IDENTIFICATION AND CHARACTERIZATION OF GENES INFLUENCING WSSV DISEASE RESISTANCE IN GIANT TIGER SHRIMP (*Penaeus monodon*) USING A TRANSCRIPTOMICS APPROACH

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FACULTY OF SCIENCE
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY/BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
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IDENTIFICATION AND CHARACTERIZATION OF GENES INFLUENCING WSSV DISEASE RESISTANCE IN GIANT TIGER SHRIMP (*Penaeus monodon*) USING A TRANSCRIPTOMICS APPROACH

ABSTRACT

The giant tiger shrimp (*Penaeus monodon*) is one of the most extensively farmed crustacean species in the world. The emergence of diseases such as the white spot syndrome virus has however become a threat to the shrimp industry. Although there have been various studies utilizing RNA-Seq, more efforts are needed to further understand the molecular mechanisms of host-virus interaction in *P. monodon*. The effect of the WSSV infection on host gene expression in the haemocytes, hepatopancreas and muscle of *P. monodon* was investigated using Illumina HiSeq 2000. The RNA-Seq of cDNA libraries was developed from survived WSSV-challenged shrimp as well as from normal healthy shrimp as controls. A comparison of the transcriptome data of the two groups showed 2644 host genes to be significantly up-regulated and 2194 genes significantly down-regulated as a result of the infection with WSSV. Among the differentially expressed genes, this study reports the first discovered HMGB, TNFSF and c-Jun in *P. monodon* as new potential candidate genes for further investigation for the development of potential disease resistance markers. This study also provided valuable information on the differential expression of giant tiger shrimp genes following WSSV infection that will help to improve understanding of host-virus interactions in this species by using 22 genes related to immune response that have been identified from transcriptome data obtained in this study. An over expression of few genes including Toll protein, C-type lectin, HMGB, Kazal-type serine proteinase inhibitor 4, haemocyte kazal-type proteinase inhibitor, caspase, penaeidin, crustin, c-Jun protein, HHAP and TNF gene were observed in the present study. In addition, a total of 36,857 and 28,527 SNPs from transcriptome of survived WSSV-challenged and control shrimp were discovered respectively after 12 days post WSSV challenge, with 95.8 %

markers successfully genotyped. Furthermore, 18 SNPs candidate gene markers for WSSV resistance were also identified in the present study. These markers include caspase, lectin, C-type lectin, heat shock proteins, chromosome-associated kinesin gene, haemocyanin and actin. These markers will be useful to assist stock improvement in the giant tiger shrimp by marker assisted selection (MAS).

Keywords: *P. monodon*, WSSV-survived transcriptome, disease resistance, HMGB, TNFSF, c-Jun

PENGENALPASTIAN DAN PENCIRIAN GEN YANG MEMPENGARUHI DAYA TAHAN PENYAKIT DALAM UDANG (*Penaeus monodon*) MENGGUNAKAN PENDEKATAN TRANSKRIPTOMIK

ABSTRAK

Udang harimau (*Penaeus monodon*) merupakan salah satu spesis krustasia yang diternak secara meluas di seluruh dunia. Kemunculan penyakit seperti 'white spot syndrome virus' (WSSV) telah menjadi ancaman kepada sektor perikanan. Walaupun telah terdapat banyak kajian menggunakan penjujukan RNA (RNA-Seq), lebih banyak usaha perlu dibuat untuk lebih memahami mekanisma molekular yang terlibat dalam interaksi antara host dan virus pada *P. monodon*. Tambahan pula, penjujukan genom dalam spesis udang masih tiada. Kesan jangkitan WSSV terhadap ekspresi gen host dalam organ haemosait, hepatopankreas dan otot udang harimau telah dikaji menggunakan penjujukan generasi baru Illumina HiSeq 2000. Penjujukan RNA dihasilkan daripada udang harimau yang terselamat dijangkiti virus WSSV dan udang sihat sebagai kumpulan kawalan. Perbandingan transkriptom kedua-dua kumpulan menunjukkan peningkatan ekspresi gen dalam kumpulan host sebanyak 2644 dan penurunan ekspresi gen host sebanyak 2194 selepas dijangkiti virus WSSV. Antara gen yang telah diekspresi melalui penjujukan RNA, kajian ini menemui gen HMGBb, TNFSF dan c-Jun dalam udang harimau yang berpotensi sebagai calon gen untuk dibangunkan sebagai penanda genetik daya tahan penyakit. Kajian ini juga menyediakan maklumat berharga mengenai pembezaan ekspresi gen dalam udang harimau yang dijangkiti virus WSSV menggunakan 22 gen imun yang telah direka bentuk berdasarkan data transkriptom dari kajian ini. Kelebihan ekspresi beberapa gen termasuk Toll protein, C-type lectin, HMGB, Kazal-type serine proteinase inhibitor 4, haemocyte kazal-type proteinase inhibitor, caspase, penaeidin, crustin, c-Jun protein, HHAP dan gen TNF dapat diperhatikan di dalam kajian ini. Tambahan lagi, sejumlah 36,857 dan 28,527 penanda genetik 'single nucleotide polymorphisms' – SNPs

diperolehi daripada transkriptom udang harimau yang terselamat dijangkiti virus WSSV dan udang kumpulan kawalan selepas 12 hari diuji dengan jangkitan virus WSSV. Sebanyak 95.8% penanda genetic SNPs berjaya di genotip. Selain itu, sebanyak 18 calon penanda genetik SNPs untuk daya tahan jangkitan terhadap virus WSSV telah dikenalpasti. Calon penanda genetik tersebut termasuk caspase, lectin, C-type lectin, heat shock proteins, chromosome-associated kinesin gene, haemocyanin dan actin. Penanda genetik ini penting untuk membantu penambahbaikan stok udang harimau melalui kaedah pemilihan berdasarkan penanda genetik (MAS).

Kata kunci: *P. monodon*, transkriptom kerintangan terhadap WSSV, rintangan penyakit, HMGB, TNFSF, c-Jun

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‘On no soul doth Allah place a burden greater than it can bear.’

Surah Al-Baqarah: Verse 286.

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I dedicate this thesis to my late grandmother, Tuan Zaimah, that has been planning to come to my graduation day, each time we met.

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LIST OF SYMBOLS AND ABBREVIATIONS

aa	:	Amino acid
bp	:	Base pair
BLASTx	:	Basic Local Alignment Tool
cDNA	:	Complementary Deoxyribonucleic acid
COG	:	Cluster of Orthologous Groups of Proteins
DEG	:	Differentially expressed genes
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleotide
DEPC	:	Diethylpyrocarbonate
EF1-A	:	Elongation factor 1-alpha
EST	:	Expressed sequence tag
FDR	:	False Discovery Rate
FPKM	:	Fragments Per kb per Million fragments
GO	:	Gene Ontology
IMD	:	Immune deficiency
KEGG	:	Kyoto Encyclopaedia of Genes and Genomes
L	:	Litre
mL	:	Mililitre
mRNA	:	Messenger RNA
NaCl	:	Sodium chloride
NCBI	:	National Centre for Biotechnology Information
ng	:	Nano gram
NGS	:	Next generation sequencing
NR	:	Non redundant

OIE	:	World Organization for Animal Health
ORF	:	Open reading frame
PCR	:	Polymerase chain reaction
qRT-PCR	:	Quantitative real time PCR
RNA	:	Ribonucleic acid
RNA-seq	:	RNA sequencing
RT-PCR	:	Reverse transcriptase PCR
SRA	:	Short read archive
TSV	:	Taura Syndrome Virus
UTR	:	Untranslated region
WSSV	:	White Spot Syndrome Virus
µM	:	Micro molar

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The demand for food supply is increasing from year to year. According to the Food and Agricultural Organization, the world population is expected to grow by over a third, or 2.3 billion people, between 2009 and 2050 (Alexandratos & Bruinsma, 2012). The authors also reported that this is a much slower rate of population growth comparing to the past four decades, with a higher growth expected to take place in the developing countries. In 2017, nearly 821 million people in the world are affected by undernourishment, or chronic food deprivation (FAO, 2018a). Even by the year 2050, the economic deprivation, starvation and malnutrition will remain unsolved in major part of the world (Bodirsky et al., 2015). Furthermore, the authors reported that in order to cater the global food demand, animal-based products will be increasingly consumed

Farming of fish, crustaceans and molluscs are one of the major economic importance in many countries. These activities supplement the food supply and also compensates the overfished or declining wild-catch fisheries (Kibenge & Gordoy, 2016). The world fish production is being dominated by capture fisheries as the total production by aquaculture was only 44.1 % in 2014 and crustaceans accounted for 17 % of fisheries product (FAO, 2016). Shrimp aquaculture industries have expanded in the early 1980s due to the growing international demand for shrimp and stagnating catches of wild shrimp (Arquitt et al., 2005). The shrimp aquaculture is dominated by two species - the giant tiger shrimp (*Penaeus monodon*) and the Pacific white shrimp (*Penaeus vannamei*) (FAO, 2009). *Penaeus vannamei* is the dominant shrimp species in aquaculture (Anderson & Valderrama, 2013). However, in Vietnam, farmers reportedly shifted from Pacific white shrimp farming to giant tiger shrimp due to the supply and demand imbalance for Pacific white shrimp (FAO, 2017a).

Although the shrimp aquaculture sector production has increased, a decline in output is being experienced by major shrimp producing countries, particularly in Asia because of shrimp disease (FAO, 2017b). The emergence of disease in fish and shrimp due to the intensive aquaculture, the global movement of aquatic animals and its products, and numerous sources of human-induced stress to aquatic environments are affecting the aquaculture industry (Walker & Winton, 2010). Farmers often rely on preventive measures as there is currently no effective treatment for viral infections (Seibert & Pinto, 2012). According to Lotz (1997), strategies to overcome the disease threats in shrimp aquaculture should be based on: (i) specific pathogen-free (SPF) and genetically improved shrimp stocks; (ii) biosecure systems including enclosed, reduced water-exchange and increased water-reuse culture systems; (iii) biosecure management practices; and (iv) co-operative industry-wide disease control strategies.

In order to overcome the disease threats in shrimp aquaculture industries, stock improvement strategies involving SPF stocks and improved strains are being developed. Most of the genetically improved livestock were developed through conventional selective breeding involving hybridization, selection and crossbreeding (Hulata, 2001). Modern genomics approach to improve livestock includes quantitative traits loci (QTL), marker assisted selection (MAS) and candidate genes (Rothschild & Ruvinsky, 2007). The genomics approach will speed genetic improvement and increase levels of production (Rothschild & Plastow, 2008). Nonetheless, the effect of genetically improved strains emphasizing desirable phenotypic traits necessary for aquaculture might correlate negatively with other fitness component (Gemmill & Read, 1998). Phenotypic traits include growth, disease resistance/tolerance, body composition and flesh of a commercial aquaculture species. Although the genetic correlation between morphological traits are more often positive, not all traits are positively correlated (Norry et al., 2000). Furthermore, the authors stated that negative correlations between

traits are least liable to evolve resulting in changes of morphology to the organisms itself.

In the present research, functional genomics approach was used to identify and characterise genes influencing disease resistance in giant tiger shrimp. According to FAO Fishery Statistic 2018 (FAO, 2018b), the total aquaculture production of *P. monodon* decreased from 710,951 tonnes (USD 5.1 billion) in 2015 to 701,081 tonnes in 2016 (USD 5.3 billion). In Malaysia, due to overexploitation of wild resources, uncontrolled breeding activities and poor stock management has resulted in the decline of total giant tiger shrimp production, and is now dominated by the introduced exotic Pacific white shrimp, a native of Mexico (Briggs et al., 2004). The intensive global adoption of domesticated and genetically improved Pacific white shrimp as the species of choice over the formerly dominant giant tiger shrimp (Wyban, 2007) is a result of a major revolution after the publication of the Bangkok Declaration (Kongkeo & Jiansan, 2000). The Pacific white shrimp was the preferable culture species due to its SPF status and was claimed to be tolerant/resistant to white spot disease (WSD) (Thitamadee et al., 2016).

The overexploitation of the giant tiger shrimp resources (Portley, 2016) has led to inbreeding and a general loss of genetic variation in cultured and wild populations, especially in the western part of Malaysia which are likely becoming genetically homogenous (Aziz et al., 2011). Since local supplies of broodstock are insufficient to meet local industry demand, the practice of importing wild broodstock is widely practiced in Malaysia. A major concern related to introductions of wild broodstock for the shrimp farming industry is potential for introductions viral infections that can affect both wild and cultured local stocks. Therefore, stock improvement strategies for the aquaculture industry should address potential disease issues and move towards the

establishment of marker assisted selective breeding programs to develop and produce elite breeders and progeny for the commercial farming industry.

Hence, the specific aims of this study were to:

i. characterise functional genes that could be involved in disease resistance pathways by using a differential transcriptome approach.
ii. monitor the molecular response of selected immune related genes involved in disease resistance pathways in shrimps triggered by white spot syndrome virus infection.
iii. identify single nucleotide polymorphisms in candidate genes associated to immune response to WSSV infection, develop a set of SNPs markers for screening of WSSV disease resistant giant tiger shrimp culture line and establish the functional role of these candidate genes.

In this thesis, names of shrimp in the genus *Penaeus* are used following the practice of the Food and Agricultural Organization and the World Organization for Animal Health (OIE) as described in detail in the report of Holthuis (1980), Flegel (2008) and Ma et al. (2011).

CHAPTER 2: LITERATURE REVIEW

2.1 *Penaeus monodon* (Fabricius, 1798) – A General Introduction

2.1.1 Distribution

Penaeus monodon, commonly known as the giant tiger shrimp is widely distributed in the Indo-West Pacific from the eastern coast of Africa to the Red Sea, Japan, Australia and Malay Archipelago (Chan, 1998). The giant tiger shrimp inhabit the coastline to depths less than 30 meter, on bottoms of sand, mud or slits. Aquaculture of the shrimp is a major commercial industry across the Malay Archipelago to Australia. This species is also cultured in many other countries around the world and constitutes a major export product to markets of the USA, EU and Japan (FAO, 2017c).

2.1.2 Morphology

The giant tiger shrimp size ranges from 2.5 to 35 cm body length. It has a well-developed rostrum that generally extends beyond eyes and always bearing more than 3 upper teeth. There are no styliform projection at the base of eyestalk and no tubercle on its inner border. Both upper and lower antennular flagella are of similar length and attached to the tip of antennular peduncle. Carapace lacks both postorbital and post antennal spines. Cervical groove generally short, always with a distance from the dorsal carapace. All 5 pairs of legs are well developed, with the fourth leg bearing a single well-developed arthrobranch (hidden beneath carapace, occasionally accompanied by a second, rudimentary arthrobranch). In males, endopod of the second pair of pleopods (abdominal appendages) with appendix masculine only. Third and fourth pleopods divided into 2 branches. Telson sharply pointed, with or without fixed and/or movable lateral spines.

The body colour of the giant tiger shrimp varies from semi-translucent to dark greyish green or reddish, often with distinct spots, cross bands and/or other markings on

the abdomen and uropods. The sexes are easily distinguished by the presence of a very large copulatory organ (petasma) on the first pair of pleopods (abdominal appendages) of males, while the females have the posterior thoracic sternites modified into a large sperm receptacle process (thelycum) which holds the spermatophores or sperm sacs (usually whitish or yellowish in colour) after mating. The eggs are small and numerous and are released directly into the water and not retained on the female abdomen. The larvae are planktonic and have the nauplius stage (Figure 2.1) (Chan, 1998).

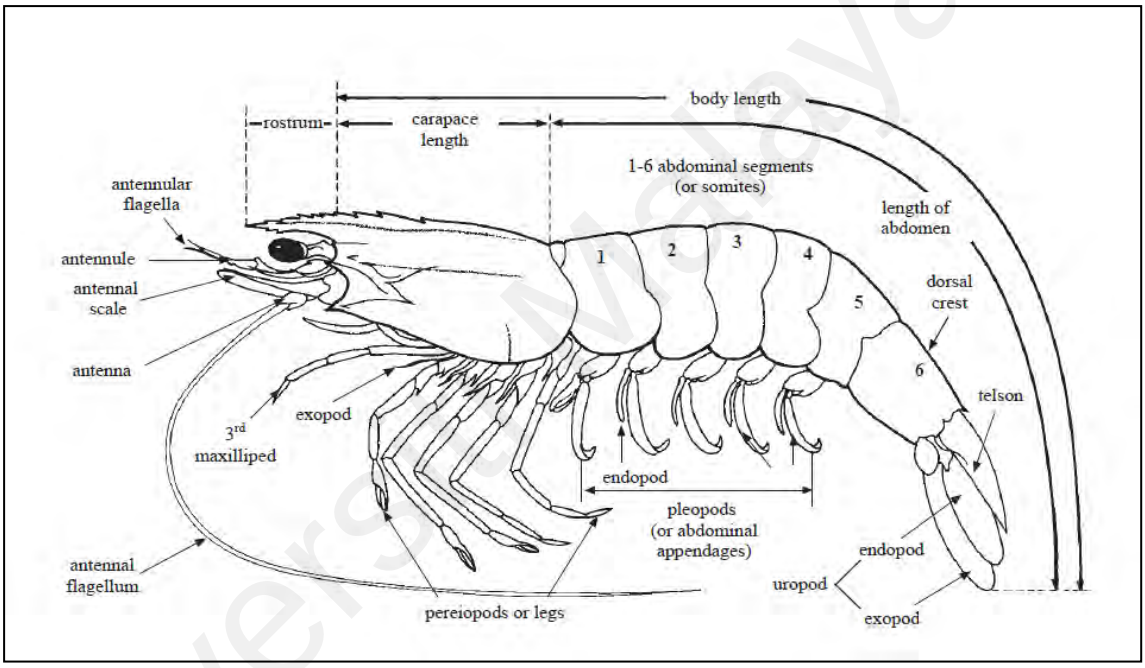


Figure 2.1: General anatomy of shrimp (Chan, 1998).

2.1.3 Life Cycle

The life cycle of species of *P. monodon* is complex (Figure 2.2). Adults generally move from shallow coastal waters to offshore and spawn at depths between 10 to 80 m. The eggs hatch within 14 to 24 hours and release very small larvae, the nauplii. The nauplius larva passes through several substages before it metamorphoses into the mysis stage. These larvae are planktonic and are carried by currents toward shore where they arrive as postlarvae (PL); this occurs about three weeks after hatching when the animals are 6 to 14 mm long and shrimp-like in appearance. The PL invade inshore brackish waters, abandon their planktonic way of life, and become bottom dwellers living in

shallow coastal areas. In these rich nursery grounds, the PL grow rapidly, develop into juveniles and, as size increases, move gradually back toward the mouths of bays or estuaries and become subadults. Soon the shrimps migrate offshore, continue growing and mate, and when they finally reach the spawning grounds, the mature females spawn and the cycle is repeated; most shrimps in these grounds are about 1-year-old, rarely older than 2 (or perhaps 3) years old (Chan, 1998).

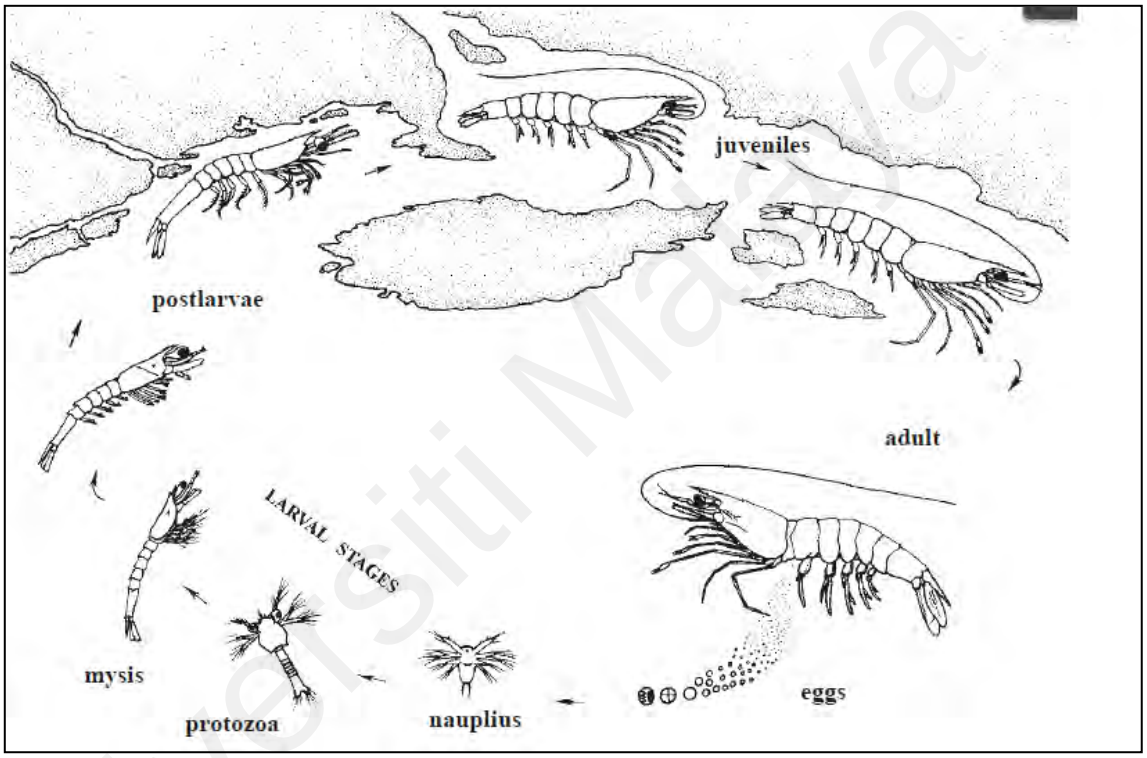


Figure 2.2: Life cycle of shrimp of genus *Penaeus* (Chan, 1998).

2.2 Disease Emergence in Shrimp Farming Industry

The shrimp farming industry has been severely affected by the emergence of diseases. The emergence of shrimp diseases has caused significant losses of global production (USD 15 billion annually), export restriction, failure of business and decreased confidence of consumers (Bondad-Reantaso et al., 2005). The emergence of disease is the result of exposure to various forms of environmental and physiological stress; handling, spawning, poor water quality or abrupt changes in temperature or salinity following viral amplification (Martínez, 2007; Walker & Winton, 2010).

The giant tiger shrimp *P. monodon* is an important aquaculture species that has been farmed for food for more than a century in Asian countries (FAO, 2017c). However, over the past century, various new diseases affecting shrimp have emerged as a result of intensive aquaculture, the increasing global movement of aquatic animals and their products, and various human-caused sources of stress to the aquatic ecosystem (Walker & Winton, 2010). Farmed penaeid shrimp are in general more susceptible to disease outbreaks than freshwater prawns (*Macrobrachium rosenbergii*) as they are farmed much more intensively (Kamalam et al., 2009). Disease outbreaks in giant tiger shrimp stocks have become a particular concern in recent years (FAO, 2017c), leading to a decline in the production of the giant tiger shrimp and its replacement in many cases by *Penaeus vannamei* – a species which is easier and simpler to farm, and which is less prone to disease problems.

Almost all shrimp virus pathogens cause very low level persistent infections and prevalence of disease can range from moderate to high in healthy farmed shrimp (Walker et al., 2001). Hence, current knowledge of shrimp viruses is needed for management and control of diseases. However, research on crustacean viruses is hampered by the limited suitable cell culture (Coelen, 1997). To date, no permanent cell line from shrimp is available (Jayesh et al., 2012)

According to Flegel et al. (2008), 60% of disease losses in shrimp aquaculture were caused by viral diseases and 20% by bacterial diseases. The remaining 20% were caused by other pathogens including parasites, fungi and due to unknown causes. According to United Nations and by the World Organization for Animal Health (OIE), the eight notifiable diseases in marine shrimp includes acute hepatopancreatic necrosis disease (AHPND), infection with infectious hypodermal and haematopoietic necrosis virus (IHHN), infection with infectious myonecrosis virus, infection with Taura

syndrome virus (TSV), infection with yellow head virus genotype 1 (YHV1), tetrahedral baculovirosis (PvSNPV), spherical baculovirosis (MBV/PemoNPV) and white spot syndrome disease (OIE, 2017).

Among all, the WSD caused by WSSV is one of the most devastating and virulent viral threat to both the Pacific white shrimp and the giant tiger shrimp (Thitamadee et al., 2016). White spot syndrome virus (WSSV), a member of the *Nimaviridae* family, is one of the eight viral pathogens causing notifiable diseases in marine shrimp (OIE, 2017), and is one of the most devastating pathogens of farmed shrimp. Until now, the methods of control for WSD still depends on preventive measures including the development and use of domestically, genetically selected SPF shrimp stocks (Moss et al., 2012). As yet there are no vaccines or other treatments available with proven efficacy against WSSV (Robinson et al., 2014). WSSV was first reported in June 1992 in cultured kuruma shrimp (*Penaeus japonicus*) in the Fujian Province of China and in nearby Taiwan (Zhan et al., 1998; Jiang, 2001). The virus can cause mass mortality (80-100%) in cultured giant tiger shrimp within 5-10 days of the first clinical signs appearing (Chou et al., 1995). In Malaysia, WSSV was first reported in 1994 and had affected 80% of farms by 1996. The virulence of WSSV increases during the rainy season (Yang et al., 2000).

2.2.1 White Spot Syndrome Virus (WSSV)

2.2.1.1 WSSV Morphology

White spot syndrome virus is an ellipsoid, bacilliform-enveloped, large double-stranded DNA virus (Wang et al., 1995). It is a member of the genus *Whispovirus*, family *Nimaviridae* which has a tail-like projection extending from one end of the WSSV virion (Tsai et al., 2004). The WSSV size ranges between 210 and 420 nm in length and 70-167 nm in diameter (Lu et al., 1997). The virion contains a rod-shaped

nucleocapsid, which encloses a DNA-protein core bounded by a distinctive capsid layer giving it a crosshatched appearance, wrapped singly into an envelope to shape the virion (Nadala et al., 1998) (Figure 2.3).

This virus has a large circular double-stranded DNA genome of 307 kbp. Analysis of the 293-kbp circular genome revealed 184 open reading frames (ORFs) of 50 amino acids or more, an unusual long ORF (18 kbp), and 9 regions along the genome with tandem repeat sequences (Figure 2.4) (van Hulten et al., 2001; Tsai et al., 2004). Genetic variation among WSSV isolates from different geographical region is evidence (Wongteerasupaya et al., 2003; de Jesús Durán-Avelar et al., 2015; Rodriguez-Anaya et al., 2016), hence, making it challenging to develop strategies to contain the disease as the genome variations of WSSV complicates it.

The WSSV viral envelope consists of at least 35 different proteins (Lin et al., 2002) with 60% the envelope proteins comprising of VP28 and VP26 (Tang et al., 2007). Both VP28 and VP26 proteins play major roles in WSSV infection into the host. VP28 envelope protein is responsible for helping the virus entry into the cytoplasm of the host as an attachment protein by binding the virus to shrimp cells (van Hulten et al., 2001; Yi et al., 2004). The VP26 protein is associated with nucleocapsid and may help WSSV to move toward the host nucleus by interacting with actin or cellular actin-binding proteins (Xie & Yang, 2005). This is achieved by interactions between WSSV VP26 with the cytoskeletal transport machinery to reach the site of transcription and replication in the host cell (Sánchez-Paz, 2010).

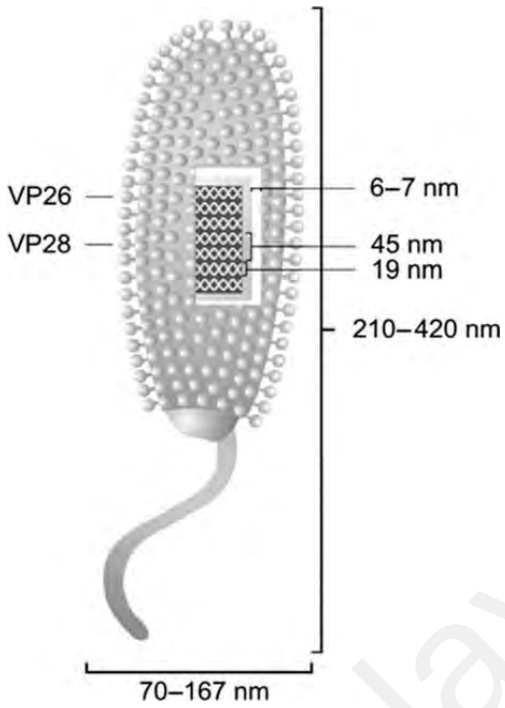


Figure 2.3: Schematic diagram of the morphology of WSSV particle (Sánchez-Paz, 2010).

