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メタデータ	言語: eng 出版者: 公開日: 2019-06-24 キーワード (Ja): キーワード (En): 作成者: Tinwongger Sasiwipa メールアドレス: 所属:
URL	https://oacis.repo.nii.ac.jp/records/1767

Doctoral Dissertation

IDENTIFICATION OF GENES ASSOCIATED WITH Vp_PirAB-LIKE TOXIN RESISTANCE IN *Litopenaeus vannamei*

March 2019

Graduate School of Marine Science and Technology
Tokyo University of Marine Science and Technology
Doctoral Course of Applied Marine Biosciences

Tinwongger Sasiwipa

Contents

Acknowledgements

First and foremost, I would like to thank my advisor, Professor Ikuo Hirono, for supporting me during these past five years and a half. Hirono sensei has provided insightful discussions and valuable guidance for my research since master's degree to PhD.

I am also thankful to Professor Hidehiro Kondo, for scientific and technical advices that were helpful in my research. I also thank the members of my PhD committee, Professor Motohiko Sano and Associate Professor Takayuki Katagiri for their suggestions.

I am heartily thankful to Mrs. Reiko Nozaki, she was my first teacher in the laboratory. Thank you so much for her patient, technical advices, and kind supports.

To the members of laboratory of Genome Sciences since 2014-2018, I would like to thank all of you for sharing your times, teaching, discussing, parties, and playing softball. My life in Japan was not boring because all of you. Especially, my tutor Keiichiro Koiwai, thank you so much for helping me along the time that I have been in Japan, in both research and living. Also, Thai's students in TUMSAT, thanks to all of you for spending your time, sharing things, and supporting.

Besides, I would like to express my thankfulness to Dr. Varin Tanasonwang, Dr. Putth Songsangjinda, Dr. Chutima Khomvilai, and Department of Fisheries, Thailand, who firstly provided me a good chance for studying in TUMSAT until I finished my PhD.

I gratefully acknowledge the Japanese Ministry of Education Culture, Sport, Science and Technology for providing my essential scholarship in PhD course.

Lastly, I would like to thank my family for their understanding and encouragement in almost six years that I have been in Japan.

Abstract

Whiteleg shrimp, *Litopenaeus vannamei* accounts for about 75% of global farmed shrimp production. They are vulnerable to viral and bacterial diseases that cause mass mortality in shrimp farms. In the last decade, shrimp farmers have suffered from an infection disease, named acute hepatopancreatic necrosis disease (AHPND). AHPND has spread to several countries in Asia and South America, resulting in a great reduction of production. AHPND is caused by a pathogenic *Vibrio parahaemolyticus* carrying a plasmid encoding Vp_PirAB-like toxin which are identified as the virulence factor. Recently, the plasmid encoding Vp_PirAB-like toxin was found in other three AHPND-causing *Vibrio* species, but not *V. parahaemolyticus*.

Vp_PirAB-like composes of two genes, Vp_PirA-like (336 bp) and Vp_PirB-like (1,313 bp), which are co-transcribed and translated to two proteins Vp_PirA-like and Vp_PirB-like. The structural topology of Vp_PirA- and B-like toxins show similarity to *Bacillus thuringiensis* delta-endotoxin or Cry toxins, insecticidal proteins. In the previous study revealed that the virulence of Vp_PirAB-like toxins, unlike the virulence of normal bacterial exotoxins, they are heat stabile and formalin-resistant. Therefore, the formalin-killed cells of *V. parahaemolyticus* AHPND-causing strain D6 (FKC-VpD6) was used to select the Vp_PirAB-like toxin-resistant *L. vannamei* by oral administration. After two weeks of feeding, only 2.5% shrimp survived, then they were collected as toxin-resistance shrimp. It is interesting to figure out how can these shrimps be resistant to toxin virulence, which may be the advantage to control an AHPND infection in shrimp farming.

Previous studies have reported that the high expressions of some immune-related genes induce protection against AHPND. On the other hand, Vp_PirAB-like toxin and Cry toxins may use similar mechanisms for insertion into host cells and share sequence similarity of receptors. The studies of Cry toxins suggested that mutations of Cry toxin-receptors are the

most common mechanism for insect resistance by altering the binding between toxin and receptor. To identify genes that are associated with Vp_PirAB-like toxin resistance, I am focusing on 1) immune-related genes that show specific response to Vp_PirAB-like toxin and 2) genetic variation of toxin-receptor candidates.

At first, stomach and hepatopancreas tissues of resistant shrimps (sur-FKC) were subjected to RNA sequencing. The differentially expressed genes (DEGs) between sur-FKC, AHPND-infected shrimp (Vp-inf), and non-treated shrimp (control) were identified. From a total of 79,591 genes, 194 and 224 DEGs were identified in the stomach and hepatopancreas transcriptomes, respectively. The expressions of ten selected DEGs were validated by qPCR. Only one gene, DN21485 (a gene homologous to *L. vannamei* anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R)) was expressed significantly more strongly in the hepatopancreas of sur-FKC than in the other groups.

Moreover, searching of Cry toxin receptor homologs was performed to identify the gene candidates of Vp_PirAB-like toxin receptors. Five Cry toxin receptors (cadherin, alkaline phosphatase, aminopeptidase N, and ATP-binding cassette transporter subfamily C) were used as query genes for homology search against *L. vannamei* control/Vp-inf transcriptome. A total of thirteen Vp_PirAB-like toxin-receptor candidates were identified.

From the results of DEG analysis, *Lv*ALF AV-R was further investigated to clarify the association of *Lv*ALF AV-R and shrimp resistant to Vp_PirAB-like toxin. In the hepatopancreas, expression of *Lv*ALF AV-R mRNA was not affected by VpD6-immersion or dietary of FKC-VpN7 (*V. parahaemolyticus* non-AHPND causing strain) but was highly induced during FKC-VpD6 feeding. Moreover, significantly higher expression of *Lv*ALF AV-R was also observed in shrimp that survived in three other trials of FKC-VpD6 selection. Histological analysis of four resistant shrimps showed that three of them exhibited normal characteristic of hepatopancreas cells without signs of AHPND.

To investigate the function of *Lv*ALF AV-R that reacts to toxin, the recombinant protein, rALF AV-R was produced for *in vitro* and *in vivo* studies. rALF AV-R bound to LPS, PGN, Gram-negative bacteria, and some Gram-positive bacteria in ELISAs. But rALF AV-R did not interact with native Vp_PirAB-like toxin in an ELISA or a Far-Western blot analysis. For *L. vannamei* orally fed rALF AV-R for 3 days, the survival rate following challenge with VpD6-immersion was not significantly different from that of shrimp fed two control diets (rGFP and PBS).

Genetic variations of Vp_PirAB-like toxin receptor candidates between resistance and other groups were analyzed. Homologous genes of pre-identified receptor candidates from control/Vp-inf were identified in sur-FKC transcriptome. Between control/Vp-inf and sur-FKC, 4 genes (cadherin-like 1, APN-like 1, ABCC2-like 1 and 2) which showed high identity but not 100%, were selected for further analysis. Validation by sequencing showed that only 4326 C/A SNP in cadherin-like 1 gene was detected in pooled cDNAs of resistant shrimp according RNA-seq data, whereas no variation was detected in the other three genes. SNP 4326 C/A was genotyped in the cDNA of resistance group (3 trials) and control/Vp-inf group. The frequencies of genotype AA, AC, and CC in resistance group were 64%, 36%, and 0%, respectively, while those of control/Vp-inf group were 17%, 0%, and 83%, respectively. In addition, SNP 4326 C/A was genotyped in genomic DNA of susceptible and resistance groups. Association analysis indicated that the genotype frequency distributions between two groups was significantly different ($P < 0.05$). In the susceptible group, the AA, AC and CC genotypes were 45%, 34%, and 21%, respectively. The frequency distributions in the resistance group were 64%, 36%, and 0%, respectively. Interestingly, the genotype CC was not observed in resistant group.

Lastly, to determine the stability of resistance phenotype, I had re-challenged the survival shrimp with dietary FKC-VpD6. The result showed that all shrimp died in 3 days after

re-challenging. These results indicate that the resistant characteristic of these collected shrimp is not permanently expressed.

In conclusion, *Lv*ALF AV-R plays an indirect role in shrimp resistant to Vp_PirAB-like toxin by promoting other shrimp molecules to directly inhibit virulence of toxin. On the other hand, the SNP 4326 C/A of cadherin-like 1 gene might be used as a genetic marker to select Vp_PirAB-like toxin-resistant shrimp, although this SNP is not directly related to the resistance mechanism.

Chapter 1

General Introduction

Since as the demand for seafood products has increased and the wild stocks has decreased year by year, aquaculture is a method for increasing the number of aquatic animals/plants, restoring habitat and replenishing wild stocks, and rebuilding populations of threatened and endanger species. The three major animal groups in aquaculture are fish, molluscs and crustaceans [Garibaldi, 1996]. FAO (2018) reported the world aquaculture production in 2016 amounted to 110.2 million tonnes, with an estimated value of 243.5 billion USD. Of the total, crustaceans shared production about 7.3%, including shrimps/prawns at amount of 5.2% (Table 1).

Table 1. Global aquaculture production in 2016 (FAO, 2018).

Aquaculture species	2016	
	quantity (t)	**value (USD x 1000)**
Aquatic plants	30,139,389	11,673,942
Crustaceans	7,862,016	57,078,984
- Freshwater crustaceans	2,280,687	2,499,854
- Crabs, sea-spiders	399,124	22,531,882
- Lobsters, spiny-rock lobsters	1,628	34,317
- Miscellaneous marine crustaceans	14	521
- Shrimps, prawns	5,182,563	32,012,410
Diadromous fishes	4,957,342	23,967,663
Freshwater fishes	46,387,932	102,293,223
Marine fishes	2,745,874	12,276,511
Miscellaneous aquatic animal products	37,967	214,565
Miscellaneous aquatic animals	938,558	6,766,010
Molluscs	17,139,140	29,201,729
Grand total	110,208,218	243,472,628

Shrimps and prawns are the most-traded product for decades and shrimp is rank second in the value terms [FAO, 2016]. The farmed shrimp accounts greater than 50% of the global shrimp production (including capture and farming). Most shrimp aquaculture are produced in China, India, Southeast Asian countries while the latter is Latin America.

Penaeid shrimps are an important group of world aquatic animals. The two main species in shrimp aquaculture are the whiteleg shrimp *Litopenaeus vannamei* and the black tiger shrimp *Penaeus monodon*. In the last decade, *L. vannamei* is the major shrimp species in shrimp farming, accounted about 4.2 million tonnes with approximated value of 24 billion USD in 2016 [FAO, 2018]. *L. vannamei* is a marine shrimp species that habitats in brackish water to seawater. It is native to the Eastern Pacific coast of Central and South America [http://www.fao.org/fishery/culturedspecies/Penaeus_vannamei/en]. Shrimp farming techniques have been improved and developed for the massive production. With the development of an intensive culture and the deterioration of ecologic environment, shrimp aquaculture has been dramatically affected by the outbreaks of bacterial, viral diseases, parasitic diseases, and the environmental and nutrition-related diseases.

Diseases in shrimp aquaculture

In the last decade of the world shrimp farming industry, several infectious diseases have been reported to cause significant losses in production and economic values. The disease losses in shrimp aquaculture are mainly caused by viral and bacterial pathogens that account for approximately 60% and 20% in total losses, respectively [Flegel, 2012]. So far, more than twenty viral diseases have been reported to affect shrimps and prawns. For penaeid shrimps, five pathogens including Taura syndrome virus (TSV), white spot syndrome virus (WSSV), yellow head virus (YHV) genotype 1, infectious myonecrosis virus (IMNV), and infectious hypodermal and hematopoietic necrosis virus (IHHNV), are the causative agents of major viral

diseases in aquaculture [Walker and Winton, 2010]. These five pathogens are currently listed by the World Organisation for Animal Health (OIE) [OIE, 2018] (Table 2).

On the other hands, *Vibrio* spp. have been reported that they are the most numerous bacterial agents of penaeid shrimp [Lightner, 1983; Flegel, 2012]. *Vibrio* spp. are the Gram-negative bacteria and ubiquitous in the marine environments, estuaries, and freshwater throughout the world. Also, they exist in the normal microflora of cultured and wild penaeid shrimp. *Vibrio* species that have been associated with shrimp diseases i.e. *V. alginolyticus*, *V. anguillarum*, *V. harveyi*, *V. nereis*, *V. penaeicida*, *V. parahaemolyticus*, *V. splendidus*, *V. vulnificus*, *V. fluvialis*, *V. damsela*, *V.* orientalis, *V. mimicus*, etc. [Song *et al*., 1993; Mohney *et al*., 1994; Lavilla-Pitogo, 1995; Genmoto *et al*., 1996; de la Pena *et al*., 2001; Aguirre-Guzman *et al*., 2005; Jayasree, 2006; Noriega-Orozco *et al*., 2007; Felix *et al*., 2011; Shruti, 2012].

