality after the WSSV challenge.
- The knockdown of *lnc164* affected the set of metabolic and immune responsive genes.

Cover Letter

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Fish & Shellfish Immunology Journal
Editorial Office

Subject: Submission of a new manuscript for evaluation

Enclosed please find a manuscript that we are submitting for consideration by Fish & Shellfish Immunology entitled "Long noncoding RNA profiling in hepatopancreas of white shrimp and its role in response to white spot syndrome virus infection".

In this manuscript, we report identification and analysis of putative long noncoding RNAs (lncRNAs) from the hepatopancreas transcriptome of healthy and white spot syndrome virus (WSSV)-infected white shrimp. We found a number of differentially expressed lncRNAs, and some of them were determined for their expression during WSSV challenge as well as in shrimp tissues. Lnc164 was chosen to be studied for its role in response to WSSV infection in *Litopenaeus vannamei* because of its significantly downregulated expression after WSSV infection and localized expression in the hepatopancreas. Using RNA interference and RNA sequencing, lnc164 most likely played a role in regulation of several metabolic and immune responsive genes resulting in the rise in the harmfulness of WSSV. Our findings make fundamental contributions to other research and we believe our manuscript will be of high interest to many of your readers.

With the submission of this manuscript, I would like to confirm that the above-mentioned manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere; and that my Institute's Prince of Songkla University representative is fully aware of this submission.

Hope you will consider this manuscript to be published in Fish & Shellfish Immunology.

Sincerely,

Ponsit Sathapondecha, Ph.D.

Figure1

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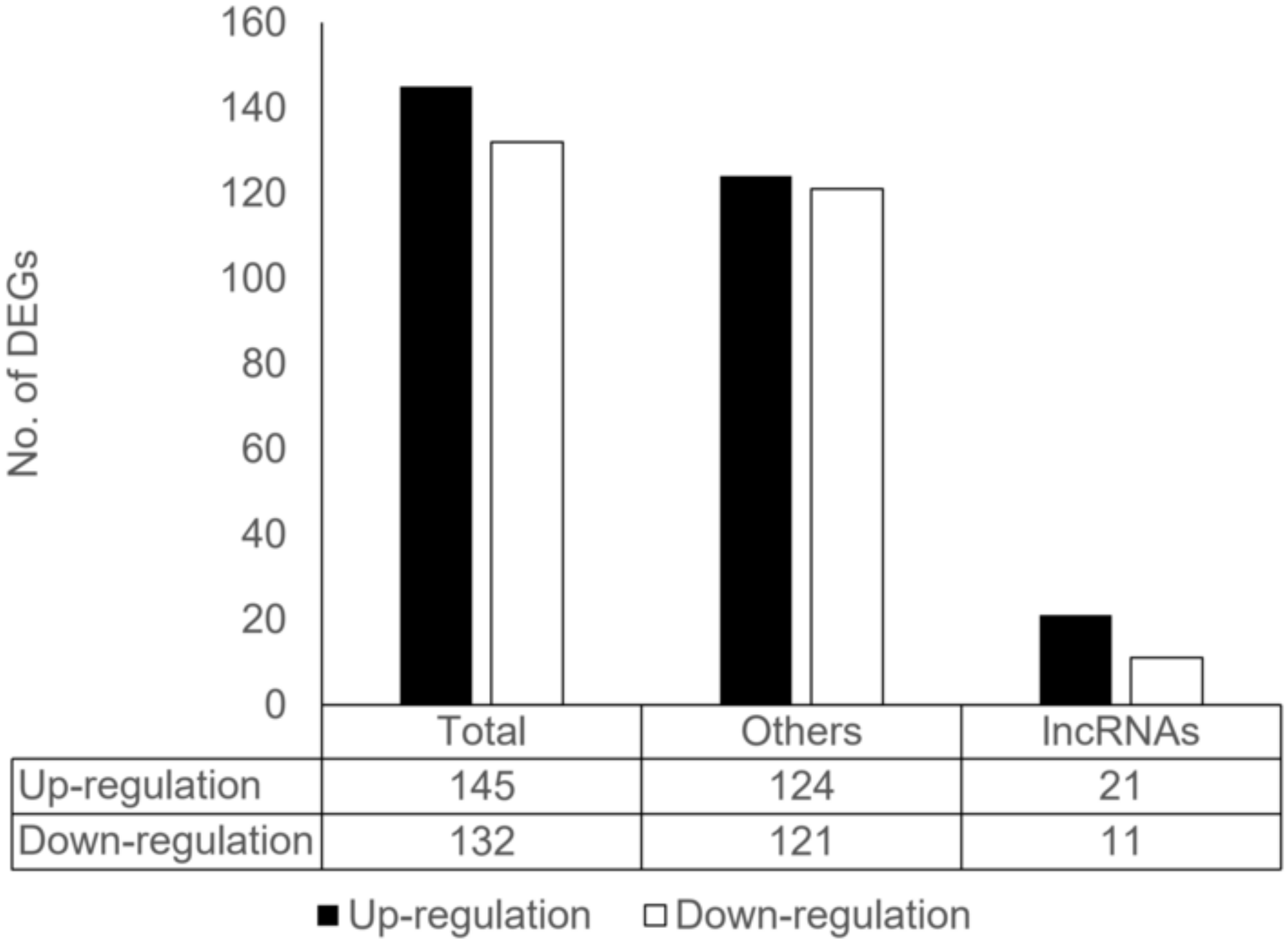