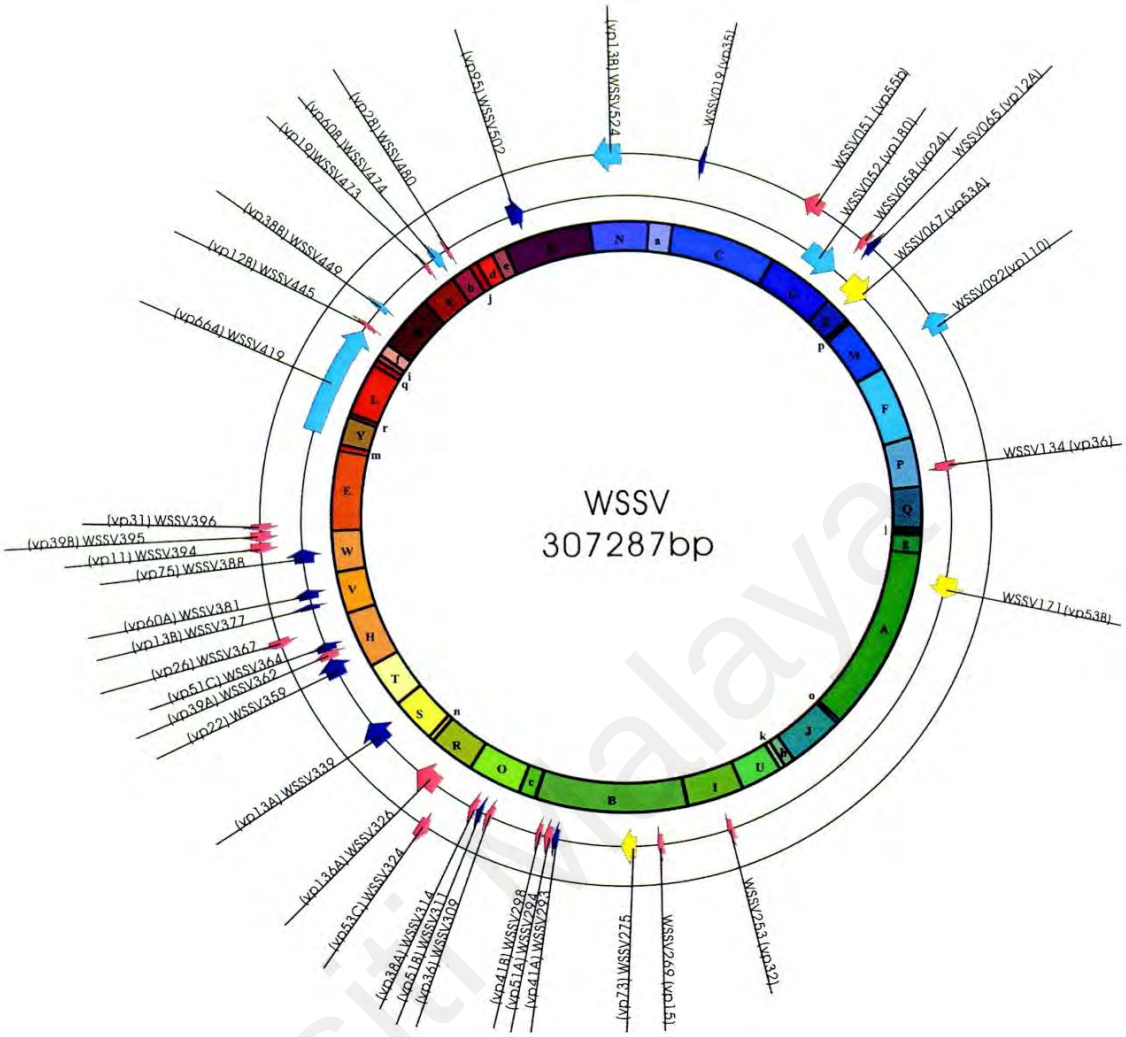


Figure 2.4: Distribution of the 39 WSSV structural protein genes in the WSSV genome (Tsai et al., 2004).

2.2.1.2 WSSV Pathogenicity, Transmission and Mode of Infection

WSSV has an extremely wide host range. Aquatic crustaceans especially decapod, including marine, brackish and freshwater prawns, crabs, crayfish and lobsters are prone to be infected with WSSV (Maeda et al., 2000). It is highly pathogenic to all species of the genus *Penaeid* (Lotz, 1997). Shrimp in the affected pond will become anorexic resulting in 80-100% mortality within 5-10 days (Chou et al., 1995). Infected shrimp will show reddish-pinkish discolouration, cease feeding, appear lethargic and congregate at the pond edges (Walker & Mohan, 2009). White spots (white calcium deposits of 0.5–2.0 mm in diameter) are commonly observed under the shell of diseased shrimp. Not all shrimp will display white spots after infection with WSSV and can only be detected by diagnostic method (OIE, 2017). The appearance of white spots also does not mean the shrimp is infected with WSSV because similar signs can occur as a result of bacterial infection, high alkalinity and other environmental conditions (Sahoo et al., 2005)

White spot syndrome virus major targets are tissue of ectodermal and mesodermal origin, especially the cuticular epithelium and subcuticular connective tissues (Wongteerasupaya et al., 1995; Chang et al., 1996). Infection from WSSV can be transmitted either vertically or horizontally by consumption of infected tissue and water-borne route. Transmission of infection can occur from apparently healthy animals in the absence of disease (Lo & Kou, 1998). According to Tsai et al. (1999), WSSV infection sometimes does not cause diseases, and it depends on the species tolerance and environmental triggers. Currently, no decapod crustacean has been reported to be resistant to WSSV, and all life stages are potentially susceptible to the virus (OIE, 2017).

During an infection, the transcription of viral genes can be divided into a cascade of three phases gene expression: immediate-early (IE), early (E) and late (L). Immediate-early (IE) genes are expressed in the absence of viral DNA replication and relied primarily on host proteins and factors for their expression. Early (E) gene expression, mainly encodes enzymes required for viral DNA synthesis, and some proteins that can regulate the expression of L genes. The expression of E gene is dependent on the preceding expression of IE genes. After viral DNA synthesis is initiated, L genes are expressed and encode enzymes and structural proteins necessary for virion assembly (Sánchez-Paz, 2010). An overview of WSSV entry and environment interactions is shown in Figure 2.5.

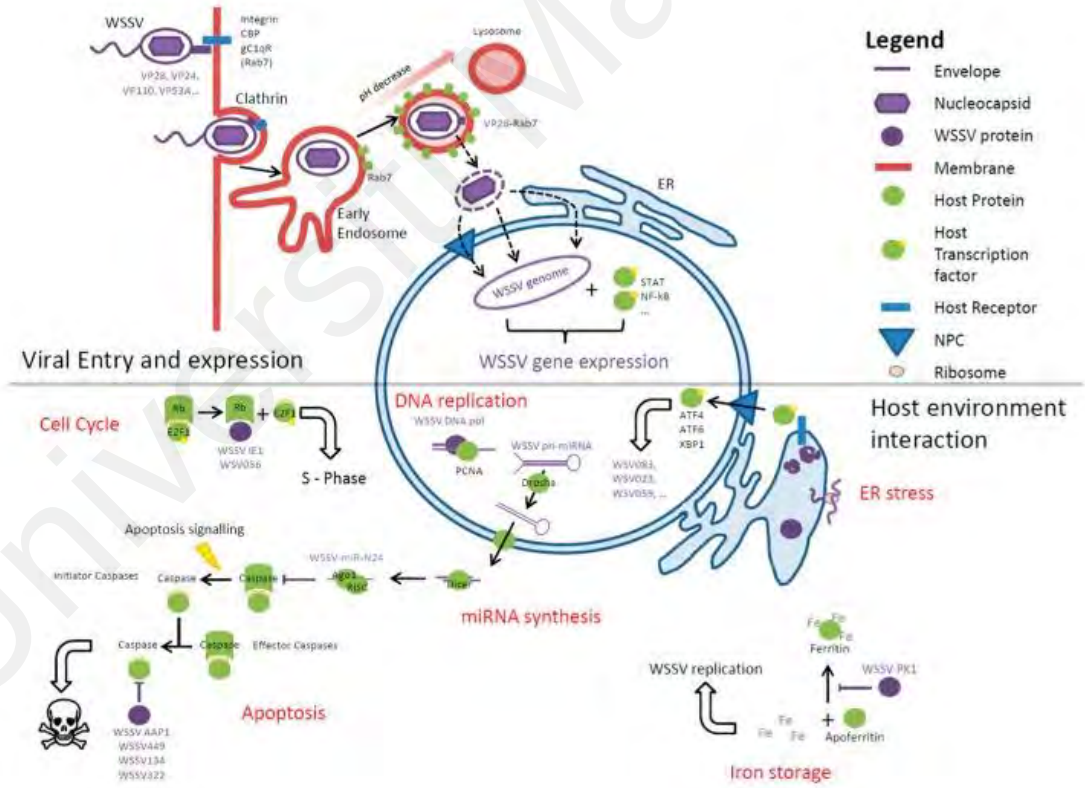


Figure 2.5: Overview of WSSV entry and environment interactions (Verbruggen et al., 2016).

2.2.2 Shrimp Immune Response Against Infection

Shrimp possess an innate immune response comprised of both cellular and non-cellular (humoral) components (Hauton, 2012). The innate immune responses are triggered by minute quantities of pathogen associated molecular patterns (PAMPs) or a viral protein antigen (Janeway & Medzhitov, 2002). Recognition of PAMPs is achieved via a spectrum of pattern recognition proteins (PRPs) of the host cell that will activate several key pathways in response to viral infection (Amparyup et al., 2013). Humoral elements are often produced in, and secreted from, circulating and fixed haemocytes and cellular responses are triggered or enhanced by factors present within the plasma (Hauton, 2012).

The humoral immune responses include clotting cascade, anti-oxidant defense enzymes like superoxide dismutase, peroxidase, catalase and nitric oxide synthase, defensive enzymes like lysozyme, acid phosphatase and alkaline phosphatase, reactive oxygen and nitrogen intermediates, antimicrobial peptides, and melanotic encapsulation (Amparyup et al., 2013). The humoral response also includes recognition to microbes and signal transduction through the various pathway (Toll pathway, IMD pathway, JAK/STAT pathway) by activation of the prophenoloxidase (proPO)-activating system. Cellular immune response of shrimp involves encapsulation, phagocytosis, coagulation, and melanization performed by different lymph gland cells (Li & Xiang, 2013).

Shrimp do not have the ability to acquire immunity and hence there is no possibility of developing vaccines for shrimps which will provide long term immunity to a specific disease (Cock et al., 2009). However, Witteveldt (2004) indicated that vaccination of shrimp against WSSV might be possible which would open the way to the design of new strategies to control WSSV and other invertebrate pathogens. Although the finding is controversial among immunologist, Cock et al. (2009) stated

that the use of vaccine might have the possibilities to stimulate the immune system and a series of non-specific responses against invading organisms.

2.3 Genetic Improvement of Stocks by Genomics Approach

The agriculture sector has advanced rapidly since the introduction and use of DNA markers in plant and animal breeding (Rafalski & Tingey, 1993). Traditionally, genetic improvement of livestock has primarily used conventional selective breeding methods (Hulata, 2000). Selective breeding is aimed to improve the genetics of animals and developed animals with improved characteristic phenotypes/traits. The diversity of phenotypic traits (e. g. growth rate, fertility, survival in different environments and resistance to disease) provides the selection of animals with superior performance in specific desirable traits (Williams, 2005). Most of the traits chosen in animal and plant genetic improvement are influenced by many genes and environmental factors (quantitative traits). Nevertheless, conventional breeding methods selection is mainly based on phenotypes, without information on which genes were selected or involved (Moniruzzaman et al., 2015).

Genomics, a study of the genomes of an organism, offers identification of genes, inter-genes relationship and its combined influence on the development and growth of the organisms (WHO, 2017). It gave a new opportunity for efficient and precise identification and selection of desirable traits. Selection and identification of desirable traits were made possible by the development of molecular markers in the 1980s. These findings enable markers related to phenotypic traits to be precisely selected from the regions within a genome that contains genes associated with specific quantitative traits, also known as quantitative trait loci (QTL)s (Collard et al., 2005). According to Norman et al. (2012), the analysis of performance traits by genome technologies are possible for genetic enhancement using QTL mapping and genome-wide association studies. The

genetic material of a species must be known in order to select a specific marker for a desirable trait and can be achieved by analyzing and sequencing the genome.

2.3.1 Next-Generation Sequencing

DNA sequencing technology has facilitated the discovery and development of the field of genomics and transformed biological and biomedical research due to the ability of the technology to sequence DNA at increasing throughput and decreasing cost (Mardis, 2017). DNA sequencing made a breakthrough in 1977 with the development of Sanger's 'chain-termination' or dideoxy technique (Sanger et al., 1977). This first-generation DNA sequencing technique has improved in the following years and contributed to the development of automated DNA sequencing machines which were used to sequence the genomes of increasingly complex species (Smith et al., 1985; Ansorge et al., 1987; Luckey & Drossman, 1990).

The second generation of DNA sequencing or pyrosequencing pioneered by (Nyrén & Lundin, 1985) utilized a luminescent method for measuring pyrophosphate synthesis. This technique could be performed using natural nucleotides and observed in real time instead of the heavily-modified dNTPs and lengthy electrophoreses used in the chain-termination (Ronaghi et al., 1998). Both Sanger's dideoxy and pyrosequencing method are 'sequence-by-synthesis' (SBS) techniques, as they both require the direct action of DNA polymerase to produce the observable output (Ronaghi et al., 1996). Pyrosequencing has evolved into the successful commercial next-generation sequencing (NGS) technology after it was licensed to 454 Life Sciences biotechnology company (Heather & Chain, 2016).

Since 2005, the NGS technologies have revolutionized biological sciences by enabling rapid discovery of genetic variation among and within individuals efficiently (Metzker, 2010). This genomics revolution has drastically reduced the cost of

sequencing by >1000 folds per base with increase outputs in an NGS platform (from less than one Gb/run in 2007 to 1500 Gb/run in 2016) (Yue & Wang, 2017). Common NGS applications include DNA sequencing (DNA-seq), RNA sequencing (RNA-seq), chromatin immunoprecipitation sequencing (ChIP-seq) and methylation sequencing (methyl-seq) (Bao et al., 2014). The NGS offers various branch of technologies that are diverse in their chemistries, specifications and capabilities. Current NGS platforms including Illumina, Roche, Ion Torrent/Life Technologies, Pacific Bioscience, Nanopore, and GenapSys, offers researchers a diverse toolbox to achieve their research objectives (Bao et al., 2014). According to Greenleaf and Sidow (2014), Illumina NGS platform has monopolized the industry becoming the most successful platform in recent years.

Illumina NGS platform uses a unique principle to amplify DNA known as bridge amplification. Bridge amplification allows replicating DNA strands to arch over to prime the next round of polymerization off neighbouring surface-bound oligonucleotides (Voelkerding et al., 2009). The sequencing library is prepared by random fragmentation of the DNA/cDNA followed by a 5' and 3'adapter ligation. Adapter-ligated fragments are then amplified by PCR. The library is loaded into a flow cell and fragments are captured on a lawn of surface-bound oligos complementary to the library adapter for cluster generation. Amplification of each fragment produces distinct neighbouring clusters of clonal populations from each of the individual original flow cell fragments (Bentley et al., 2008). After cluster generation is complete, the template is sequenced in a sequence-by-synthesis (SBS) method using fluorescent reversible terminator-bound dNTPs and allow the detection of single bases as they are incorporated into DNA template strands. The presence of reversible terminator-bound dNTPs during each sequencing cycle creates natural competition; minimizes

incorporation bias and greatly reduces raw error rates even within repetitive sequence regions and homopolymers compared to other NGS platforms (Ross et al., 2013).

Improvement of stocks utilizing genomics approaches have successfully been applied in both plants and animals. In plant, genomic selection and association mapping were applied in rice (*Oryza sativa*) (Spindel et al., 2015), coffee (*Coffea arabica* L.) (Aggarwal et al., 2007), apple (*Malus domestica* Borkh) (Potts et al., 2014), soybean (*Glycine max* (L.) Merr.) (Vuong et al., 2007), tomato (*Lycopersicon esculentum* Mill.) (Foolad, 2007) and sugarcane (*Saccharum officinarum*) (Arruda & Silva, 2007). Genomic selection for livestock improvement was successfully applied in dairy cattle (*Bos taurus* sp.) (Veerkamp & Beerda, 2007; Fortes et al., 2013) dairy sheep (*Ovis aries*) (Duchemin et al., 2012; Legarra et al., 2014), chicken (*Galus galus*) (Lahav et al., 2006), Atlantic salmon (*Salmo salar*) (Moen et al., 2009) and abalone (Hayes et al., 2007). Genomic approach for improvement of livestock enable breeding strategy based on marker-assisted selection and facilitate rapid genetic gain related to economic traits (Moniruzzaman et al., 2015). According to Yue & Wang (2017), various researches have published draft genome sequences of over 24 aquaculture species (e.g. Pacific oyster, common carp, grass carp, European sea bass), however no genome sequence has been reported in shrimp species although efforts are being made to sequence shrimp species, such as *P. vannamei* and *P. monodon*. Huang et al. (2011) suggested the reason for the absence of genome sequences in shrimp may be due to high contents of repeat sequences in the genomes of shrimp species.

Various techniques have been applied to study shrimp immune responses to viral infections, including cDNA microarray technology (Aoki et al., 2011; Lu et al., 2011; Shekhar et al., 2015) and next generation sequencing (Clavero-Salas et al., 2007; Zeng et al., 2013; Chen et al., 2013). However, although a number of immune-related proteins

in shrimp have been identified detailing the interactions between viruses and the host innate immune system using these two techniques, the interactions between WSSV and the host intracellular environment have received less attention. Research on WSD infection has been complicated by the lack of well-annotated genomic resources for host species (Verbruggen et al., 2016). WSSV is also known to infect most shrimp tissues and organs (Pradeep et al., 2012), including both immune-related and non-immune cells; and the expression of these different cell types to the pathogenesis of the virus is likely to be different (Leu et al., 2007). Hence, this present study focuses not only on immune-related tissues (hepatopancreas and haemocytes), but also investigates the host-virus interaction of non-immune-related tissue (muscle) and intracellular environment.

Large-scale and detailed assessments of the transcript abundance and transcript structure in host tissues can be obtained from the sequencing of the transcriptome (Grabherr et al., 2011). Thus, applying gene expression profiling to the interactions between the virus and shrimp can provide insights into the mechanisms through which WSSV suppresses and destabilizes host defence responses (Chen et al., 2013). By providing a partial description of the transcribed regions in a target organism, transcriptome analysis can be a resource for the identification and mining desirable gene traits (Leu et al., 2011). Specifically, by using the Illumina HiSeq 2000 Platform, the present study aims to provide valuable information on the differential gene expressions of *P. monodon* challenged with WSSV and to identify disease resistance genes for breeding purposes.

2.3.2 Gene Expression Analysis and Molecular Immune Response in Functional Genomics

Gene expression analysis is an important functional genomics tool that has given a profound impact on biological research. Gene expression analysis, which involves the analysis of mRNA, can give significant information about functional genomics itself

(Gasperskaja & Kučinskas, 2017). Moreover, the study of gene expression can provide insights into cellular processes (Alberts et al., 2002). The biological mechanisms involved in pathogenicity and immune response of an organism, either resistance or susceptible, could be identified and understood, based on the resulting cascades of gene expression changes (Robledo et al., 2016). Research based on gene expression to assess immune response after pathogen challenge is valuable due to the widespread of severe epidemic diseases in shrimp aquaculture (Wang et al., 2007). As a response to a potential pathogen, invertebrates rely on innate immunity due to the lack of an adaptive immune system (Iwanaga & Lee, 2005). Various studies pertaining to the innate immune response of crustacean have been conducted extensively. However, most of the host-virus interaction focuses on a few genes from major immune related organs. In the present study, in addition to focusing on major immune related organs with the application of a high-throughput gene expression analysis, the antiviral response of the cascade of gene expression changes could also provide further insight and new findings related to the crustacean innate immunity.

The massive economic losses resulting from virus epidemics can be control by the establishment of effective methods related to the emerging infectious diseases (Tassanakajon et al., 2013). Thus, to establish effective control measures, essential information is required to understand the immune response of shrimp against invading microbes (Zeng & Lu, 2009). Shrimp rely on the innate immune responses to fight against invading microbes due to the lack of an adaptive immune system (Bachère et al., 2004). The innate immune responses involve the cellular immune reactions including phagocytosis, encapsulation, apoptosis and nodule formation; and the humoral responses including antimicrobial peptides, the prophenoloxidase (proPO) system, proteinase inhibitors (Iwanaga & Lee, 2005). Response of host genes toward virus infection have been studied in various species of penaeid shrimp including Chinese

white shrimp (*Penaeus chinensis*) (Wang et al., 2006), Pacific white shrimp (Yeh et al., 2009), kuruma shrimp (*Penaeus japonicus*) (Rojtinnakorn et al., 2002) and Indian shrimp (*Penaeus indicus*) (Yoganandhan et al., 2003). These studies provided a framework to further investigate the mechanism of interaction between the host and the virus.

Real time quantitative PCR (qPCR) has been commonly used to determine gene and/or transcript numbers present within samples (Smith & Osborn, 2009). This 'gold standard' for quantification of gene expression is a robust, highly reproducible and sensitive method (Nolan et al., 2013) and can offer an overview of interaction/response of host genes toward virus infection (Zhao et al., 2003). However, this method produces low throughput data with high cost and is a tedious technique (Sung Jang et al., 2011). Thus, to overcome the need to produce a high throughput and robust gene expression data, molecular biology was introduced to Fluidigm microfluidic dynamic arrays gene expression system.

The Fluidigm microfluidic dynamic arrays gene expression system uses the integrated fluidic circuits (IFC) (Pieprzyk & High, 2009). This system allows for possible performance of routine qPCR analysis for thousands of reactions in a single run (Sung Jang et al., 2011). IFC was able to move molecules of biological samples and reagents in a variety of arrays through tens of thousands of microfluidic controlled valves & interconnected channels (Thorsen et al., 2002). The development of IFC technology has transformed our understanding of the field of molecular biology and this technology has been applied in gene expression studies, rare mutation detection, digital haplotyping and absolute quantification of nucleic acid sequence (Melin & Quake, 2007; Ramakrishnan et al., 2013). Thus, by utilizing the IFC technology, a series of differentially expressed immune related genes in the haemocytes, hepatopancreas and

muscles of the giant tiger shrimp infected with WSSV could be identified. These results will give a broad and comprehensive understanding of the host viral defense system.

2.3.3 Marker Assisted Selection (MAS)

The NGS technologies have been widely used for the genome-wide association studies related to gene expression and transcript profiles, also known as RNA-seq or transcriptome. RNA-seq allows the profiling of gene expression levels in the sample of interest. In addition, RNA-seq can also be used to analyze transcript boundaries and intron/exon junctions, discover novel transcripts and novel alternative splice variants, profiling of noncoding RNA, nascent transcripts, and ribosome-associated mRNA. Thus, it has the potential to immensely increase our understanding of the different roles of RNA and of the various levels of regulation of gene expression (Bunnik & Le Roch, 2013). RNA-seq offers a resource for gene identification and mining SNPs for desirable traits because they provide a partial description of the transcribed regions in a target organism (Leu et al., 2011).

Functional genomics approaches have been applied for a variety of aquaculture species to help identify suites of genes that are associated with stress response (Prunet et al., 2008; Sánchez et al., 2011; Zhang et al., 2012), metabolism with dietary influence (Leaver et al., 2008; Xiong et al., 2014; Xu et al., 2017a), immunity (Xia & Yue, 2010; Pereiro et al., 2012; Köbis et al., 2013; Zhang et al., 2014) and traits of commercial interest (Ma et al., 2014; Lv et al., 2015; Robledo et al., 2016). These approaches can be utilized to develop markers from genes themselves. DNA markers that are linked to important genes may be used as molecular tools for marker-assisted selection in a breeding scheme (Ribaut & Hoisington, 1998).

Gene identification and mining SNPs for desirable traits are achievable with RNA-seq, by providing a partial description of the transcribed regions in a target

organism (Leu et al., 2011). The cost effective and rapid generation of a large amount of data derived from the transcriptomic study has enabled identification of SNPs marker for MAS (Kumar et al., 2012). Since there are no vaccines or other treatments available with proven efficacy against WSSV (Robinson et al., 2014), MAS breeding will provide an efficient and precise identification and selection of desirable trait.

Marker-assisted selection is a method whereby a phenotype is selected on the genotype of a marker (Collard et al., 2005). According to Moniruzzaman et al. (2015), MAS offer advantages as it can be used in both sexes of a species at any age and reduces generation interval of a breeding scheme. Moreover, MAS is more efficient, effective, reliable and cost-effective compared to the more conventional breeding methodology because it involves using the presence/absence of a marker as a substitute for or to assist in phenotypic selection (Collard et al., 2005).

MAS has been widely used in livestock animals including sheep, cow and bull (Biscarini et al., 2015) and has now been successfully applied in aquaculture (Dunham et al., 2014) including MAS-enhanced flounder and salmon (Ozaki et al., 2012). The use of gene sequences derived from ESTs or gene analogues, also known as the 'candidate gene approach,' holds much promise in identifying the actual genes that control the desired traits (Cato et al., 2001). These methods can also be utilized to identify single nucleotide polymorphisms (SNPs). High throughput SNP genotyping can be used to test the association of candidate SNPs with phenotypes of interest (Dunham et al., 2014).

A candidate gene approach used in the present study will enable the identification and characterization of specific functional mutations that affect variation in a specific phenotype (De-Santis & Jerry, 2007). Gene variations identified in these candidate genes and their association with economic traits may help to improve breeding programs (Guo et al., 2017). In addition, this approach is directed at genes that have

clear roles in controlling special traits, hence dramatically decreases the genotyping workload (Du et al., 2014). Thus, through this method, identification of genes influencing disease resistance in *P. monodon* can be made possible. Furthermore, negative impacts on the environment can be decreased by eliminating or at least reducing the use of both antibiotics and chemical treatments that are normally needed to control disease outbreak (Cock et al, 2009).

2.3.3.1 Single Nucleotide Polymorphisms (SNPs)

SNPs have been used to screen genomes for markers linked to QTL traits. A SNP is a single nucleotide variation at a given position in the genome between members of a species (Marth et al., 1999) that shows codominant inheritance (Vignal et al., 2002). In general, SNPs are abundant across the genomes of species and occur in coding and non-coding regions, so they have the potential to influence phenotypic variation in an individual. Thus, trait-genotype associations can be investigated effectively (Beuzen et al., 2000).

The high throughput sequencing and genotyping have led to the establishment of numerous data sets of the model and non-model organism genomes and transcriptomes (Ellegren, 2014; Karimi et al., 2017). Sequencing and genotyping, smaller more targeted portions of the genome are easier and can provide genome-wide sequence information which can be used to characterize population and selection pressure parameters as well as provide evolutionary insights (Luikart et al., 2003). Single nucleotide polymorphisms are a useful starting point to scan large and disparate regions of the genome due to their abundance in both coding and non-coding regions, co-dominant nature, and lack of ambiguity (Williams et al., 2010). Single nucleotide polymorphisms are relatively genetically stable than other mutations, with the least frequent mutation in the biological genome of 1% or greater (Zhao et al., 2018). It is deemed as the best markers for genetic

variation resource in population studies and genome mapping since its covers 90% of variations between individuals (Frohlich et al., 2004). Single nucleotide polymorphisms can be divided into coding-region SNPs (cSNPs), perigenic SNPs (pSNPs) and intergenic SNPs (iSNPs) based on their positions in the genomes (Rafalski, 2002a). These may directly influence the gene translation or gene transcription (Rafalski, 2002a).

Although SNPs are considered as the marker of choice for molecular genetics in recent years, one major drawback is the information content per SNP marker is lower than highly polymorphic microsatellite markers. Five SNP markers provide similar information to one microsatellite marker (Beuzen et al., 2000). This is due to the bi-allelic nature of these markers as compared to the multi-allelic microsatellites (Vignal et al., 2002). However, the advantages of SNPs markers surmount its shortfall of being less polymorphic than microsatellite markers due to the occurrence of homoplasy in microsatellites. Homoplasy is the occurrence of microsatellite alleles of identical size but with a different evolutionary origin, making them less suitable for association studies (Viard et al., 1998). Besides, SNPs are also liable to high-throughput automated analysis due to its bi-allelic mutation (Lindblad-Toh et al., 2000). Liu et al. (2012) proposed few considerations in the development of molecular markers from SNP candidates that include: 1) identification and exclusion of duplicated genes (paralogs) from molecular markers by genotyping at an individual level; 2) SNPs do not necessarily result in phenotypic changes; and 3) correlation of a SNP with phenotypic changes might be weak, by which phenotype may be influenced by multiple genes. Thus, the SNP candidates and their relationship with phenotypes should be validated by biological experiments, such as challenge test for disease resistance.