Recently, the new disease threats of cultivated shrimps for both *L. vannamei* and *P. monodon*, have been reported, including acute hepatopancreatic necrosis disease (AHPND) which is caused by the unique isolates of *V. parahaemolyticus* [Tran *et al*., 2013], covert mortality disease (CMD) by covert mortality nodavirus (CMNV) [Zhang *et al*., 2014] and hepatopancreatic microsporidiosis (HPM) by a microsporidian parasite (*Enterocytozoon hepatopenaei*) [Tourtip, 2005; Tangprasittipap *et al*., 2013].

Network of Aquaculture Centres in Asia-Pacific (NACA) (2018) has reported the diseases prevalence in the Asia-Pacific region in April-June 2018. The reported diseases of shrimps/prawns are listed in Table 2.

Table 2. List of shrimps/prawns diseases in the Asia-Pacific region reported by NACA (2018).

OIE-listed diseases	Non OIE-listed diseases
1. Infection with Taura syndrome virus (TSV)	1. Hepatopancreatic microsporidiosis caused by *Enterocytozoon hepatopenaei* (HPM-EHP)
2. Infection with white spot syndrome virus (WSSV)	2. Viral covert mortality disease (VCMD) of shrimps
3. Infection with yellow head virus (YHV) genotype 1	3. *Spiroplasma eriocheiris* infection
4. Infection with infectious hypodermal and haematopoietic necrosis virus (IHHNV)	
5. Infection with infectious myonecrosis virus (IMNV)	
6. Infection with *Macrobrachium rosenbergii* nodavirus (white tail disease)	
7. Infection with *Hepatobacter penaei* (Necrotising hepatopancreatitis)	
8. Acute hepatopancreatic necrosis disease (AHPND)	

Acute hepatopancreatic necrosis disease (AHPND)

Acute hepatopancreatic necrosis disease, AHPND or widely known as early mortality syndrome (EMS), is an infection disease in shrimp farming. AHPND typically has affected to juvenile shrimp by causing mass mortality (up to 100%) within 30-35 days post stocking in the grow-out pond. The unique strains of *V. parahaemolyticus* that carrying plasmid harboring the virulent gene was firstly identified as the causative agent of AHPND. The disase has spread to several countries in Asia and Latin America. In 2009, the AHPND outbreak was firstly reported in shrimp farming in China, then in Viet Nam (2010), Malaysia (2011), Thailand (2012), Mexico (2013) and the Philippines (2014) [Lightner *et al*., 2012; Leaño and Mohan, 2012; Flegel, 2012; FAO, 2013; Gomez-Gil *et al*., 2014; Nunan *et al*., 2014; Dabu *et al*., 2017]. In 2013, shrimp production of the large producer that affected by AHPND decreased more than 50% compare with 2012, which caused economic losses about 1 billion USD [FAO, 2013; De Schryver *et al*., 2014]. Recently, a disease outbreak has been reported in shrimp farms in

Bangladesh which the isolated *V. parahaemolyticus* from the outbreak farms showed AHPND-positive strains [Eshik *et al*., 2018]. Also, the presence of AHPND has been reported in Texas, USA [Dhar *et al*., 2018].

The clinical symptoms consist of lethargy, low speed in growth rate, spiral swimming and empty gut. Infected shrimp present the abnormalities of hepatopancreas. Typical gross signs of AHPND such as pale or white coloration of hepatopancreas tissue, emaciated and may not squash easily by finger [Lightner *et al*., 2013]. Histopathological analysis of the hepatopancreas of shrimp infected with AHPND showed the sloughing of hepatopancreas tubule epithelial cells and hepatopancreas tubules surrounded by hemocytic infiltration.

The unique virulent strains of *Vibrio parahaemolyticus* harboring a ~70-kbp plasmid that contains genes encode homologous of the *Photorhabdus* insect-related toxins (PirAB), have been characterized as the causative agent of AHPND [Kondo *et al*., 2014; Yang *et al*., 2014; Gomez-Gil *et al*., 2014; Devadas *et al*., 2018]. Moreover, the pathogenic *V. parahaemolyticus* associated with AHPND also was isolated from shrimp in South America and the draft genome sequence showed the presence of plasmid containing Vp_PirAB-like toxin gene [Restrepo *et al*., 2016]. *V. parahaemolyticus* is a Gram-negative bacterium that is generally found in marine environments and seafood. *V. parahaemolyticus* AHPND-causing strains unlike the human pathogenic strains, lack thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) and type III secretion system [Letchumanan *et al*., 2014]. To distinct the AHPND-causing strain of *V. parahaemolyticus* from another, a part of Vp_PirAB-like toxin gene was used to design the specific primer sets for detection by PCR [Tinwongger *et al*., 2014; Dangtip *et al*., 2015; Sirikharin *et al*., 2015].

The other *Vibrio* species causing AHPND also have been discovered, such as *V. harveyi* [Kondo *et al*., 2015], *V. owensii* [Liu *et al*., 2015], *V. campbellii* [Dong *et al*., 2017] and *V. punensis* [Restrepo *et al*., 2018] by all these strains contain the plasmid encoding the Vp_PirAB-like toxin. In addition, the Vp_PirAB-like toxin gene have been detected in the

bacterial strains which were isolated from diseased shrimp before AHPND outbreak occurred in China (2009) that including, *V. campbellii* isolated in Thailand before 2005 [Wangman *et al*., 2018] and the Gram-positive bacterium *Micrococcus luteus* isolated in Mexico since 2006 [Durán-Avelar *et al*., 2018].

OIE (2018) has reported two shrimp species of whiteleg shrimp (*L. vannamei*) and black tiger shrimp (*P. monodon*) as the susceptible host species. Whereas, the fleshy prawn (*P. chinensis*) and kuruma shrimp (*Marsupenaeus japonicus*) were classified in the host species with incomplete evidence for susceptibility such as lacking the histological analysis to confirm the AHPND signs in hepatopancreas tissue. *V. parahaemolyticus* AHPND-causing strain did not exhibit an enterotoxicity in rabbit ileal loops [Tinwongger *et al*., 2016]. The artificial challenge tests of *V. parahemolyticus* AHPND-causing strains to the ridgetail white prawn (*Exopalaemon carinicauda*) [Ge *et al*., 2017] and the Catarina scallop (*Argopecten ventricosus*) [Mendoza-Maldonado *et al*., 2018] have shown the susceptibility and signs of AHPND in the hepatopancreas by histopathology. These suggest that the Vp_PirAB-like toxin has wide range of host susceptibility in crustacean species but not in vertebrate, including human. On the other hand, Tran *et al*. (2013) reported that the frozen AHPND-infected shrimp could not transmit the AHPND virulence to susceptible shrimp.

Khimmakthong and Sukkarun (2017) have demonstrated the spreading of *V. parahaemolyticus* AHPND-causing strain in tissues of *L. vannamei* shrimp, which was mostly spread at 6 h after exposure in the gills, hepatopancreas, intestine, muscle, and hemolymph. After that small amounts of bacteria was detected in the hepatopancreas and intestine. However, histopathological sections of hepatopancreas tissue showed abnormalities at 1 min-72 h after exposure, suggesting that although amount of *V. parahaemolyticus* reduced somehow, their toxins still caused damage to shrimp.

Vp_PirAB-like toxin

Bacterial toxins are identified as potential virulence factors of numerous pathogenic bacteria that cause damage to susceptible host cells and cells death finally [Wu *et al*., 2008]. Bacteria produce two types of toxins which include exotoxins and endotoxins [Todar, 2006]. Endotoxins are the integral part of the cell walls of Gram-negative bacteria, lipopolysaccharides (LPS) which are released on bacterial death and not protein. Exotoxins are toxin proteins that are secreted by living cells of Gram-positive and Gram-negative bacteria or released during lysis of the cell. Bacterial toxin proteins might be encoded by bacterial chromosomal genes, plasmids or phages.

Vp_PirAB-like toxin gene was firstly found in the plasmid DNA of pathogenic *V. parahaemolyticus* causing AHPND [Kondo *et al*., 2014; Gomez *et al*., 2014; Yang *et al*., 2014]. Because this virulent gene is homologous to the *Photorhabdus* insect-related proteins (PirAB), then it was named as Vp_PirAB-like toxin or others also called $PirAB^{Vp}$. The binary PirAB toxin is produced by *Photorhabdus luminescens* (a Gram-negative proteobacterium) and exhibits the cytotoxicity and insecticidal activity [Li *et al*., 2014].

Vp_PirAB-like compose of two genes, Vp_PirA-like (336 bp) and Vp_PirB-like (1,317 bp). Both genes are co-transcribed to be one messenger RNA as polycistronic mRNA and translated to two proteins of Vp_PirA-like and Vp_PirB-like (Figure 1). Vp_PirA-like gene is translated to 111 amino acid residues and Vp_PirB-like is translated to 438 amino acid residues with the predicted protein size of 13 kDa and 50 kDa, respectively. The protein expression of Vp_PirA- and B-like proteins were detected in the bacterial cells of pathogenic strains, and a part were secreted into an extracellular environment, which could detect in the bacterial culture medium [Lee *et al*., 2015; Tinwongger *et al*., 2016], indicating that the Vp_PirAB-like toxin is the bacterial exotoxin.

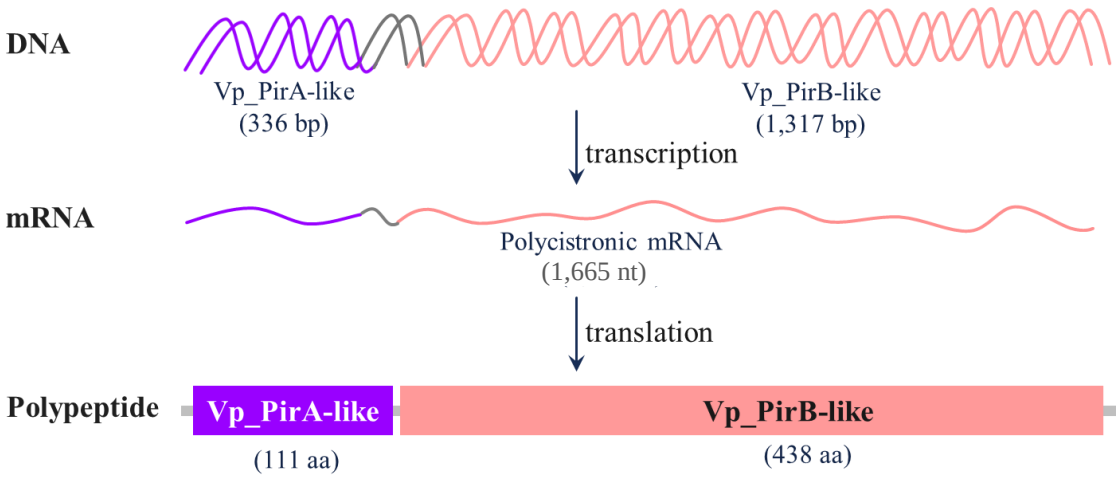

Figure 1. Schematic represents the transcription and translation of Vp_PirAB-like toxin.

Several studies have suggested that the expression of both Vp_PirA-like and Vp_PirB-like toxins are necessary to cause AHPND in susceptible shrimp. The deletion mutant of a part of Vp_PirAB-like gene [Tinwongger *et al*., 2016 ; Kanrar and Dhar, 2018] or an entire Vp_PirAB-like gene [Han *et al*., 2017] caused the loss of AHPND virulence. The co-administration of recombinant Vp_PirA- and B-like toxins by reverse gavage to *L. vannamei* caused high mortality and massive sloughing of hepatopancreatic tubule epithelial cells, characteristic of AHPND, whereas Vp_PirB-like alone induced some sloughing and caused low mortality [Lee *et al*., 2015; Sirikharin *et al*., 2015]. The requirement of both toxins to cause AHPND is supported with orally challenged with diet soaked with both recombinant toxins, while each toxin alone did not cause mortality or present AHPND signs in the hepatopancreas cells [Lee *et al*., 2015]. In addition, the reverse gavage or injection shrimp with secreted Vp_PirAB-like toxin which was partially purified from the culture supernatant of *V. parahaemolyticus* AHPND-causing strains, showed mass mortality and AHPND signs [Sirikharin *et al*., 2015; Boonchuen *et al*., 2018]. Recently, Hao *et al*. (2019) have examined the host range of Vp_PirAB-like toxin to shrimp (*L. vannamei*), crab (*Eriocheir sinensis*),

European seabass (*Dicentrarchus labrax*), bloodworm (*Nereis succinea*) and mosquito (*Aedes albopictus*) and results demonstrated that only *L. vannamei* showed high mortality, whereas, less than 3% mortality was observed in mosquito and no mortalities were observed in other three species. This result indicates that the Vp_PirAB-like toxin may be a host-specific, especially in shrimp/prawn.