	Total	Others	lncRNAs
Up-regulation	145	124	21
Down-regulation	132	121	11

Figure2

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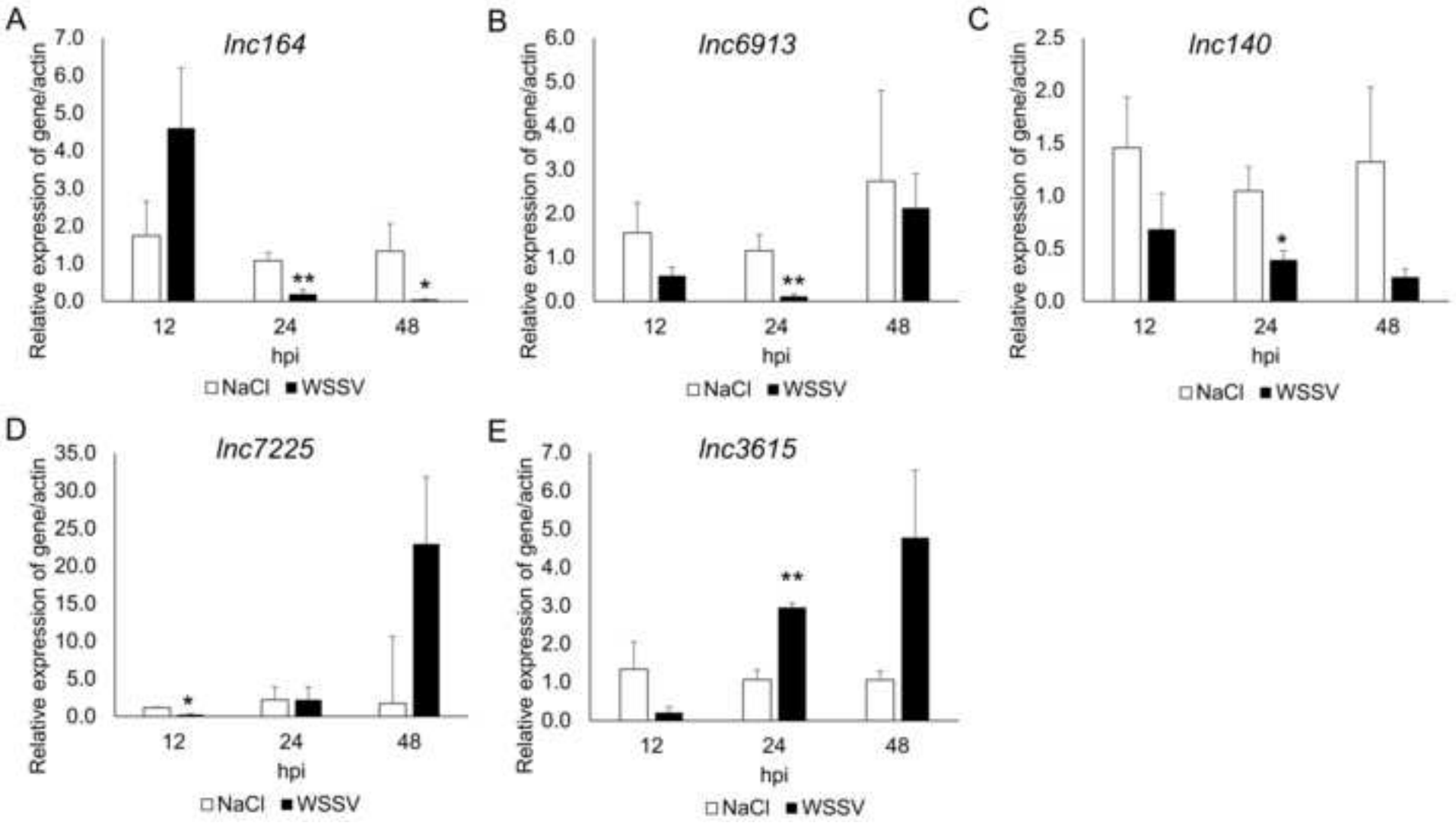

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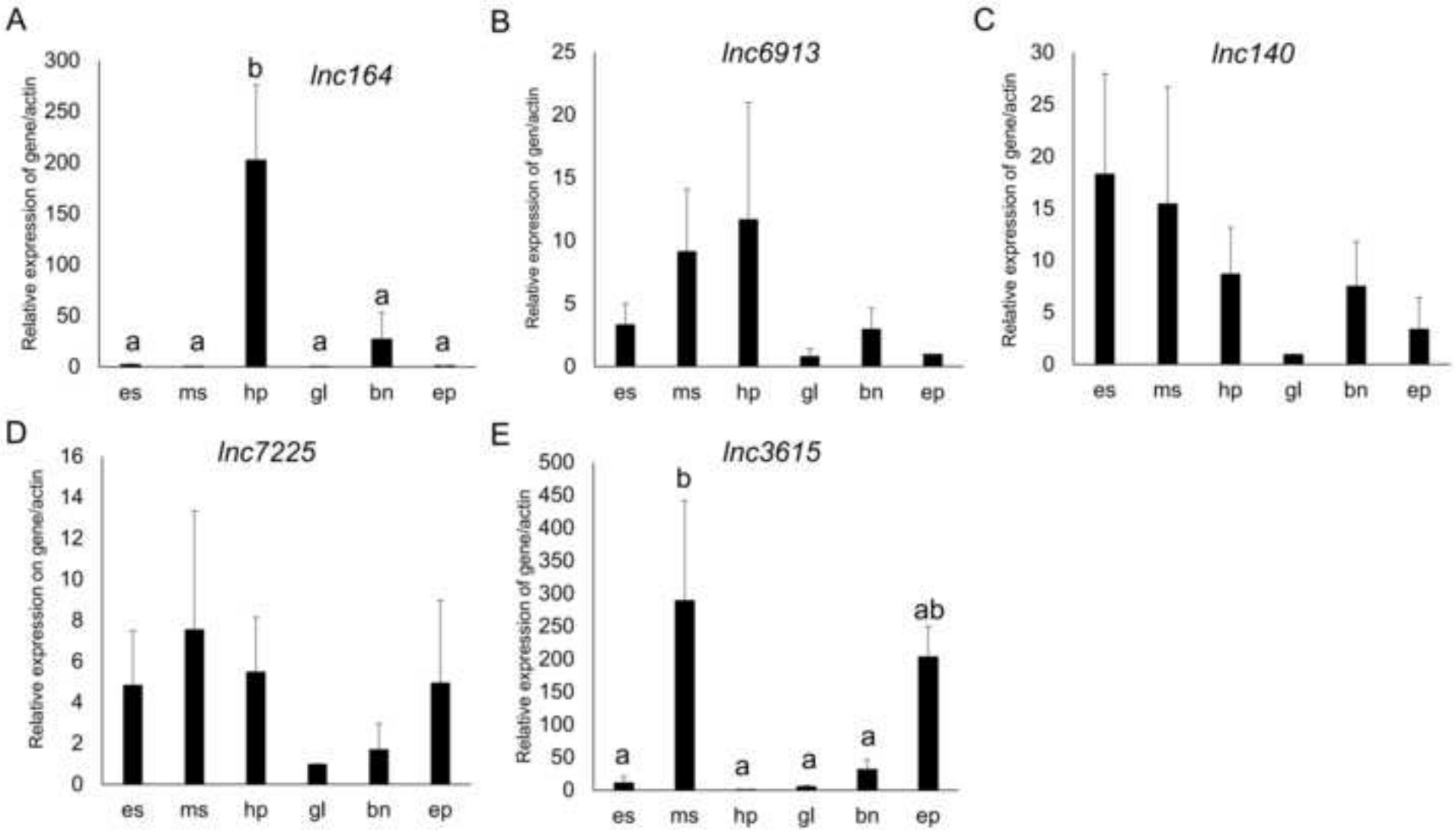