The rapid development of SNPs genotyping and improvement of relevant databases for SNPs, has made SNPs as a marker of choice for molecular biology studies. Single nucleotide polymorphisms have been used to investigate differences between individuals, populations and species (Williams et al., 2010). It is extensively applied for genetic maps construction, association studies between genes, product traceability and identification of breed and individual (Zhao et al., 2018). In addition, SNPs has been proven useful for studies involving susceptibility to disease and disease conditions in human (Nagrani et al., 2017; Fang et al., 2018; Li et al., 2018). These markers have also been significantly applied in aquaculture for construction of genetic linkage map, finding QTL for economically important traits such as growth, body weight, resistance to stress and disease and for marker assisted selection and its assessment of genomic selection (Wenne, 2018).

Research involving disease resistance is frequently studied as disease represents a major challenge and bottleneck in aquaculture (Yue, 2014). Studies involving SNPs for disease resistance have been made in several aquaculture species such as catfish (*Ictalurus punctatus x I. furcatus*) (Geng et al., 2015), Atlantic salmon (*Salmo salar*) (Holborn et al., 2018), Japanese flounder (*Paralichthys olivaceus)* (Shao et al., 2015), common carp (*Cyprinus carpio)* (Kongchum et al., 2011) and rainbow trout (*Oncorhynchus mykiss*) (Campbell et al., 2014). In crustacean, *P. vannamei* is one of the most extensively studied marine shrimp species. Several gene-based SNP linkage maps for this species has been constructed, revealing basic genomic architecture for this species and lays the foundation for future shrimp genomics studies (Du et al., 2010; Yu et al., 2015). Another study involving SNPs discovery in the transcriptome of this species was also discovered and will be useful for genome-wide association studies, especially in the identification of genetic markers for economically important traits (Yu et al., 2014). SNPs polymorphisms related to WSSV-resistance was discovered in the

ALF gene of *P. vannamei* and provide theoretical support for the selection of WSSV-resistant varieties of this species (Liu et al., 2014)

As in *P. monodon*, a high density linkage map has been developed based on cSNPs performed using combinations of four populations samples from India, including one population of survivors of a severe WSSV infection during pond culture (Baranski et al., 2014). The authors reported a total of 3959 SNPs that were mapped to 44 linkage groups, corresponding to the haploid chromosome number in this species. The female map was 28% longer than the male map with a 1.6 higher recombination rate observed for female compared to male meioses. However, they were unable to set up rigorous experiments to compare sequence between different group of populations in their study. Another study by Robinson et al. (2014) discovered nine QTL that were significantly associated with several hours of survival after WSSV infection in the Indian *P. monodon*. Several SNPs associated with QTL showed homology with putative immune functions including genes affecting the ubiquitin-proteasome pathway, lymphocyte-cell function, heat shock proteins, the TOLL pathway, protein kinase signal transduction pathways, mRNA binding proteins, lectins and genes affecting the development and differentiation of the immune system.

Although these studies managed to map QTL in *P. monodon*, more information is needed to increase expressed sequence tags and DNA marker resources to sufficiently cover the genome and for the validation of markers that have been discovered in *P. monodon*. Hence, in the present study, the comparative transcriptome profiling analysis between the survived WSSV-challenged and control *P. monodon* will increase the chance of detecting SNPs variants that are associated with resistance to WSSV and SNPs that occur in transcribed genes. Transcriptome sequencing can be used as a cheaper alternative to identify SNPs located in transcribed regions. Thus, markers

assisted selection based on candidate gene approach could be achieved. Marker assisted or also known as genomic selection is a suitable approach for resistance to WSSV trait due to the low heritability of this trait and its negative correlation with another selected trait such as growth.

2.3.4 Specific Pathogen Free (SPF) vs Specific Pathogen Resistance/Tolerance (SPR/SPT)

Disease prevention and control in shrimp aquaculture industry have led to a substantial increase in the use of chemical treatments and veterinary medicines such as antibiotics and prebiotics (Wang et al., 2008a). Most shrimp farmers have little understanding about safety and handling chemical products and they believe that antibiotics help prevent and treat viral disease outbreaks (Holmström et al., 2003). Viral disease outbreaks often result in escalating antibiotics use (Lebel et al., 2016). Nomoto (2005) has questioned the effectiveness of antimicrobial agents as a preventive measure due to extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria. Moreover, the use of chemical treatments in preventing and treating disease outbreak is often associated with environmental problems (Wang & Xu, 2004).

Therefore, to overcome the problems related to the use of chemicals in aquaculture, it is essential to use specific pathogen-free shrimp species that are free of specific significant pathogens (Lotz, 1997). Specific pathogen free shrimp are a special stock of shrimp that are kept in SPF facilities under a rigorous monitoring system (Barman et al., 2012). These SPF shrimp are bred under controlled conditions and have passed through a rigorous quarantine and disease screening process to maintain their freedom from specific pathogens (Briggs et al., 2004). SPF shrimp are free of specific pathogens but are not resistant to the specific pathogens or infections (Lotz, 1997).

Since the SPF shrimp are susceptible to infection by one or several specific pathogens, the need to develop shrimp that are more resistant to viral infection could equally lead to effective disease treatments for shrimp (Verbruggen et al., 2016). Specific pathogen resistant (SPR) shrimp are those that are not susceptible to infection by one or several specific pathogens, and specific pathogen tolerant (SPT) shrimp are those that are intentionally bred to develop tolerance to disease caused by one or several specific pathogens (Barman et al., 2012). A further advantage of SPR/SPT shrimp is the minimal negative impact on the environment as neither antibiotics nor chemical treatments are normally needed to enhance control of viral infection (Cock et al., 2009). According to Barman et al. (2012), SPF shrimp can possibly be developed as SPR/SPT species.

In the mid-1990s, the Taura Syndrome Virus (TSV) epidemic in Ecuador and Colombia have led to the development of TSV SPR *P. vannamei*. One of the major producers in Colombia, C.I. Oceanos S.A. initiated a program to select the survivors from TSV infected ponds and use them as parents for the next generation by mass selection scheme. This program has successfully increased the commercial pond survival rates within two to three generations (Cock et al., 2009). Following the successful program, improved domesticated line of *P. vannamei* and *P. stylirostis* broodstock has been produced throughout the America and some Asian countries including China, Thailand, Indonesia and Malaysia (Barman et al., 2012).

Hence, the need to develop shrimp that is resistant to viral disease, particularly WSSV will help the commercial farming industry to effectively treat the emergence of a disease. Until now, no commercial SPR/SPT *P. monodon* strain is available for the industry. Although few studies on WSSV resistant *P. monodon* have been carried out (Cock et al., 2009; Robinson et al., 2014), the frequency of resistance genes appears to

be very low, and there may be sources of resistance that are not included in the initial populations of the study. Furthermore, Cock et al. (2009) also highlight the difficulties encountered when there is a negative correlation between two or more desired traits. In addition, most of the markers established by those studies are not validated within a population of black tiger shrimp. Thus, the present study attempt to establish and identify markers related to WSSV-resistant black tiger shrimp and its validation in a selected population.

CHAPTER 3: MATERIALS AND METHODS

3.1 Generation of Transcriptome in the Survived-White Spot Syndrome Virus Challenged Giant Tiger Shrimp, *Penaeus monodon*

An overview of the experimental procedures involved in the generation of transcriptome in the survived-white spot syndrome virus challenged giant tiger shrimp is presented in Figure 3.1.

3.1.1 White Spot Syndrome Virus Propagation and Preparation of Virus Inoculation

Juvenile giant tiger shrimp from local wild broodstock were used for the propagation of WSSV. These shrimp (15-20g body weight), collected from a local commercial farm, were first tested by PCR to ensure that they were negative for WSSV (Kimura et al., 1996). The propagation of WSSV was achieved by feeding the juvenile shrimp minced WSSV infected muscle tissue in 40-L glass aquaria (n = 10). During propagation, the salinity of the seawater was reduced drastically from 30 ppt to 15 ppt (at 28.0± 1.0 ^{o}C) to induce stress and ensure successful infection with 100% mortality. All the dead shrimp were then frozen to -80^{o}C and tested for WSSV using OIE primer pairs VP28 F (5'TACTCAGTCGACACCACCATGGATCTTTCTTTC'3) and VP28 R (5'TACTCACTGCAGTTACTCGGTCTCAGTGCCA3') (Kimura et al., 1996).

White spot syndrome virus inoculation was prepared using the positively infected propagated shrimp, based on a method described by Supamattaya et al. (1998). Muscle tissues from the infected shrimp were homogenized and lysed in a TN Buffer (20mM Tris.HCl, 0.4M NaCl; pH 7.4) and tissue homogenate was collected and centrifuged at 3000g for 10 minutes (4^{o}C). The supernatant was filtrated through a 0.20 µM sterile microfilter and the virus stock solution was stored at -80^{o}C until used. The WSSV copy numbers in the stock solution were determined by a method described by Mendoza-Cano and Sánchez-Paz (2013) using primer pairs VP28-140Fw

(5'AGGTGTGGAACAACACATCAAG'3) and VP28-140Rv (5'TGCCAACTTCATCCTCATCA'3).

Crude viral extract prepared earlier was diluted serially to 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} to determine the lethal dose (LD_{50}). Juvenile giant tiger shrimp (15-20g body weight), obtained from Balik Pulau, Penang, Malaysia and checked by PCR to ensure they were negative for WSSV (Kimura et al., 1996), were maintained in 40-L glass aquaria (10 individuals per tank) containing seawater at a salinity of 30 ppt (at 28.0± 1.0ºC). An LD_{50} test was carried out based on Tassanakajorn et al. (2006), by injecting 100 µL of each dilution factor of the virus into the ventral 3rd abdominal segment of the shrimp using a 1 mL syringe (29 G). Control animals were injected with 100 µL PBS. These LD_{50} experiments were carried out for 7 days, during which the shrimp were monitored for activity and changes in behaviour.

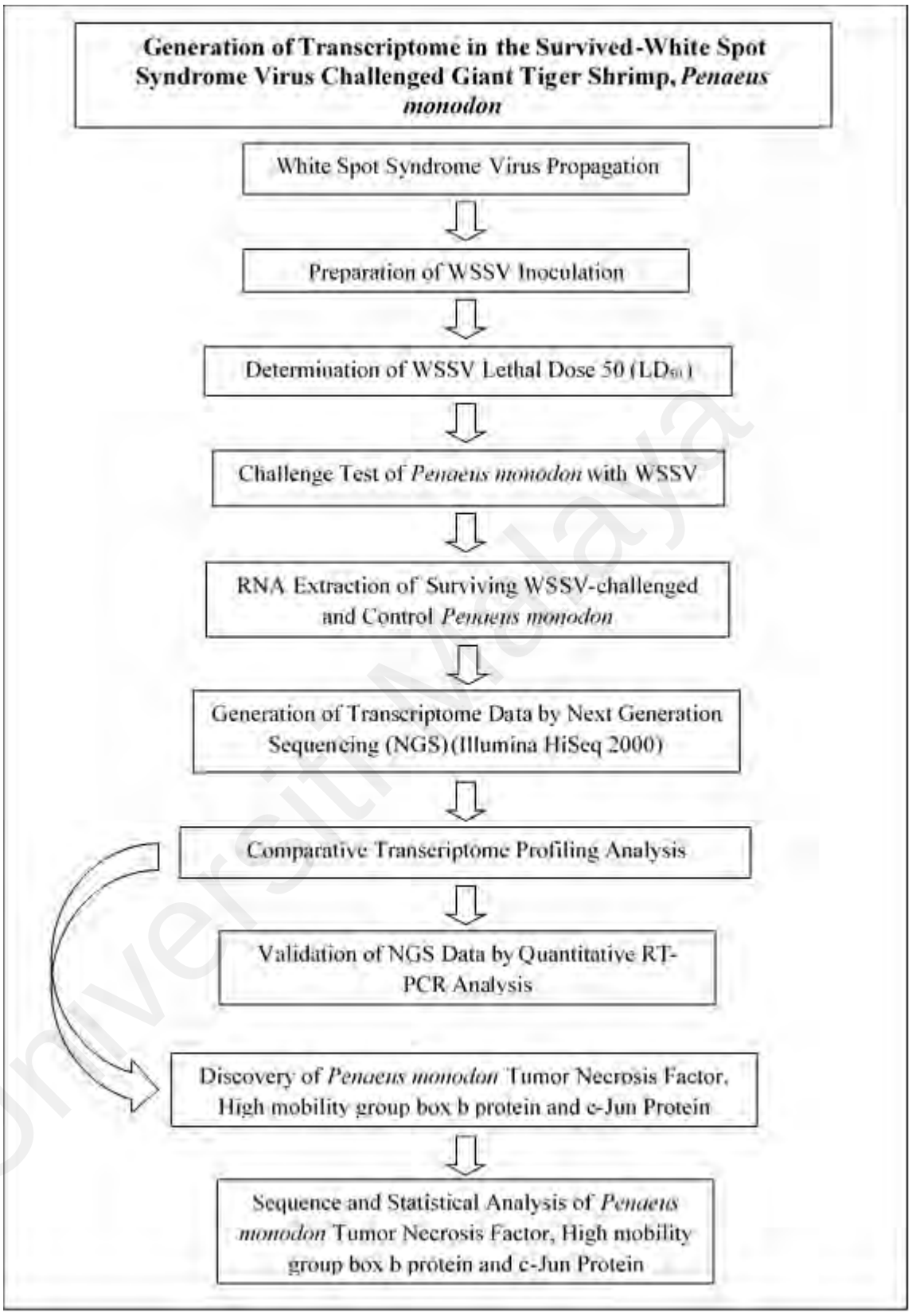


Figure 3.1: Experimental procedures involved in the generation of transcriptome in the survived-white spot syndrome virus challenged giant tiger shrimp, *Penaeus monodon*.

3.1.2 Challenge with WSSV

The giant tiger shrimp used in the challenge test experiment originated from Mozambique, Africa, and were maintained on a commercial farm in Balik Pulau, Penang, Malaysia, for breeding purposes. Eighty juvenile shrimp from the F4 generation (age 60 days, 15-20g body weight) were acclimatised for 1 week at ambient temperature prior to the challenge test to allow them to recover from the transportation stress and were fed twice daily with commercial postlarval feed. The salinity of water was 30 ppt with a pH of 7.4–7.6. The water was renewed at a daily rate of 20%. All the shrimp were checked as being WSSV negative by PCR (Kimura et al., 1996).

The experiment was performed using eight 40-L tanks with 10 individuals per tank. The shrimp were challenged with WSSV in seven tanks and one tank was the negative control tank. Artificial seawater (Forty Fathoms Marine Mix, Baltimore, MD) at 28±1.0ºC and with a salinity of 30 ppt was used in all the tanks and the tanks were equipped with air diffusers to provide sufficient aeration as well as with water filters to maintain a clean and healthy environment. Each tank was covered with green netting to contain aerosol and minimize water temperature fluctuations.

All the shrimp in the seven challenge tanks were injected with 100 µL of WSSV (4.11×10^5 viral copies/µL) in the ventral 3rd abdominal segments using a 1 mL syringe (29 G). For the negative control group, the shrimp were injected with 100 µL PBS. All the shrimp were fed with commercial shrimp pellets twice a day. The tanks were checked three times daily for moribund or dead animals. After exposure to the virus, dead shrimp were removed daily, to reduce cannibalism. These dead shrimps were frozen immediately at -80ºC. The remaining WSSV-challenged shrimp were maintained in the tanks until mortality ceased at day 12 post challenge. The live shrimp at that point were counted as survivors. All the survivors were dissected, snap frozen and stored at -

80ºC, and subsequently tested for the presence of WSSV by PCR (Kimura et al., 1996) and qPCR (Mendoza-Cano & Sánchez-Paz, 2013).

3.1.3 Generation of Transcriptome Data by Next Generation Sequencing (Illumina HiSeq 2000)

Total RNA from hepatopancreas, haemolymph and muscle of the surviving and control shrimp were isolated using an RNA Isolation Kit (Macherey's-Nagel, Germany) according to the manufacturer's protocol. The RNA was quantified by UV absorbance at 260 nm, and its quality was assessed by electrophoresis in 1% agarose gel. An equal amount of high-quality total RNA from each individual was then pooled for sequencing. A library construction and sequencing run were carried out by the Beijing Genome Institute (Hong Kong) on an Illumina HiSeq 2000 platform.

The raw sequencing reads were quality trimmed, and adaptor sequences were removed before assembly. The filtered high-quality sequences (cleaned reads) were *de novo* assembled using Trinity with default parameters (Grabherr et al., 2011). The overall assembly was produced by assembling the combined sequence data from both the surviving WSSV-challenged shrimp and the control samples. For functional annotation analysis, all the unigenes (result sequences) were compared with sequences in NCBI non-redundant (nr) protein and the Swiss-Prot, KEGG and COG databases using BLASTX programs (E-value<0.00001) (Kanehisa et al., 2008). The genes were tentatively identified according to the best hits against known sequences. Functional annotation in gene ontology terms (GO) was produced using a BLAST2GO program (http://www.BLAST2go.org/) (Conesa et al., 2005).

In order to analyse the differential gene expression, the transcript levels were measured as FPKM (Fragments Per Kilobase of exon model per Million mapped reads) values to determine the relative abundance of each gene in the transcript. A statistical

comparison between two different libraries was conducted using SOAP2.21 (http://soap.genomics.org.cn/soapaligner.html) (Audic & Claverie, 1997; Mortazavi et al., 2008). An FDR (false discovery rate) of 0.001 was used as the threshold of the p-value in the multiple tests to judge the significance of the difference in gene expression (Storey, 2002). Genes were considered differentially expressed in a given library when the p-value was less than 0.001 and a greater than two-fold change in expression between libraries was observed.

3.1.4 Validation of NGS Data & Comparative Transcriptome Profiling Analysis

Using the same RNA samples as for the Illumina HiSeq 2000 sequencing, 10 genes were chosen for quantitative RT-PCR analysis, to validate the Illumina HiSeq 2000 sequencing data (Table 3.1). First strand cDNA was synthesized from 1 µg of RNA using the ImProm-II™ Reverse Transcriptase (Promega). The qPCR reaction mixture consisted of 2X Power SYBR Green PCR Master Mix, each of the forward and reverse primers, and 1 µL of template cDNA. Primer sets were designed using the Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012).

The expression levels of the selected genes were evaluated using the high-throughput microfluidic RT-qPCR platform BioMark™ (Fluidigm 192.24 dynamic array systems) (Fluidigm Corporation, CA, USA). The sample reaction mixtures were produced in a final volume of 5 µl containing 1.25 µl of preamplified cDNA (diluted 1:5), 2.5 µl of 2X TaqMan Gene Expression Master Mix (Applied Biosystems), 0.25 µl of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.25 µl of 20X EvaGreen (Biotium) and 0.75 µl of 1X TE buffer. Primer reaction mixtures were produced in the same volume of 5 µl, containing 2.5 µl of 2X Assay Loading Reagent (Fluidigm), 1.25 µl of 20 µM of forward and reverse primer mix, and 1.25 µl of 1X TE buffer. Both sample and primer reaction mixtures were loaded into a dynamic array chip

that was subsequently placed on the HX IFC controller for loading and mixing. After approximately 50 minutes, the chip was transferred to the BioMark™ Real-Time PCR System.

The cycling program used consisted of 10 minutes at 95ºC, followed by 40 cycles of 95ºC for 15 seconds and 1 minute at 60ºC. A melting curve analysis was performed after the RT-qPCR was completed, collecting fluorescence between 60–95ºC at 0.5ºC increments. The resulting data were analyzed using the BioMark™ Real-time PCR analysis software to obtain Ct values. The elongation factor (EF) gene was selected as an endogenous reference gene. The results were presented as changes in relative expression normalized to the arithmetic mean of the Ct values of the reference gene (Livak & Schmittgen, 2001). Statistical significance was determined by a one-way ANOVA, followed by Tukey's test at $p < 0.05$.

Table 3.1: Primers used for real-time RT-qPCR validation of transcriptome data, showing nucleotide sequence and amplicon size.

Target Gene	Primer	Primer Sequence (5'- 3')	Amplification Size
Heat shock protein 10	hsp10F	ACCTTCCCTGTGAGGACCTT	113
	hsp10R	TTTGTTCCCCTGTTCGACCG	
Heat shock protein 60	hsp60F	CAGTCCTGGCTCGCACTATT	97
	hsp60R	TCCACGGCCAACATAACTCC	
Heat shock protein90	hsp90F	GGAGACGCTCAACAAATGGC	182
	hsp90R	AGACTCTGCAAACCGTACCC	
Caspase	cascF	GCGAGCATCGTAGTCGAGTT	87
	cascR	GCACGAGGTTTTGTTCGCAT	
Carcinin like protein	carcF	ACATCGTAGCAGCACTTGGA	122
	carcR	GAAGTTCACGACGGCGACT	
Anti-lipopolysaccharide factor isoform 3	alf3F	CTACAAGGGGAGGATGTGGTG	85
	alf3R	CTTTCCAGCTACCCCGGAC	
Hemocyte homeostasis-associated protein	hhapF	TTTCCTTCGGTGGGTCATCG	78
	hhapR	AGTGCAAATCGTGCAACACC	
Crustacean hematopoietic factor	chfF	GTGCCCAATTTCTTCCACGTC	133
	chfR	GTGAAGGATGCACACCCGA	
Hepatopancreas kazal-type proteinase inhibitor 1A1	hepkpiF	ACTCTGGCAATTGGCTCGTT	81
	hepkpiR	GAGAACTACGACCCCGTGTG	
Kazal-type serine proteinase inhibitor 4	ksp14F	CGCCAGGCTAATACCTCCTC	74
	ksp14R	ACGGCGTGACCTACTCTAAC	

3.1.5 Sequence and Statistical Analysis of *Penaeus monodon* Tumor Necrosis Factor, High Mobility Group Box b Protein and c-Jun Protein

The nucleotide sequences were analyzed to search for open reading frame (ORF) by ORF Finder (NCBI) (https://www.ncbi.nlm.nih.gov/orffinder/). The ORF translated amino acid sequences were search against the NCBI non-redundant (nr) protein database using BLASTp program (E-value< 0.00001) (Kanehisa et al., 2008). The protein orthologs were identified according to the best hits against known sequences. Multiple sequence alignments of the protein orthologs were generated using the Clustal Omega (McWilliam et al., 2013).

The amino acids under selective pressure were detected by the ratio of the rate of non-synonymous substitutions (dN) to the rate of synonymous substitutions (dS) for each codon, calculated with Selecton web server (Stern et al., 2007), based on M8 evolutionary model which allows for positive selection. Additionally, the dS and dN variances: Var(dS) and Var(dN), were estimated respectively. With this information, dN/dS was calculated and the null hypothesis of no selection (H0: dN = dS) versus the positive selection hypothesis (HA: dN > dS) using the Z-test: $Z = (dN-dS) / \sqrt{(Var (dS) + Var (dN))}$ was tested. Z tests calculations were performed using the MEGA software (Tamura et al., 2013).

3.2 Survivability and Immune Response of *Penaeus monodon* Experimentally Infected with White Spot Syndrome Virus Infection

An overview of the experimental procedures involved in the survivability and immune response of *P. monodon* experimentally infected with WSSV infection is presented in Figure 3.2.

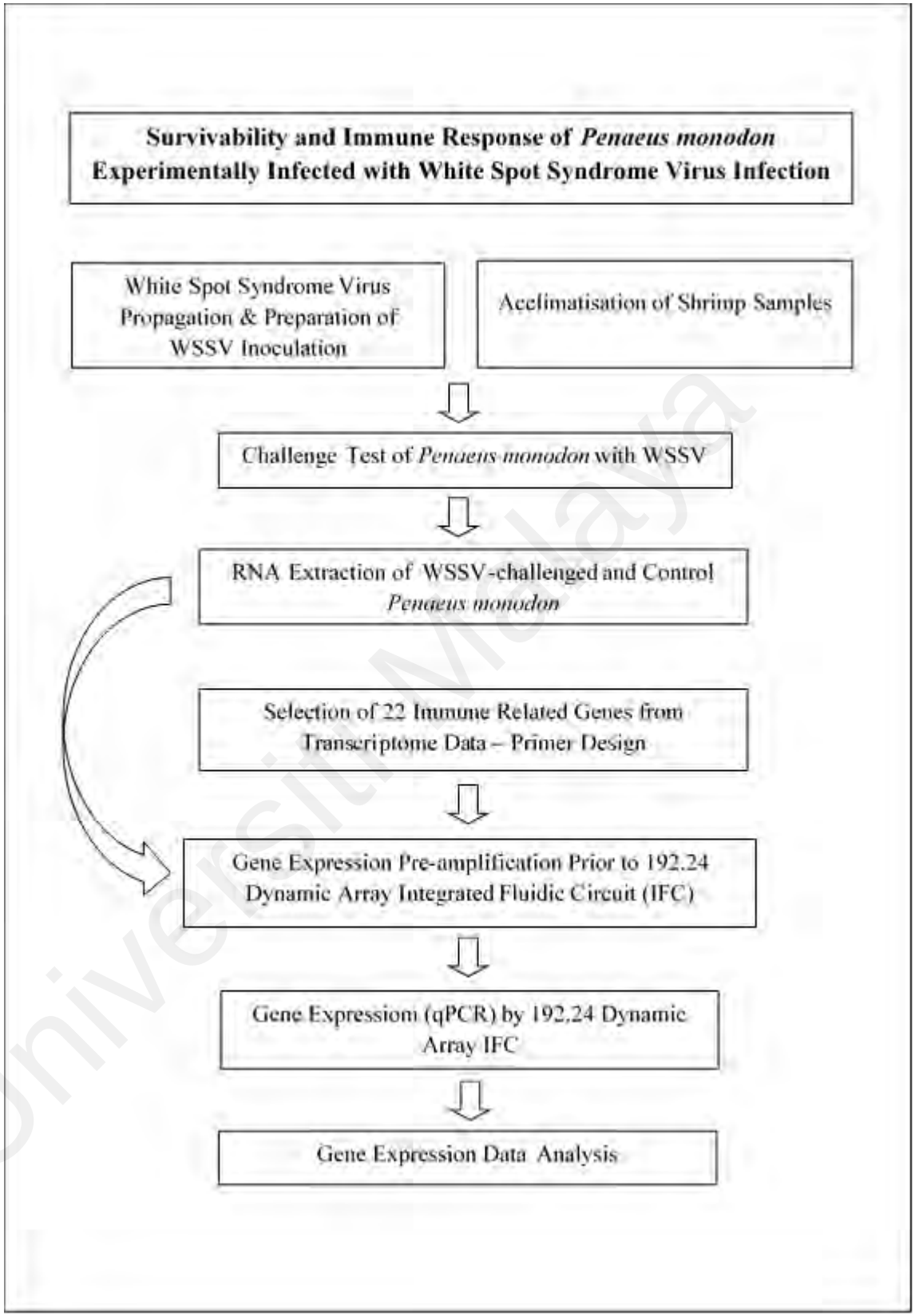


Figure 3.2: Experimental procedures involved in the survivability and immune response of the giant tiger shrimp (*Penaeus monodon*) experimentally infected with white spot syndrome virus infection.

3.2.1 Shrimp Samples

Juvenile giant tiger shrimp, bred from local wild caught broodstock were used for the propagation of WSSV. These shrimp (15-20g body weight) collected from a local commercial farm were tested WSSV negative by PCR (Kimura et al., 1996). The giant tiger shrimp that were used in the challenge test experiment originated from Mozambique, Africa and were maintained in a commercial farm in Balik Pulau, Penang, Malaysia for breeding purposes. Ninety juvenile shrimp from the F4 generation (age 60 days) were acclimatised for 1 week at ambient temperature prior to the challenge test to allow recovery from transport stress and were fed twice daily with commercial postlarval feed. The salinity of water was 30 ppt with pH 7.4–7.6. Water was renewed at a daily rate of 20%.

3.2.2 White Spot Syndrome Virus Propagation and Preparation of Virus Inoculation

The propagation of WSSV was achieved by feeding juvenile shrimp with minced WSSV infected muscle tissue in 40-L glass aquaria. During propagation, the salinity of the seawater was reduced drastically from 30 ppt to 15 ppt (at 28.0± 1.0 ºC) to induce stress and ensure successful infection. All dead shrimp were frozen in -80ºC and tested for WSSV using OIE primer pairs VP28 F (5'TACTCAGTCGACACCACCATGGATCTTTCTTTC'3) and VP28 R (5'TACTCACTGCAGTTACTCGGTCTCAGTGCCA3') (Kimura et al., 1996).

White spot syndrome virus inoculation was prepared using the positively infected propagated shrimp. Virus inoculation preparation was based on a method described by Supamattaya (1998). Muscle tissues of infected shrimp were homogenised and lysed in TN Buffer (20mM Tris.HCl, 0.4M NaCl; pH 7.4) and tissue homogenate was collected and centrifuged at 3000g for 10 minutes (4ºC). The supernatant was

filtrated through a 0.20 µM sterile microfilter and the virus stock solution was stored at -80°C until used.