Apart from the virulence of Vp_PirAB-like toxin, some researcher groups have used the recombinant protein of Vp_PirA-like toxin as immunostimulant. The intraperitoneal injection of Vp_PirA-like toxin could enhance the humoral immune response and some immune genes in Pacific red snapper *Lutjanus peru* [Reyes-Becerril *et al*., 2016] and the bath-immersion of Vp_PirA-like to juvenile shrimp caused lower mortality after immersion with *V. parahaemolyticus* AHPND-causing strain, compared with control [Campa-Córdova *et al*., 2017]

Previous study has reported the crystal structures of Vp_PirA- and B-like which showed high similarity to the Cry protein, one of toxins produced by *Bacillus thuringiensis* [Lee *et al*., 2015]. The active Cry toxins have three functional domains, composing of the pore-forming domain I, the receptor-binding domain II and the sugar-binding domain III [Pardo-Lopez *et al*., 2013; Adang *et al*., 2014]. The structure of Vp_PirA-like is corresponding to Cry toxin domain III which is related in receptor recognition, whereas the N-terminal and C-terminal of Vp_PirB-like are corresponding to Cry toxin domain I and domain II, respectively [Lee *et al*., 2015]. Cry toxins are insecticidal proteins which widely used as biological control the insect pests in agriculture and they have been classified in at least 70 different Cry toxin groups, according to their amino acid sequences [Bravo *et al*., 2013].

Until now, the mode of action of the Vp_PirAB-like toxin has not been elucidated. However, the similarity in structure between Vp_PirAB-like toxin and Cry toxins provides strong evidence that they may use similar mechanisms to invade into host cells and cause cell death.

Shrimp's hepatopancreas

The hepatopancreas or midgut gland is one of organs in the digestive tract of crustaceans. Generally, the digestive of decapod crustaceans is divided into 3 parts, foregut, midgut, and hindgut. The hepatopancreas is placed in the midgut and normally accounts for about 2-6% of total body weight. It is considered as a major organ in decapods for metabolic functions e.g. synthesis and secretion of digestive enzymes, production of mineral reverses and organic substances, absorption of digested dietary products, role in distribution of stored reserves during the intermoult period [Ceccaldi, 1989]. Additionally, hepatopancreas tissue has combined many of the functions of liver, pancreas, and intestine in vertebrates, although they are not homologs [van Weel, 1974].

Caceci *et al*. (1988) have reported the histology and ultrastructure of the hepatopancreas of *L. vannamei* which are similar that seen in other decapods. Shrimp's hepatopancreas compose of blind tubules containing primarily four types of cells: E- (embryonic), F- (fibrillar), B- (blister-like), and R- (resorptive/absorption) cells, which play individual roles in digestion. An example of histopathological section of shrimp's hepatopancreas is shown in Figure 2.

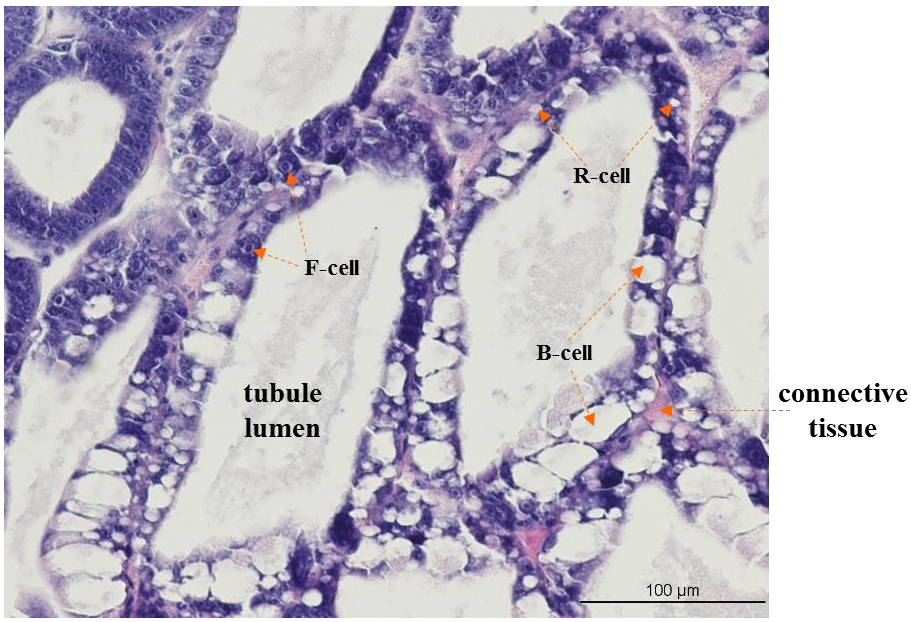

Figure 2. Histopathological section of hepatopancreas tissue from an apparently healthy *L. vannamei* (H&E staining).

Previous studies have revealed the importance of hepatopancreas to health, growth, and survival of the shrimp in culturing. Vogt *et al*. (1985) have advocated use of the hepatopancreas R-cells to monitor the nutrition value of different diets in *P. monodon*. Also, histological changes of the hepatopancreas could be observed under various conditions such as different salinities [Li *et al*., 2008], contamination of aflatoxin B_1 [Boonyaratpalin *et al*., 2001], the presence of petroleum compounds [Sreerum and Menon, 2005; Abdelmeguid *et al*., 2009], and a ubiquitous metal cadmium [Boudet *et al*., 2015]. These indicate that the hepatopancreas can be used as an indicator organ for nutrition quality, stress, and toxicity assessment in shrimp. Moreover, the hepatopancreas has function involving in the reproductive system in shrimp. It is the site of extraovarian vitellogenin production, then the synthesized vitellogenin is transported to the ovary via the hemolymph [Tiu *et al*., 2008].

Shrimp's hepatopancreas is the target organ of AHPND, which show damages in the hepatopancreas cells after infection. Also, other shrimp diseases have caused the histological changes in the hepatopancreas e.g. baculoviruses that infecting the epithelium cells of the hepatopancreas [Lightner, 1985], hepatopancreatic parvovirus (HPV) [Lightner, 1993], hepatopancreatic microsporidiosis (HPM) [Tourtip *et al*., 2009], and pathogenic *V. harveyi* [Soonthornchai *et al*., 2010]. These results suggest that hepatopancreas tissue is a target organ of several infectious diseases caused by bacteria, virus, and parasite.

Nevertheless, the hepatopancreas plays roles in the innate immunity for defense against various pathogens in invertebrates. Alday-Sanz *et al*. (2002) reported that in *P. monodon*, hepatopancreas cells were capable to engulf the cell debris and soluble bacterial cells of *V. vulnificus* from the gut lumen, then the bacterial antigens were released into hemolymph. In penaeid shrimp, expression of immune-related gene mRNAs were up-regulated in the hepatopancreas against some pathogen-associated molecular patterns (laminarin, LPS and poly I:C), viral infections, and bacterial infections [Pan *et al*., 2005; Ji *et al*., 2009; Wang *et al*., 2009; Chai *et al*., 2010; Zeng *et al*., 2013]. A hepatopancreas-specific C-type lection (one of

shrimp's pattern recognition receptors) has been reported in *P. monodon* and *Fenneropenaeus chinensis* [Ma *et al*., 2008; Sun *et al*., 2008].

The above-mentioned studies indicate the importance of hepatopancreas tissue in shrimp survivability, either normal condition or abnormal conditions (e.g. pollution, stress, and infection).

Shrimp immune defense

Because shrimps are the commercially important animals, understanding of their immune defenses are greatly needed to reduce the economic losses from disease outbreaks in aquaculture. It is well known that invertebrates rely entirely on innate immune system as the primary mechanism of defense against parasites and pathogens. In contrast, vertebrates use both innate immunity and adaptive immunity. The adaptive immune system are highly specific to particular pathogen that induced them to create the immunological memory after an initial response, which can prolong the protection against same pathogen [Alberts *et al*., 2002]. Innate immune system in penaeid shrimps consists of humoral and cellular responses to protect themselves from disease agents that invading into internal tissues. Humoral responses include antimicrobial peptides (AMPs) that are largely synthesized in the hemocytes, melanization by prophenoloxidase (proPO) system, and other signaling pathways. The cellular responses include apoptosis, phagocytosis, nodule formation and encapsulation [Tassanakajon *et al*, 2018].

On the other hand, many studies have demonstrated that the enhancement of immune responses is capable to improve disease resistance. Orally administration of algae extracts could induce shrimp immune activities which have effect on vibriosis resistance [Hou and Chen, 2005; Huang *et al*., 2006] and WSSV resistance [Supamattaya *et al*., 2005]. Also, treatment shrimp with probiotics *Bacillus* spp., increased immune responses and disease resistance [Li *et al*., 2009; Tseng *et al*., 2009; Zokaeifar *et al*., 2012]. These indicate that the

immune stimulation in shrimps is an advantage to control disease outbreaks in shrimp aquaculture, instead of using chemical agents and antibiotics.

Objectives and outline of the thesis

In my previous study, feeding the commercial diet supplemented with formalin-killed cells (FKC) of *V. parahaemolticus* AHPND-causing strain (FKC-VpD6) to *L. vannamei* showed massive mortality up to 100% in a week, but no mortality was observed in feeding FKC of *V. parahaemolyticus* non AHPND-causing strain [Tinwongger *et al*., 2016]. This suggests that the Vp_PirAB-like toxin is formalin-resistant and heat stabile because the supplemented feed was dried with high temperature incubation (60 °C) . Therefore, the FKC-VpD6 diet has been used for selection of shrimp resistant to Vp_PirAB-like toxin. After daily feeding for 2 weeks, only 2% of total shrimp survived. It is interesting to investigate "How these shrimp can survive?" and "What molecules are involved in this resistant mechanism?".

Chapter 1: The importance of shrimp aquaculture and the current shrimp diseases that affect the global shrimp production are introduced. Especially, bacterial disease named acute hepatopancreatic necrosis disease (AHPND) which is newly emerging disease that caused mass mortalities and large economic losses in shrimp farming. The functions of shrimp hepatopancreas tissue are discussed to provide its importance for survivability. Also, the shrimp immunity in relevance to disease resistances are mentioned briefly.

Chapter 2: Identification of novel genes that involving in the Vp_PirAB-like toxin resistance mechanism by comparison of RNA sequencing among three groups, toxin resistance, 24h-infected AHPND, and non-treated shrimp to identify the differentially expressed genes and toxin-receptor candidates.

Chapter 3: Investigation of the relation between resistant shrimp and *L. vannamei* anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R) gene, which was highly expressed in

the hepatopancreas of resistant shrimp in the previous chapter, including *in vitro* and *in vivo* functional assays of *Lv*ALF AV-R.

Chapter 4: Examination of the possibility of toxin resistance caused by the mutation of toxin receptor candidates and its relation to the resistance phenotype.

And lastly, Chapter 5: Summary of the results from the three main studies that identifying the genes associated with resistance to Vp_PirAB-like toxin in *L. vannamei*, functional insights of *Lv*ALF AV-R related to Vp_PirAB-like toxin resistant mechanism, possibility of genetic variation involving in resistance, and conclusions of how *Lv*ALFAV-R may be used in shrimp protection against Vp_PirAB-like toxin and association of genotypic variation in cadherin-like gene to the resistance phenotype.