Figure4

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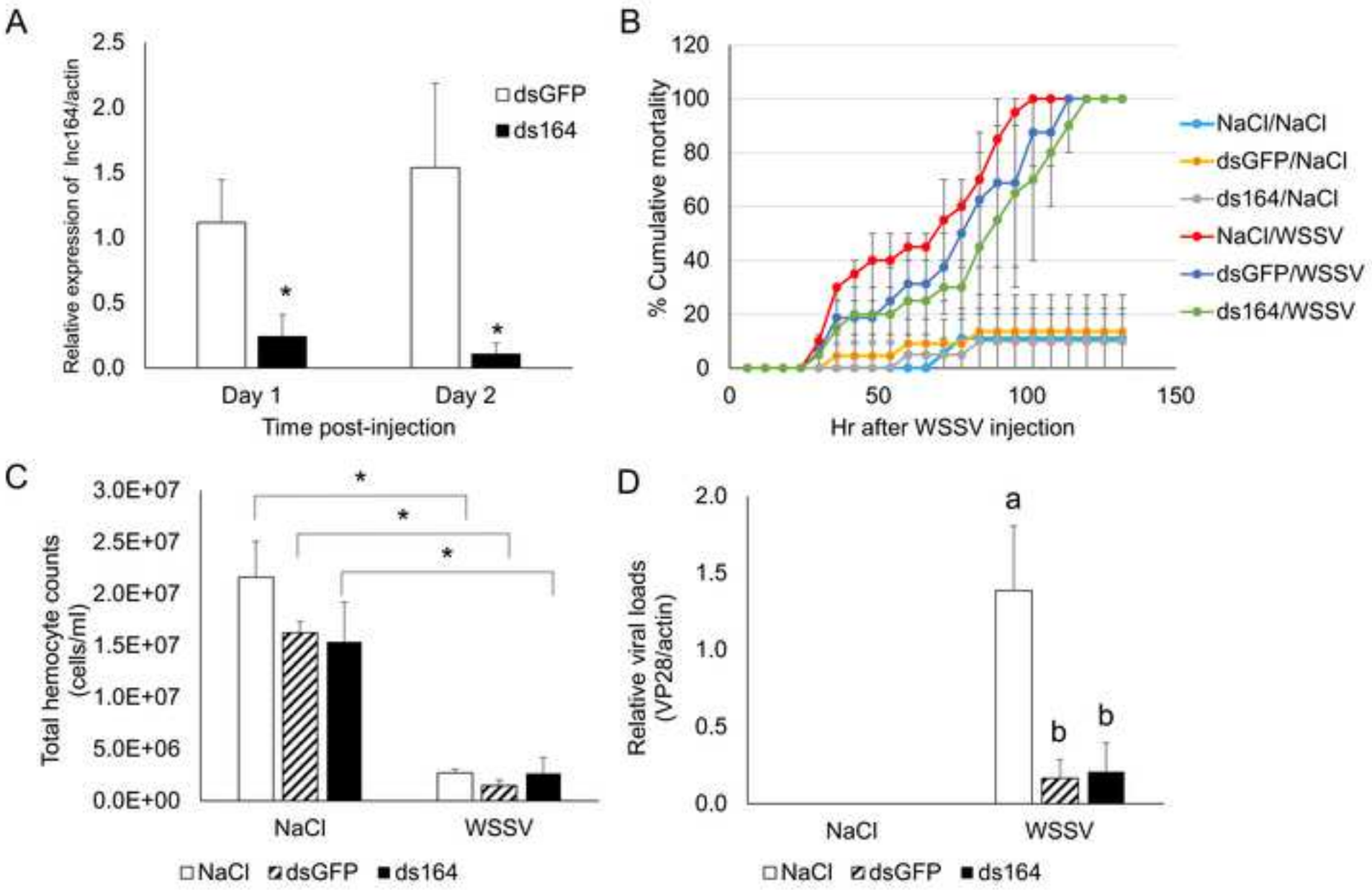

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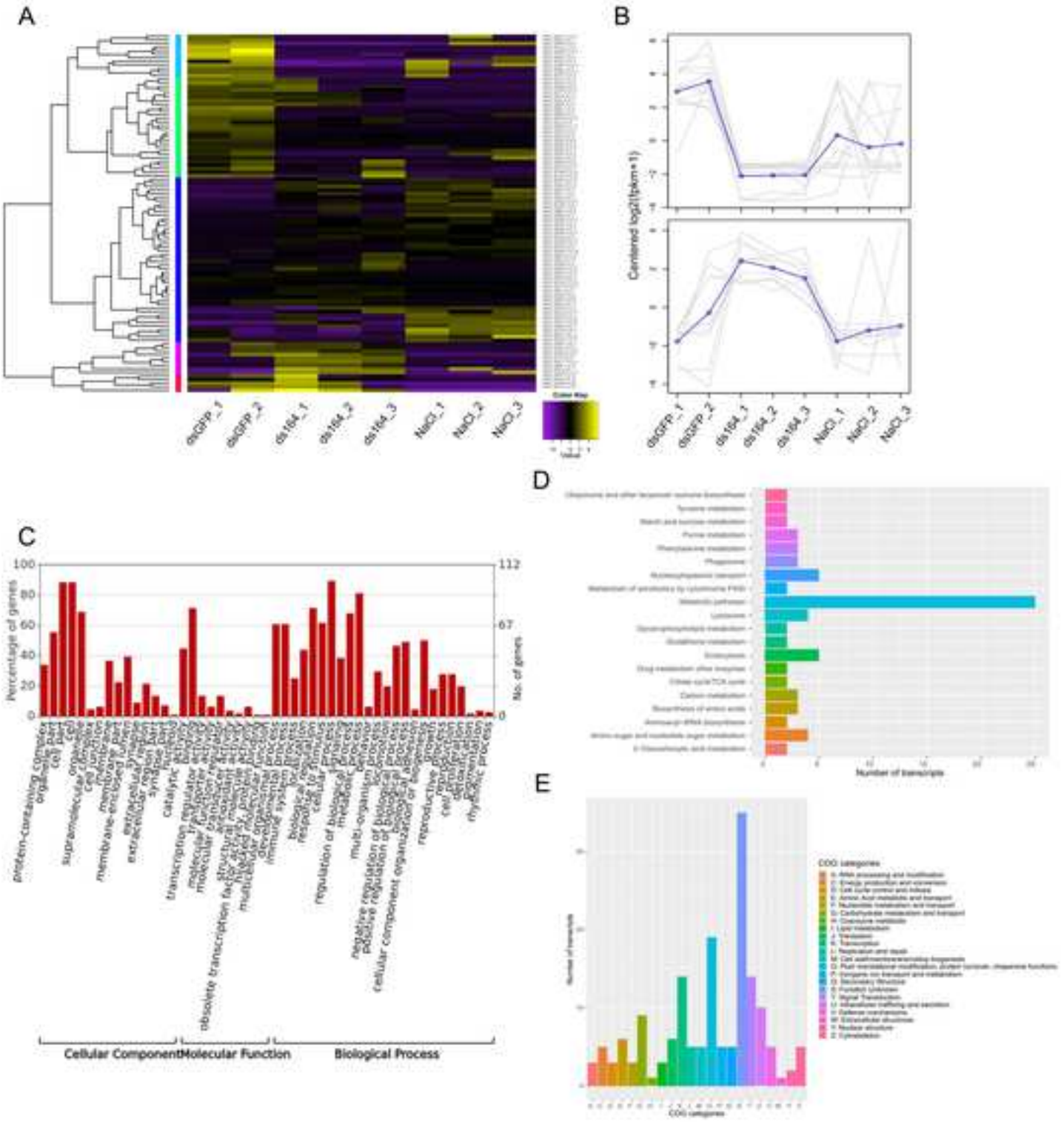