3.2.3 Molecular Response of Selected Immune Related Genes in Shrimp Triggered by WSSV Infection

The experimental challenge consisted of one 40-L negative control tank and six 40-L WSSV-challenged tanks (10 individuals per tank). Artificial seawater (Forty Fathoms Marine Mix, Baltimore, MD) at 28±1.0°C and salinity of 30 ppt was used in the challenge test. All tanks were equipped with air diffuser to provide sufficient aeration and water filter to maintain a clean and healthy environment. Each tank was covered with green netting to maintain aerosol and minimize water temperature fluctuations. All shrimp of the six challenge tanks (n = 60) were injected with 100 µL of WSSV stock solution (4.11 x 10^5 viral copies/µL) into the ventral 3rd abdominal segment of the shrimp using a 1mL syringe (29 G). Control animals (n = 10) were injected with 100 µL PBS. Shrimp were fed with commercial pellet shrimp diet twice a day. All tanks were checked thrice daily for moribund or dead animals.

To reduce cannibalism in the experimental tanks, dead shrimp were removed daily after virus exposure and throughout the test period until the study was terminated on day 12. Hepatopancreas, muscle tissue and haemolymph were collected from the WSSV challenged and control shrimp at 0, 6, 12, 24, 48 hours post infection (hpi) and 12 days post infection (dpi). The time point 0 hpi refer to the time point at which the samples were dissected and collected immediately after being injected with 100 µL of WSSV stock solution. For each time point, samples were collected in triplicate. Haemolymph was collected using a 1mL syringe (30 G needle) pre filled with 100 µL anticoagulant. All tissue was snap frozen and stored at -80°C. Total RNA from all tissue was extracted using TRIzol reagent (Qiagen) and first strand cDNA was synthesized from 1 µg of RNA using ImProm-II™ Reverse Transcriptase (Promega). RNA was

quantified by UV absorbance at 260 nm and its quality was assessed by electrophoresis in 1% agarose gel. All tissue were tested for presence of WSSV by PCR (Kimura et al., 1996) and qPCR (Mendoza-Cano & Sánchez-Paz, 2013).

For quantitative RT-PCR analysis, 22 immune related genes were chosen from the initial Illumina HiSeq 2000 transcriptome data as described in Chapter 3/Section 3.1.3 (Table 3.2). Primer sets were designed using the Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012).

3.2.3.1 Gene Expression Pre-amplification

Prior to qPCR, primers (assays) mix was prepared by pooling each of 100 µM assays with DNA suspension buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA; TEKnova) and were stored in -20ºC until needed. The final concentration of each assay in the pooled mix was 500 nM. Then, pre-amplification of cDNA was prepared by mixing 1 µL TaqMan PreAmp Master Mix (2X), 0.5 µL of 24-pooled assay mix (0.2X) and 1.25 µL of cDNA to the final volume of 5 uL. The pre-amplification PCR was performed at 95ºC for 2 minutes, 18 cycles at 95ºC for 15 seconds and then 60ºC for 4 minutes. After pre-amplification of PCR, the unincorporated primers were removed by adding 4 U/ uL Exonuclease I to the pre-amplification products. The mix was incubated at 37ºC for 30 minutes and 80ºC at 15 minutes. The products were diluted 1:5 with TE Buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA; TEKnova) and stored at -20°C until needed.

3.2.3.2 Analysis of Gene Expression by 192.24 Dynamic Array IFC

qPCR was carried out using the 192.24 Dynamic Array IFC (Fluidigm Corporation, CA, USA) following the manufacturer's protocol (Spurgeon et al., 2008). The sample reaction mixtures were performed in a final volume of 5 µl containing 1.25 µl of preamplified cDNA (diluted 1:5), 2.5 µl of 2X TaqMan Gene Expression Master Mix (Applied Biosystems), 0.25 µl of 20X DNA Binding Dye Sample Loading Reagent

(Fluidigm), 0.25 µl of 20X EvaGreen (Biotium) and 0.75 µl of 1X TE buffer. Primer reaction mixtures were made in total volume of 5 µl containing 2.5 µl of 2X Assay Loading Reagent (Fluidigm), 1.25 µl of 20 µM of forward and reverse primer mix and 1.25 µl of 1X TE buffer. Both sample and primer reaction mixtures were loaded into the dynamic array chip that was subsequently placed on the HX IFC controller for loading and mixing. After approximately 50 minutes, the chip was transferred to the BioMark™ Real-Time PCR System.

The cycling program used consisted of 10 minutes at 95ºC followed by 40 cycles of 95ºC for 15 seconds and 1 minute at 60ºC. Melting curve analysis was performed after completed RT-qPCR collecting fluorescence between 60–95ºC at 0.5ºC increments. The data was analyzed with Real-Time PCR Analysis Software in the BioMark instrument (Fluidigm Corporation, CA, USA) to obtain Ct values. The elongation factor (EF) gene was selected as endogenous reference gene. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, Inc.). Results were presented as changes in relative expression normalized to the arithmetic mean of the Ct values of the reference gene (Livak & Schmittgen, 2001). Relative gene expression values were determined using the the $2^{-\Delta\Delta CQ}$ Livak method (Livak & Schmittgen, 2001). Statistical significance was determined by one-way ANOVA or Welch ANOVA followed by Tukey's test at $p < 0.05$.

Table 3.2: Primers used for real-time RT-qPCR gene expression analysis providing, nucleotide sequence and amplicon size.

Target Gene	Primer	Primer Sequence (5'- 3')	Amplification Size
Heat shock protein 10	hsp10F	ACCTTCCCTGTGAGGACCTT	113
	hsp10R	TTTGTTCCCCTGTTCGACCG	
Heat shock protein 60	hsp60F	CAGTCCTGGCTCGCACTATT	97
	hsp60R	TCCACGGCCAACATAACTCC	
Heat shock protein 90	hsp90F	GGAGACGCTCAACAAATGGC	182
	hsp90R	AGACTCTGCAAACCGTACCC	
Caspase	cascF	GCGAGCATCGTAGTCGAGTT	87
	cascR	GCACGAGGTTTTGTTCGCAT	
Carcinin like protein	carcF	ACATCGTAGCAGCACTTGGA	122
	carcR	GAAGTTCACGACGGCGACT	
Anti-lipopolysaccharide factor isoform 3	alf3F	CTACAAGGGGAGGATGTGGTG	85
	alf3R	CTTTCCAGCTACCCCGGAC	
Haemocyte homeostasis-associated protein	hhapF	TTTCCTTCGGTGGGTCATCG	78
	hhapR	AGTGCAAATCGTGCAACACC	
Crustacean hematopoietic factor	chfF	GTGCCCAATTTCTTCCACGTC	133
	chfR	GTGAAGGATGCACACCCGA	
Hepatopancreas kazal-type proteinase inhibitor 1A1	hepkpiF	ACTCTGGCAATTGGCTCGTT	81
	hepkpiR	GAGAACTACGACCCCGTGTG	
Haemocyte kazal-type proteinase inhibitor	haekpiF	TGTGTGTGGCTCTGATGGAA	85
	haekpiR	GTGGAAAGTGACACTCACGC	
Kazal-type serine proteinase inhibitor 4	ksp14F	CGCCAGGCTAATACCTCCTC	74
	ksp14R	ACGGCGTGACCTACTCTAAC	

Table 3.2, continued.

Target Gene	Primer	Primer Sequence (5'- 3')	Amplification Size
Haemocyanin	HaemoF	ATGTGGATGGTGTTGCTCGT	132
	HaemoR	CGTCAATGCCACGCTCATTC	
C-type lectin	C-TlecF	ACGGCACCAACACGACTATT	154
	C-TlecR	TGGCAGACGAAGGAGAGGTA	
penaeidin 3	Pen3F	CCAAGGATACAAGGGCGGTT	110
	Pen3R	GGCATGAAGTGCAACCAGTG	
crustin type I	Crus-1F	AGCGCCCTCCATCAATTTCA	121
	Crus-1R	GCCGTCTTCTCCCAAACGTA	
Toll Protein	TollF	TGCCACACCCACAAGAACTTA	137
	TollR	CCAGTTACTCGCATGCACAAAA	
Tumor Necrosis Factor Superfamily	TNFSF-F	CGGTGTGGCAAGAAGGAGAC	124
	TNFSF-R	CTCTATGCGCAGGTGCTGT	
Ras-related C3 botulinum toxin substrate 1	RC3F	GCAAGACGTGCATGTTGATCT	85
	RC3R	GGGGCCGAATAGTTGTCGAA	
Crustin 2	Crus-2F	CGAAATACAGATCTTCCCGGC	78
	Crus-2R	TGCTACATCGACAGCGACT	
Actin 2	Act-2F	TTGCTGGTCGTGACCTTACC	126
	Act-2R	CAAGGGCGATGTAGCAAAGC	
High mobility group box b protein	HMGBb-F	GTTGCTTGGCCACATCACCT	97
	HMGBb-R	AGAGCGTTGTCTGCCTTCTTC	
c-Jun protein	c-Jun-F	GAAAACGCTCAAGGGCGAGA	128
	c-Jun-R	GCGTTACGAAGGGGATCTGG	

3.3 Identification of Candidate Genes for Disease Resistance in *Penaeus monodon* Survivability against the White Spot Syndrome Virus

An overview of the experimental procedures involved in the identification of candidate genes for disease resistance in *P. monodon* survivability against the WSSV is presented in Figure 3.3.

3.3.1 SNPs Analysis from Transcriptome of WSSV-survived and Control Shrimp

3.3.1.1 Detection and Filtering of SNPs – Bioinformatics Analysis

Based on the transcriptome data generated from the WSSV-survived and control shrimp (Chapter 3/Section 3.1.3), SNPs were detected and filtered using SOAPsnp (Li et al., 2009). The cleaned reads obtained from the RNA-seq were aligned with the transcripts that were assembled by Trinity, and the duplicated reads and multi-mapped reads were filtered. Subsequently, the alignment results were sorted according to the transcripts' positions. SOAPsnp was used for SNPs calling based on the sorted data, and initial raw prediction results were obtained. After further filtering based on their quality values, sequencing depths and SNPs separation distances, final SNPs prediction results were acquired.

3.3.2 SNPs Validation and Genotyping

3.3.2.1 Primer Design

A total of 96 SNPs markers were chosen as candidate genes for disease resistance traits from the comparison between both WSSV-survived and control shrimp transcriptome. Two criteria were used to select sequences for the design of primers from transcript sequences of WSSV-survived *P. monodon* transcriptome dataset. The contigs must contain SNPs identified in the initial Illumina HiSeq 2000 RNA-seq and have a putative function related to immune response. All selected contigs were annotated

provisionally to identify exon, intron and untranslated regions via BLASTx and ORF searches of public databases.

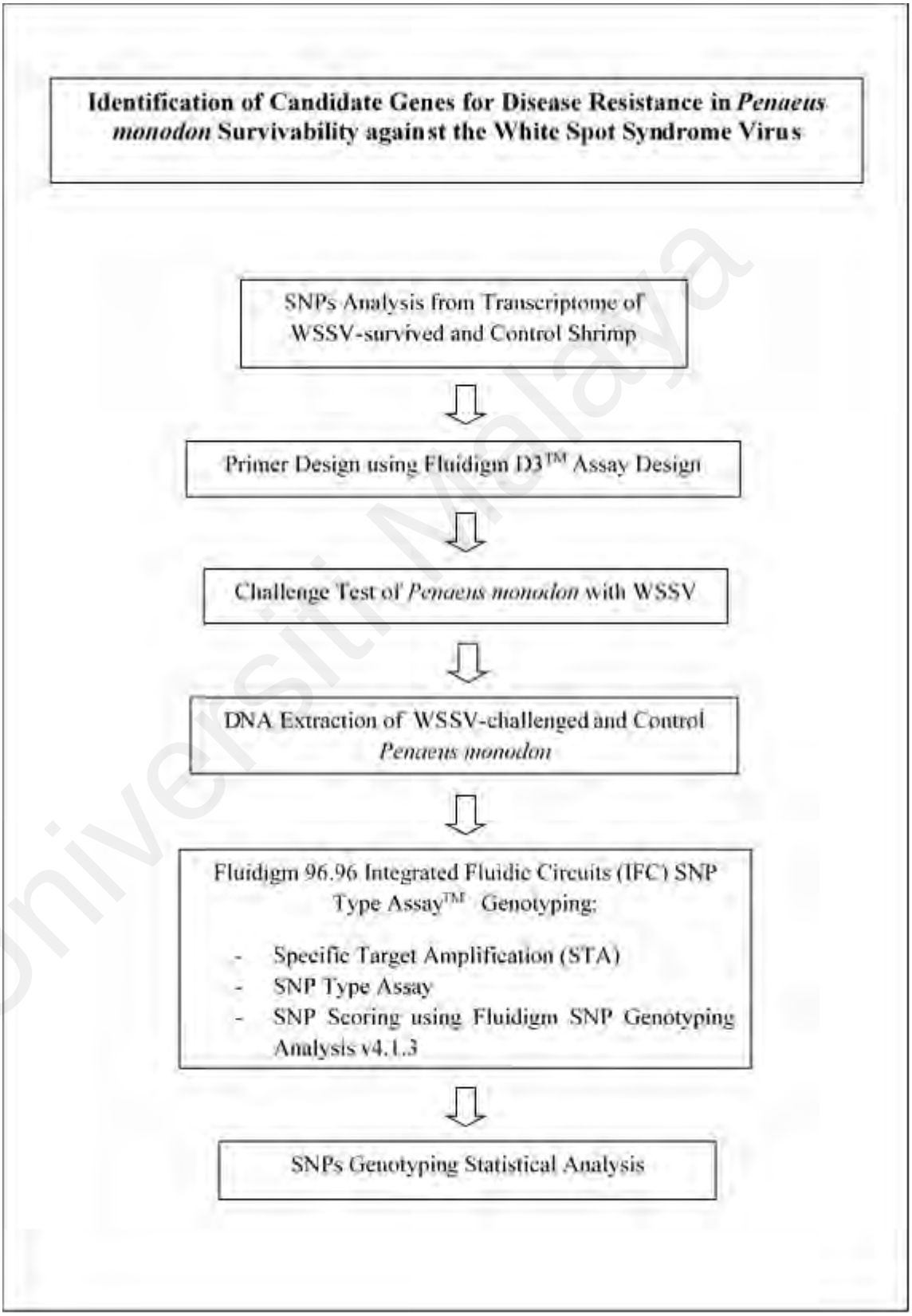


Figure 3.3: Identification of candidate genes for disease resistance in the giant tiger shrimp (*Penaeus monodon*) survivability against the white spot syndrome virus.

Primers were design using Fluidigm D3™ Assay Design (https://d3.fluidigm.com/; Fluidigm, South San Francisco, CA, USA). The following target sequence criteria were employed to design primers for the SNP type assays: 1-length of the target sequences: a minimum of 60 bp (including both upstream and downstream of the target SNP site) and a maximum of 250 bp. For SNPs, only one was present in the target sequence. For insertions/deletions (In/Dels), the length of the In/Del was shorter than 10 bp. The G/C content of the target sequence was < 65%. Primer information is listed in Appendix A. Each assay consisted of three types of primers: a specific target amplification (STA) primer, a locus-specific primer (LSP) primer, and two allele-specific primer (ASP) (Wang et al., 2009a).

3.3.2.2 Shrimp Population, Challenge Test and Isolation of Genomic DNA

A test panel consisting of 167 giant tiger shrimp samples was used to validate the SNPs primers. These included 43 tiger shrimp samples collected from Balik Pulau, Penang (Mozambique; F4 generation; age 60 days; 15-20g body weight) and 124 samples from Manjung, Perak (Madagascar x Local; F1 generation; age 60 days; 15-20g body weight). For SNPs association study, a total of 200 samples, characterised as fast growth shrimp (N = 100) and slow growth shrimp (N = 100) from a selected line reared in Manjung, Perak were sampled for the study. All samples were experimentally challenged with WSSV as described in Chapter 3/Section 3.1.2. Muscle tissue and pleopod were dissected and stored in -20^{o}C prior to genomic DNA isolation.

DNA Extraction was performed by using EasyPure^{R} Marine Animal Genomic DNA Kit (TransGen Biotech, Beijing, China) following the manufacturer's protocol. Approximately 30 mg of minced tissue was placed into a 1.5 ml sterile microcentrifuge tube and was added with 200 µL of Lysis Buffer 8 and 20 µL of RNAse before being vortexed and incubated at room temperature for 2 minutes. Then, 20 µL of Proteinase K

was added into the microcentrifuge tube and mixed thoroughly by vortexing and samples were incubated at 55ºC until lysis was completed. After lysis, 1.5X volume of Binding Buffer 8 was added and mixed thoroughly before being transfer into a spin column and collection tube. The samples were centrifuged at 12 000 g for 30 seconds and flow-through was discarded. A volume of 500 µL of Clean Buffer 8 was added to the spin column and centrifuged at 12 000 g for 30 seconds before flow-through was discarded. This step was repeated twice. Later, 500 µL of Wash Buffer 8 was applied to the spin column, centrifuged at 12 000 g for 30 seconds and flow-through was discarded. This step was also repeated twice. Then, to completely remove residual Wash Buffer 8, the spin column was centrifuged at 12 000 g for 2 minutes. The spin column was later transferred into a new sterile 1.5 ml microcentrifuge tube and was added with 50 µL of Elution Buffer. The tube was incubated at room temperature for 2 minutes and was centrifuged as 12 000 g for 1 minute to elute the genomic DNA. Eluted genomic DNA was stored in -20ºC until use. All the genomic DNA was quantified by UV absorbance at 260/280 nm, and its quality was assessed by electrophoresis in 1% agarose gel.

3.3.2.3 Fluidigm 96.96 Integrated Fluidic Circuits (IFC) SNP Type Assay™ Genotyping

Specific Target Amplification (STA)

Specific target amplification (STA) for the enrichment of the amplicon including the targeted SNP sequences, was performed to increase the probability of success of the SNP type assay. First, a 10X SNPtype STA primer pool was prepared to comprise a mixture of 2 µL of STA primer for each of the 96 markers, 2 µL of LS primer for each of the 96 markers, and 16 µL of DNA suspension buffer (TE buffer; pH 8.0; Invitrogen, Waltham, MA, USA). For each of the samples, STA was performed using Bio-Rad C1000 Thermal Cycler (Ramsey, Minnesota, United States) in a total volume of 5 µL

per reaction, which contained 2.5 µL of 2X Multiplex PCR Master Mix (Biotechrabbit, Hennigsdorf, Germany), 0.5 µL of the 10X SNPtype STA primer pool, 0.75 µL of PCR-certified water and 1.25 µL of genomic DNA with the following PCR profile: pre-denaturation at 95°C for 15 minutes followed by 14 cycles of a 2-step amplification at 95°C for 15 seconds and 60°C for 4 minutes. Then, the SNPtype STA products were diluted to 1:100 in DNA suspension buffer (TE buffer; pH 8.0; Invitrogen, Waltham, MA, USA) and stored in -20ºC until use.

SNP Type Assay

To perform the SNP type assays using the 96.96 IFC, the 10X assays mix and sample mix were prepared. The 10X assay mix contained 2 µL of 2X Assay Loading Reagent, 1.2 µL of PCR-certified water, and 1.0 µL of the assay primer mix (prepared in a master mix for 96.96 IFC), which was comprised of 3 µL of each AS primers, 8 µL of each LS primers, and 29 µL of DNA suspension buffer (TE buffer; pH 8.0; Invitrogen, Waltham, MA, USA). Subsequently, 4 µL of the 10X assay mix were combined with 1 µL of the assay primer mix in each well of a 96-well plate.

The sample mix was prepared by mixing sample pre-mix for 96.96 IFC contained 360 µL of 2X Fast Probe Master Mix (Biotium, Fremont, CA, USA), 36 µL of 20 SNP Type sample loading reagent, 12 µL of 60X SNP Type reagent, 4.3 µL of 50X ROX dye (Invitrogen, Life Technologies, Waltham, MA, USA), and 7.7 µL of PCR-certified water. Then, the sample mix was prepared by mixing 2.5 µL of each STA product and 3.5 µL of the sample pre-mix in each well of a 96-well plate.

Finally, 4 µL of each 10X assays mix and 5 µL of each sample mix were loaded into the 96 assay inlets and 96 sample inlets of the 96.96 IFC, respectively. The SNP type assays were performed in series using two machines, the HX IFC controller (Fluidigm, South San Francisco, CA, USA) and the BioMark™ Real-Time PCR System

(Fluidigm, South San Francisco, CA, USA) according to the manufacturer's instructions (Wang et al., 2009a).

Scoring of SNPs

In each SNP type assay, two types of fluorescence, FAM (red, Y axis) and HEX (green, X axis), were analyzed and each fluorescence was linked to each SNP. Three different genotypes (XX, XY, and YY) were identified using Fluidigm SNP Genotyping Analysis version 4.1.3 (Fluidigm, South San Francisco, CA, USA).

3.3.3 Statistical Analysis

To estimate the individual SNPs from the Fluidigm 96.96 IFC SNP genotyping, the allele frequencies, Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were calculated in GENEPOP v4.3 with 1000 iterations and 100 Markov Chain approximations (Raymond & Rousset, 1995; Rousset, 2008). A sequential Bonferroni to correct for multiple comparisons on the expected and observed heterozygosity were adjusted for all locus (Rice, 1989).

CHAPTER 4: RESULTS

4.1 Transcriptome in the Survived-White Spot Syndrome Virus Challenged Giant Tiger Shrimp, *Penaeus monodon*

4.1.1 Viral Copies Number and LD_{50}

The WSSV stock solution contained 4.11 x 10^{11} viral copies/µL. For determination of LD_{50}, WSSV stock solution with dilution factor 10^{-6} was chosen to use for challenge test (4.11 x 10^5 viral copies/µL). According to Chen et al. (2013), a dose of about 1×10^5 WSSV copies/g are sufficient to cause 100% mortality in 5–7 days.

4.1.2 Detection of WSSV in Surviving Giant Tiger Shrimp

Overall, only five shrimp survived the WSSV challenge test. Based on the PCR and qPCR detection method described earlier using OIE primer pairs VP28 and VP28-140, all the samples that were challenged with white spot syndrome virus and survived were shown to be negative for the infection (Figure 4.1). The amounts of WSSV DNA in all samples were obtained by plotting Ct values onto the standard curve of each known amounts of WSSV DNA based on the qPCR detection method (Figure 4.2). The Ct value of all samples that were challenged and survived were less than 10 showing negative presence of WSSV DNA.

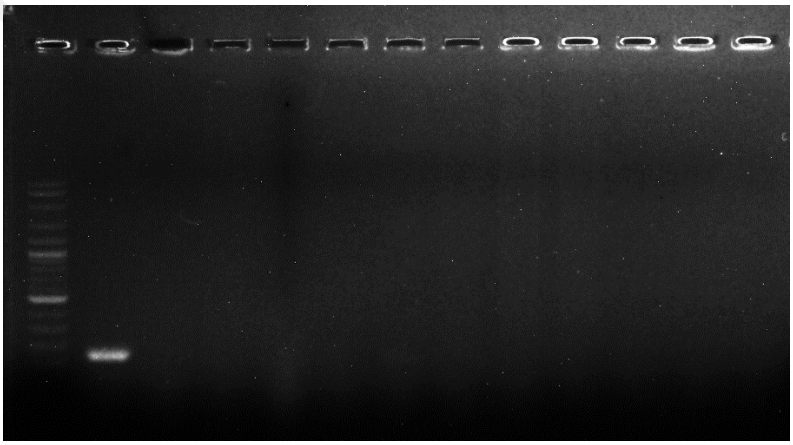


Figure 4.1: Detection of white spot syndrome virus in the surviving WSSV-challenged and control shrimp samples by PCR (Lane 1: ladder; lane 2: positive control; lanes 3-6: survived shrimp samples; lanes 7-12: control shrimp samples; lane 13: negative control).

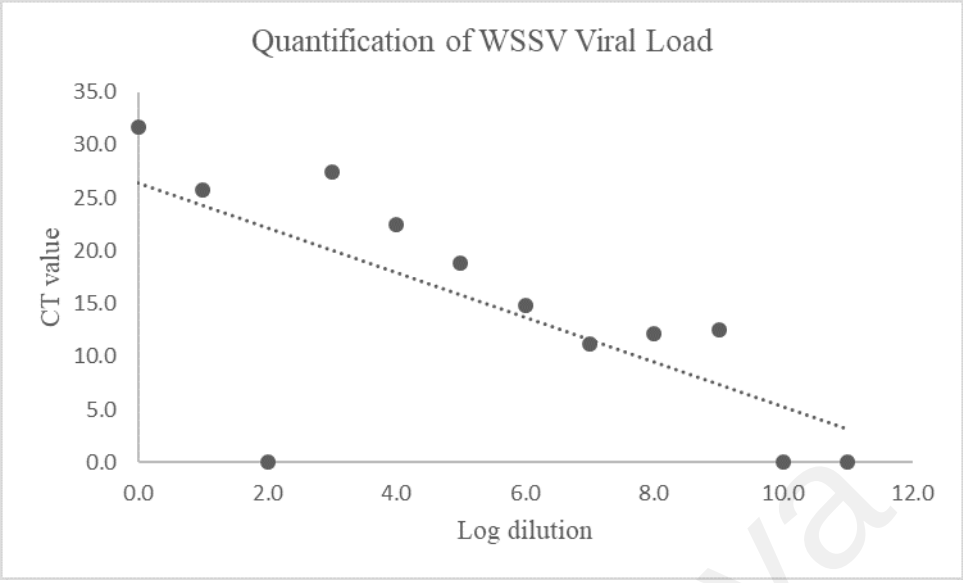


Figure 4.2: A standard curve for each dilutions of known amounts of WSSV DNA based on the qPCR detection method. Twelve consecutive dilutions (dilution factor 1:10) were prepared containing from 1012 to 101 copies/reaction.

4.1.3 Transcriptome Result

4.1.3.1 Sequencing and *de novo* Assembly

cDNA libraries from mRNAs extracted from the hepatopancreas, haemolymph and muscle tissue of surviving WSSV-challenged shrimp and control shrimp were subjected to a run on the Illumina HiSeq 2000 sequencing instrument, resulting in 55,692,118 and 56,206,168 raw reads respectively. A total of 49,488,606 high-quality cleaned reads were obtained in the survived WSSV-challenged library with a total of 49,589,106 high-quality cleaned reads were obtained in the control library after removal of repetitive and low-quality reads. After *de novo* assembly by Trinity, a total of 43, 730 unigenes with an average length of 810 bp and N50 length of 1667 bp were obtained from the survived WSSV-challenged library. In the control library, a total of 44, 755 unigenes with an average of 760 bp and N50 length of 1504 bp were obtained. (Table 4.1).

Table 4.1: Summary reads of Illumina HiSeq 2000 in surviving WSSV-challenged and control giant tiger shrimp cDNA libraries.

	Survived WSSV-challenged giant tiger shrimp	Control giant tiger shrimp
Total sequenced cDNA	55,692,118	56,206,168
Cleaned reads	49,488,606	49,589,106
Total unigene after assembly	43, 730	44, 755
Unigene average length	810	760
N50 length	1667	1504

A total of 37,223 unigenes with an average length of 760 bp and N50 length of of 1504 bp were harvested from the combined reads of the two libraries. Based on a BLASTX similarity search of all unigenes against the NCBI non-redundant (NR) protein databases (cut off e-value<0.0000), 15,486 unigenes showed significant matches in the UniProtKB/Swiss-Prot database and 17,458 unigenes in the NR database. The species distribution of the best match result for each sequence is shown in Figure 4.3. The *P. monodon* unigenes showed 10.8% matches with *Daphnia pulex* sequences, followed by *Tribolium castenum* (5.6%) and *Pediculus humanus corporis* (4.0%).

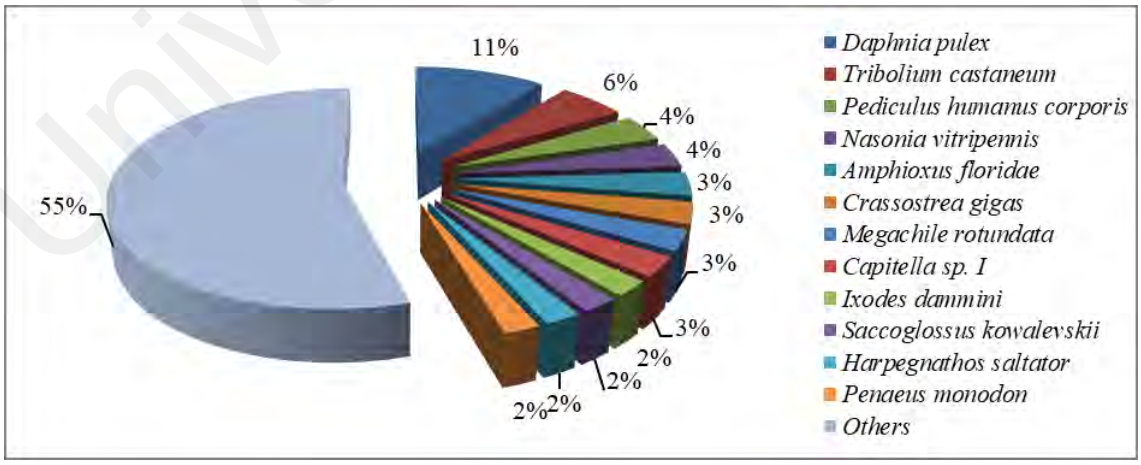


Figure 4.3: Summary reads of Illumina HiSeq 2000 in surviving WSSV-challenged and control giant tiger shrimp cDNA libraries. The figure shows the species distribution of the unigene BLASTX results against the NCBI non-redundant protein databases, with a cutoff E value of 10^{25}. Different colours represent different species. Only species with proportions of more than 1% are shown.