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Chapter 2

Transcriptome analysis of *Litopenaeus vannamei* resistant to Vp_PirAB-like toxin

Abstract

Vp_PirAB-like toxin is the virulent factor of acute hepatopancreatic necrosis disease (AHPND), which is an emerging disease in shrimp farms. Vp_PirA- and B-like toxin genes are coded in plasmid DNA of the specific pathogenic strains of *Vibrio parahaemolyticus* and other *Vibrio* species that caused AHPND. The structures of Vp_PirA- and B-like proteins are similar to *Bacillus thuringiensis* Cry toxins, insecticidal protein. Vp_PirAB-like toxin is heat-stabile and formalin-resistant. Therefore, the formalin-killed cells of *V. parahaemolyticus* AHPND-causing strain D6 (FKC-VpD6) was used to select the Vp_PirAB-like toxin-resistant *Litopenaeus vannamei* by oral administration. Stomach and hepatopancreas tissues of shrimps that survived for two weeks (sur-FKC) were subjected to RNA sequencing. The differentially expressed genes (DEGs) between sur-FKC, AHPND-infected shrimp, and normal shrimp were identified and separated by tissue. In the stomach, 194 DEGs were identified and in the hepatopancreas, 224 DEGs were identified. The expressions of DEGs were validated by qPCR of ten genes. Only one gene, DN21485 (a gene homologous to *L. vannamei* anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R)) was expressed more strongly in the hepatopancreas of sur-FKC than in the other groups. Moreover, analysis of Cry toxin receptor homolog was performed to identify the gene candidate of Vp_PirAB-like toxin receptor. Cry toxin receptors including cadherin, alkaline phosphatase, aminopeptidase N, and ATP-binding cassette transporter subfamily C were used as query genes to blast against *L. vannamei*

control/Vp-inf transcriptome. A total of 13 Vp_PirAB-like toxin-receptor candidates was identified.

[A part of Chapter 2 has been published under title "Identification of an anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R) related to Vp_PirAB-like toxin resistance in *Litopenaeus vannamei*" in *Fish & Shellfish Immunology*, Vol. 84, p 178-188, 2019.]

1. Introduction

Whiteleg shrimp, *Litopenaeus vannamei* is one of the most economically important shrimp species that provide the highest production. *L. vannamei* accounts for about 75% of global farmed shrimp production [FAO, 2018]. In the last decade, a new emerging disease named acute hepatopancreatic necrosis disease (AHPND) has occurred in Asia and Latin America, resulting in a great reduction of shrimp production [FAO, 2013].

The causative agent of AHPND is the unique strains of *Vibrio parahaemolyticus* harboring plasmid that carrying the virulent gene named Vp_PirAB-like [Kondo *et al*., 2014; GomezGil *et al*., 2014; Yang *et al*., 2014]. Vp_PirAB-like genes encoding toxin proteins (Vp_PirA-like and Vp_PirB-like), which were later characterized as the virulence factor of AHPND [Lee *et al*., 2015; Tinwongger *et al*., 2016]. Recently, the plasmid encoding Vp_PirAB-like toxin was found in other *Vibrio* species causing AHPND such as *V. harveyi*, *V. owensii*, and *V. campbellii* [Kondo *et al*., 2015; Xiao *et al*., 2017; Dong *et al*., 2017]. Interestingly, Durán-Avelar *et al*. (2018) reported that this Vp_PirAB-like toxin gene was also detected in the plasmid DNA of Gram-positive bacteria *Micrococcus luteus*, which was isolated from the hepatopancreas of *L. vannamei* in Mexico since 2006, before AHPND outbreak occurred (2012). Also, Wangman *et al.* (2018) found Vp_PirAB-like toxin plasmid in pathogenic *V. campbellii* isolated from diseased shrimp before 2005 in Thailand. Hence, the further studies for controlling of AHPND-infection should focus on the patholigical mechanisms of Vp_PirAB-like toxin, not bacterial species.

My previous study revealed that the virulence of Vp_PirAB-like toxin unlike the virulence of normal bacterial exotoxins, remained in non-living bacterial cells after the cells were treated with formalin and heated at 60 °C for 2 h [Tinwongger *et al*., 2016]. The artificial challenges by immersion with mutant strains of *V. parahaemolyticus* showed that both Vp_PirA- and B-like toxins are necessary to cause AHPND in shrimp [Lee *et al*., 2015; Tinwongger *et al*., 2016]. Although the reverse gavage of recombinant Vp_PirB-like toxin

alone to *L. vannamei* showed some mortality [Sirikharin *et al*., 2015], it is unnatural route of infection. The binary toxin Vp_PirAB-like caused damage in shrimp hepatopancreas and signs of AHPND in histopathological analysis, including hemocytic infiltration and sloughing of tubule epithelial cells [Tran *et al*., 2013].

In addition, previous study reported that the structural topology of Vp_PirA- and B-like toxins showed high similarity to that of *Bacillus thuringiensis* delta-endotoxin or Cry insecticidal toxins, although their sequences showed low identical (less than 10%) [Lee *et al*., 2015]. The Vp_PirA-like corresponding to Cry domain III (function: receptor recognition), whereas N-terminal of Vp_PirB-like corresponding to Cry domain I (function: pore-forming) and the C-terminal corresponding to Cry domain II (function: receptor binding) [Lin *et al*., 2017]. It raises the high possibility that Vp_PirAB-like toxins may use similar mechanisms of Cry toxins, which need to bind specific membrane proteins for entering host cells and cause cells death [Gómez *et al*., 2007].

Since penaeid shrimps are lacking an adaptive immunity, they use humoral and cellular innate immune responses to fight against invading microorganisms [Tassanakajon *et al*., 2013]. Junprung *et al*. (2017) reported the non-lethal heat shock could induce *L. vannamei* tolerance to AHPND infection, which may be mediated by the induction of heat shock proteins (HSPs) and the upregulation of other immune-related genes. Shrimp fed diet supplemented with red seaweed extract (*Gracilaria fisheri*) which exhibited agglutination and growth inhibition to *V. parahaemolyticus* AHPND-causing strain, also showed improved survival rate after immersion test [Boonsri *et al*., 2017].

Transcriptome analysis by RNA-Seq is a powerful tool in order to identify new genes, single nucleotide polymorphisms (SNP) marker, and analysis of differentially expressed genes (DEGs) etc. This technique has been widely used to understand mechanisms of pathogens and the immune responses in fish and shrimp [Santos *et al*., 2014; Sudhagar *et al*., 2018]. Example

in *L. vannamei*, genes associated with resistance to Taura syndrome virus (TSV) have been identified by RNA-seq [Sookruksawong *et al*., 2013].

Selection of Vp_PirAB-like toxin resistance in shrimp is an interesting strategy to develop the breeding program and new biological treatments instead of antibiotic usage. In this study, *L. vannamei* were fed the commercial diet supplemented with formalin-killed cells of *V. parahaemolyticus* AHPND-causing strain for selection of toxin-resistant shrimp. To identify genes involved in conferring toxin resistance, the hepatopancreas and stomach mRNAs of survival shrimp were sequenced with a next generation sequencer. The resistant-transcriptome was analyzed together with the transcriptomes of non-treated shrimp and AHPND-infected shrimp.

2. Materials and Methods

2.1 Experimental shrimp and Vp_PirB-like toxin resistant shrimp selection

Juvenile *L. vannamei* shrimp were purchased from IMT engineering Inc. (Niigata Prefecture, Japan) and stocked in a 500-L tank with aerated, recirculating artificial seawater (25 ppt) at 28 °C.

Two hundred healthy shrimp (approximately 1-2 g body weight) were reared in a 100-L plastic tank with aerated, recirculating artificial seawater (28 ppt) at 28 °C. Shrimp were fed commercial pellets containing 5% (w/w) of formalin-killed cells (FKCs) of *V. parahaemolyticus* AHPND-causing strain D6 (FKC-VpD6) [Kondo *et al.*, 2014]. The FKC diet was prepared as previously reported [Tinwongger *et al.*, 2016]. Shrimp were fed twice per day (a total of 5% of body weight) and checked daily to remove dead shrimp from the tank. After 2 weeks of feeding, all surviving shrimp were collected, and their hepatopancreas and stomach were dissected.

AHPND-infected shrimp were prepared using five *L. vannamei* (approximately 4-6 g body weight) immersed with *V. parahaemolyticus* AHPND-causing strain FP11_PirAB-like [Tinwongger *et al.*, 2016] at a final concentration of $1x10^6$ CFU/ml. Three shrimp were collected at 24 h after immersion, and their hepatopancreas and stomach were dissected. The hepatopancreas and stomach were also collected from three non-treated shrimp which were apparently healthy (approximately 4-6 g body weight), as a control group. All the tissue samples were temporarily stored at -80 °C for total RNA isolation.

2.2 RNA-Seq library preparation

Total RNA from the hepatopancreas and stomach of the three groups was isolated using RNAiso reagent (Takara, Japan), treated with RQ1 RNase-free DNase (Promega, USA) and purified with RNeasy Mini Kit (Qiagen, Germany). The concentration was measured with a Qubit RNA HS Assay Kit (Life Technologies, Thermo Fisher Scientific Inc., USA). The total

RNA of each tissue was pooled by group. The purity and integrity of total RNAs were assessed using a 2100 Bioanalyzer (Agilent Technologies, Inc.). Four μg of pooled RNA was used to construct a cDNA library by following the protocols of Illumina Truseq Stranded mRNA Library Prep Kit (Illumina, USA). Six cDNA libraries from the pooled RNA samples obtained from the hepatopancreas and stomach of surviving shrimp (sur-FKC), AHPND-infected shrimp (Vp-inf) and non-treated shrimp (control) were sequenced with Illumina Miseq sequencer (USA).

2.3 *De novo* assembly of RNA-Seq

The raw reads of RNA-Seq were cleaned up by trimming and removing the adapter. The cleaned reads were combined across all samples and subjected to *de novo* assembly using Trinity software to obtain a single RNA-Seq data set [Grabherr *et al.*, 2011]. The RNA-Seq files have been submitted to the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under GEO Series accessions number GSE104715.

2.4 Differential expression analysis and gene ontology

The original RNA-Seq reads of each sample were aligned by bowtie. Transcript abundance relative to Trinity-assembled transcripts was estimated by RSEM software. To identify differentially expressed genes, all samples were pairwise compared by edgeR. Between two groups comparison, genes that were differentially expressed at *P*-values < 1e-5 and expression level greater than 4-fold were selected. The differentially expressed genes at FDR (false discovery rate) value of less than 1e-5 were considered as the significantly differentially expressed genes (DEGs). The DEGs were analyzed using BLAST2GO software [Conesa *et al.*, 2005]. The sequences were aligned to NCBI non-redundant protein database with default parameters by BLASTX (E-value cutoff < 1e-5). Annotated DEGs that were identified as genes from microorganisms and vertebrates were discarded. Gene ontology was

annotated by BLAST2GO with default parameters in BLAST (E value cutoff 1e-3). The genes were grouped as described previously [Tassanakajon *et al*., 2013].

2.5 Validation of differentially expressed genes by quantitative PCR (qPCR)

One μg of total RNA from individual shrimp (the same sample used for RNA-Seq) was used to synthesize cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). To validate the results of DEGs, 10 genes were selected to perform quantitative PCR by StepOnePlus (Applied Biosystems, USA) using Thunderbird™ SYBR® qPCR Mix. The primers were designed using Primer Express 3.0.1 (Applied Biosystems, USA) and are listed in Table 1. The qPCR conditions were 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s and additional of dissociation curve analysis.

The gene expression level of selected genes was analyzed using the comparative CT method (ΔΔCT) [Schmittgen and Livak, 2008]. The elongation factor-1 alpha (EF-1α) was used as a reference gene for relative quantifications.

2.6 Prediction of Cry toxin receptor homologs

RNA sequence data were reassembled by RNA-Seq of control and Vp-inf. groups were co-assembled to create control/Vp-inf transcriptome. The putative Cry toxin receptor proteins which have previously been identified in insect were selected from NCBI database. The selected receptors were three cadherin genes, two alkaline phosphatase (ALP) genes, two aminopeptidase N (APN) genes, and two ATP-binding cassette transporter subfamily C (ABCC) genes (all 9 selected genes are listed in Table 2). The amino acid sequences of these identified receptors were searched against transcriptome of control/Vp-inf. (supposed to be normal shrimp) by BLAST+ program with TBLASTN mode. The top hit genes of each receptor were selected as Cry toxin receptor homologs. The nucleotide sequences of all Cry toxin

receptor homologs were predicted the coding sequence by using open reading frame finder (https://www.ncbi.nlm.nih.gov/orffinder/).

Table 1. List of primers used in this study.