Figure6

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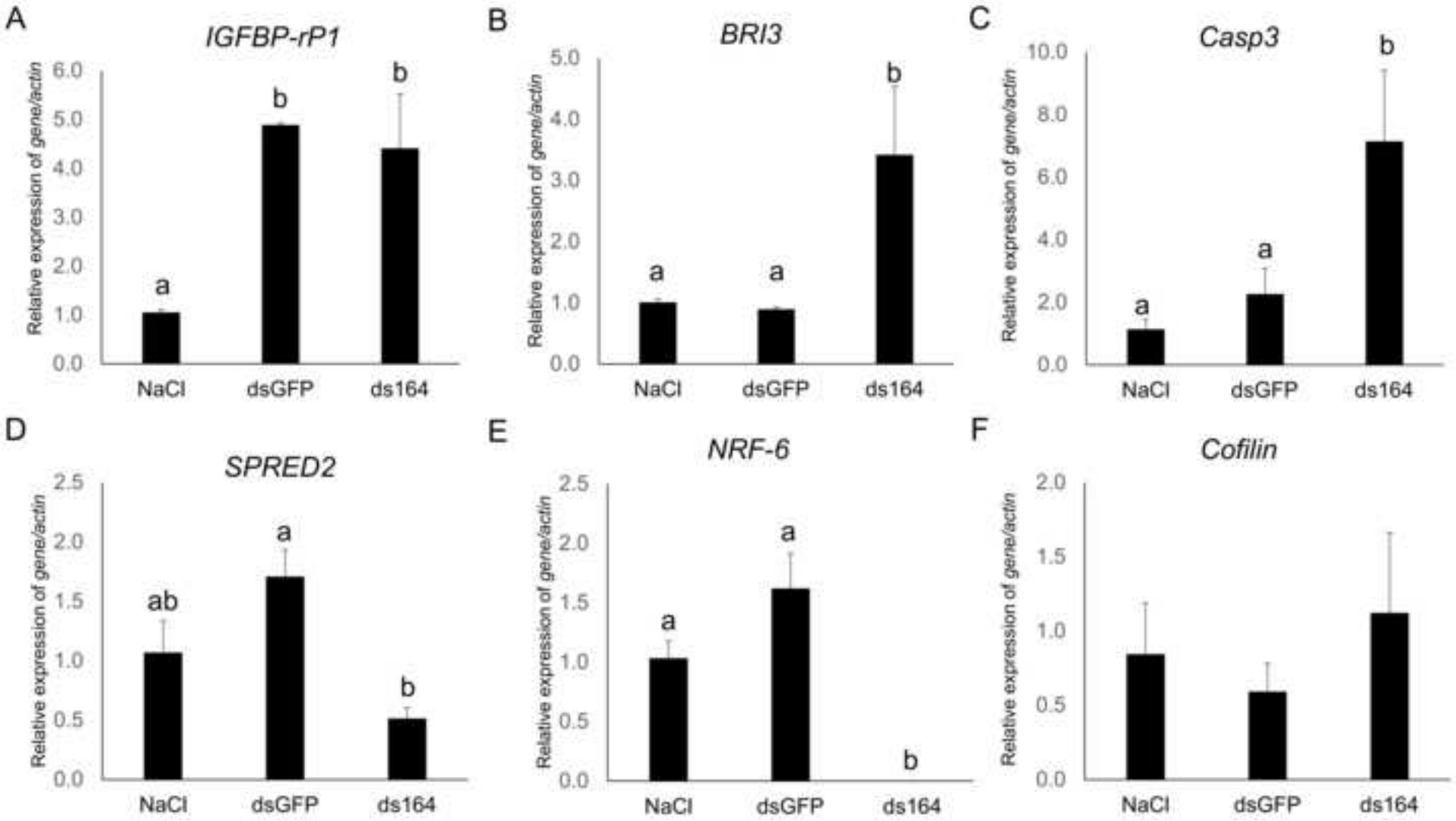