4.1.4 Transcriptome Comparison Between WSSV-infected and Uninfected Shrimp.

4.1.4.1 Gene Ontology Assignments of Differentially Expressed Genes

In total, 4572 unigenes (55.51%) of the differentially expressed unigenes between the survived WSSV-challenged and control shrimp were mapped to biological processes, 2234 unigenes (27.12%) were mapped to cellular components, and 1431 unigenes (17.37%) were mapped to molecular functions (Figure 4.4). With regard to the gene ontology (GO) assignment to biological processes in the differentially expressed genes, most were involved in cellular processes (15.77%), metabolic processes (12.31%) and single-organism processes (12.20%). GO assignments for the cellular component genes were associated with cells (22.83%), parts of cells (22.83%) and cell organelles (15.17%). Additionally, most of the GO assignments of the molecular function genes were associated with catalytic activity (46.12%) or binding (36.55%), with a smaller proportion associated with transporter activity (7.69%).

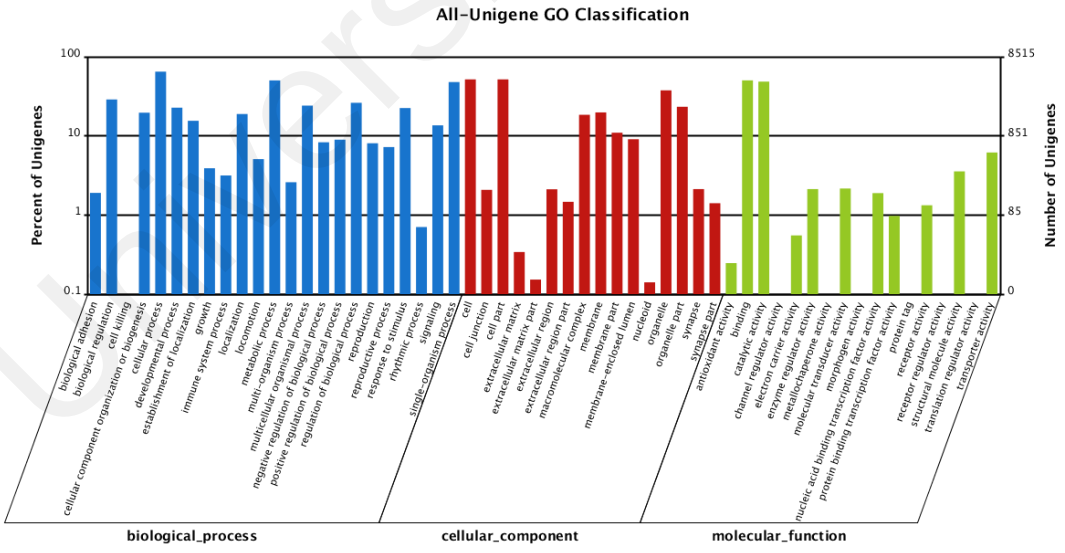


Figure 4.4: Gene Ontology (GO) classification of putative functions of unigenes from surviving WSSV-challenged and uninfected (control) giant tiger shrimp. The x axis shows subgroups of molecular functions from GO classification and the y axis shows the number of the meatched unigenes.

4.1.4.2 Identification of Differentially Expressed Genes

Based on significant differences in the expression of relative transcript abundance between the survived WSSV-challenged and uninfected control shrimp unigenes, 2,644 host genes were significantly up-regulated and 2,194 genes were significantly down-regulated by infection with WSSV. A scatter plot was generated for FPKM values from the treatment group (Figure 4.5). The qRT-PCR results confirmed the data obtained from the Illumina HiSeq 2000 sequencing analysis, showing similar trends in the up- and down-regulation of host genes (Figure 4.6).

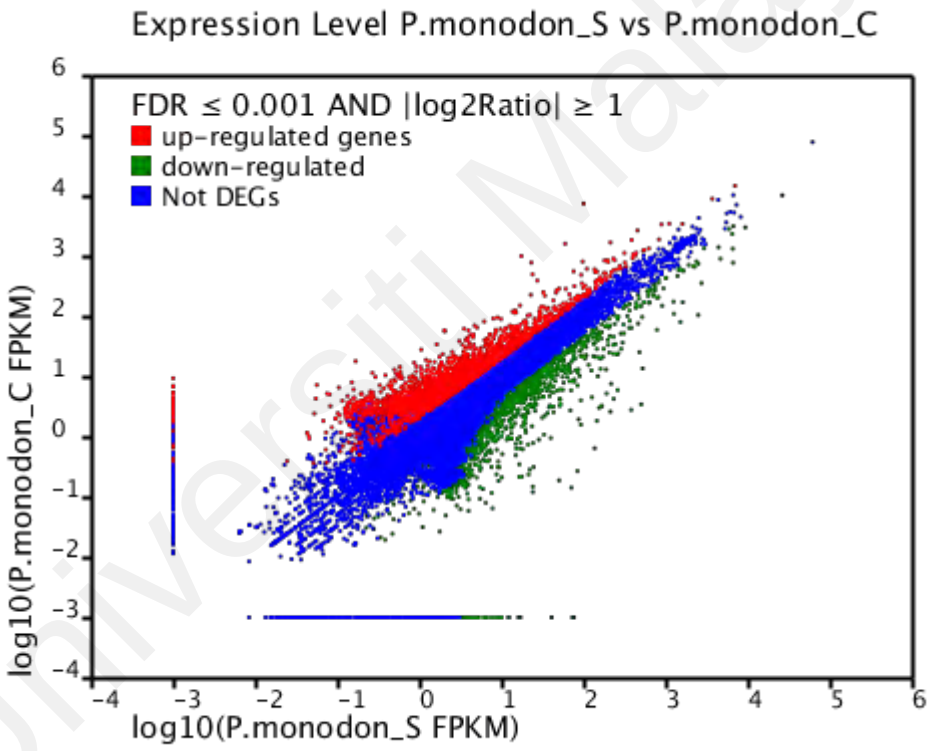


Figure 4.5: Scatter plot showing gene expression levels from giant tiger shrimp. Average FPKM values after WSSV-challenge correlated to average FPKM values for each gene in normal conditions.

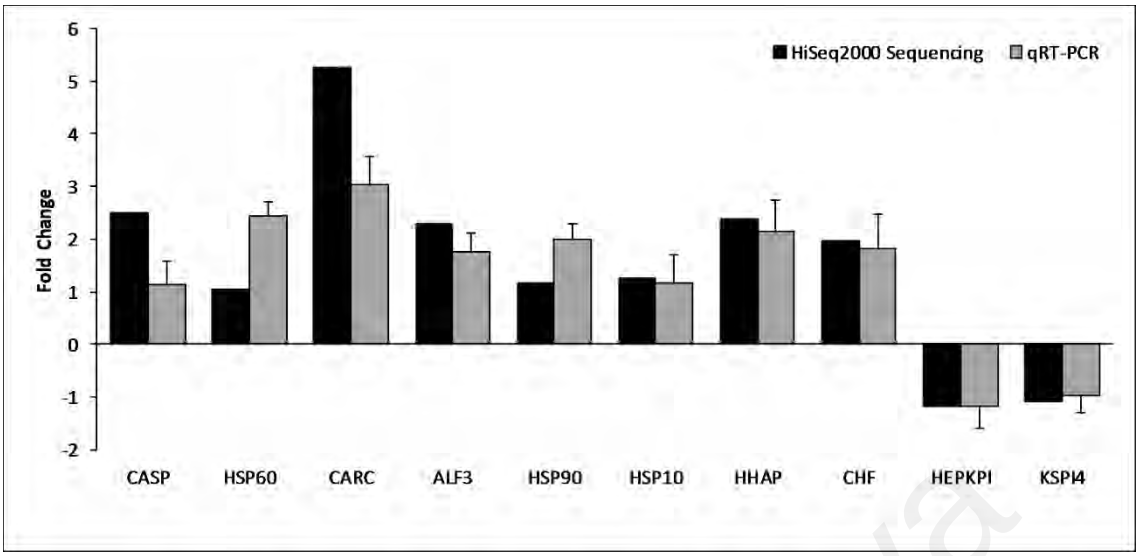


Figure 4.6: Comparison of expression profiles of selected genes as determined by Illumina HiSeq 2000 sequencing (black) and qRT-PCR (grey) in WSSV-challenged shrimp. Target gene abbreviations are as follows: CASP – caspase, HSP60 – heat shock protein 60, CARC – carcinin, ALF3 – anti-lipopolisaccharide factor-3, HSP90 – heat shock protein 90, HSP 10 – heat shock protein 10, HHAP – haemocyte homeostasis-associated protein, CHF – crustacean hematopoietic factor, HEPKPI – hepatopancreas kazal-type proteinase inhibitor 1A1 and KSPI4 – kazal-type serine proteinase inhibitor 4.

4.1.4.3 KEGG Pathway Analysis of the Differentially Expressed Genes

All the differentially expressed genes related to virus infection of the host gene were characterized by mapping them against the referential canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The most abundant categories were associated with amino sugar and nucleotide sugar metabolism, ubiquinone and other terpenoid-quinone biosynthesis, fatty acid metabolism, biosynthesis of secondary metabolites and folate biosynthesis (Table 4.2).

Table 4.2: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for differentially expressed genes (DEGs) in WSSV-challenged giant tiger shrimp (P value < 0.05).

KEGG Pathway	DEGs with Pathway Annotation
Amino sugar and nucleotide sugar metabolism	30 (1.4%)
Ubiquinone and other terpenoid-quinone biosynthesis	10 (0.47%)
Fatty acid metabolism	14 (0.66%)
Biosynthesis of secondary metabolites	86 (4.03%)
Folate biosynthesis	8 (0.37%)
Phagosome	27 (1.26%)
Cysteine and methionine metabolism	18 (0.84%)
Inositol phosphate metabolism	14 (0.66%)
Caffeine metabolism	8 (0.37%)
Glycerophospholipid metabolism	19 (0.89%)

4.1.5 Candidate Genes Involved in *Penaeus monodon* Immune Response

Several genes were found to be differentially expressed in the WSSV-challenged shrimp compared to the uninfected controls. Immune-related genes discovered in this study were the high mobility group box b protein (HMGBb), tumor necrosis factor superfamily (TNFSF), c-Jun protein, as well as a series of Kazal type serine proteinase inhibitors including the haemocyte kazal type proteinase inhibitor and hepatopancreas kazal type proteinase inhibitor. We also found immune-related genes involved in shrimp defence against invading pathogens in the various pathway such as MAPK signaling pathway, apoptosis pathway, toll-like receptors pathway and the prophenoloxidase activation system pathway. Genes associated with the host intracellular environment in response to WSSV were also identified (Table 4.3).

Table 4.3: Candidate genes selected in the surviving WSSV-challenged giant tiger shrimp**.**

Category	Homologous Function	Fold Changes in Gene Expression
MAPK Signalling Pathway	Heat shock protein 21	3.47
	Anti-lipopolysaccharide factor isoform 3	2.30
	chaperonin 10	1.25
Apoptosis Pathway	Caspase	2.49
	Cathepsin L	-1.21
Toll-like Receptor Pathway	Toll protein	1.02
	Crustin type 1	2.13
Prophenoloxidase Activation Pathway	C-type lectin	1.04
	Haemocyanin	6.21
	Haemocyte homeostasis-associated protein	2.38
Signal Transduction Pathway	Tumor necrosis factor superfamily (TNFSF)	1.34
	High mobility group box b protein (HMGBb)	1.75
	c-Jun Protein	1.54
Proteinase & Proteinase Inhibitors	Kazal type serine proteinase inhibitors (SPIs)	-1.08
	Haemocyte kazal type proteinase inhibitor	-1.64
	Hepatopancreas kazal type proteinase inhibitor	-1.18
Intracellular Genes	Plasmolipin	2.25
	FAD oxidoreductase	8.65
	G protein alpha subunit	2.47
	Peritrophin	-7.64
	Sodium/potassium-transporting ATPase subunit beta	-3.25

4.1.6 Characterisation and Sequence Analysis of *Penaeus monodon* Tumor Necrosis Factor, High Mobility Group Box b Protein and c-Jun Protein

The *P. monodon* TNF (PmTNF) gene was 1177 bp long, containing a 453 bp ORF encoding 150 amino acids. Bioinformatics analysis of PmTNF sequence with the BlastP program revealed that the deduced amino acid sequence of PmTNF exhibited similarities with the tumor necrosis factor of other species (Figure 4.7). PmHMGBb gene was 2657 bp long, containing a 2601 bp ORF encoding 866 amino acids. The deduced amino acid sequence of PmHMGBb exhibited similarities with the high mobility group box b of other species (Figure 4.8). The PmcJun gene was 1807 bp long, containing a 879 bp ORF encoding 292 amino acids. The deduced amino acid sequence of PmcJun exhibited similarities with the c-Jun of other species (Figure 4.9).

Conserved motifs were observed between the gene sequences orthologs of PmHMGBb and PmcJun. Interestingly, the multiple sequence alignment of PmTNF between species revealed nonsynonymous mutations among its orthologs. To further investigate the nonsynonymous mutations observed in PmTNF orthologs have been shaped by positive selection pressure, the presence of sites under positive selection was calculated by the ratio of nonsynonymous to synonymous substitutions (dN/dS) per codon. However, the nonsynonymous polymorphic sites showed no evidence of positive selection.

A phylogenetic tree was also constructed to gain insights into the evolutionary relationship between the various animal tumor necrosis factor, high mobility group box b and c-Jun sequences (Figure 4.10). In general, the tree shows that all the shrimp TNFSF, HMGBb and c-Jun each were clustered within a single clade.

Figure 4.7: Multiple alignment analysis of amino acid sequences of PmTNF. The fully conserved amino acid residues in these sequences are indicated by '*'. Conservation between groups of strongly similar properties are indicated in ':'. A '.' indicates conservation between groups of weakly similar properties. The species names and GenBank accession numbers of TNF sequences used in this study are listed in Table 4.4.

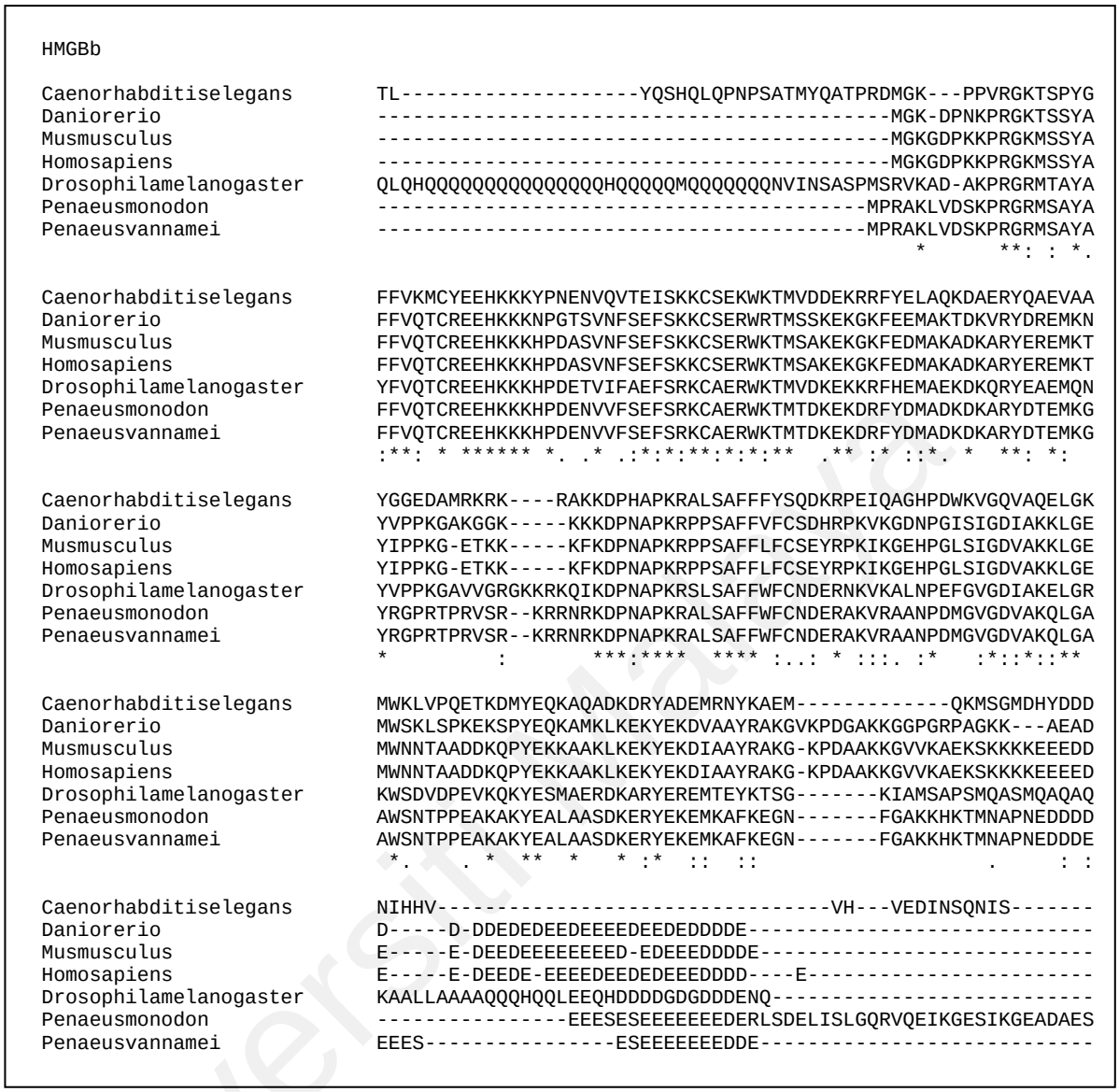


Figure 4.8: Multiple alignment analysis of amino acid sequences of PmHMGBb and its homologs. The fully conserved amino acid residues in these sequences are indicated by '*****'. Conservation between groups of strongly similar properties are indicated in ':'. A '.' indicates conservation between groups of weakly similar properties. The species names and GenBank accession numbers of HMGBb sequences used in this study are listed in Table 4.4.

Figure 4.9: Multiple alignment analysis of amino acid sequences of PmcJun and its homologs. The fully conserved amino acid residues in these sequences are indicated by '*'. Conservation between groups of strongly similar properties are indicated in ':'. A '.' indicates conservation between groups of weakly similar properties. The species names and GenBank accession numbers of c-Jun sequences used in this study are listed in Table 4.4.

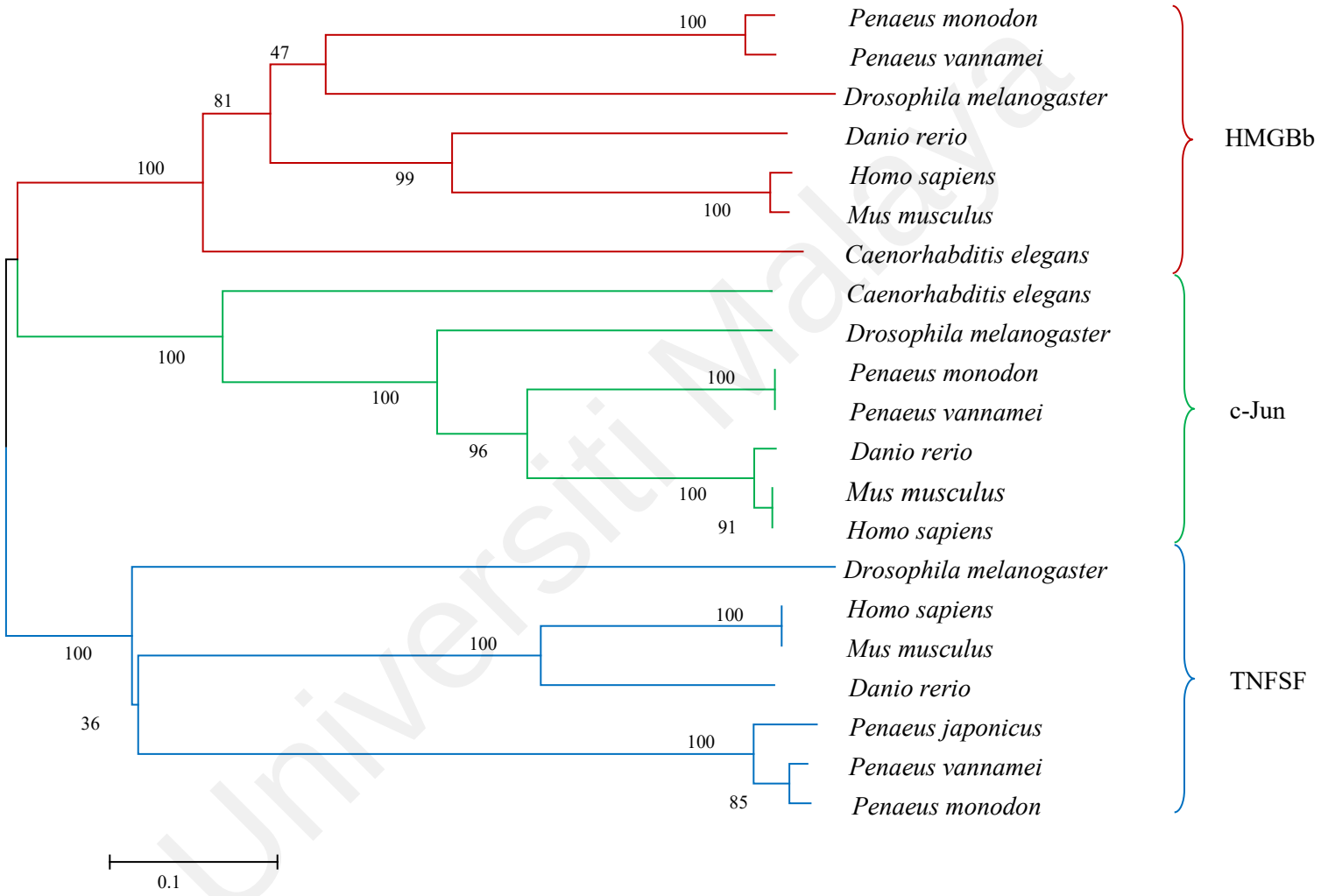


Figure 4.10: A phylogenetic tree of HMGBb, c-Jun and TNF homologs from the giant tiger shrimp (*Penaeus monodon*) and other species were constructed using MEGA 6.0 with the neighbor-joining method. Numbers at tree nodes refer to percent bootstrap values after 1000 re replicates. The bar (0.1) indicates the genetic distance. The accession numbers of the selected HMGBb, c-Jun and TNF sequences are listed in Table 4.4.

Table 4.4: Amino acid identity comparison of the PmTNF, PmHMGBb and PmcJun with other known homologues.

Gene	Species	GenBank Accession Number	Amino Acid Identity (%)
TNFSF	*Penaeus monodon*		-
	Penaeus vannamei	AEK86525.1	96.0
	Penaeus japonicus	BAJ10320.1	91.0
	Drosophila melanogaster	NP724878.2	36.0
	Danio rerio	NP001108537.1	26.0
	Mus musculus	NP001171408.1	27.0
	Homo sapiens	NP001005609.1	27.0
HMGBb	*Penaeus monodon*		-
	Penaeus vannamei	ADQ43367.1	99.0
	Drosophila melanogaster	NP_727960.1	67.0
	Danio rerio	NP_001032501.1	56.0
	Mus musculus	NP_034569.1	58.0
	Homo sapiens	NP_002119.1	58.0
	Caenorhabditis elegans	NP_001022600.1	45.0
c-Jun	*Penaeus monodon*		-
	Penaeus vannamei	AIB53746.1	99.0
	Drosophila melanogaster	NP_476586.1	36.0
	Danio rerio	NP_956281.1	45.0
	Mus musculus	NP_034721.1	43.0
	Homo sapiens	NP_002219.1	43.0
	Caenorhabditis elegans	NP_001122643.1	40.0

4.2 Immune Response of *Penaeus monodon* Experimentally Infected with White Spot Syndrome Virus Infection

4.2.1 Fludigm 192.24 Dynamic Array IFC System

In total, the giant tiger shrimp dynamic array IFC run resulting in 4608 high quality data. A heat map of the gene expression analysis obtained from the dynamic array IFC system is shown in Figure 4.11.

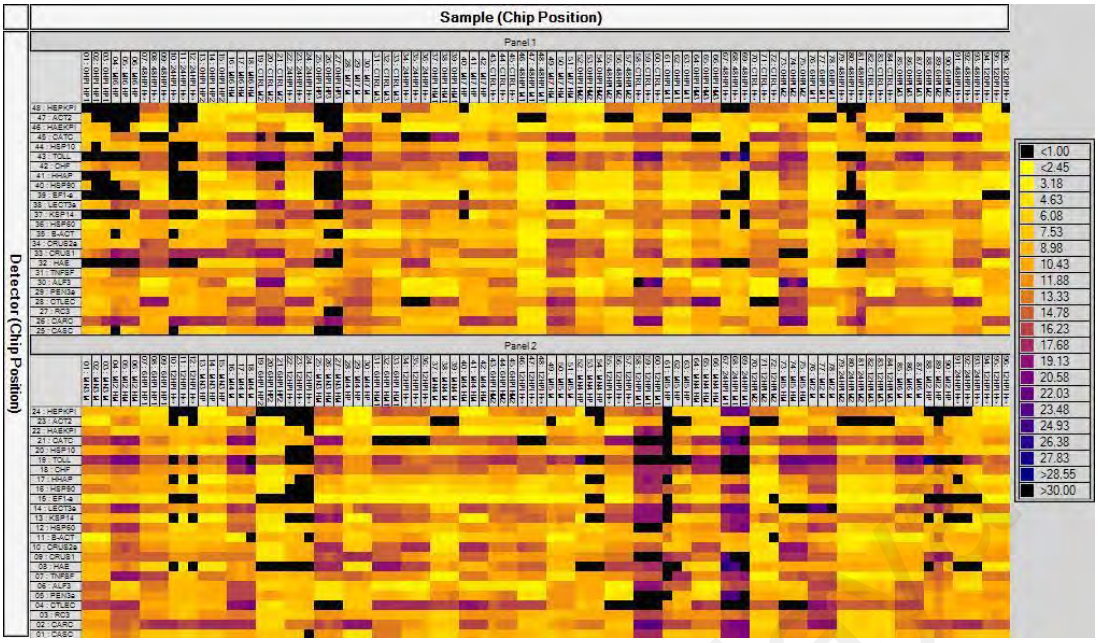


Figure 4.11: Heat map of a 192.24 dynamic array obtained after thermal cycling of the IFC. Each of the squares represents 1 reaction chamber from the chip. The color indicates the Ct value according to the legend shown on the right.

4.2.2 Immune Response of *Penaeus monodon* to WSSV Infection

4.2.2.1 Gene Expression of Immune Related Genes in Hepatopancreas

After challenge with WSSV, several genes in the hepatopancreas showed changes in expression level compared with the control shrimp. A summary of the up- and downregulation of these genes in comparison with control shrimp is shown in Table 4.5.

Based on the graph in Figure 4.12, the toll protein, HHAP and KSPI4 increased significantly ($p<0.5$) immediately after challenge with WSSV (0 hpi). However, in all these three genes the expressions decreased significantly at 6 hpi after infection. Both toll protein and HHAP expressions were highest at 0 hpi. However, the expression of HHAP increased again at 48 hpi. KSPI4 also showed almost the same up-and downregulation pattern with toll protein and HHAP, but only reached it peaks significantly at 48 hpi.

Table 4.5: A summary of gene expression in hepatopancreas of WSSV-challenged *Penaeus monodon* at 0, 6, 12, 24, 48 hpi and 12 dpi in comparison with control shrimp.

Gene	**Time Point**					
	0 hpi	6 hpi	12 hpi	24 hpi	48 hpi	12 dpi
Toll protein	↑	↑	↑	↑	↓	↓
HHAP	↑	↑	↑	↑	↑	↓
KSPI4	↑	↓	↑	↑	↑	↓
HSP90	↑	↑	↑	↑	↑	↓
C-type lectin	↑	↑	↑	↑	↑	↑
HSP 10	↑	↑	↑	↑	↑	↑
HSP 60	↑	↑	↑	↑	↑	↑
HMGB	↓	↑	↓	↓	↑	↓
TNFSF	↑	↑	↓	↑	↑	↑
HaeKPI	↑	↑	↑	↑	↑	↓
Caspase	↑	↑	↑	↑	↑	↓

Arrow shown (↑); gene being upregulated and (↓) gene being downregulated.