Target gene		**Sequences (5′-3′)**	**Length (bp)**
DN3154	Fw	AGGTGGATACGGCGGAAAC	19
	Rv	CGAAGGATCGCCTGTACGA	19
DN6348	Fw	GGACCTTCACCTTACCGATTTG	22
	Rv	GAAGAAGCCCAGGAGGAGGAT	21
DN13245	Fw	GGGCACGCTCCTCTCCAT	18
	Rv	TTTACCCCGCGTGACTTCTT	20
DN13839	Fw	ACGGCAACTTCAACTACGACTTC	23
	Rv	GAGCGCCGACAGCTGAGA	18
DN13910	Fw	GGAGCTGTTAGGACACTACTGCAA	24
	Rv	ACATGCGACCCCTGAAATACA	21
DN18811	Fw	CCGGCAGGAAGGCAGAA	17
	Rv	GGGCGTGGACGTATCCTATG	20
DN20905	Fw	GCTGCAGGCCCTCTTCTTCT	20
	Rv	TTGCCTCCTTCCCACATCTT	20
DN21485	Fw	TGGCGGTGTTCCTGGTGG	18
	Rv	GTTCCTCCACAGCCCAACG	19
DN21632	Fw	GCGACGACCTTCCCTTCA	18
	Rv	AGACATAGCGCTGGCACACA	20
DN22624	Fw	CGGAGACAAGAGCAACTTCATATG	24
	Rv	GGGCTGGGTCTCGCTCAT	18
EF-1α	Fw	TGGCTACTCACCTGTGCTTG	20
	Rv	CCAGCTCCTTACCAGTACGC	20

Table 2. List of identified Cry toxin receptor proteins in several insect species.

Name	Species	Accession number	Reference
Cadherin	1. *Bombyx mori*	BAA99404.1	Ikawa *et al*., 2000
	2. *Manduca sexta*	AAG37912.1	Dorch *et al*., 2002
	3. *Tenebrio molitor*	ABL86001.2	Fabrick *et al*., 2009
Alkaline phosphatase (ALP)	1. *B. mori*	BAA34926.1	Itoh *et al*., 1999
	2. *Heliothis virescens*	ACP39712.1	Perera *et al*., 2009
Aminopeptidase N (APN)	1. *M. sexta*	Q11001.1	Knight *et al*., 1995
	2. *Aedes aegypti*	AAK55416.1	unpublished
ATP-binding cassette transporter subfamily C (ABCC)	1. *B. mori*	BAK82126.1	Atsumi *et al*., 2012
	2. *H. virescens*	ADH16740.1	unpublished

3. Results

3.1 Illumina sequencing and *de novo* assembly

Of a total of 200 *L. vannamei*, only four survived after feeding with formalin-killed cells of *V. parahaemolyticus* AHPND causing-strain D6 (FKC-VpD6) for 2 weeks. Raw reads were obtained from the stomach and hepatopancreas: 21,485,676 from the control group, 24,930,246 from the Vp-inf group, and 18,507,162 from the sur-FKC group. The assembled transcriptome generated 94,135 transcripts with an average size of 905 bp, representing 79,591 genes (Table 3).

3.2 Identification of DEGs in the hepatopancreas and stomach

The DEGs were identified based on combining all reads across 6 samples. The differential expression levels are shown in the heatmap (Figure 1). In the stomach, gene expression of control group showed more similarities to sur-FKC group, but in the hepatopancreas, control group showed more similarities to the Vp-inf group than to the sur-FKC group. The pairwise comparison of DEGs between the three groups of control, Vp-inf and sur-FKC from each tissue were analyzed and displayed with Venn diagram (Figure 2). In the hepatopancreas, a total of 17 DEGs were found between the control and Vp-inf, 137 DEGs were found between the control and sur-FKC and 165 DEGs were found between Vp-inf and sur-FKC (Figure 2A). In the stomach, a total of 110 DEGs were found between the control and Vp-inf, 48 DEGs were found in the control and sur-FKC and 92 DEGs were found in the Vp-inf and sur-FKC (Figure 2B).

The numbers of DEGs that were high-expressed or low-expressed among three groups comparisons are shown in Figure 3. In the hepatopancreas, there were 13, 25, and 24 high-expressed genes in the DEGs of Vp-inf vs control, sur-FKC vs control, and sur-FKC vs Vp-inf, respectively. In the stomach, there were 102, 43, and 48 high-expressed genes in the DEGs of Vp-inf vs control, sur-FKC vs control, and sur-FKC vs Vp-inf, respectively.

3.3 Gene annotation and gene ontology of differential expressed genes

Of the 218 annotated differentially expressed genes in the hepatopancreas, there were 161 genes of known proteins (Supplementary Table 1), 37 genes of hypothetical or uncharacterized proteins and 20 genes of unknown proteins. Of the 187 annotated differentially expressed genes in the stomach, there were 91 genes of known protein (Supplementary Table 2), 34 genes of hypothetical or uncharacterized proteins and 62 genes of unknown proteins. The immune-related genes found in the DEGs were manually grouped into six functions for the hepatopancreas (antimicrobial peptides, oxidase stress, proteinases/proteinase inhibitors, pattern recognition proteins, blood clotting system and other immune molecules) (Table 4) and four functions for the stomach (antimicrobial peptides, proteinases/proteinase inhibitors, pattern recognition and other immune molecules) (Table 5). The main gene ontology (GO) categories were catalytic activity in the hepatopancreas (Figure 4A) and structural molecular activity in the stomach (Figure 4B).

3.4 Validation of mRNA expression

Ten of the most highly differentiated genes that were involved in the immune response were selected to validate the expression level by qPCR. The expression level of 8 genes were examined in the hepatopancreas. The expression of four of these genes (DN13245, DN21485, DN20905 and DN13245) were consistent with the RNA sequence data in all comparisons (Figure 5A). Of the three genes that were examined in the stomach, only one gene (DN3154) showed similar expression to the RNA sequence data (Figure 5B).

3.5 Identification of Cry toxin receptor homologs

Total of thirteen receptor homologs were identified from the transcriptome of control/Vp-inf *L. vannamei* (Table 6). Three genes were homologous to cadherin, two genes

were homologous to ALP, five genes were homologous to APN, and three genes were homologous to ABCC2.

Table 3. Summary of reads in control, Vp-inf and sur-FKC transcriptomes.

Trinity Outputs	**control**		**Vp-inf**		**Sur-FKC**	
	St[*1]	**HP**[*2]	**St**	**HP**	**St**	**HP**
Number of raw reads	13,516,128	7,969,548	10,328,124	14,602,122	9,564,080	8,943,082
Total trinity 'genes'	79,591					
Total trinity 'transcripts'	94,135					
Percent GC	44.72					
Transcript contig N50	1,777					
Median contig length (base)	422					
Average contig (base)	905.37					
Total assembled bases	85,226,619					

([*1] St = stomach and [*2] HP = hepatopancreas)

Table 4. Differentially expressed genes of the hepatopancreas involved in immune response of sur-FKC compared to Vp-inf and control.

Gene ID	Gene homologous [species]
Antimicrobial peptides	
TRINITY_DN13910_c0_g1	anti-lipopolysaccharide factor isoform 6 [*Penaeus monodon*]
TRINITY_DN19681_c1_g1	anti-lipopolysaccharide factor [*Litopenaeus stylirostris*]
TRINITY_DN21485_c0_g1	anti-lipopolysaccharide factor AV-R isoform [*Litopenaeus vannamei*]
Oxidase stress	
TRINITY_DN21942_c1_g1	glutathione peroxidase 3 [*Penaeus monodon*]
TRINITY_DN23533_c0_g2	PREDICTED: UDP-glucuronosyltransferase-like isoform X2 [*Hyalella azteca*]
Proteinases/Proteinase inhibitors	
TRINITY_DN17668_c0_g2	metalloproteinase [*Culex quinquefasciatus*]
TRINITY_DN19077_c1_g1	cathepsin l [*Litopenaeus vannamei*]
TRINITY_DN19194_c0_g1	PREDICTED: lysosomal aspartic protease-like isoform X2 [*Octopus bimaculoides*]
TRINITY_DN19227_c0_g1	serine proteinase inhibitor-2 [*Eriocheir sinensis*]
TRINITY_DN19522_c0_g1	collagenolytic serine protease [*Fenneropenaeus chinensis*]
TRINITY_DN19537_c0_g1	N-acetylated-alpha-linked acidic dipeptidase 2 [*Crassostrea gigas*]
TRINITY_DN19991_c0_g1	hematopoietic prostaglandin D synthase [*Penaeus monodon*]
TRINITY_DN20381_c0_g1	PREDICTED: deoxynucleoside triphosphate triphosphohydrolase SAMHD1-like [*Saccoglossus kowalevskii*]
TRINITY_DN20844_c0_g1	PREDICTED: UDP-glucose 4-epimerase-like isoform X2 [*Hyalella azteca*]
TRINITY_DN21632_c3_g11	kazal-type serine protease inihibitor 3 [*Fenneropenaeus chinensis*]
TRINITY_DN21632_c3_g3	kazal-type serine protease inihibitor 2 [*Fenneropenaeus chinensis*]

Gene ID	Gene homologous [species]
TRINITY_DN21632_c3_g7	kazal-type serine protease inihibitor 4 precursor [*Fenneropenaeus chinenesis]*
TRINITY_DN21897_c2_g1	carboxypeptidase B, partial [*Litopenaeus vannamei*]
TRINITY_DN23230_c0_g1	serine proteinase inhibitor-3 [*Eriocheir sinensis*]
TRINITY_DN23273_c0_g1	PREDICTED: lysosomal alpha-mannosidase-like [*Hyalella azteca*]
TRINITY_DN24617_c0_g2	serine proteinase inhibitor [*Litopenaeus vannamei*]
TRINITY_DN2553_c0_g1	hepatopancreas kazal-type proteinase inhibitor [*Penaeus monodon*]
TRINITY_DN2664_c0_g1	serine proteinase inhibitor B3 [*Penaeus monodon*]
TRINITY_DN2976_c0_g1	clip domain serine proteinase 2 [*Penaeus monodon*]
TRINITY_DN5608_c0_g1	serine proteinase inhibitor B3 [*Litopenaeus vannamei*]
Pattern recognition proteins	
TRINITY_DN12106_c0_g1	C-type lectin [*Litopenaeus vannamei*]
TRINITY_DN12136_c0_g1	C-type lectin 2 [*Fenneropenaeus chinensis*]
TRINITY_DN13245_c0_g1	C-type lectin 1 [*Fenneropenaeus chinensis*]
TRINITY_DN14395_c0_g1	C-type lectin 4 [*Litopenaeus vannamei*]
TRINITY_DN17381_c0_g3	C-type lectin 3 [*Fenneropenaeus chinensis*]
TRINITY_DN17381_c0_g9	C-type lectin 5 [*Fenneropenaeus merguiensis*]
TRINITY_DN18924_c0_g1	beta-1,3-glucan binding protein [*Litopenaeus vannamei*]
TRINITY_DN19588_c0_g2	C-type lectin [*Procambarus clarkii*]
TRINITY_DN20189_c0_g1	beta-1,3-glucan-binding protein precursor [*Astacus astacus*]
TRINITY_DN20254_c5_g1	C-type lectin domain-containing protein [*Fenneropenaeus chinensis*]
TRINITY_DN20837_c0_g1	C-type lectin [*Eriocheir sinensis*]
TRINITY_DN21575_c1_g12	C-type lectin [*Litopenaeus vannamei*]
TRINITY_DN21893_c0_g2	C-type lectin 3 [*Fenneropenaeus merguiensis*]
TRINITY_DN22624_c0_g2	C-type lectin 5 [*Fenneropenaeus chinensis*]
TRINITY_DN23093_c1_g1	C-type lectin receptor [*Scylla paramamosain*]
TRINITY_DN23093_c1_g15	C-type lectin [*Procambarus clarkii*]
TRINITY_DN26534_c1_g7	beta-1,3-glucan-binding protein precursor [*Astacus astacus*]

Gene ID	Gene homologous [species]
TRINITY_DN49777_c0_g1	C-type lectin [*Litopenaeus vannamei*]
Blood clotting system	
TRINITY_DN22504_c0_g1	PREDICTED: proclotting enzyme-like [*Metaseiulus occidentalis*]
Other immune molecules	
TRINITY_DN10390_c0_g1	metallothionein [*Litopenaeus vannamei*]
TRINITY_DN10870_c0_g1	metallothionein [*Penaeus monodon*]
TRINITY_DN12536_c0_g1	peritrophin [*Macrobrachium nipponense*]
TRINITY_DN19406_c1_g2	ENSANGP00000021035-like, partial [*Litopenaeus vannamei*]
TRINITY_DN21118_c0_g13	hemocyanin subunit L2, partial [*Litopenaeus vannamei*]
TRINITY_DN21690_c0_g1	PREDICTED: macrophage mannose receptor 1-like [*Hyalella azteca*]
TRINITY_DN22197_c3_g2	tick legumain [*Haemaphysalis longicornis*]
TRINITY_DN22645_c0_g1	peritrophin [*Fenneropenaeus chinensis*]
TRINITY_DN22859_c0_g2	PREDICTED: leucine-rich repeats and immunoglobulin-like domains protein 1 [*Parasteatoda tepidariorum*]
TRINITY_DN24015_c3_g2	hemocyanin subunit L1, partial [*Litopenaeus vannamei*]
TRINITY_DN24015_c3_g29	hemocyanin subunit L2, partial [*Litopenaeus vannamei*]
TRINITY_DN24015_c3_g32	hemocyanin, partial [*Penaeus monodon*]
TRINITY_DN24015_c3_g36	hemocyanin subunit L2, partial [*Litopenaeus vannamei*]
TRINITY_DN24015_c3_g8	hemocyanin subunit L1, partial [*Litopenaeus vannamei*]
TRINITY_DN25815_c4_g2	chitinase [*Charybdis japonica*]
TRINITY_DN25815_c4_g3	chitinase 1 precursor [*Litopenaeus vannamei*]
TRINITY_DN42332_c0_g1	ENSANGP00000021035-like, partial [*Litopenaeus vannamei*]
TRINITY_DN6348_c0_g1	TSPAN8 [*Litopenaeus vannamei*]
TRINITY_DN8066_c1_g1	ENSANGP00000021035-like, partial [*Litopenaeus vannamei*]
TRINITY_DN8066_c1_g4	ENSANGP00000021035-like, partial [*Litopenaeus vannamei*]

Table 5. Differentially expressed genes of the stomach involved in immune response of sur-FKC compared to Vp-inf and control.