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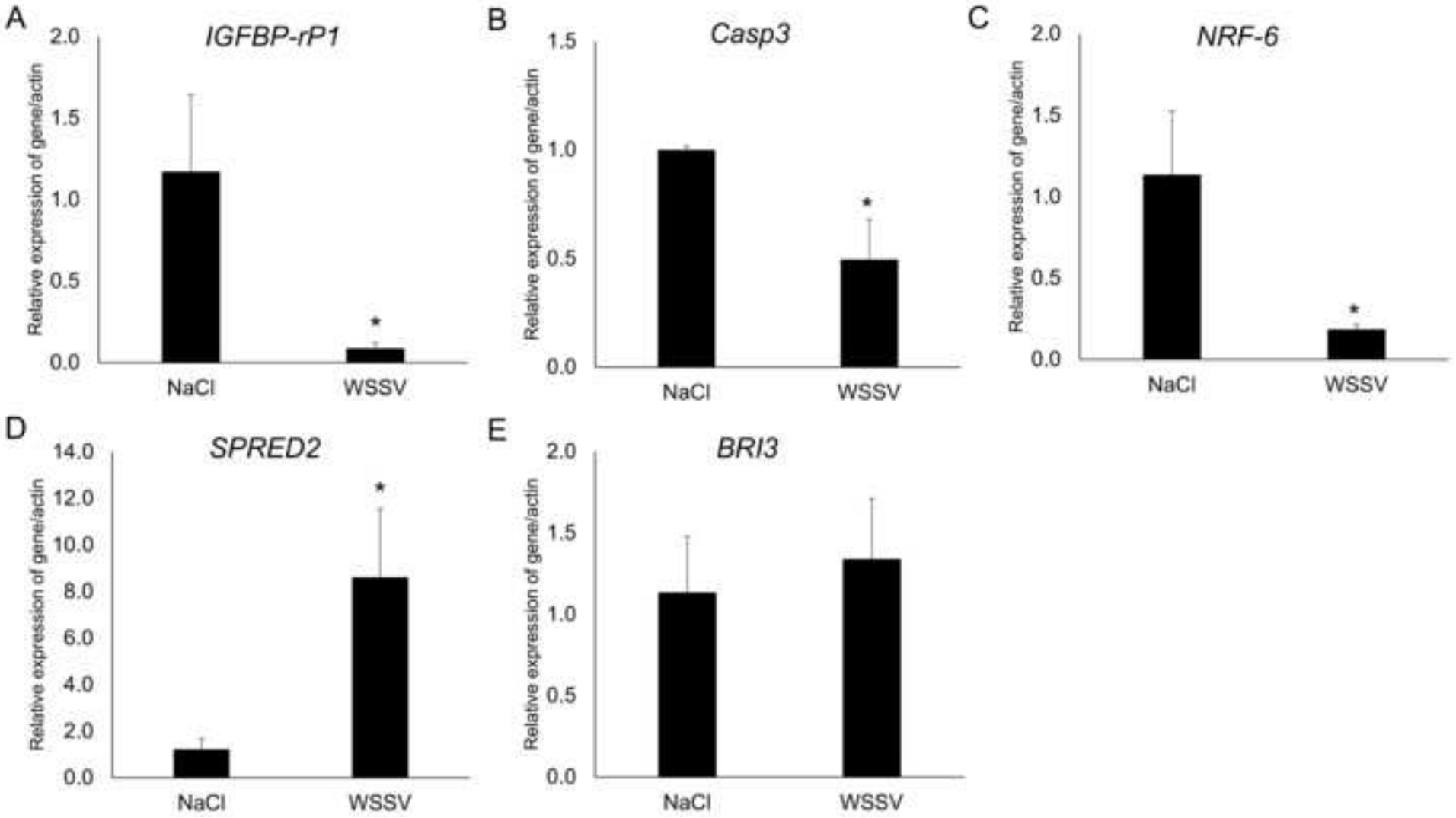

Supplementary material 1

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Supplement S1.xlsx

Proceeding I

Wirasit, I., Udomkit, A., and Sathapondecha, P., 2023. Identification of Long Noncoding RNAs Associated to White Spot Syndrome Virus Infection in White Shrimp, *Litopenaeus vannamei*. The 8th RSU International Research Conference on Sciences and Technology 2023, April, 451–457

Identification of Long Noncoding RNAs Associated to White Spot Syndrome Virus Infection in White Shrimp, *Litopenaeus vannamei*

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Abstract

Long non-coding RNA (lncRNA) is a noncoding RNA that lacks of the capability for protein coding, usually over 200 nucleotides in length. It is reported to play significant roles in various physiological processes, including immune regulation, reproduction and development. Although several transcriptomes have been studies in response to viral infections in many organisms, the role of lncRNAs in viral responses has not been elucidated in shrimps. Therefore, this study aimed to identify and classify putative lncRNAs related to white spot syndrome virus (WSSV) infection in white shrimp. The RNA sequencing data of gills from WSSV infection experiment were used to identify lncRNAs. Among 226,797 of *de novo* assembled transcripts, 3,235 transcripts were differentially expressed upon WSSV infection, and 1,628 of them were identified as putative lncRNAs. At 24 h post-WSSV infection, 692 putative lncRNAs were highly expressed, while expressions of the rest 936 lncRNAs were decreased in gills of *L. vannamei*. Furthermore, the differentially expressed lncRNAs were classified based on their location on genome. The 860 and 191 of putative lncRNAs were sense and antisense lncRNAs, respectively, while 15 and 144 of them were promoter-and 3′ UTR-associated lncRNAs, respectively. Other 336 and 82 lncRNAs were classified as intronic and intergenic lncRNAs, respectively. The findings revealed an association between *L. vannamei* lncRNAs and WSSV infection.

Keywords**:** *long noncoding RNA, Litopenaeus vannamei, White spot syndrome virus*

1. Introduction

Long noncoding RNAs (lncRNAs) are transcripts over 200 nucleotides in length, which are a large and functionally diverse class among noncoding RNAs (ncRNAs) or RNAs that lack of the capability to encode for proteins (Aliperti et al., 2021; Bridges et al., 2021; Kazimierczyk et al., 2020). The significance of lncRNAs as a new biomolecule become greater as there are many reports about the lncRNAs engage in many biological processes and play regulatory functions in transcription, translation, splicing, protein localization, imprinting, cellular structure integrity, cell cycle and apoptosis, stem cell pluripotency and reprogramming, and heat shock response (Aliperti et al., 2021; Ma et al., 2013). LncRNAs have been found to play important roles in immune system of organisms. For instance, in human, the lncRNA *Morrbid*, *HOTAIRM1* or *lnc-DC* involved in the development and differentiation of dendritic and myeloid cells8 (Kotzin et al., 2016; Zhuang et al., 2018). Nevertheless, studies of immune-related lncRNAs in invertebrate species are still scarce.

White shrimp (*Litopenaeus vannamei*), one of most economically important crustaceans has been facing to deadly pathogens so far (Gucic et al., 2013). White spot syndrome virus (WSSV) is a double-stranded DNA virus causing white spot disease in a wide range of crustaceans including white shrimp, and it can spread very quickly in saline waters which tends to lead the 100% mortality in a shrimp pond (Leu et al., 2008; Santos et al., 2018). In white shrimp, the number of transcriptome study has been gradually increased as it is an effective way to analyze number of genes expressed in organisms under certain conditions (Casamassimi et al., 2017; Lowe et al., 2017). In particular, identification and functional study of genes involved in immune system of the host against the infection of pathogens could be beneficial to provide the key for accurate therapeutic treatments or any other possible solutions. In recent, transcriptome

studies have uncovered the alteration of gene expression patterns in *L. vannamei* upon WSSV infection in various conditions. Millard et al. (2021) discovered amounts of mRNAs and miRNAs which were differentially expressed following WSSV infection. However, the lncRNA transcript profiles induced by WSSV infection have not been characterized so far.

In this study, identification of lncRNAs associated with WSSV infection in white shrimps was performed, and differential expressions of lncRNAs were investigated. Herein, this research also classified types of the putative lncRNAs depending on their localization on a genome. This study provided novel insights into lncRNAs related to antiviral immune defense of invertebrate.