A fluctuating gene expression levels was observed in HSP 90, HSP 60, HSP 10 and C-type lectin. Expression level of HSP 90 reached it peaks at 6 hpi (27 fold), but decreased at 12 hpi (3 fold) before increasing again at 24 hpi (18 fold). Both C-type lectin and HSP 10 also increased at 6 hpi (48 fold and 17 fold respectively) before being downregulated at 12 hpi (10 fold respectively), then it increased once more and reached its peak at 48 hpi (52 fold and 42 fold respectively) after challenged with WSSV. A fluctuating recovery can be observed in HSP 60 when the gene expression increased at 6 hpi (78 fold), then decreased at 12 hpi (22 fold) before extremely increased and showed highest activity than that of control group at 24 hpi (408 fold). In HMGB, the expression level peaks at 6 hpi and decreased afterwards until it reached same expression as of the control group. The gene expression level in TNFSF, HaeKPI and caspase increased from 0 hpi until it reached highest expression level at 48 hpi with WSSV treatment.

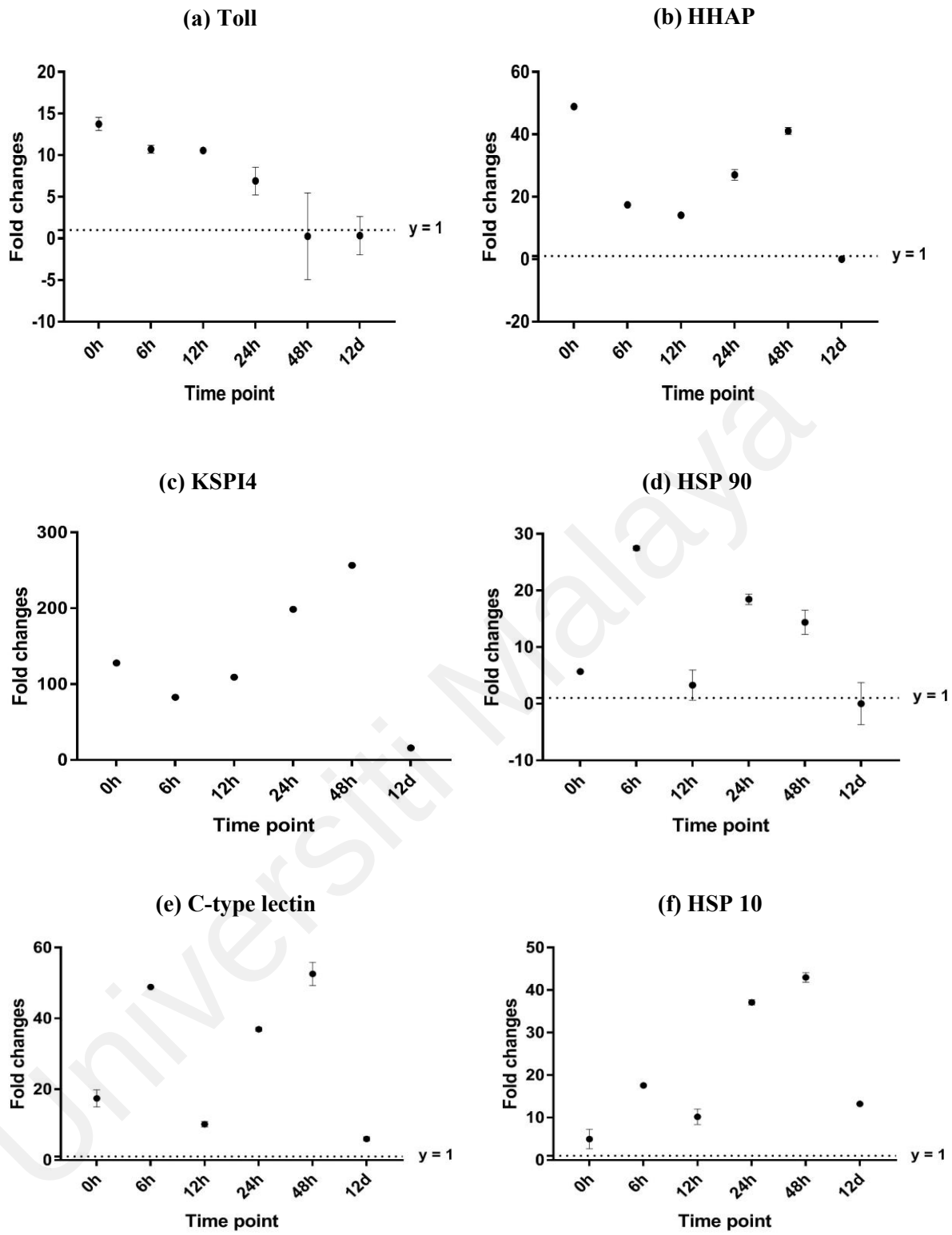
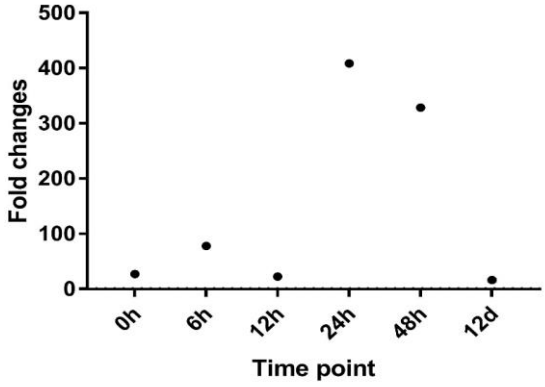
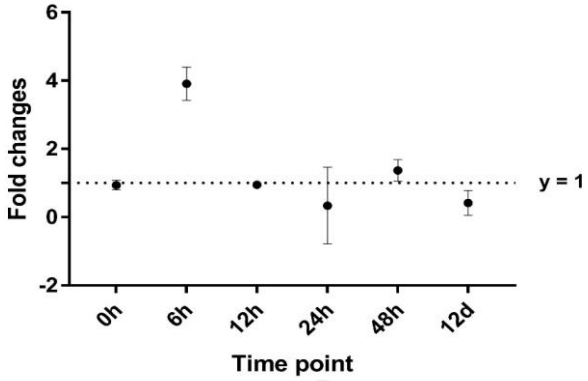


Figure 4.12: Analysis of gene expression profile in hepatopancreas of control and WSSV-challenged giant tiger shrimp (*Penaeus monodon*) by microfluidic dynamic array at 0, 6, 12, 24, 48 hours and 12 days post-injection in (a) Toll protein, (b) HHAP, (c) KSPI4, (d) H HSP 90, (e) C-type lectin, (f) HSP 10, (g) HSP 60, (h) HMGB, (i) TNFSF, (j) HaeKPI and (k) caspase. Each dot represents the mean fold change of the normalized expression levels of the replicates (N=3). Data (mean ± SE) with (*) are significant at ($p < 0.05$). Axis y = 1 indicates control group at log 0.

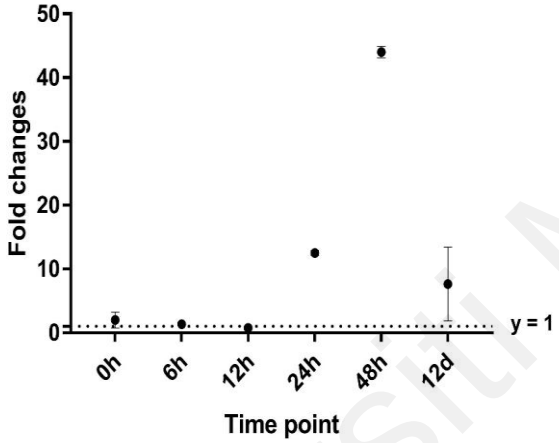
(g) HSP 60



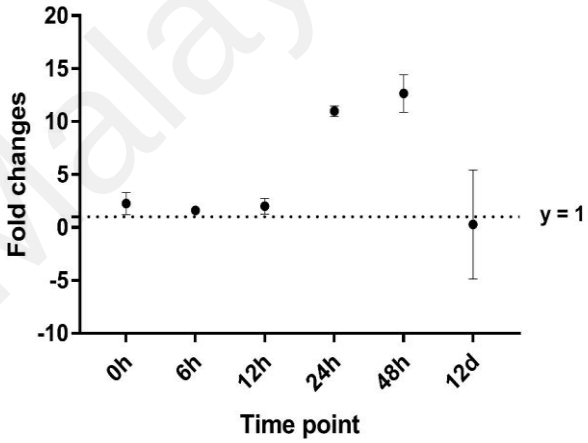
(h) HMGB



(i) TNFSF



(j) HaeKPI



(k) Caspase

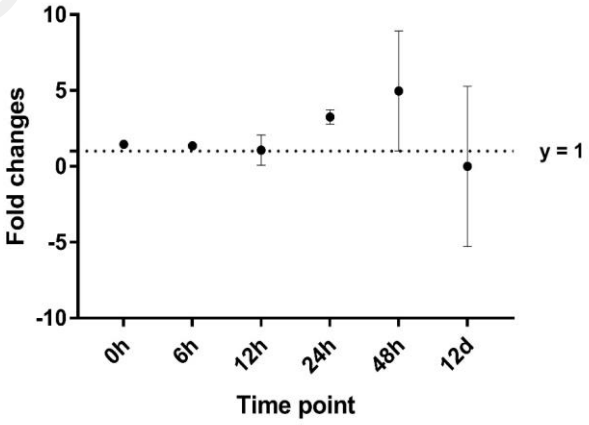


Figure 4.12, continued.

4.2.2.2 Gene Expression of Immune Related Genes in Haemocytes

Several genes showed trends in up- and downregulation of gene expression in haemocytes of the giant tiger shrimp after challenge with WSSV. A summary of the up- and downregulation of those genes compared to control shrimp is shown in Table 4.6.

Table 4.6: A summary of gene expression in haemocytes of WSSV-challenged *Penaeus monodon* at 0, 6, 12, 24, 48 hpi and 12 dpi in comparison with control shrimp.

Gene	Time Point					
	0 hpi	6 hpi	12 hpi	24 hpi	48 hpi	12 dpi
KSPI4	↑	↑	↓	↓	↓	↓
Crustin 1	↑	↑	↑	↓	↑	↓
HMGB	↑	↑	↓	↑	↓	↓
Caspase	↓	↓	↑	↓	↓	↓
TNFSF	↓	↓	↑	↓	↑	↓
c-Jun	↓	↓	↓	↑	↑	↑

Arrow shown (↑); gene being upregulated and (↓); gene being downregulated.

Based on the graph in Figure 4.13, the kazal SPI 4 were extremely upregulated (54 fold) and reach the highest point at 0 hpi with WSSV. Crustin 1 gene regulation were upregulated immediately after infection with WSSV (0 hpi) but decreased at 6 hpi before reaching its highest point at 12 hpi. Interestingly, the gene expression level of crustin 1 dropped lower than that of control shrimp at all time point but dramatically increased at 12 hpi by 9.4 fold. HMGB gene expression started to increase immediately after WSSV infection and reached its peaks at 6 hpi by 68 fold, before its reached the lowest expression at 12 hpi. This gene expression started to increased again at 24 hpi by 17 folds and decreased again by 5 folds at 48 hpi.

Caspase was only upregulated at 12 hpi by 4 folds and maintained its expression below control group at all time point. In TNFSF, the gene expression level slightly decreased below the control group shrimp at 0 hpi and 6 hpi before fairly increased above the control shrimp group at 12 hpi. The expression of TNFSF gene in infected

shrimp then decreased again below the control shrimp expression level at 24 hpi and reach it maximum expression level at 48 hpi by 2.5 fold. In c-Jun, the gene was down-regulated below control shrimp group gradually from 0 hpi to 12 hpi but started to increase at 24 hpi until it reached its peak at 48 hpi by 4.7 fold.

4.2.2.3 Gene Expression of Immune Related Genes in Muscle Tissue

In muscle tissue of WSSV-challenged giant tiger shrimp several genes showed a fascinating pattern of expression level after WSSV challenge. A summary of the up- and downregulation of those genes compared to control shrimp is shown in Table 4.7.

Table 4.7: A summary of gene expression in muscle tissue of WSSV-challenged *Penaeus monodon* at 0, 6, 12, 24, 48 hpi and 12 dpi in comparison with control shrimp.

Gene	**Time Point**					
	0 hpi	6 hpi	12 hpi	24 hpi	48 hpi	12 dpi
HSP 10	↑	↑	↑	↑	↑	↓
Penaeidin 3	↓	↓	↑	↑	↑	↓
Crustin 2	↑	↑	↑	↑	↑	↓
TNFSF	↑	↑	↑	↑	↑	↓
HSP90	↑	↑	↑	↑	↑	↓
c-Jun	↓	↓	↓	↑	↑	↑

Arrow shows (↑); gene being upregulated and (↓); gene being downregulated.

Based on the graph in Figure 4.14, heat shock protein 10 expression increase from 0 hpi to 12 hpi by 9.6 fold before decreasing to 3 fold at 24 hpi. This gene suddenly increased again at 48 hpi by 7 fold. Penaeidin 3 gene expression was downregulated at 0 and 6 hpi. Its expression then extremely increased at 12 hpi by 21 fold before fluctuated again and decrease significantly to 9 fold at 24 hpi. Fascinatingly, the gene expression of penaeidin 3 was upregulated again to 15 fold at 48 hpi. Crustin 2 expression level was slightly upregulated upon infection with WSSV and continue to increase at 6 hpi by 21 fold. It expression level suddenly decreased at 12 hpi by 11 fold before increasing again at highest level by 74 fold at 24 hpi.

The TNFSF gene expression level started to increase from 0 hpi until it reached maximum expression 48 hpi by 24 fold. However, a sudden decrease in expression level of TNFSF was observed after WSSV infection at 24 hpi. The HSP 90 gene also showed a fluctuating gene expression level when the gene expression increased from 0 hpi to 6 hpi by 35 fold. The expression of HSP 90 gene then started to decrease from 12 hpi and dropped to 9 fold at 24 hpi before sharp increase at 48 hpi by 40 fold. C-Jun protein was down-regulated below control shrimp group from 0 hpi to 12 hpi but started to increase at 24 hpi until it reached its peak at 48 hpi by 5 fold.

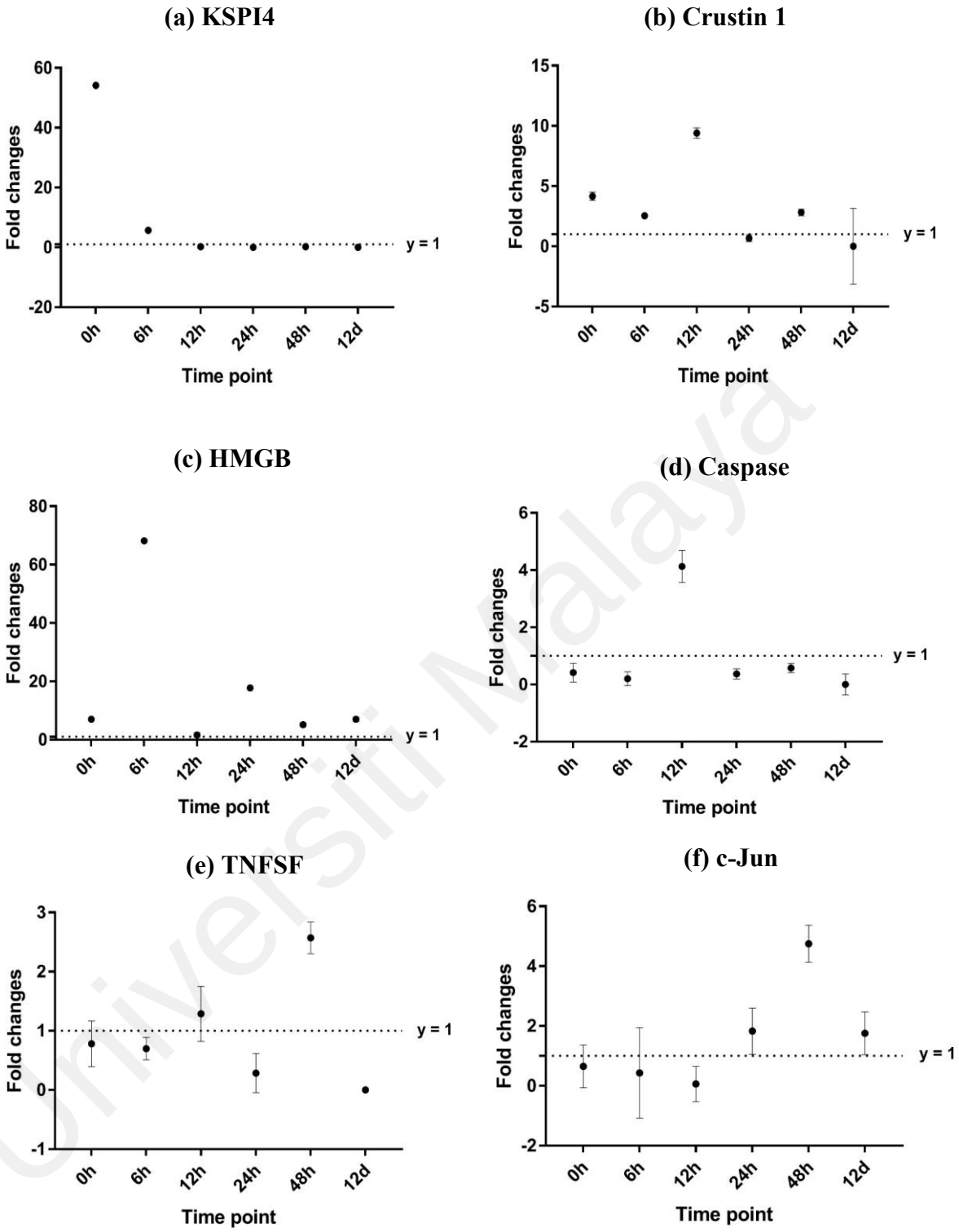


Figure 4.13: Analysis of gene expression profile in haemocytes of control and WSSV-challenged giant tiger shrimp (*Penaeus monodon*) by microfluidic dynamic array at 0, 6, 12, 24, 48 hours and 12 days post-injection in (a) KSPI4, (b) Crustin 1, (c) HMGB, (d) Caspase, (e) TNFSF and (f) c-Jun. Each dot represents the mean fold change of the normalized expression levels of the replicates (N=3). Data (mean ± SE) with (*) are significant at ($p < 0.05$). Axis y=1 indicates control group at log 0.

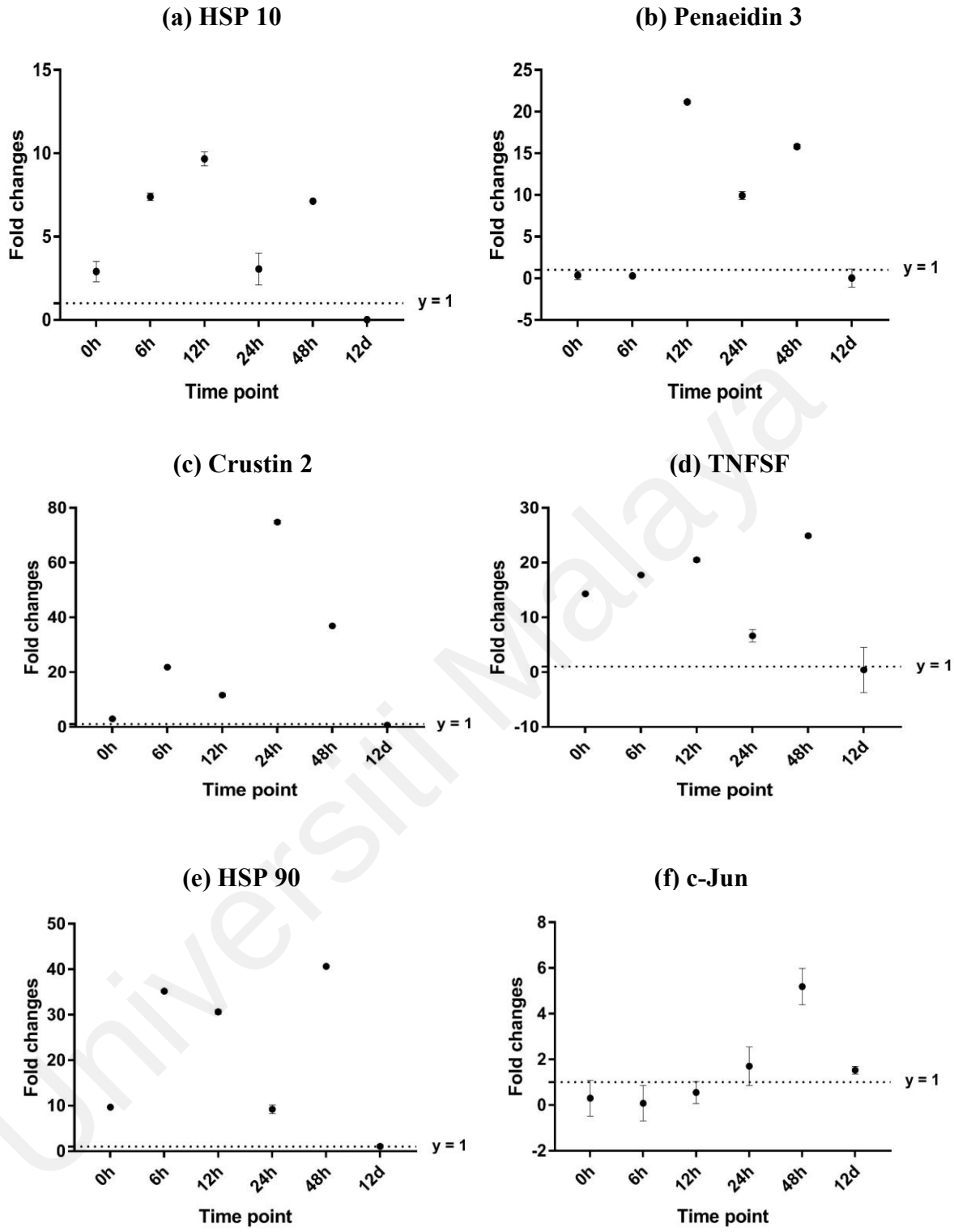


Figure 4.14: Analysis of gene expression profile in muscle tissue of control and WSSV-challenged giant tiger shrimp (*Penaeus monodon*) by microfluidic dynamic array at 0, 6, 12, 24, 48 hours and 12 days post-injection in (a) HSP 10, (b) Penaeidin 3, (c) Crustin 2, (d) TNFSF, (e)HSP 90 and (f) c-Jun. Each dot represents the mean fold change of the normalized expression levels of the replicates (N=3). Data (mean ± SE) with (*) are significant at ($p < 0.05$). Axis y = 1 indicates control group at log 0.

4.3 Candidate Genes for Disease Resistance in *Penaeus monodon* Survivability against the White Spot Syndrome Virus

4.3.1 SNPs Analysis from Transcriptome of WSSV-survived and Control Shrimp

Overall, after unigene sequence assembly, a total of 36,857 and 28,527 SNPs were identified from transcriptome of WSSV-survived and control shrimp respectively after 12 days post WSSV challenge. Of all the SNPs identified in the WSSV-survived shrimp, 23,803 were putative transitions (Ts) and 13,054 were putative transversion (Tv), with a mean observed Ts/Tv ratio of 1.82. The SNP types A – G and C – T were most common in the WSSV-survived transcriptome (Figure 4.15)

In control shrimp, 18,484 SNPs identified across the transcriptome were Ts, and 10,043 SNPs were Tv. The mean observed Ts/ Tv ratio is 1.84. Both SNP types A – G and C – T were most common in the control shrimp.

4.3.2 SNPs Validation and Genotyping

A total of 96 SNPs were chosen as candidate markers related for immune response against WSSV infection from the comparison between both survived WSSV-challenged and control shrimp transcriptome. Three different genotypes (XX, XY, and YY) representing the different SNPs genotype were identified across all samples (Figure 4.16). A call map view from the dynamic array IFC, displaying SNP fingerprints of each of the genotyped *P. monodon* individual is shown in Figure 4.17.

Overall, most of the SNPs in all 96 genes were transition (58) and the other is transversion (38) (Figure 4.18). Among the 96 genes characterized, the most common SNP mutation types were C – T (37%) and A – G (23%), while incidences of other SNP types were significantly lower: G – T (15%), A – T (13%), C – G (6%) and A – C (6%) (Figure 4.19). However, four SNPs markers, CTLec_CL143.Contig1_All, ALFi4_2_Unigene5232_All, Haemocyanin_18_CL1190.Contig4 and

Haemocyanin_34_CL1190.Contig4 failed to produce any results in the IFC SNPs genotyping across all individual.

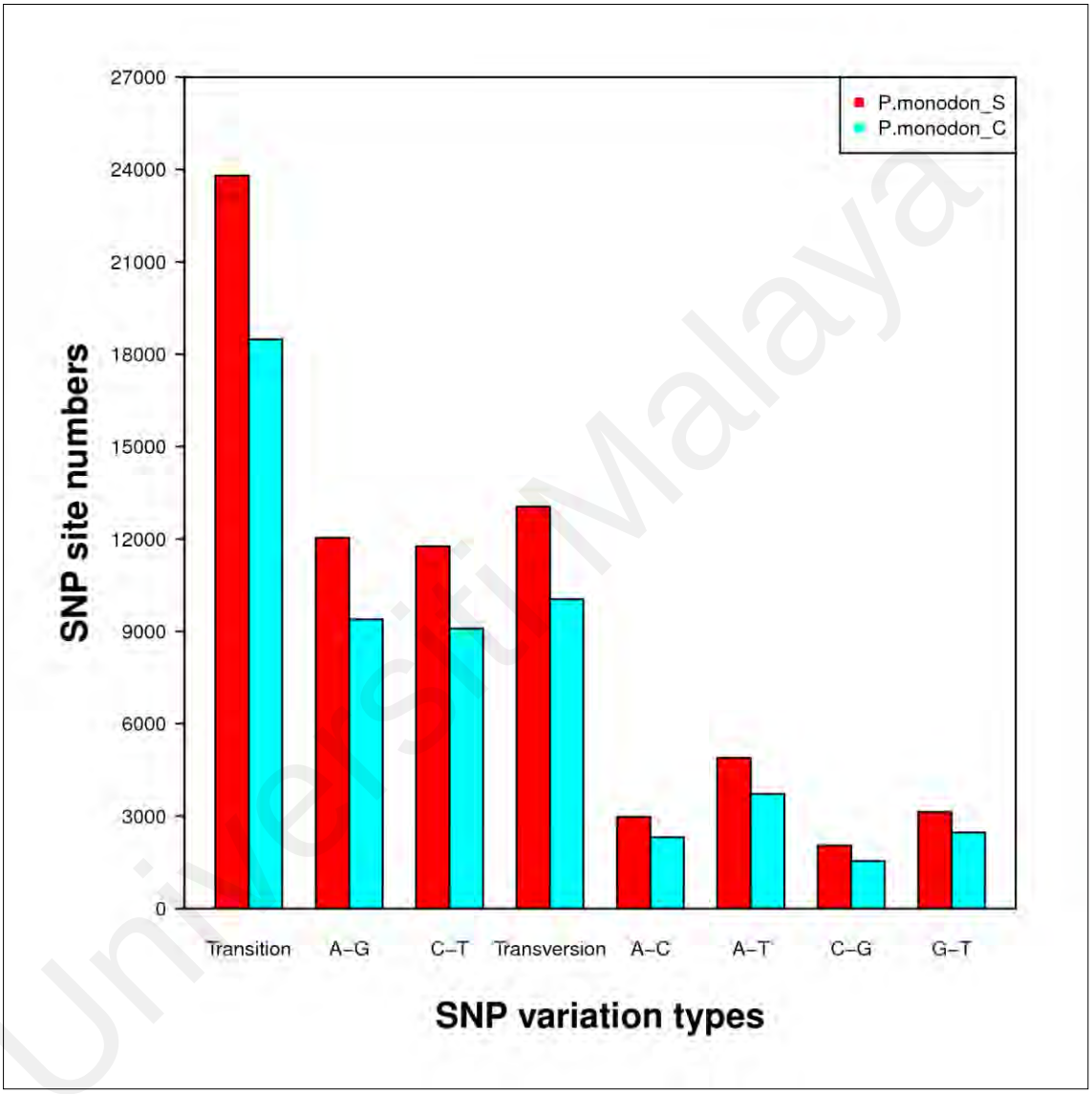


Figure 4.15: Distribution of putative single nucleotide polymorphisms in the giant tiger shrimp *(Penaeus monodon)* sequences.