Gene ID	Gene homologous [species]
Antimicrobial peptides	
TRINITY_DN13910_c0_g1	anti-lipopolysaccharide factor isoform 6 [*Penaeus monodon*]
TRINITY_DN19375_c0_g1	antimicrobial peptide type 2 precursor IIb [*Pandalopsis japonica*]
TRINITY_DN21223_c0_g13	anti-lipopolysaccharide factor isoform 2 [*Penaeus monodon*]
Proteinases/Proteinase inhibitors	
TRINITY_DN18468_c0_g1	serine proteinase inhibitor-3 [*Eriocheir sinensis*]
TRINITY_DN19886_c0_g5	clip domain serine proteinase 2 [*Penaeus monodon*]
Pattern recognition proteins	
TRINITY_DN23894_c0_g1	techylectin-5B isoform [*Tachypleus tridentatus*]
TRINITY_DN23894_c0_g2	fibrinogen-like protein [*Fenneropenaeus merguiensis*]
TRINITY_DN23894_c0_g3	fibrinogen-like protein [*Fenneropenaeus merguiensis*]
TRINITY_DN3091_c0_g1	C-type lectin 4 [*Danaus plexippus*]
Other immune molecules	
TRINITY_DN18887_c1_g4	thymosin beta [*Penaeus monodon*]
TRINITY_DN22229_c0_g6	strongly chitin-binding protein-1 [*Procambarus clarkii*]
TRINITY_DN22833_c1_g32	crustacyanin subunit C [*Fenneropenaeus merguiensis*]
TRINITY_DN23191_c2_g1	strongly chitin-binding protein-1 [*Procambarus clarkii*]
TRINITY_DN25601_c0_g1	PREDICTED: mucin-17-like [*Hyalella azteca*]
TRINITY_DN25995_c0_g1	PREDICTE: mucin-19-like isoform X1 [*Hyalella azteca*]
TRINITY_DN21823_c0_g1	interferon regulatory factor [*Litopenaeus vannamei*]

Table 6. List of *Litopenaeus vannamei* genes homologous to the Cry toxin receptors.

Receptor genes	control/Vp-inf.	
	Gene ID	**Length* (aa.)**
cadherin	1. DN14090_c0_g1_i1	1,760
	2. DN23271_c0_g1_i1	1,825
	3. DN22247_c0_g1_i2	991
ALP	1. DN17552_c0_g1_i1	570
	2. DN8155_c0_g1_i1[p]	542
APN	1. DN20883_c0_g1_i1	993
	2. DN19107_c0_g1_i4	938
	3. DN18022_c0_g1_i1	917
	4. DN23067_c0_g1_i1	867
	5. DN21738_c0_g1_i1[p]	821
ABCC2	1. DN21605_c0_g2_i3	1,452
	2. DN23110_c0_g1_i1	1,537
	3. DN18494_c0_g1_i1	1,436

* Length of predicted coding sequence; p = partial coding sequence

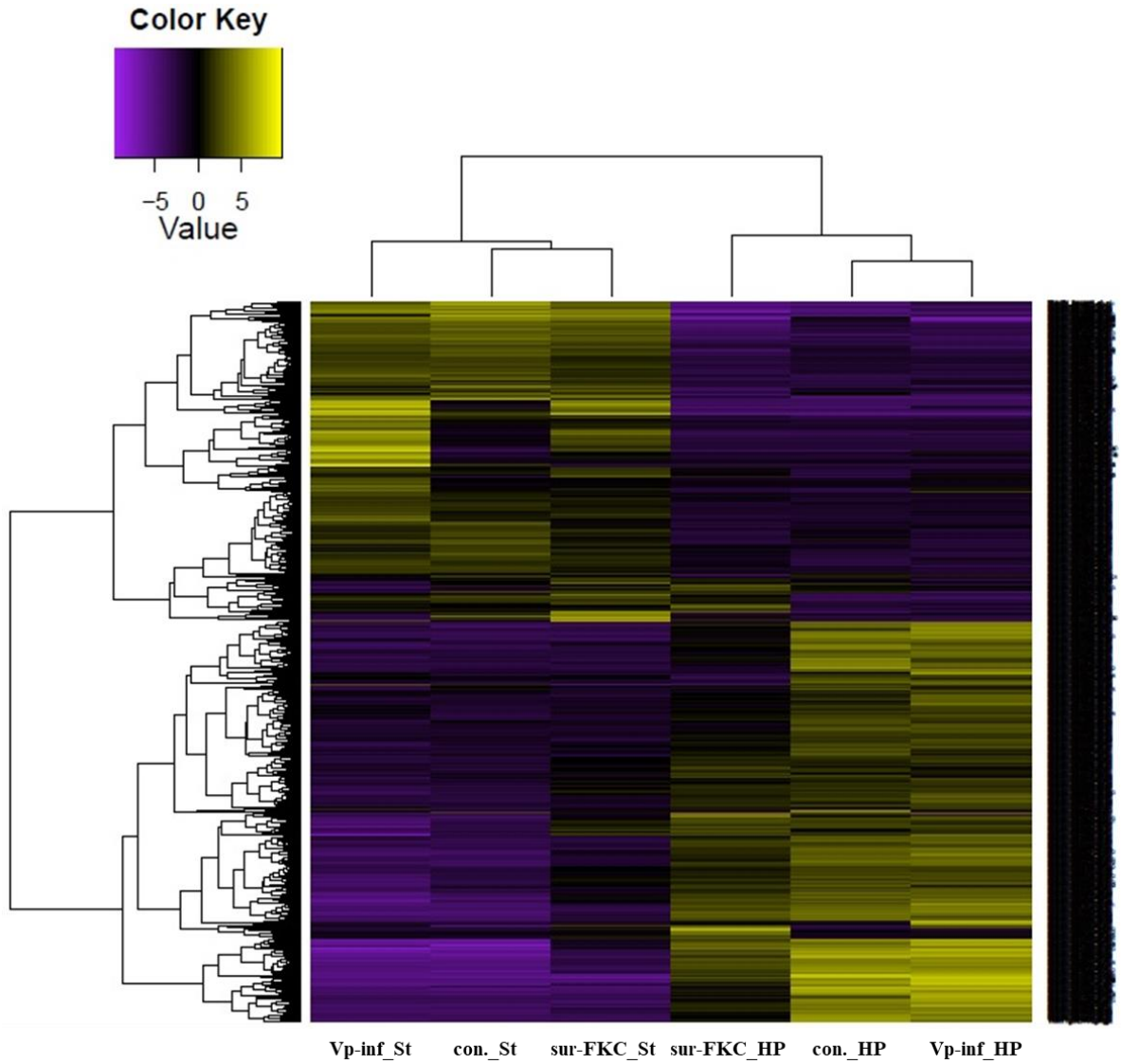

Figure 1. Heat map of expression level of DEGs across all samples. Yellow represents the higher expression and purple represents the lower expression. ($P < 10^{-5}$ and fold change > 4).

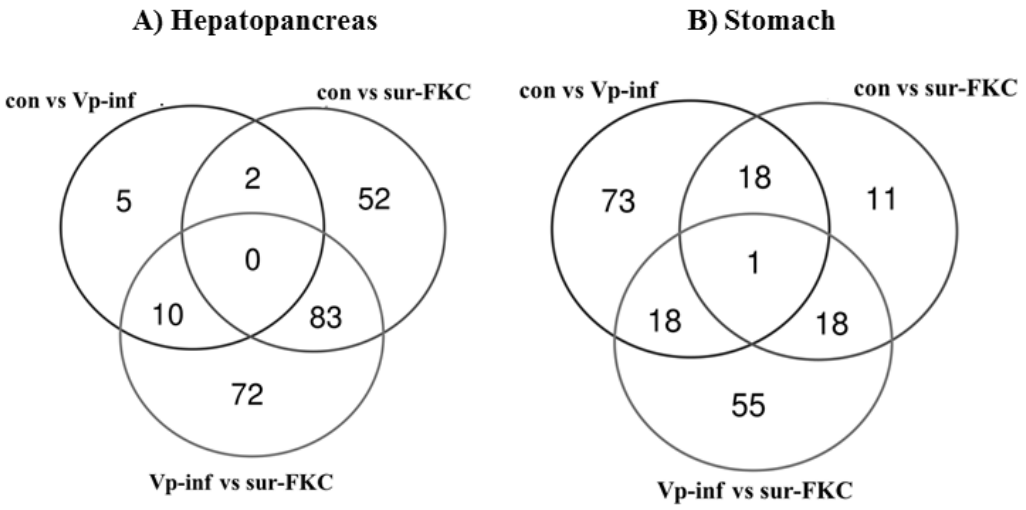

Figure 2. Venn diagram showing the number of differentially expressed genes by pairwise comparison among 3 groups of control, Vp-inf and sur-FKC in the hepatopancreas (**A**) and stomach (**B**). Venn diagrams were drawn by http://bioinformatics.psb.ugent.be/webtools/Venn/.

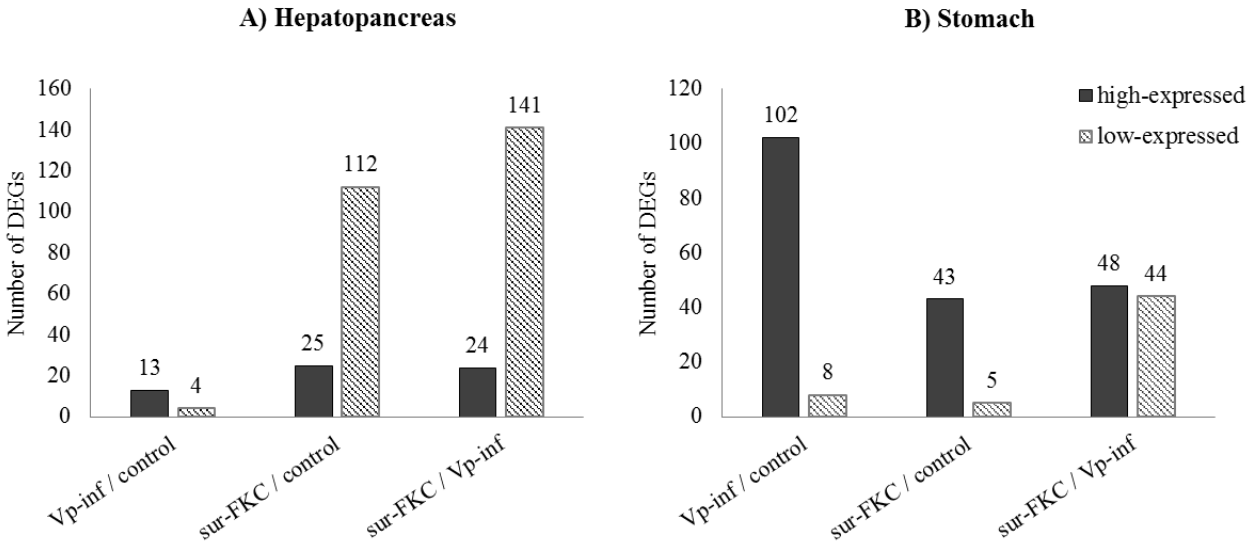

Figure 3. Numbers of high-expressed and low-expressed genes of DEGs in Vp-inf or sur-FKC compared to control and sur-FKC compared to Vp-inf in the hepatopancreas (**A**) and stomach (**B**).