2. Objectives

To identify lncRNAs differentially expressed in *L. vannamei* against WSSV infection

3. Materials and Methods

3.1 Transcriptome assembly and gene differential expression analysis

The RNA seq data derived from gills of WSSV-challenged and control shrimps at 24 h post-infection was retrieved from NCBI database (BioProject No. PRJNA716175; Millard et al., 2021). The raw reads were examined for their qualities by FastQC program, and their adaptors and low quality sequence were removed using Trimmomatic program (Bolger et al., 2014). The clean reads were used to generate *de novo* assembled transcriptome using Trinity package (version 2.9.1) with default parameters (Grabherr et al., 2011). After using CD-HIT-EST program to remove duplicate sequences with 95% identity, the quality of the assembled transcriptome was determined by TrinityStat and BUSCO program. The read sets were analyzed for differentially expressed gene (DEG) by mapping to the transcriptome and estimating transcript abundance using Bowtie2 (Langmead & Salzberg, 2012) and RSEM (Li & Dewey, 2011), respectively. Next, DESeq2 package was used for identifying DE features with default parameters of FDR < 0.01, p-value < 0.05, and |log2(fold-change)| > 1 (Love et al., 2014).

3.2 *In silico* identification and classification of long noncoding RNA related to WSSV infection in *L.vannamei* gills

To identify putative lncRNAs, the DEG was in-house analyzed by BLASTx and BLASTn against the arthropod database from NCBI with an e-value of 1e-20, and all positive hit was discarded. Then, filtered transcripts were analyzed with other non-coding RNAs databases to remove non-related non-coding RNAs and pseudogenes (Figure 1). Finally, the remaining transcripts were predicted for putative lncRNAs by coding potential assessment tools (CPAT; http://lilab.research.bcm.edu/cpat/). The differentially expressed lncRNAs were predicted as sense and antisense lncRNAs, promoter-associated lncRNAs, 3′ UTR-associated lncRNAs, intronic lncRNAs, and intergenic lncRNAs during alignment and coding potential prediction steps as shown in Figure 1.

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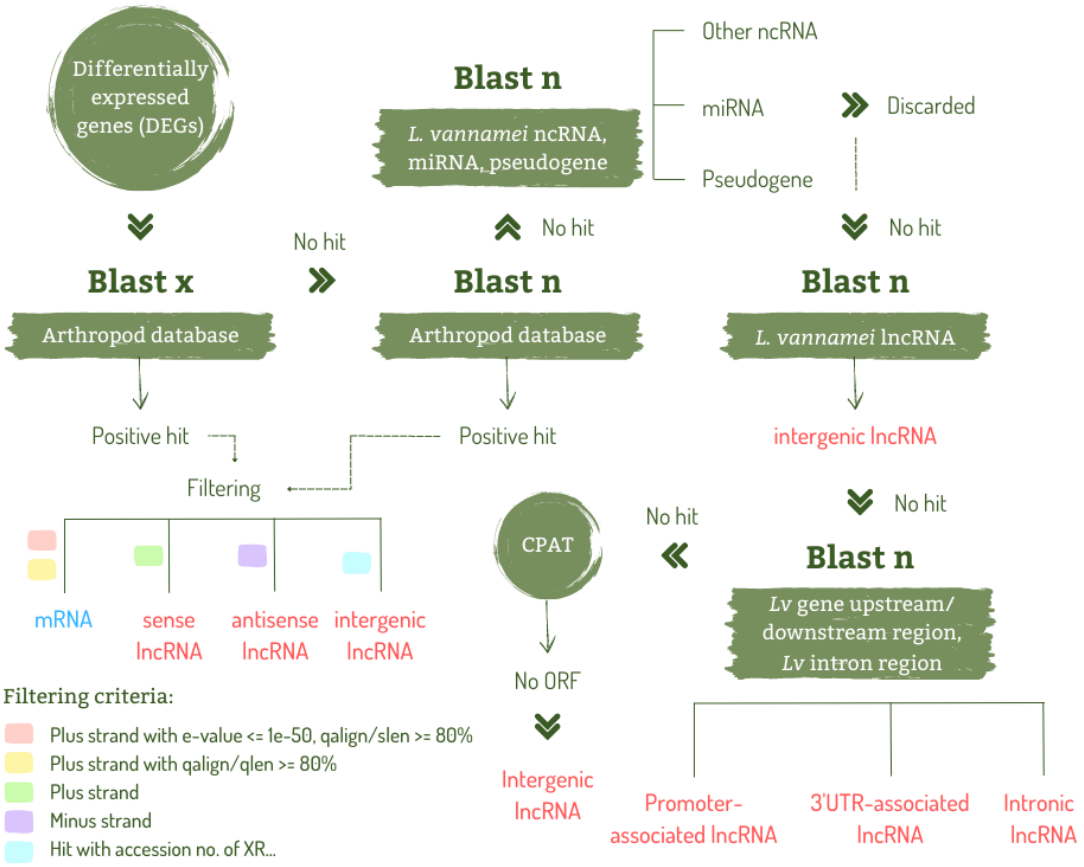

Figure 1 Schematic diagram of identification of putative lncRNAs

4. Results and Discussion

4.1 *In silico* identification of lncRNAs involved in WSSV infection in *L. vannamei* gills

Using RNA sequencing and miRNA sequencing, the transcription of mRNAs and miRNAs in *L. vannamei* gills was investigated over 36 h following WSSV infection (Millard et al., 2021). The research discovered a total of 6,192 mRNA transcripts and 27 miRNAs which were significantly induced by WSSV infection. Silencing of an immune priming gene Dscam by a novel shrimp miRNA (Pva-pmiR-78) might prevent the WSSV invasion. Nonetheless, studies about lncRNA expression profiles upon WSSV infection remain elucidated.

By using the same RNA-seq data provided by Millard et al. (2021), a total of 226,797 unigenes of *de novo* assembled transcriptome were obtained with a GC content of 43.64%, N50 of 929 bp and an assembled completeness of 96.8% (Table 1). After statistically analyzing the expression profile between control and WSSV-infected group, a total of 3,235 transcripts were significantly induced after WSSV infection (Table 1).

Notably, a total of 1,232 transcripts which made up a 38.1% of the total DEGs were mRNA. After filtering the transcripts subjected to protein coding sequences, pseudogenes, and other noncoding RNAs, the remaining 1,628 transcripts (50.3%) were identified as putative lncRNAs (Table 1). Additionally, of the 1,628 differentially expressed putative lncRNAs, 692 and 936 were up- and down-regulated at 24 h post-WSSV infection, respectively (Figure 2).