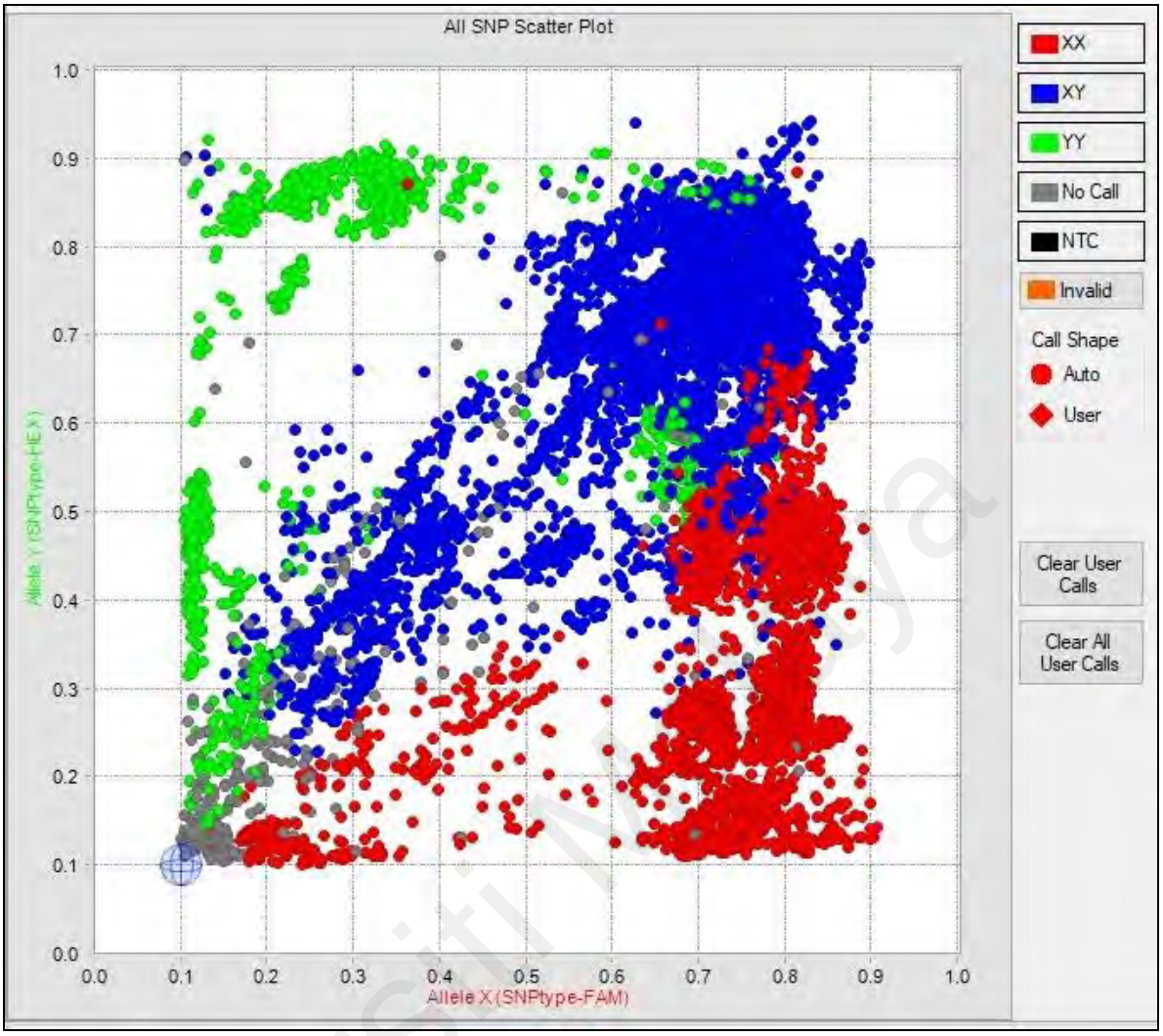


Figure 4.16: A SNP scatter plot of each *Penaeus monodon* individuals genotyped by dynamic array IFC.

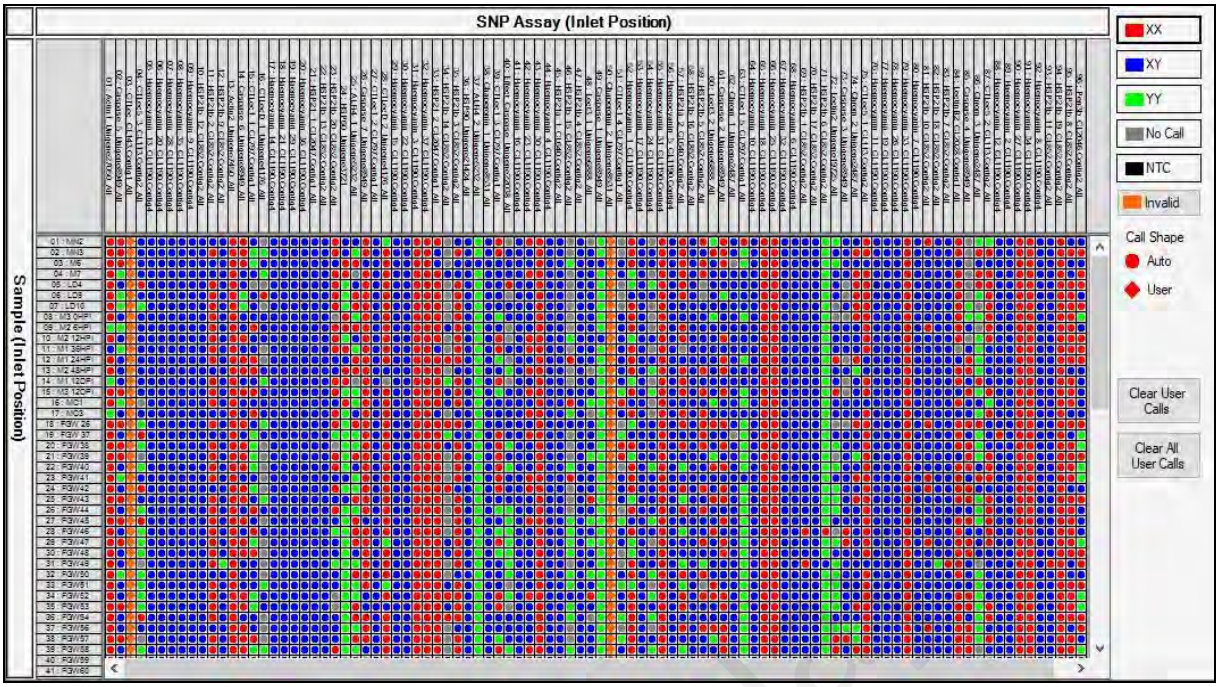


Figure 4.17: Call map view from the dynamic array IFC, displaying SNP fingerprints of genotyped *Penaeus monodon*. The map view shows the computer-generated image of the genotype calls for each of the individual reaction chambers. Each column represents data from one assay that correlated to the SNP genotyping assay loaded from each assay inlet.

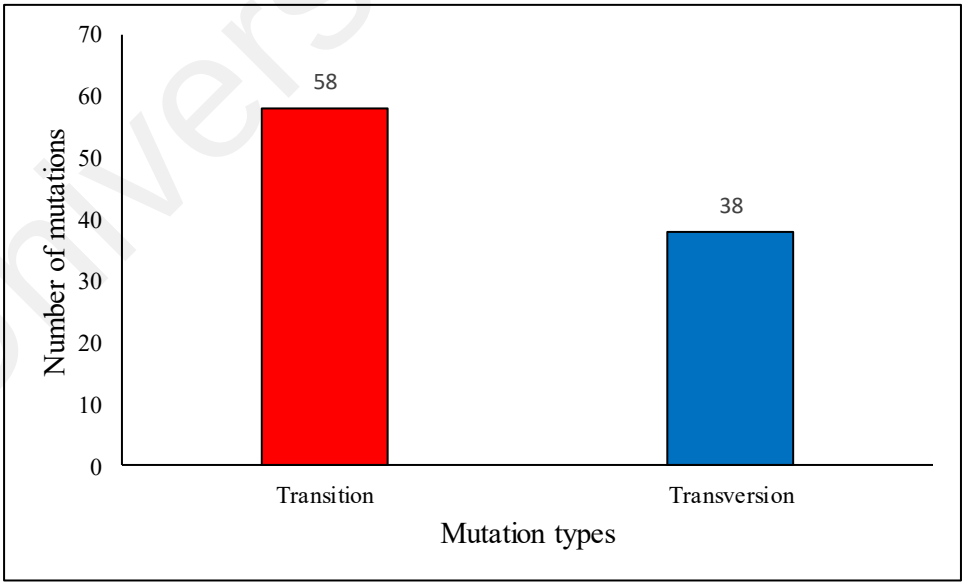


Figure 4.18: Total number of transition and transversion class of SNPs in 96 selected candidate genes of *Penaeus monodon.*

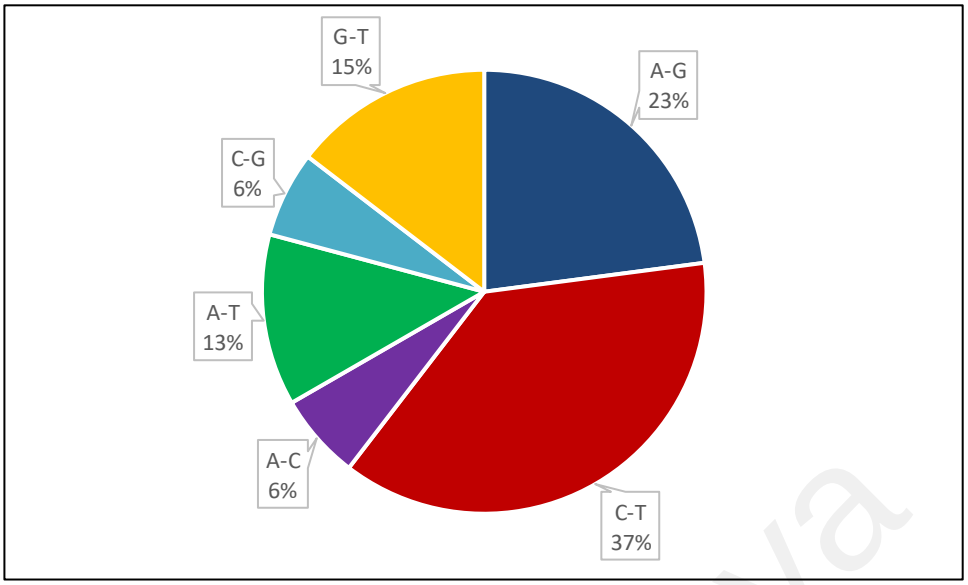


Figure 4.19: Percentage of polymorphism variation of SNPs in 96 selected candidate genes of *Penaeus monodon*.

4.3.3 Statistical Analysis

The allele frequencies, Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) for each individual SNPs from the Fluidigm 96.96 IFC SNP genotyping is shown in Appendix B. Based on the statistical analysis, only 18 SNPs does not deviate from HWE and were not in linkage disequilibrium. In total, 64 SNPs in the present study deviate from HWE and 50 SNPs were linked. All 18 SNPs that could be applied as potential candidate genes were listed in Table 4.8.

Table 4.8: List of potential candidate genes associated with WSSV-resistance in *Penaeus monodon*.

Locus / SNPs ID	Gene
Caspase_1_Unigene8949_All	Caspase
Caspase_2_Unigene8949_All	Caspase
Caspase_5_Unigene8949_All	Caspase
Caspase_7_Unigene8949_All	Caspase
Effect_Caspase_Unigene22038_All	Effector Caspase
LectinB2_CL2028.Contig1_All	Lectin B
Lect3_1_Unigene6888_All	Lectin 3
CTLec1_1_CL797.Contig1_All	C-type Lectin 1
CTLec1_3_CL797.Contig1_All	C-type Lectin 1
CTLec5_1_CL113.Contig2_All	C-type Lectin 5
CTLec5_3_CL113.Contig2_All	C-type Lectin 5
HSP21b_20_CL852.Contig2_All	HSP21
HSP21b_2_CL852.Contig2_All	HSP21
HSP21_2_CL2047.Contig1_All	HSP21
Chaperonin_2_Unigene8531_All	HSP 10
Chrom_2_Unigene2487_All	Chromosome-associated kinesin KIF4A
Haemocyanin_24_CL1190.Contig4	Haemocyanin
Actin2_Unigene7450_All	Actin 2

CHAPTER 5: DISCUSSION

5.1 Transcriptome in the Survived-White Spot Syndrome Virus Challenged Giant Tiger Shrimp, *Penaeus monodon*

The WSSV is a major disease in penaeid shrimp aquaculture, which has caused massive cumulative losses to the industry (estimated at 6 billion USD) since its emergence in 1992 (Lightner et al., 2012). Until now, there has been no effective treatment available, and the industry has mainly relied on good management practices to contain and reduce infection (Kongkeo, 2005; Verbruggen et al., 2016).

Various studies have been carried out on virus infection pathways and the shrimp immune system (Robalino et al., 2007; Zeng & Lu, 2009; Chen et al., 2013; Xue et al., 2013). However, most of these have been on shrimp 3 days after they were infected with WSSV. The present study, in contrast, investigated shrimp that had survived for at least 12 days after challenge with WSSV and the interactions between WSSV and the host intracellular environment. This gave an opportunity to compare, via transcriptome analysis, the gene expression in shrimp that had survived WSSV infection with that of healthy shrimp, to gain a better understanding of the host-virus interactions. In the process, five potential candidate genes associated with disease resistance against WSSV were identified: HMGB, TNFSF, c-Jun and a series of Kazal type serine proteinase inhibitors (haemocyte kazal type proteinase inhibitor and hepatopancreas kazal type proteinase inhibitor). The present study is, to our knowledge, the first to discover HMGB, TNFSF and c-Jun in *P. monodon*.

HMGB plays an important role in the signal-transducing antiviral immune response (Yanai et al., 2012). Indeed, it has been recognized as the universal sentinel of nucleic-acid-mediated innate immune responses (Yanai et al., 2009). In mammals, there are four members of the HMGB family, all of which (HMGB1-4) function as

chaperones influencing multiple processes in chromatin including transcription, replication, recombination, DNA repair and genomic stability (Stros, 2010). There are various members of HMGB in lower vertebrates and invertebrates (Rao & Su, 2015). In fishes, HMGB1, HMGB2 and HMGB3 are present in cartilaginous fish and bony fish (Moleri et al., 2011), while two types of HMGB are found in white shrimp (*P. vannamei*): HMGBa and HMGBb (Chen et al., 2011). The multiple alignment of HMGBa and HMGBb in white shrimp is similar to that of HMGB1 and HMGB2 in human (*Homo sapiens*), house mouse (*Mus musculus*), African clawed frog (*Xenopus laevis*), grass carp (*Ctenopharyngodon idella*), Zebrafish (*Danio rerio*), American dog tick (*Dermacentor variabilis*), western predatory mites (*Metaseiulus occidentalis*) and lice (*Pediculus humanus corporis*), with the presence of two DNA binding domains (A box and B box) as well as a tail (Chen et al., 2011). The sequence similarity between HMGB genes in different species might give an indication on the function of the gene itself that needs further elucidation. Homologous genes, particularly orthologous genes, are generally assumed to retain equivalent functions in different organisms and to share other key properties (Gabaldón & Koonin, 2013). A study by Chen et al. (2014a) on white shrimp found that HMGBb expression levels were up-regulated when induced by a pathogen-associated molecular pattern (PAMP). They suggested that the release of HMGBa and HMGBb occurs naturally during cell necrosis, and that it occurs in shrimp haemocytes in response to PAMP. Further research is required on the role of this gene and into the mechanisms through which it acts in innate immunity in shrimps, particularly in *P. monodon* against WSSV infection.

The Janus family tyrosine kinase and signal transducer and activator of transcription (JAK/STAT) signaling pathway have been proven to be very important in antiviral immunity in both vertebrates and invertebrates (Glenney & Wiens, 2007; Sonar & Lal, 2015). However, little is known about the function of this signaling

pathway in the antiviral immunity of shrimp; and in particular about the TNFSF, one of the potential STAT regulator genes (Wen et al., 2014). To date, only a small number of TNFSF members have been identified in shrimp, including in *P. japonicus* (Mekata et al., 2010) and *P. vannamei* (Wang et al., 2012). A homology analysis of the kuruma shrimp TNF (MjTNF) showed 30.7% and 26.7% identities with fruit fly (*Drosophila melanogaster*) Eiger and human (*Homo sapiens*) ectodysplasin A (Mekata et al., 2010). Ectodysplasin-A plays an important role in mammalian development (Harris et al., 2008). Further studies of giant tiger shrimp TNF will provide more information and should lead to a better understanding of shrimp inflammatory responses.

The sequence polymorphisms detected in PmTNF discovered in the present study could have consequences on the differential susceptibility of the survived giant tiger shrimp. A high polymorphism in two AMP families (Cg-Defs and Cg-Prp), in Cg-Toll and in glutathione reductase genes was also discovered in the Pacific oyster (*Crassostrea gigas*) that showed resistance to summer mortalities (Schmitt et al., 2013). Similarly, resistance to Vibrio infection was observed in green mud crab (*Scylla paramamosain*) with different variants of the Sp-Toll gene (Lin et al., 2012). The presence of the polymorphic sites found in PmTNF might contribute to the improvement of the disease resistance to WSSV in giant tiger shrimp. However, more work with a larger sample size should be oriented to correlate the contribution of this gene towards improved immune response in giant tiger shrimp.

C-Jun, a member of the Jun family along with JunB and JunD, is a major substrate of c-Jun N-terminal kinase (JNK). C-Jun participates in regulating gene transcription in response to various stimuli, including cytokines, stress signals, and bacterial and viral infection (Yao et al., 2015). C-Jun was first reported in 1987 (Maki et al., 1987) and has recently been reported to be involved in WSSV gene transcription (Shi et al., 2012; Yao

et al., 2015). Yao et al. (2015) found that, during the process of WSSV infection, the transcription levels of *P. vannamei* c-Jun (Lvc-Jun) were up-regulated, suggesting that WSSV infection could enhance both the expression and phosphorylation levels of Lvc-Jun. The same authors further stated that increased levels of Lvc-Jun along with the aggravation of viral infection indicated a notable positive correlation between Lvc-Jun activation and viral infection. Another study by Li et al. (2015), on the interaction of *P. vannamei* encoding the full-length c-Fos protein (Lvc-Fos) and Lvc-Jun, found that silencing of Lvc-Fos or Lvc-Jun in shrimp caused lower mortality and virus loads under WSSV infection, suggesting that Lvc-Fos and Lvc-Jun could be engaged in WSSV replication and pathogenesis. In contrast, our findings suggest that, while c-Jun was upregulated in surviving WSSV-infected shrimp, the activation of this gene in cases of WSSV infection may trigger a sequence of immune-related genes and immune pathways to help the shrimp to survive the disease. In summary, more studies on the interaction of immune-related genes are needed to elucidate their role in immune responses against pathogens, particularly in surviving shrimp.

An optimal environment is needed for a virus to replicate in a host cell. Virus will dwell within a host cell and alter the host cell pathways by making it beneficial for virus replication (Sanchez & Lagunoff, 2015). In response to the presence of the virus, the host cellular environment will deteriorate, e.g., through draws on energy for anabolic reactions, demand for essential nutrients, and accumulation of non-host proteins, making it less conducive for viral replication (Verbruggen et al., 2016). Changes of intracellular environment in host cells to reduce the ability of the virus to replicate is essential for survival (Mothes et al., 2010). In the present study, the host intracellular environment response to WSSV infection was observed and the specific genes involved were also recognized. Those genes include plasmolipin, G protein alpha subunit and peritrophin.

Plasmolipin is a membrane-bound 18-kDa proteolipid protein and consists of four transmembrane segments (Miller et al., 2008). It is an amphipathic protein, which participates in transmembrane ion movement including H^+, Ca^{2+} channels, and possibly in Na^+, K^+-ATPase transport function (Cochary et al., 1990). The biological function of plasmolipin is not known; but the *in vitro* formation of a voltage dependent K^+ channel by plasmolipin suggests its ion channel function *in vivo* (Fischer & Sapirstein, 1994). Plasmolipin proteins were first reported from the canine and bovine kidney plasma membranes (Pérez et al., 1997) and its homologues are found in ion homeostasis-dependent tissue, such as the apical surface of the kidney tubular cells and the myelin sheaths of the nervous system in the brain (Fischer & Sapirstein, 1994). In crustaceans, plasmolipin was first reported in *P. monodon* and is abundance in most tissue (Vatanavicharn et al., 2012).

A study by Miller et al. (2008) on the Asian wild mouse (*Mus caroli*) confirmed that plasmolipin (PLLP; TM4SF11), is a receptor for *M. caroli* endogenous retrovirus (McERV) but was not expressed in the mouse cell types. In *P. monodon*, two isoforms of plasmolipin, *Pm*PLP1 and *Pmp*LP2, was upregulated in haemocytes after infection with yellow head virus (YHV) and WSSV (Vatanavicharn et al., 2012). They proposed that *Pm*PLP may be required for the viral entry into shrimp haemocytes. In addition, *PM*PLP1 was postulated to be an YHV receptor, but with no evidence after a gene knock-down by dsRNA. In contrast, in the present study, plasmolipin was upregulated in the survived-WSSV shrimp, suggesting its potential role in defense response. However, the function of this gene needs further evaluation to verify it involvement in shrimp immune response.

Heterotrimeric guanine nucleotide binding proteins (G proteins) play a vital role in transmembrane signaling process as they mediate the effects of neurotransmitters,

numerous hormones or sensory stimuli by coupling their transmembranous receptors to various effectors like enzymes and ion channels (Wettschureck & Offermanns, 2005). G protein, activated by G protein–coupled receptors (GPCRs), are composed of three subunits, namely α, β, and γ (Offermanns & Simon, 1996). Activation of G protein α subunit will release guanosine diphosphate (GDP) and bind guanosine triphosphate (GTP) to G_{α}, resulting in dissociation of the $G_{\alpha\beta\gamma}$ heterotrimer. Both $G_{\beta\gamma}$ and GTP–G_{α} can activate downstream effectors (Kehrl, 1998). The G protein α subunit is a part of chemokine receptors signaling that is widely expressed on a variety of immune cells (Bennett et al., 2011). The biological function of these receptors is based on the receptors itself, either constitutive or inflammatory, predominantly involved in development and homeostasis, or in host response to infection (Johnson et al., 2005). These proteins are widely investigated in vertebrates (Du & Macara, 2004; Wettschureck & Offermanns, 2005; Tsvetanova et al., 2015; Kamp et al., 2016; Toro-Tapia et al., 2017).

In invertebrates, research on the G proteins mainly focuses on neurotransmission, energy metabolism, longevity and stress resistance, germ cell migration and developmental regulation (Dong & Zhang, 2012). However, Dong and Zhang (2012) also reported limited research on the immune function of these proteins in invertebrates. In the roundworm (*Caenorhabditis elegans*), the G protein-coupled receptors (GPCRs) have been demonstrated to be involved in the regulation of the innate immune response via neural and non-neural mechanisms (Liu & Sun, 2017). The (GPCR) DCAR-1 in *C. elegans* was required for the response to fungal infection and wounding (Zugasti et al., 2014). DCAR-1 acted in the epidermis to regulate the expression of antimicrobial peptides via p38 mitogen-activated protein kinase pathway. A putative GPCR found in the red swamp crayfish (*Procambarus clarkii*) HP1R gene, was required to defend against bacterial challenge (Dong & Zhang, 2012). Silencing of HP1R gene by RNA

interference in crayfish demonstrated high bacterial burden and decreased total haemocytes count in response to bacterial challenge. A study by Xu et al. (2017b) on G protein alpha signaling in Arabidopsis demonstrated that Arabidopsis Gα (GPA1) is a key component of a new immune signaling pathway activated by bacteria-secreted proteases. Therefore, the upregulation of the G protein α subunit established in the present study is an indication of a response of innate immunity in crustacean to viral infection. However, this remains speculative as more research is needed to confirm the functions of G protein in crustacean innate immune system.

According to a study by Xie et al. (2015), the *P. vannamei* peritrophin-like protein (*Lv*PT) was found to interact with VP37, an envelope protein of the white spot syndrome virus. Further studies using the yeast two-hybrid (Y2H) library that was constructed using cDNA obtained from the stomach and gut of *P. vannamei* revealed that *Lv*PT could also interact with other WSSV envelope proteins such as VP32, VP38A, VP39B, and VP41A. VP37, found on the outside of the virion WSSV membrane protein complex (Chang et al., 2010), played a major role in WSSV infection (Wu et al., 2005). This envelope protein interacts with receptors or assisting proteins on the peritrophic membrane (PM), possibly peritrophin, that can enable the WSSV to break through the physical barrier of the PM (Xie et al., 2015). However, PM only allows particles smaller than 20 nm to pass through the membrane (Martin et al., 2006), wherein, the width and length of WSSV is 70–150 nm and 250–380 nm, respectively (Lu et al., 1997). Xie et al., 2015 postulated on the presence of receptors or assisting proteins in the stomach and gut that can enable the WSSV to break through the physical barrier of the PM. The PM aids in digestion and forms a protective barrier to prevent the invasion of bacteria, viruses and parasites (Lehane, 1997).

Peritrophin is a type 3 protein of the PM matrix that has been extensively studied in various organisms such as the red flour beetle (*Tribolium castaneum*) (Jasrapuria et al., 2010), the green bottle fly (*Lucilia cuprina*) (Elvin et al., 1996), the common cutworm (*Spodoptera litura*) (Chen et al., 2014b), African malaria mosquito (*Anopheles gambiae*) (Shen & Jacobs-Lorena, 1998), Chinese mitten crab (*Eriocheir sinensis*) (Huang et al., 2015) and ridgetail prawn (*Exopalaemon carinicauda*) (Wang et al., 2013a). This secretory protein was mainly expressed in stomach and gills of the Chinese white shrimp (*Penaeus chinensis*) (Du et al., 2006). The discovery of a peritrophin-like gene (*Es*PT) obtained from *E. sinensis* revealed that it could bind to different microbes, and enhanced the clearance of *Vibrio parahaemolyticus in vivo* (Huang et al., 2015). However, in *E. carinicauda*, a peritrophin-like protein (*Ec*PT) might be involved in WSSV infection as silencing of *Ec*PT by dsRNA interference led to higher survival rate of shrimp against WSSV challenge (Wang et al., 2013a). Hence, the downregulation of peritrophin gene observed in the present study, might give an indication on the immune response of shrimp against WSSV infection and possibly increase the survival rate.

Among the differentially expressed genes found in this study, several had been previously reported to be involved in shrimp responses to WSSV, such as C-type lectin (Gross et al., 2001; Dhar et al., 2003; Goncalves-Soares et al., 2012), caspase (Wongprasert et al., 2007; Rijiravanich et al., 2008; Chen et al., 2013), haemocyanin (Leu et al., 2007; Wang et al., 2008b; James et al., 2010) and haemocyte homeostasis-associated protein (Prapavorarat et al., 2010). A detailed description of these immune-related genes functions and its gene expression in response to WSSV infection is clarified in Section 5.2.

In the present study, the shrimp surviving at 12 days post infection were found to be clear of the virus. Venegas et al. (2000) demonstrated a quasi-immune response in *P.*

japonicus that survived for 32 days after a series of WSSV infections. In this case, the shrimp that were reared collectively and those reared individually were 72 % and 100 % WSSV negative respectively when tested by PCR. Venegas et al. (2000) also hypothesized the presence of a 'neutralizing factor' (or non-specific binding factor) in the haemolymph of shrimp that survived after 17 days of the challenge with WSSV. The neutralizing factor could not be detected at the end of the 17-day survival period, but it again emerged following secondary exposure to the virus. However, the authors could not confirm the phenomenon of acquired resistance or a 'quasi-immune response' in *P. japonicus* to WSSV.

Clearance of WSSV was also observed by PCR in an experimentally injected giant freshwater prawn (*M. rosenbergii)* (Sarathi et al., 2008). The giant freshwater prawn is known to act as an asymptomatic carrier for the virus (Hossain et al., 2001). Sahul Hameed et al. (2000) found that WSSV pathogenicity for juveniles and adults of *M. idella*, *M. lamerrae*, and *M. rosenbergii* pointed to the former two species being more susceptible to WSSV infections than *M. rosenbergii*, which has a high level of tolerance for the disease. *Macrobrachium rosenbergii* recovered and survived without any mortality at 5 days post infection over the 100-day period of the experiment. However, the mechanism of resistance involved in this species remains unknown. Further work is thus needed to determine the exact mechanisms of clearance and resistance against WSSV.