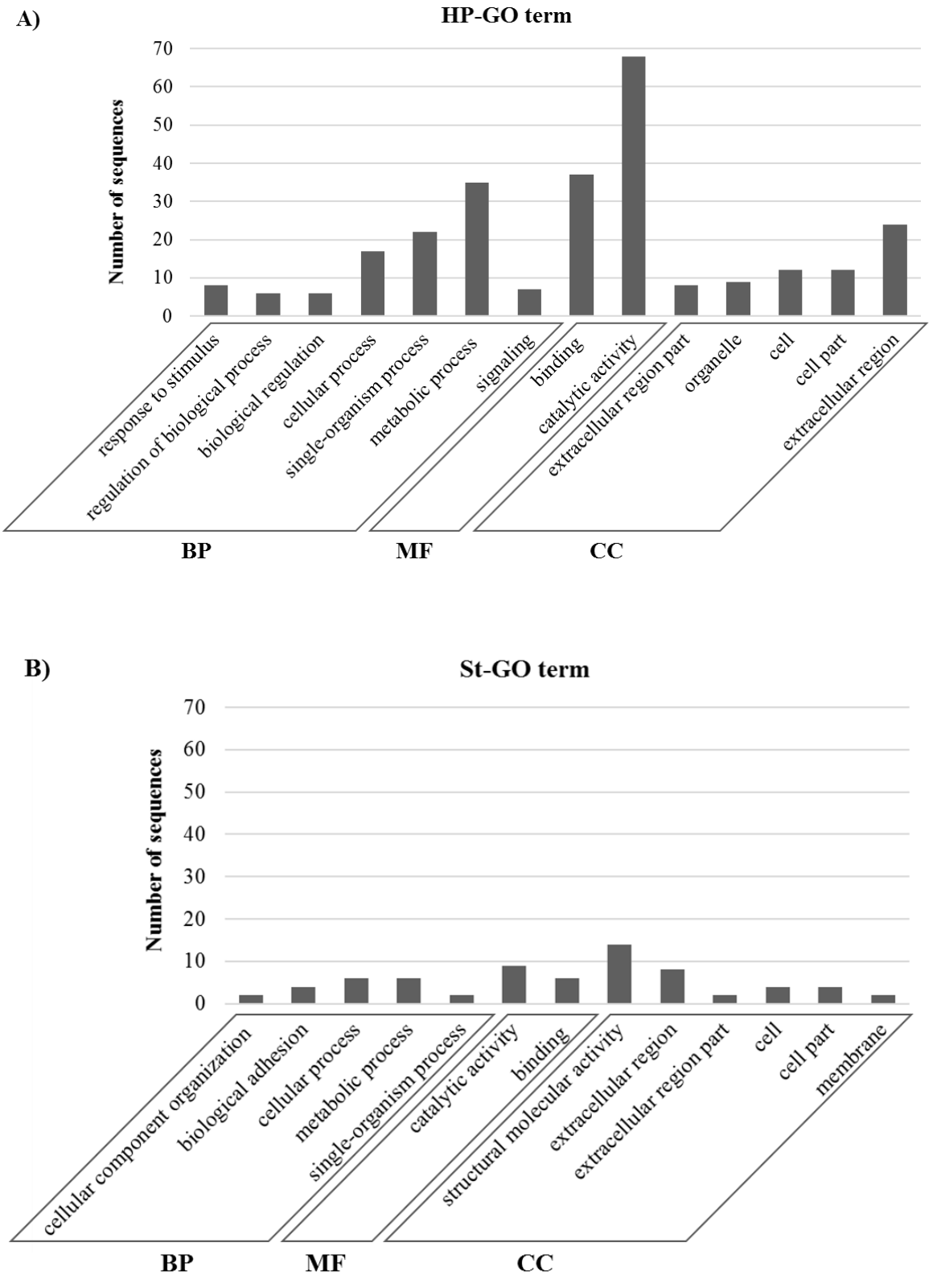

Figure 4. Gene ontology (GO) classification of differentially expressed genes in the hepatopancreas (**A**) and stomach (**B**). Categories of GO are classified into three groups of biological process (BP), molecular function (MF) and cellular component (CC).

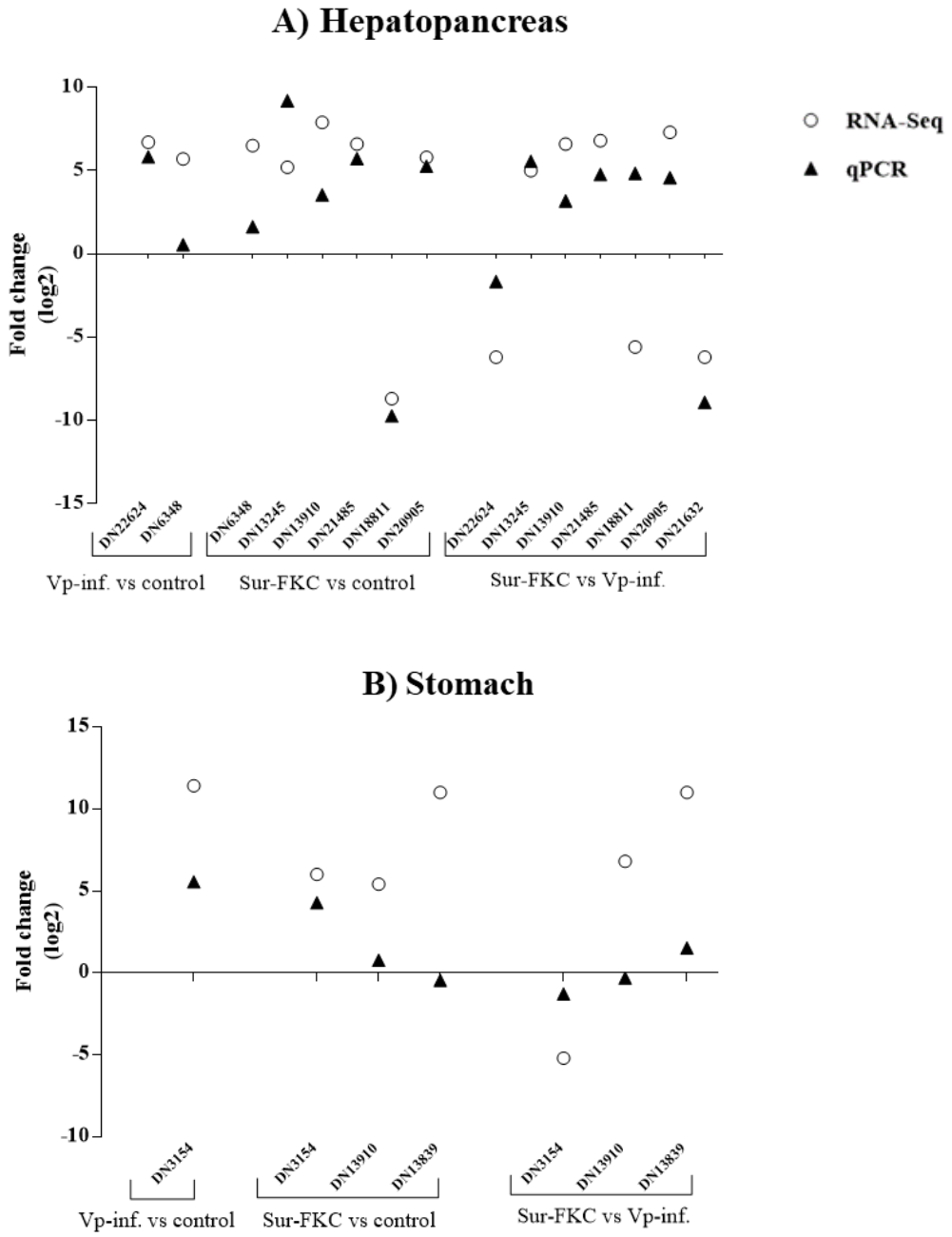

Figure 5. Expression levels of selected DEGs measured by RNA sequencing and qPCR in the hepatopancreas (**A**) and stomach (**B**). For each tissue, pairwise comparisons are made among the three groups of Vp-inf, sur-FKC and control.

Discussion

AHPND is one of serious problems in penaeid shrimp farming. The causative agents are the bacteria that carrying Vp_PirAB-like toxin plasmid. Since the toxin plasmid have been found in several bacterial species, this suggesting that the toxin plasmid might be transmitted among related species via conjugative transfer or other horizontal gene transfer mechanisms. In the worst case scenario, the toxin-carrying plasmid could be transferred to common microbial flora in shrimp ponds.

To prevent and control AHPND outbreak, I focused on the action of Vp_PirAB-like toxin and defense mechanisms against toxin in shrimp. Although, the toxin is secreted into extracellular environment, most of toxin proteins are accumulated in bacterial cells [Tinwongger *et al*., 2016]. The use of inactivated cells of *V. parahaemolyticus* AHPND-causing strains is unlike another bacterial strains, which have been used to enhance protection against several bacterial diseases in aquatic animals and are developed to be function as vaccine [Pridgeon and Klesius, 2012].

Present study, the FKC-VpD6 supplemented diet was used to feed *L. vannamei* for 2 weeks to select shrimp that is highly resistant to Vp_PirAB-like toxin. After daily feeding for 5 days, over 90% of shrimp died (data not shown), suggesting that the toxin amount in the FKC-VpD6 diet was a lethal dose. The surviving shrimp after FKC-VpD6 treatment were supposed to be the Vp_PirAB-like toxin resistant shrimp. The hepatopancreas and stomach of resistant shrimp were collected for RNA-Seq analysis, because the hepatopancreas is the target organ that exhibit the signs of AHPND. Also, Vp_PirA-like and Vp_Pir_B-like toxins were observed in hepatopancreas, stomach and hemolymph of *L. vannamei* immersed with *V. parahaemolyticus* AHPND-causing strain [Lai *et al*., 2015].

The obtained *L. vannamei* transcriptomes were firstly analyzed the differential expression of mRNAs among three groups, especially genes that are related to the shrimp immune system. Apart from physical barrier which is the first line of defense mechanism in

shrimp to protect themselves from disease agents [Aguirre-Guzman *et al*., 2009], shrimp use innate immunity that is consisting of cellular responses (e.g., phagocytosis and encapsulation) and humoral immune responses (e.g., antimicrobial peptides (AMPs), pattern recognition proteins (PRPs), blood clotting, proteinase/proteinase inhibitors) [Rowley and Powell, 2007; Tassanakajon *et al.*, 2013]. Our transcriptome analysis showed that PRPs and proteases/ protease inhibitors were the most abundant immune-related genes found in DEGs of the hepatopancreas. Similar results were reported for *Macrobrachium rosenbergii* in response to *V. parahaemolyticus* [Rao *et al.*, 2015] and in the ridgetail white prawn *Exopalaemon carinicauda* infected with an AHPND strain of *V. parahaemolyticus* [Ge *et al.*, 2017]. PRPs such as lectins and beta-1,3-glucan binding protein (LGBP) were found to be activated after bacterial infection in penaeid shrimp. AMPs such as penaeidin, crustin, and anti-lipopolysaccharide factor (ALF) are also shrimp immune molecules that are produced in response to bacterial infection [Somboonwiwat *et al*., 2005; De la Vega *et al*., 2008; Shockey *et al*., 2009; Hipolto *et al*., 2014; Du *et al*., 2015; An *et al*., 2016]. Moreover, some AMPs specially exhibit antimicrobial activity against AHPND-causing strains of *V. parahaemolyticus* [Supungul *et al*., 2017; Visetnan, *et al*., 2017]. Furthermore, a previous study showed that the upregulations of *Lv*HSP70, *Lv*HSP90, *Lv*Crustin, and *Lv*ProPO mRNAs were involved with AHPND tolerance in *L. vannamei* [Junprung *et al.*, 2017], but I did not observe the high expression of these genes in the sur-FKC transcriptome.