Many transcriptome studies focus on lncRNAs as important regulators in viral infections regarding their differential expressions upon viral infection (W. Liu & Ding, 2017; Peng et al., 2010). In WSSV

infection, 6,544 lncRNAs were identified from hepatopancreas of kuruma shrimp, *Penaeus japonicas*, and 457 from them were significantly induced by WSSV infection (Zhang et al., 2022). Studies on shrimp lncRNAs are still limited to the identification and differential expression of lncRNAs and their responsible genes so far. Thus further studies should focus on the regulatory mechanisms of lncRNAs, especially in the host immune defense system and viral-host interaction.

Table 1 Statistics of *de novo* assembled transcriptome of shrimp during 24 h WSSV infection in gills

General information	
SRA accessions for control group	SRR14027734- SRR14027737
SRA accessions for WSSV-infected group	SRR14027701-SRR14027702, SRR14027704-SRR14027705
Total assembled bases	139,979,357
Number of transcripts	226,797
GC content (%)	43.64%
Average contig length (bp)	617.20
Contig N50 (bp)	692
Assemble completeness (%)	96.8
Differential expression of genes	
No. of differentially expressed transcripts	3,235
No. of differentially expressed mRNA	1,232
No. of differentially expressed lncRNA	1,628
lncRNA up-regulated during 24 h WSSV infection	692
lncRNA down-regulated during 24 h WSSV infection	936

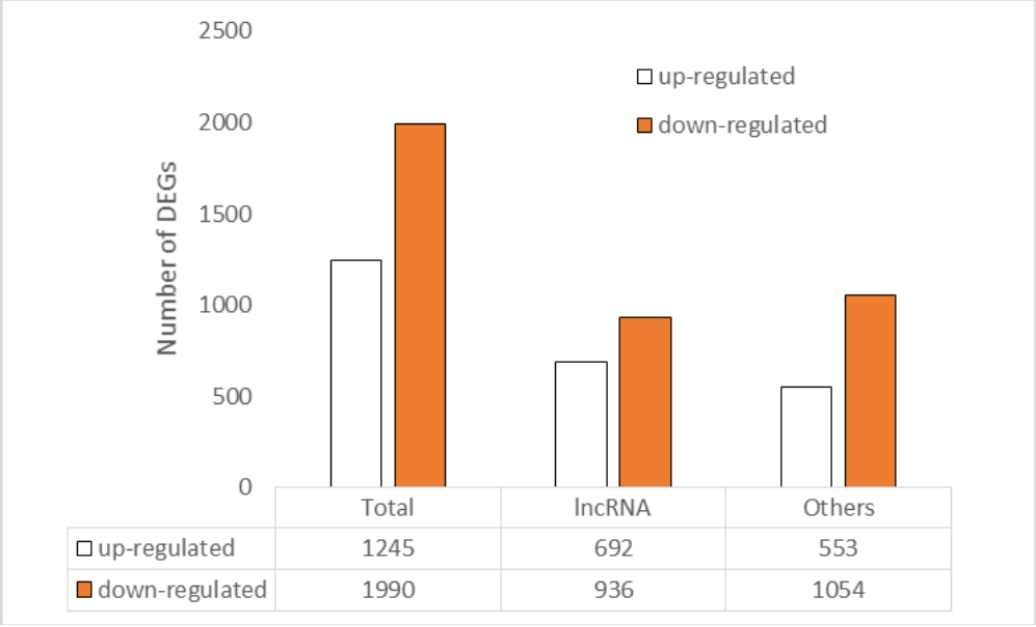

Figure 2 *In silico* analysis of differentially expressed lncRNAs. *De novo* assembled transcriptome was generated and DEG was analysis. Number of DEG composing of total transcripts, putative lncRNA, and others were shown

4.2 Classification of DE lncRNAs upon WSSV infection

The differentially expressed lncRNAs were classified based on their location on genome (Table 2). Among 1,628 differentially expressed lncRNAs, 860 and 191 were found to be transcribed from the same or

opposite strand of protein coding genes, known as sense and antisense lncRNA, respectively. Apart from that, 15 and 144 lncRNAs were located within 1,000 upstream and downstream of coding genes, which were classified as promoter-associated and 3′ UTR-associated lncRNAs, respectively. The remaining lncRNAs were mapped with the intron region, returning 336 lncRNAs classified as intronic lncRNA. Apart from that, the remaining 82 transcripts matched with the lncRNAs on NCBI database, or *L. vannamei* lncRNA genome database, or they contained no ORF predicted by CPAT subjected to intergenic lncRNAs**.**

Based on the results, lncRNAs involving in WSSV infection were identified, and the classification was demonstrated. However, more understanding about the mechanism of lncRNAs regulate genes involved in host immune defense against infection remains elucidated. Understanding the genomic location of lncRNAs is relevant to the prediction of the possibility of lncRNA regulatory functions. Sense lncRNAs have been reported to comprise the majority of the lncRNAs, albiet poorly studied for their function regarding the difficulty in differentiating their sequences from their overlapping coding genes (Perez et al., 2021). In, a small planktonic crustacean, *Daphnia magna*, the sense lncRNA *DAPALR* reactivated the transcription of mRNA by canceling the suppression caused by a specific repressor (Perez et al., 2021). In this study, most of the identified lncRNAs involving viral infection belonged to sense lncRNA subclass, which took up 52.8% of the total lncRNAs.

In human, the cyclin D1 promoter-associated lncRNA represses transcription through an RNA binding protein TLS (Kurokawa 2011). Apart from that, a large proportion of antisense lncRNAs have been reported in many research studiesto have many regulatory functions over specific genes to modulate different signaling pathways (B. Liu et al., 2021). Intronic lncRNAs has been explored only in a small portion, regarding their function (Ma et al., 2013). A large number of lincRNAs have been identified with various regulation functions such as transcriptional regulation, translational control, splicing regulation, other post-transcriptional regulation, etc. (Ma et al., 2013). In this research, 82 lincRNAs which might regulate the function of their neighboring genes were found. In addition, these findings visualized the function of lncRNAs in WSSV infection, which might be mostly associated with transcriptional modulation which up- or down-regulated their adjacent or their co-expressed genes.

Table 2 Classification of lncRNAs induced by WSSV infection

lncRNA subclasses	**No. of lncRNAs**
Sense lncRNAs	860
Antisense lncRNAs	191
Promoter-associated lncRNAs	15
3′ UTR-associated lncRNAs	144
Intronic lncRNAs	336
Intergenic lncRNAs	82

5. Conclusion

From 3,235 DEGs, 1,628 lncRNAs have been identified in gills of *L. vannmei* infected with WSSV. The classification of putative lncRNAs returned sense lncRNAs as the major subclass, made up to 52.8% of the total putative lncRNAs, reflecting their regulatory function on expression of their adjacent genes. Further studies on lncRNAs should emphasize their functions in immune defense of host against viral infection and the mechanisms of lncRNAs regulating other coding genes should be clarified. Nevertheless, the research successfully discovered a number of lncRNAs which somehow might be associated with WSSV infection in *L. vannamei*.