Breeding for disease resistance of Penaeid shrimp based on the mass selection procedure, has been successfully carried out for the Taura Syndrome Virus in *P. vannamei* (Argue et al., 2002). Cuéllar-Anjel et al. (2012) also reported significant WSSV-resistance in a Pacific white shrimp Panamanian breeding program, again based on the mass selection procedure. The mass selection procedure has been shown to be

capable of effectively selecting desirable traits for breeding purposes. However, this procedure requires progeny testing schemes with longer generation intervals than is the case with genomic selection (Van Grevenhof et al., 2012). The utilization of transcriptomic studies in the present study could help the development of genomics toolkits and assist stock improvement in the giant tiger shrimp by marker assisted selection (MAS). The transcriptome data produced by the present study could also assist the discovery of SNPs directly associated with desirable traits (Yu et al., 2014). Moreover, only 2.13% of the unigenes in this study matched *P. monodon* sequences in the GenBank non-redundant database, while 36,852 new unigenes were discovered, significantly broadening our knowledge of the *P. monodon* transcriptome.

5.2 Immune Response of *Penaeus monodon* Experimentally Infected with White Spot Syndrome Virus Infection

The white spot syndrome virus has a wide reservoir range that includes marine shrimp, crab, freshwater prawn and lobster (Rajendran et al., 1999). Besides causing mortality, shrimp infected with WSSV will also results in a latent infection, thus becoming a reservoir for this virus. According to Pradeep et al. (2012), WSSV infects most tissues. At an early stage, WSSV infect the stomach, gills, cuticular epidermis and the connective tissue of the hepatopancreas and progress to the lymphoid organ, antennal gland, muscle tissue, hematopoietic tissue, heart, hindgut and parts of the midgut during late stage of infection (Pradeep et al., 2012). Thus, in the present study, by incorporating various organs that are involved in the WSSV infection, the expression and progress of host cell defence against WSSV could be observed.

The immune status of shrimp will greatly influence shrimp resistance to pathogens (Martínez, 2007). Most immune response study on host-virus interaction in penaeid shrimp only focuses on the major organ for immunity that include hepatopancreas and haemocytes (Wongpanya et al., 2007; Ji et al., 2009; James et al.,

2010; Goncalves et al., 2014; Rubio-Castro et al., 2016). In the present study, the muscle tissue, a non-vital organ for immunity, was also included together with the hepatopancrease and haemocytes to have a broad overview of WSSV infections in the giant tiger shrimp. Moreover, 22 immune-related genes were selected to study the host-virus interaction in giant tiger shrimp infected with WSSV by microfluidic dynamic array, providing a comprehensive summary of the cascade of genes respond towards virus infection as discussed at the end of the chapter.

The immune response of shrimp towards virus infection will activate several immune molecules that are part of the humoral innate immune response. These immune molecules participate in the major immune reaction against invading pathogens in shrimp including pattern recognition receptor, protein and proteinase inhibitors, antimicrobial peptides, apoptosis pathway, signal transduction pathway and chaperons (Tassanakajon et al., 2013). The pattern recognition receptors (PRRs) are the first line of defense in shrimp innate immunity (Wang & Wang, 2013). PRRs recognized pathogen-associated molecular patterns (PAMPs) which are derived from viruses, pathogenic bacteria, pathogenic fungi and parasitic protozoa (Wang et al., 2013b). Toll protein, C-type lectin and HMGB are involved in the first line defense as PRRs (Medzhitov & Janeway, 1997; Weis et al., 1998).

In the present study, the expression of Toll protein after infection with WSSV was significantly upregulated immediately after infection. The expression level of Toll protein gradually decreases after 6 hpi until the end of the experiment. This finding, however, contradicts with a study by Arts et al. (2007), that found lack of regulation of PmToll in *P. monodon* against WSSV challenge. However, despite the absence of regulation of PmToll in the former study, there is a potential for PmToll involvement in the immune response to WSSV. According to Takeuchi & Akira (2010), depending on

the TLRs and cell types involved, recognition of PAMPs by Toll-like receptor leads to transcriptional upregulation of distinct genes. Stimulation of Toll protein by PAMPs also leads to the activation of signaling pathways that are capable to induce antimicrobial responses through the production of antimicrobial peptides and other innate immune genes (Medzhitov & Janeway, 1997; De Gregorio et al., 2002).

An *in vitro* study by Luo et al. (2003) showed that PmAV, a type of C-lectin found in WSSV-challenged *P. monodon,* demonstrated a strong antiviral activity in fish cells. Whereas a study by Zhao et al. (2009) on c-type lectin from the shrimp *P. vannamei* (LvCTL), found that this gene possesses anti-white spot syndrome virus activity. Similar with our findings, the LvCTL1 protein expression increased steadily at 6 hpi and remain at a high level until 48 hpi (Zhao et al., 2009). The same author suggest that a high level of C-type lectin may bind to WSSV and neutralize the virus. The present study also reveals that C-type lectin expression dropped at 12 hpi and increased again at 24 hpi, suggesting a possible secondary immune response which was also reported by (Wang et al., 2009b). C-type lectins serve an important role in innate immunity through opsonization and non-self immune recognition in invertebrates (Holmskov et al., 1994). C-type lectin has a diverse function in various processes of cell including cell adhesion, pathogen recognition, endocytosis, agglutination and phagocytosis (Kerrigan & Brown, 2009).

High-mobility-group-box (HMGB) protein family is the most abundant and ubiquitous non-histone chromatin binding proteins in eukaryotic cells (Ueda & Yoshida, 2010). This protein can be secreted into the extracellular environment as a signaling molecule when cells are under stress. It also acts as DNA chaperones influencing multiple processes in chromatin such as transcription, replication, recombination, DNA repair and genomic stability (Stros, 2010). There are various

members of HMGB in lower vertebrates and invertebrates (Rao & Su, 2015). Two types of HMGB are found in white shrimp (*P. vannamei*): HMGBa and HMGBb (Chen et al., 2011). A study by Chen et al. (2014a) in white shrimp found that HMGBb expression levels were up-regulated when induced by a pathogen-associated molecular pattern (PAMP). They suggested that the release of HMGBa and HMGBb occurs naturally during cell necrosis, and that it occurs in shrimp haemocytes in response to PAMP. The innate immune system also plays an important role in the acquired immune response and is equally important in higher vertebrates (Magnadóttir, 2006). In grass carp (*C. idella*), two HMGB2 homologue genes, CiHMGB2a and CiHMGB2b, are widely expressed with different abundances in 15 tissues, and are induced by Grass Carp Reovirus (GCRV) and viral/ bacterial PAMP challenge. Both genes demonstrate significant antiviral activities and provide strong evidence to support the essential roles of HMGB in innate immunity (Yang et al., 2013). The expression of HMGBb in the present study was elevated at 6 hours post infection with WSSV. The result indicates that this gene plays an important role as the first line of defense in shrimp innate immunity. Further research is needed on this gene and the mechanisms through which it acts in shrimp innate immunity, particularly against WSSV infection.

Proteinases are key components of the innate cellular immunity that involved in several proteolytic cascade such as apoptosis and melanization (Tassanakajon et al., 2013). Protease inhibitors function to prevent extreme activation of cascades, prevent consequent damage to host tissue and inhibit protease of invading pathogens into host (Tassanakajon et al., 2013). Both proteinase and protease inhibitor are regulated by the prephonoloxidase-activation (proPO) system (Cerenius & Söderhäll, 2004). Three type of proteinase and protease inhibitors were utilized in the present study in order to monitor its regulation in WSSV-infected shrimp including Kazal-type serine proteinase inhibitor 4 (KSPI4), haemocyte kazal-type proteinase inhibitor (HaeKPI) and caspase.

All three genes in the present study showed the same pattern of gene expression in hepatopancreas of WSSV-challenged shrimp, in which the genes regulation increased gradually from 6 hours post infection until 48 hour post infection.

In the present study, kazal type SPIs were observed to be up-regulated in surviving WSSV-infected shrimp in the early stages of infection. Kong et al. (2009) reported that haemocytes from wild WSSV-infected oriental white shrimp*, P. chinensis*, expressed higher levels of the Kazal type SPIs transcript compared to healthy shrimp. Another study by Zeng and Lu (2009) similarly found increased levels of serine proteinase inhibitors in the haemocytes of the crayfish (*Procambarus clarkii*) within 72 hours of WSSV infection, by suppression subtractive hybridization and cDNA microarrays. Moreover, in the present study, Kazal type SPIs were found to be down-regulated in late stage of infection of the surviving WSSV-infected tiger shrimp (12 days post infection). These results suggest that there might be a complexity of factors and/or mechanisms at play relating to the modulation of Kazal-like gene expression and pathogen defense.

There have been a few studies in recent years on Kazal type serine proteinase inhibitors (SPIs) to investigate the structure, activity and regulation of these genes in response to virus infection. Donpudsa et al. (2010) found that Kazal type SPIs function to limit and control the extent of proteinase activities in immune processes. During the development of infectious diseases, many invasive pathogens produce extracellular proteinases as virulent factors to help penetrate through the physical barriers of the host (Christeller, 2005). Consequently, host organisms produce proteinase inhibitors to defend against such pathogens and combat microbial proteinases (Rimphanitchayakit & Tassanakajon, 2010). Both proteinase and protease inhibitor are capable of eliminating invading pathogens by catalytic event in the host cell.

Another major component of the innate immune defense is antimicrobial peptides (AMPs) (Tincu & Taylor, 2004). AMPs provide an immediate and rapid response to invading microorganisms (Bartlett et al., 2002) by disrupting the membrane integrity of the cell target (Rosa & Barracco, 2010). These 'natural antibiotics' are efficient against broad range of microorganisms including Gram-positive and Gram-negative bacteria, yeast, filamentous fungi, protozoans and enveloped viruses (Horne et al., 2005; Rivas et al., 2009; Aoki & Ueda, 2013). According to Bahar & Ren (2013), AMPs neutralize invading viruses in host cell by integration in either the viral envelope and host cell membrane. The integration of AMPs into viral envelopes cause membrane instability and preventing the viruses to infect cell and decrease its ability to bind into host (Robinson et al., 1998; Sitaram & Nagaraj, 1999; Belaid et al., 2002).

In the present study, various type of AMPs were found to be involved in the defence mechanism against WSSV including penaiedin, crustin and c-Jun protein. Results of the present study suggested that crustin 2 was triggered immediately after infection with WSSV and the enhanced expression resulted from increased immune response to eliminate pathogen. Unlike crustin 2, a decrease of expression in penaeidin 3 gene immediately after exposure to WSSV was observed before the expression spike at 12 hour post infection. Jose et al. (2010) speculated that an apparent decrease in expression of most immune-related genes after initial hours of infection is temporary of WSSV infection in shrimp primary culture. A sharp increase of both crustin 2 and penaeidin 3 were also observed at 12 and 24 hour post infection with WSSV. The spike of gene expression might be for the clearance of viruses by both AMPs since VP28, a WSSV envelope protein, that was highly expressed in host gene from 12 to 24 hour during infection (Khadijah et al., 2003; Jose et al., 2010).

Yao et al. (2015) found that, during the process of WSSV infection, the transcription levels of *P. vannamei* c-Jun (Lvc-Jun) were up-regulated, suggesting that WSSV infection could enhance both the expression and phosphorylation levels of Lvc-Jun. The same authors further stated that increased levels of Lvc-Jun along with the aggravation of viral infection indicated a notable positive correlation between Lvc-Jun activation and viral infection. In agreement with Yao et al. (2015), our findings suggest that, while c-Jun was upregulated in surviving WSSV-infected shrimp, the activation of this gene in cases of WSSV infection may trigger a sequence of immune-related genes and immune pathways to help the shrimp survive the disease. Another study by Li et al. (2015), on the interaction of *P. vannamei* encoding the full-length c-Fos protein (Lvc-Fos) and Lvc-Jun, found that silencing of Lvc-Fos or Lvc-Jun in shrimp caused lower mortality and virus loads under WSSV infection, suggesting that Lvc-Fos and Lvc-Jun could be engaged in WSSV replication and pathogenesis. In sum, more studies on the interaction of immune-related genes are needed to elucidate further their role in immune responses against pathogens, particularly in surviving shrimp after an infection.

In the present study, the expression of HHAP was elevated during WSSV infection. However, the expression level of HHAP reduced from 6 to 12 hpi. The reduced expression of HHAP observed might be due to the WSSV genome replication stage at 6 to 12 hour post infection as reported by (Chen et al., 2016), that suppressing the haemocyte regulator producing haemocytes during apoptosis. The HHAP is involved in the apoptosis pathway of an immune system by maintaining the haemocyte level in the blood circulating system (Prapavorarat et al., 2010). During apoptosis, the number of circulating haemocytes is significantly reduce that potentially can affect immunity against viral infection (Wang & Zhang, 2008). Thus, the presence of HHAP is important to regulate haemocytes as a response against virus infection. Prapavorarat et al. (2010) identified HHAP as being highly up-regulated at both the transcript and

protein levels, in WSSV-infected shrimp haemocytes. They confirmed that silencing HHAP gene in *P. monodon* by dsRNA-interference (RNAi) caused damage to shrimp haemocytes and a severe drop in the numbers surviving the disease. The HHAP play a major role in the control of haemocyte homeostasis in shrimp during viral infection (Apitanyasai et al., 2015). Although studies on HHAP have been limited, it seems reasonable to conclude that the up-regulation of HHAP is an important mechanism to control circulating haemocyte levels in crustaceans during viral infection.

Signal transduction pathways including Toll, Immunodeficiency (IMD) and the Janus kinase-signal transducer and activator of transcription (JAK/STAT) are involved in the immune responses and have essential roles in shrimp immunity (Tassanakajon et al., 2013). This pathway involves the binding of extracellular signaling molecular and ligands to cell-surface receptors that trigger the events inside the cell. It occurs when an extracellular signaling molecular, such as a kind of pathogen-associated molecular patterns (PAMPs) or a viral protein antigen (cause), activates a cell-surface receptor. In turn, this receptor alters intracellular molecular creating a response. A second messenger transmits the signal into the cell, triggers a physiological response. The combination of messenger with receptor causes a change in the conformation of the receptor, known as receptor activation. The signal can be amplified and one signaling molecular can cause many responses (Li & Xiang, 2013).

The tumor necrosis factor receptors are a potential STAT regulator gene. This gene activates caspase via the extrinsic pathway by promoting the oligomerization of caspases at the intracellular domain of the membrane-spanning TNFR (Salvesen, 2008; Menze et al., 2010). Mekata et al. (2010) discovered a high level of expression of the MjTNF gene following stimulation with peptidoglycan and polycytidylic acid in lymphoid organs cells in *P. vannamei*. A high expression level of MjTNF was also

observed *in vivo* 2 h and 4 h after stimulation with lipopolysaccharide and *Vibrio penaeicida* respectively (Mekata et al., 2010). A study by Wang et al. (2012) on the isolation and characterization of the *P. vannamei* TNF superfamily (LvTNFSF) gene found that it contains an N-terminal transmembrane segment and a conserved C-terminal TNF domain with 26.9% and 24.8% sequence identity with *Drosophila melanogaster* Eiger (DmEiger) and *Homo sapiens* ectodysplasin A (HsEDA), respectively. LvTNFSF also similarly responded to viral infection in a similar manner to MjTNFSF. LvTNFSF was upregulated at 3 and 24 h post-injection with WSSV in the hepatopancreas, suggesting that LvTNFSF may participate in host immune responses against pathogens, especially viruses and Gram-positive bacteria (Wang et al., 2012).

Overall, a schematic diagram of immune response of *P. monodon* infected with WSSV in the present study is shown in Figure 5.1. During WSSV infection, non-self molecules that act as pathogen-associated molecular patterns (PAMPs) in WSSV are recognized by the appropriate pattern-recognition proteins (PRPs) (HMGBb and C-type lectin) of the host cell, P. monodon. This event triggers the activation cascade of several serine proteinase and proteinase inhibitor (KPi4, HAEKPI, cathepsin C and caspase) that involved in several proteolytic cascade such as phagocytosis and melanization. The resulting production of PRPs leads to apoptosis event and haemocytes homeostasis are achieved by HHAP regulations in the haemocytes. Simultaneously, the unfolded protein response (UPR) (HSPs) are initiated as a cellular stress response from the recognition of PAMPs. PRPs consequently activates the JAK/STAT signaling pathway (TNF). Subsequently, the activation of the serine proteinase cascade leads to the synthesis of the antimicrobial peptides (AMPs) (c-Jun, penaeidin and crustin) as a response to WSSV infection for virus elimination.

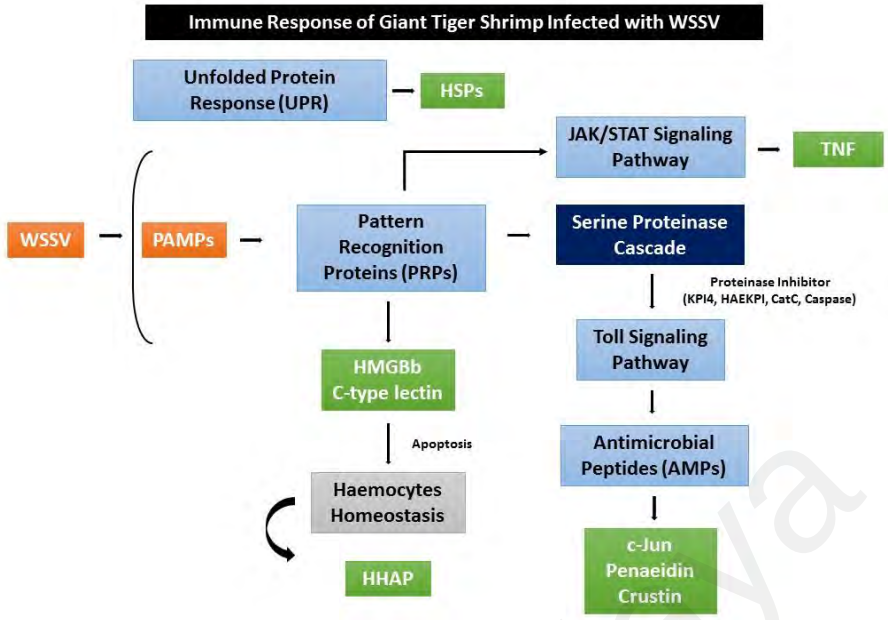


Figure 5.1: A schematic diagram of immune response of *Penaeus monodon* infected with WSSV as seen in the present study.

5.3 Candidate Genes for Disease Resistance in *Penaeus monodon* Survivability against the White Spot Syndrome Virus

In the present study, a genome-wide SNP resource of *P. monodon* using the comparative transcriptome profiling analysis method and SNPs genotyping panel was developed using WSSV-survived shrimp and control shrimp. To our knowledge, the present study is the first report on the generation of a large panel of SNPs for WSSV-survived *P. monodon*. This approach has significantly increased and improved expressed sequence and DNA marker resources for *P. monodon*. Moreover, the potential advantages of selected SNPs or candidate genes for application of marker assisted selection based on selected immune related genes such as caspase, lectin, C-type lectin, heat shock proteins, chromosome-associated kinesin gene, haemocyanin and actin discovered in the present study have potential to be applied as candidate markers for the development of WSSV resistance *P. monodon*.

A mean observed transition/transversion (Ts/Tv) ratio of 1.82 was observed in the transcriptome of the survived WSSV-challenged shrimp. The Ts/Tv ratio was slightly lower than the 2.0-2.2 genome-wide Ts/Tv ratio (DePristo et al., 2011) and was generally lower than the 3.0 whole exome Ts/Tv ratio (Bainbridge et al., 2011). The expected Ts/Tv ratio varies between species and may simply be an artefact of the specific loci that were examined (Smith et al., 2005). However, partly as a result, the exact expected Ts/Tv in RNA-seq data was tricky to conclude as transcriptomes represents a subset of the exome and a quasi-complete set of transcribed genes and other noncoding RNAs (Ku et al., 2012).

Considering that the variant detected using RNA-seq is suggested to high false positive rates (Guo et al., 2017), a proper SNP filtering strategy are recommended to reduce the error rates (Reumers, 2012). To eliminate the false positive variants, only SNPs that reside within the target region of the contigs with a minimum sequencing depth of ten were selected. Moran et al. (2017) demonstrated that increasing the minimum read depth supporting the variants from 8 to 15 reads increased the specificity to almost 85%. Higher read depth could ensure that most of expected SNPs in the sequenced population could be detected and allow the discovery of infrequent alleles in highly expressed genes (Lopez-Maestre et al., 2016). Additionally, gene expression values were also used to filter the variants. Genes with an FPKM value less than 1 were excluded from the SNPs calling procedure. Variant that is located within an expressed gene may have an impact at the biological level (O'Brien et al., 2015).

Posterior to SNPs filtering, a linkage-based genotype can contribute a lot to the accurate SNP and genotype calling (Liu et al., 2012). Altmann et al. (2012) also suggest assessment of deviations from the Hardy–Weinberg equilibrium (HWE) to minimize SNP calling artifacts. Thus, to further validate SNPs loci discovered in the

survived WSSV-challenged transcriptome, 96 SNP markers were identified and genotyped through Fluidigm's IFC SNP Type Genotyping. Among the 96 SNP markers, 95.8 % markers were successfully being genotyped. This rate was higher compared to the giant freshwater prawn (75%) (Jung et al., 2014) and the Pacific white shrimp (76%) (Yu et al., 2014). The high success rate for SNPs validated in the present study implies most SNPs predicted in this study are true SNPs. All SNPs that were selected in the present study were not in the dbSNP database, implying newly discovered SNPs.

However, after assessment of deviations from HWE, only 18 SNPs were chosen as markers for survivability against WSSV. Single nucleotide polymorphisms that were in Hardy-Weinberg equilibrium were moderately polymorphic, likely due to ascertainment bias in SNP discovery caused by using *P. monodon* population with moderate genetic variations. According to Krawczak (1999), it is critical to consider that SNP informativity may vary significantly between populations in the development of efficient SNP-based markers systems. Due to this, SNP markers that were identified in the present study need to be validated using large and diverse population to predict phenotype so that it can be used in routine screening for MAS (Collard et al., 2005). Nonetheless, markers developed in the present study still can be used for the genetic differentiation of *P. monodon* and with other species (Williams et al., 2010) due to its successful cross amplification against the Manjung, Perak F1 breeding line.

The SNPs identified in the present study represent a potential markers for survivability against WSSV in *P. monodon*. This is due to the application of transcriptome sequencing that enables the identification of SNPs located in transcribed regions and could be affecting the expression of a gene (Lopez-Maestre et al., 2016). Single nucleotide polymorphisms markers selected in the present study were involved in the innate immune response. Those candidate genes are caspase, effector caspase,

lectin B, lectin 3, C-type lectin 1, C-type lectin 5, HSP 21, HSP 10, chromosome-associated kinesin KIF4A gene, haemocyanin and actin 2.

In the present study, 96 SNPs were identified for SNPs genotyping, most of it were in linkage disequilibrium, only 18 were chosen as candidate genes for WSSV-resistance gene. As mentioned by Rafalski (2002b), the information on SNPs is most useful when several closely spaced SNPs completely define haplotypes in the region are examined. Moreover, SNP haplotypes can overcome the loss of information (due to the bi-allelic nature of the markers) when testing for the association between complex phenotypic traits and candidate loci (Vignal et al., 2002). Single nucleotide polymorphisms that were in Hardy-Weinberg equilibrium were moderately polymorphic, likely due to ascertainment bias in SNP discovery caused by using *P. monodon* population with moderate genetic variations. According to Krawczak (1999), it is critical to consider that SNP informativity may vary significantly between populations in the development of efficient SNP-based markers systems. Due to this, SNPs markers that were identified in the present study need to be validated using large and diverse population to predict phenotype so that it can be used in routine screening for MAS (Collard et al., 2005). Nonetheless, markers developed in the present study can still be used for the genetic differentiation of *P. monodon* with other species (Williams et al., 2010). All SNPs detected in the present study were in the coding region (cSNPs). Some of the SNPs were synonymous SNPs that cannot change the protein structure of a gene directly. However, Yu et al. (2017) proved that synonymous SNPs could affect the biological process by: 1) synonymous SNPs and some functional SNPs might be in strong linkage disequilibrium; 2) allele-specific differences in mRNA folding might influence the splicing, transcription and regulation; and 3) the codon usage bias can affect the protein translation and folding.

The innate immune system involves multiple molecules that take part in defending the host against WSSV infection. Therefore, multiple SNPs were chosen for the present study, to enable the detection of more potential candidate genes and SNPs which might affect WSSV-resistance. Previous study by Zhao et al (2012) identified a total of 13 SNPs in the C-terminus of hemocyanin (HcSC) from the shrimp *P. vannamei*. The authors found that these polymorphisms could be modulated by environmental stresses such as temperature and pathogen infection. These mutations were found to be associated with shrimp resistance to pathogens. In addition to haemocyanin, heat shock proteins and actin were also selected as candidate genes for WSSV-resistance. Five SNPs in the coding region of the HSP 70 gene in *P. vannamei* was detected by Zeng et al. (2008) to be associated with a Taura syndrome virus (TSV)-resistance trait in *P. vannamei*. However, future studies involving a larger set of individuals need to be examined to determine if an association exists between HSP70 polymorphism and the TSV-resistance trait for this study. Another study by Robinson et al. (2014) characterised nine immune related SNPs including heat shock proteins and lectins showing associations with hours of survival post WSSV infection in *P. monodon*. All the candidate gene SNPs discovered in the present study were new gene SNPs set for WSSV immune response in *P. monodon*. Hence, the present study provides new significant information for a better understanding of the genetic basic for disease response and potential resistance in shrimp and will assist in MAS for WSSV-resistance *P. monodon*.

CHAPTER 6: CONCLUSION

In summary, the present study obtained over 37,223 unigenes, and a total of 4839 differentially expressed genes, in *P. monodon* challenged with WSSV, providing additional genomic resources for research on *P. monodon.* Most of the genes found in this study have not been studied extensively. Samples were taken from different tissues chosen from WSSV survivors as an effort to discover as many genetic variabilities within expressed genes as possible. This would ensure that a large number of polymorphisms would be available for development of a genetic marker. The molecular responses and defence mechanisms of *P. monodon* against WSSV obtained from this study's comparative transcriptome analysis of shrimp surviving WSSV challenge and uninfected shrimp could help the development of effective strategies to prevent WSD. This gave an opportunity to compare, the gene expression in shrimp that had survived WSSV infection with that of healthy shrimp, as to gain a better understanding of the host-virus interactions. Moreover, three genes including HMGB, TNFSF and c-Jun identified in the present study are the first to be reported in *P. monodon*. The large number of transcripts and molecular markers obtained in this study should provide a strong basis for future genomic research on shrimp.

A cascade of responses against WSSV infection were involved in protecting the host. In total, 22 immune-related genes were selected to study the host-virus interaction, in giant tiger shrimp infected with WSSV, by microfluidic dynamic array, providing a comprehensive summary of the cascade of genes responds towards virus infection. Understanding shrimp defence mechanisms in combination with different strategies can contribute to improving disease management. The tissue specific transcripts being identified in the present study can be implicated in the shrimp immune responses as candidate genes and improve strategies for disease management.

Moreover, over expression of few genes in the present study including Toll protein, C-type lectin, HMGB, Kazal-type serine proteinase inhibitor 4, haemocyte kazal-type proteinase inhibitor, caspase, penaeidin, crustin, c-Jun protein, HHAP and TNF gene could hypothetically improve disease resistance in *P. monodon*. Nevertheless, the function of these immune genes and proteins are poorly understood and require further studies to unveil their function in the shrimp immune system.

Apart from that, 18 SNP candidate gene markers for WSSV resistance were also identified in the present study. Those markers include caspase, lectin, C-type lectin, heat shock proteins, chromosome-associated kinesin gene, haemocyanin and actin. However, SNPs were not detected in either three newly discovered genes in the *P. monodon* transcriptome. This could be due to the large genome size of this species (estimated 2.2 Gb) (You et al., 2010) and the lack of well-annotated genome sequences for *P. monodon*, limiting the study of structural variation that could have important influences on traits (Baranski et al., 2014). Nevertheless, the present study successfully identified a total of 36,857 and 28,527 SNPs from transcriptome of WSSV-survived and control shrimp respectively after 12 days post WSSV challenge, with 95.8 % markers successfully genotyped. Thus, these present data is adding more information on the genomic resources for *P. monodon* research. Markers developed in the present study could also be applied to supplement genetic evaluations for *P. monodon*.

The efficacy of the candidate genes in the present study to be used for marker assisted selection in improving resistance to WSSV should be further evaluated. These markers will need to be validated and tested with large sample numbers using multiple populations before being applied for stock improvement. While deviation of HWE and significant LD were observed in the SNPs between the different population of WSSV-challenge *P. monodon*, these results will require detailed confirmation as to whether

these polymorphisms are a random effect because of chance or are selection signature for a specific trait. Furthermore, future study for the identification of genes affecting disease resistance should be directed at fine mapping of the genes involved paired with comprehensive experiments to elucidate their function. Nevertheless, the transcriptome data generated in the present study offer an invaluable resource for future functional studies to select or manipulate genes that influence disease resistance trait for future *P. monodon* stock improvement programmes.

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