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7. References

Aliperti, V., Skonieczna, J., & Cerase, A. (2021). Long non-coding rna (Lncrna) roles in cell biology, neurodevelopment and neurological disorders. *Non-Coding RNA*, *7*(2). https://doi.org/10.3390/ncrna7020036

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics (Oxford, England), 30(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bridges, M. C., Daulagala, A. C., & Kourtidis, A. (2021). LNCcation: lncRNA localization and function. *Journal of Cell Biology*, *220*(2), 1–17. https://doi.org/10.1083/JCB.202009045

Casamassimi, A., Federico, A., Rienzo, M., Esposito, S., & Ciccodicola, A. (2017). Transcriptome Profiling in Human Diseases: New Advances and Perspectives. *International Journal of Molecular Sciences 2017, Vol. 18, Page 1652*, *18*(8), 1652. https://doi.org/10.3390/IJMS18081652

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., ... & Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature biotechnology, 29(7), 644-652

Gucic, M., Cortés-Jacinto, E., Civera-Cerecedo, R., Ricque-Marie, D., & Martínez-Córdova, L. R. (2013). Apparent carbohydrate and lipid digestibility of feeds for whiteleg shrimp, Litopenaeus vannamei (Decapoda: Penaeidae), cultivated at different salinities. *Revista de Biologia Tropical*, *61*(3). https://doi.org/10.15517/rbt.v61i3.11935

Kazimierczyk, M., Kasprowicz, M. K., Kasprzyk, M. E., & Wrzesinski, J. (2020). Human long noncoding RNA interactome: Detection, characterization and function. *International Journal of Molecular Sciences*, *21*(3). https://doi.org/10.3390/ijms21031027

Kotzin, J. J., Spencer, S. P., McCright, S. J., Kumar, D. B. U., Collet, M. A., Mowel, W. K., Elliott, E. N., Uyar, A., Makiya, M. A., Dunagin, M. C., Harman, C. C. D., Virtue, A. T., Zhu, S., Bailis, W., Stein, J., Hughes, C., Raj, A., Wherry, E. J., Goff, L. A., … Henao-Mejia, J. (2016). The long non-coding RNA Morrbid regulates Bim and short-lived myeloid cell lifespan. *Nature 2016 537:7619*, *537*(7619), 239–243. https://doi.org/10.1038/nature19346

Kurokawa R. (2011). Promoter-associated long noncoding RNAs repress transcription through a RNA binding protein TLS. Advances in experimental medicine and biology, 722, 196–208. https://doi.org/10.1007/978-1-4614-0332-6_12

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. Nature methods, 9(4), 357-359

Leu, J. H., Tsai, J. M., & Lo, C. F. (2008). White Spot Syndrome Virus. *Encyclopedia of Virology*, 450–459. https://doi.org/10.1016/B978-012374410-4.00776-7

Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC bioinformatics, 12, 1-16

Liu, B., Xiang, W., Liu, J., Tang, J., Wang, J., Liu, B., Long, Z., Wang, L., Yin, G., & Liu, J. (2021). The regulatory role of antisense lncRNAs in cancer. *Cancer Cell International*, *21*(1). https://doi.org/10.1186/s12935-021-02168-4

Liu, W., & Ding, C. (2017). Roles of LncRNAs in viral infections. In *Frontiers in Cellular and Infection Microbiology* (Vol. 7, Issue MAY). https://doi.org/10.3389/fcimb.2017.00205

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, *15*(12), 1–21. https://doi.org/10.1186/s13059-014-0550-8

Lowe, R., Shirley, N., Bleackley, M., Dolan, S., & Shafee, T. (2017). Transcriptomics technologies. *PLoS Computational Biology*, *13*(5). https://doi.org/10.1371/JOURNAL.PCBI.1005457

Ma, L., Bajic, V. B., & Zhang, Z. (2013). On the classification of long non-coding RNAs. In *RNA Biology* (Vol. 10, Issue 6). https://doi.org/10.4161/rna.24604

Millard, R. S., Bickley, L. K., Bateman, K. S., Farbos, A., Minardi, D., Moore, K., Ross, S. H., Stentiford, G. D., Tyler, C. R., van Aerle, R., & Santos, E. M. (2021). Global mRNA and miRNA analysis reveal key processes in the initial response to infection with WSSV in the pacific whiteleg shrimp. *Viruses*, *13*(6). https://doi.org/10.3390/v13061140

Peng, X., Gralinski, L., Armour, C. D., Ferris, M. T., Thomas, M. J., Proll, S., Bradel-Tretheway, B. G., Korth, M. J., Castle, J. C., Biery, M. C., Bouzek, H. K., Haynor, D. R., Frieman, M. B., Heise, M., Raymond, C. K., Baric, R. S., & Katze, M. G. (2010). Unique signatures Of long noncoding RNA expression in response to virus infection And altered innate immune signaling. *MBio*, *1*(5). https://doi.org/10.1128/mBio.00206-10

Perez, C. A. G., Adachi, S., Nong, Q. D., Adhitama, N., Matsuura, T., Natsume, T., Wada, T., Kato, Y., & Watanabe, H. (2021). Sense-overlapping lncRNA as a decoy of translational repressor protein for dimorphic gene expression. *PLoS Genetics*, *17*(7), 1–18. https://doi.org/10.1371/journal.pgen.1009683

Santos, C. A., Andrade, S. C. S., Teixeira, A. K., Farias, F., Kurkjian, K., Guerrelhas, A. C., Rocha, J. L., Galetti, P. M., & Freitas, P. D. (2018). Litopenaeus vannamei Transcriptome profile of populations evaluated for growth performance and exposed to White Spot Syndrome Virus (WSSV). *Frontiers in Genetics*, *9*(APR), 1–6. https://doi.org/10.3389/fgene.2018.00120

Zhang, Y., Yao, N., Zhang, C., Sun, X., Huang, J., Zhao, B., & Li, H. (2022). LncRNA-mRNA integrated profiling analysis in response to white spot syndrome virus in hepatopancreas in Penaeus japonicus. *Fish and Shellfish Immunology*, *129*(August), 251–262. https://doi.org/10.1016/j.fsi.2022.08.061

Zhuang, L., Tian, J., Zhang, X., Wang, H., & Huang, C. (2018). Lnc-DC regulates cellular turnover and the HBV-induced immune response by TLR9/STAT3 signaling in dendritic cells. *Cellular & Molecular Biology Letters*, *23*(1). https://doi.org/10.1186/S11658-018-0108-Y

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