Interestingly, of the 10 DEGs validated by qPCR in this study, only one gene DN21485 which is homologous to *L. vannamei* anti-lipopolysaccharide AV-R isoform (*Lv*ALF AV-R) [Jimenez-Vega and Vargas-Albores, 2007], showed significantly higher expression in the hepatopancreas of sur-FKC shrimp, compared to those in Vp-inf and control. *Lv*ALF AV-R might be constantly expressed in the hepatopancreas before infection or it was induced by toxin and constantly expressed, resulting in toxin resistance. This result is supported by the hepatopancreas transcriptome of *E. carinicauda*, which a gene homologous to *M. resenbergii*

ALF3 showed higher expression in AHPND-surviving group post 48 h of injection, than AHPND-infected and non-infected groups [Ge *et al*., 2017].

On the other hand, the cause of AHPND-resistance may be due to mutation of some genes that affect the action of toxin for host invasion as presented in Cry toxins-resistant insects. The resistance mechanisms to Cry toxins in different lepidopteran insects have been widely studied and shown that reduction of binding between toxin and receptors is the most common mechanism, which caused by mutation in receptors (cadherin, alkaline phosphatase, aminopeptidase N or ATP-binding cassette transporter subfamily C) [Pardo-Lopez *et al*., 2012; Peterson *et al*., 2017]. Therefore, the candidate toxin receptors have been identified from *L. vannamei* control/Vp-inf transcriptome, which is assumed as a representative of normal shrimp.

The cause of toxin resistance remains unclear whether involved with the up-regulation of *Lv*ALF AV-R mRNA or mutation of toxin receptor genes. Consequently, the further studies are necessary to clarify these two hypotheses.

Supplementary Table 1 Annotated differentially expressed genes in the hepatopancreas among control, Vp-inf and sur-FKC groups of *Litopenaeus vannmaei* .

	Gene ID	Size (bp)	E-value	Description	Similarity	Accesssion No.	Fold change (log2)*		
							0h/Vp-inf	0h/sur-FKC	Vp-inf/sur-FKC
1	TRINITY_DN10390_c0_g1	445	6.89E-08	metallothionein [Litopenaeus vannamei]	100.0%	AFC76410.1		4.036	
2	TRINITY_DN10694_c0_g1	1165	4.56E-97	Phosphoethanolamine N-methyltransferase [Daphnia magna]	73.9%	JAN57234.1			4.429
3	TRINITY_DN10870_c0_g1	381	5.38E-15	metallothionein [Penaeus monodon]	93.6%	ADQ28316.1	5.047		-4.610
4	TRINITY_DN11878_c0_g1	596	2.59E-94	preprochymotrypsin 1 [Litopenaeus vannamei]	86.9%	CAA47046.1			6.113
5	TRINITY_DN12106_c0_g1	426	2.53E-09	C-type lectin [Litopenaeus vannamei]	77.6%	AGV68681.1		5.405	
6	TRINITY_DN12136_c0_g1	1024	7.70E-12	C-type lectin 2 [Fenneropenaeus chinensis]	42.6%	ACJ06428.1		7.176	10.947
7	TRINITY_DN12536_c0_g1	1034	2.24E-22	peritrophin [Macrobrachium nipponense]	70.7%	ADB44903.1			4.648
8	TRINITY_DN13245_c0_g1	685	6.86E-10	C-type lectin 1 [Fenneropenaeus chinensis]	76.9%	ABA54612.1		-5.180	-4.964
9	TRINITY_DN13601_c0_g2	2497	0	mitochondrial ATP synthase subunit alpha precursor [Litopenaeus vannamei]	99.6%	ADC55251.1		6.377	8.034
10	TRINITY_DN13910_c0_g1	726	2.00E-52	anti-lipopolysaccharide factor isoform 6 [Penaeus monodon]	91.0%	AER45468.1		-7.911	-6.601
11	TRINITY_DN14322_c0_g2	710	1.39E-09	zinc proteinase [Astacus astacus]	78.0%	CAB43519.1		4.920	
12	TRINITY_DN14395_c0_g1	814	2.76E-49	C-type lectin 4 [Litopenaeus vannamei]	68.2%	AKA64754.1			5.734
13	TRINITY_DN14784_c0_g1	1980	0	beta-1,3-glucan-binding protein precursor [Astacus astacus]	59.5%	AHK23026.1	-5.884		
14	TRINITY_DN15555_c0_g1	1313	1.04E-81	D-beta-hydroxybutyrate dehydrogenase, mitochondrial [Daphnia magna]	63.6%	JAN57575.1		5.949	6.138
15	TRINITY_DN15619_c0_g1	963	8.76E-08	trypsin [Daphnia pulex]	58.5%	EFX75427.1		5.345	
16	TRINITY_DN15674_c0_g1	1056	2.70E-08	AChain A, 1.2a Resolution Structure Of A Crayfish Trypsin Complexed With A Peptide Inhibitor, Sgti	69.9%	4BNR_A, 4BNR_B, 2F91_A		5.149	
17	TRINITY_DN15862_c0_g1	2073	0	beta-1,3-glucan-binding protein precursor [Astacus astacus]	84.4%	AHK23026.1	-6.119		
18	TRINITY_DN16056_c0_g1	875	3.40E-88	JHE-like carboxylesterase 1 [Pandalopsis japonica]	66.8%	ADZ96217.1			4.053
19	TRINITY_DN16256_c0_g1	3521	4.76E-07	GM22957 [Drosophila sechellia], leucine-rich repeat domain	48.1%	EDW56146.1, XP_002039404.1	-5.370		5.013
20	TRINITY_DN16670_c0_g1	1185	1.07E-30	PREDICTED: cytochrome b5-like [Dendroctonus ponderosae], bark beetle	80.0%	XP_019761362.1	6.279		-6.225
21	TRINITY_DN17189_c1_g1	918	8.48E-33	Heme-binding protein 2, partial [Daphnia magna]	57.2%	JAN72319.1		4.715	5.520
22	TRINITY_DN17381_c0_g3	537	1.46E-59	C-type lectin 3 [Fenneropenaeus chinensis]	85.8%	ACJ06431.1		6.296	6.656
23	TRINITY_DN17381_c0_g9	586	8.36E-75	C-type lectin 5 [Fenneropenaeus merguiensis]	84.9%	AGS42196.1		4.980	
24	TRINITY_DN17668_c0_g2	1009	6.77E-66	metalloproteinase [Culex quinquefasciatus]	64.3%	XP_001850227.1,		4.306	
25	TRINITY_DN18811_c0_g1	692	5.57722E-33	Apolipoprotein D [Daphnia magna]	53.6%	JAN90051.1		8.673	5.623
26	TRINITY_DN18924_c0_g1	1297	0	beta-1,3-glucan binding protein [Litopenaeus vannamei]	100.0%	AAW51361.1			5.082
27	TRINITY_DN19069_c0_g1	1410	0	triacylglycerol lipase [Litopenaeus vannamei]	99.0%	ACU57197.1		5.963	4.970
28	TRINITY_DN19077_c1_g1	360	4.13E-39	cathepsin l [Litopenaeus vannamei]	100.0%	CAA68066.1		4.475	6.433
29	TRINITY_DN19077_c1_g10	410	1.03E-95	cathepsin l, partial [Litopenaeus vannamei]	100.0%	CAA59441.1		4.681	5.967
30	TRINITY_DN19077_c1_g2	257	8.94E-38	cathepsin l, partial [Litopenaeus vannamei]	100.0%	CAA59441.1		5.874	7.317
31	TRINITY_DN19077_c1_g5	958	0	cathepsin l, partial [Litopenaeus vannamei]	99.6%	CAA59441.1		4.619	6.371
32	TRINITY_DN19194_c0_g1	1453	1.57E-114	PREDICTED: lysosomal aspartic protease-like isoform X2 [Octopus bimaculoides]	66.1%	XP_014771656.1			5.058
33	TRINITY_DN19227_c0_g1	2128	8.43E-162	serine proteinase inhibitor-2 [Eriocheir sinensis]	80.3%	AJR22372.1		-5.942	
34	TRINITY_DN19259_c0_g1	8809	0	PREDICTED: MAM and LDL-receptor class A domain-containing protein 2-like isoform X1 [Parasteatoda tepidariorum]	31.0%	XP_015914845.1		4.588	
35	TRINITY_DN19406_c1_g2	264	6.46E-32	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	94.7%	ABD65299.1		5.966	6.665
36	TRINITY_DN19406_c1_g3	264	6.46E-32	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	94.7%	ABD65299.1		5.187	5.334
37	TRINITY_DN19406_c1_g4	252	1.41E-40	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	100.0%	ABD65299.1		5.811	5.253
38	TRINITY_DN19406_c1_g5	314	1.08E-39	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	100.0%	ABD65299.1		5.301	5.426
39	TRINITY_DN19406_c1_g7	517	3.82E-43	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	94.6%	ABD65299.1		5.893	5.296
40	TRINITY_DN19406_c1_g8	403	1.97E-39	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	95.6%	ABD65299.1		4.481	4.604
41	TRINITY_DN19522_c0_g1	1112	0	collagenolytic serine protease [Fenneropenaeus chinensis]	92.9%	ACV97157.1		6.032	5.553
42	TRINITY_DN19537_c0_g1	2681	1.74E-178	N-acetylated-alpha-linked acidic dipeptidase 2 [Crassostrea gigas]	60.4%	EKC20430.1			5.747
43	TRINITY_DN19588_c0_g2	496	6.04E-28	C-type lectin [Procambarus clarkii]	64.0%	AGI92548.1		4.646	4.503
44	TRINITY_DN19681_c1_g1	983	7.89E-68	anti-lipopolysaccharide factor [Litopenaeus stylirostris]	98.4%	AAY33769.1		-5.078	-4.986
45	TRINITY_DN19973_c1_g1	1125	5.56E-84	PREDICTED: D-beta-hydroxybutyrate dehydrogenase, mitochondrial-like isoform X2 [Ciona intestinalis]	62.5%	XP_018669489.1			5.343
46	TRINITY_DN19991_c0_g1	1188	2.52E-73	hematopoietic prostaglandin D synthase [Penaeus monodon]	72.9%	AFJ11392.1			5.861
47	TRINITY_DN20119_c1_g1	1100	8.12E-65	MOSC domain-containing protein 1, mitochondrial [Acromyrmex echinatior]	57.7%	EGI57317.1		5.693	5.129
48	TRINITY_DN20189_c0_g1	18801	4.00E-110	beta-1,3-glucan-binding protein precursor [Astacus astacus]	30.0%	AHK23026.1			5.937
49	TRINITY_DN20213_c1_g3	2342	0	amylase [Litopenaeus vannamei]	100.0%	AIJ02078.1		4.813	4.054
50	TRINITY_DN20226_c0_g1	1138	3.92E-56	pancreatic lipase-related protein [Mizuhopecten yessoensis]	53.0%	ACA23976.1		6.171	

	Gene ID	Size (bp)	E-value	Description	Similarity	Accesssion No.	Fold change (log2)*		
							0h/Vp-inf	0h/sur-FKC	Vp-inf/sur-FKC
51	TRINITY_DN20254_c5_g1	941	2.75E-32	C-type lectin domain-containing protein [Fenneropenaeus chinensis]	55.4%	AGL93170.1		4.194	
52	TRINITY_DN20323_c0_g1	2712	0	beta-1,3-glucan-binding protein precursor [Astacus astacus], crayfish	69.8%	AHK23026.1	-5.753		
53	TRINITY_DN20381_c0_g1	1872	2.00E-117	PREDICTED: deoxynucleoside triphosphate triphosphohydrolase SAMHD1-like [Saccoglossus kowalevskii]	44.0%	XP_006813924.1		4.092	
54	TRINITY_DN20592_c0_g1	924	8.25E-39	JHE-like carboxylesterase 1 [Pandalopsis japonica]	53.3%	ADZ96217.1			4.602
55	TRINITY_DN20687_c0_g1	1305	8.98E-51	LUCB_OPLGRRecName: Full=Oplophorus-luciferin 2-monooxygenase non-catalytic subunit; Flags: Precursor	50.0%	Q9GV46.1		6.555	6.828
56	TRINITY_DN20837_c0_g1	1231	4.04E-17	C-type lectin [Eriocheir sinensis]	50.4%	ADK66338.1			4.627
57	TRINITY_DN20844_c0_g1	2034	0	PREDICTED: UDP-glucose 4-epimerase-like isoform X2 [Hyalella azteca]	80.0%	XP_018015063.1		-4.734	-4.826
58	TRINITY_DN20905_c0_g1	1605	0	innexin 2 [Penaeus monodon]	98.6%	ACC62172.1		-5.818	-7.027
59	TRINITY_DN20968_c0_g1	1465	9.82E-146	innexin 7 [Homarus americanus]	73.4%	AIT97138.1		-5.472	-5.121
60	TRINITY_DN21089_c1_g9	278	2.24E-21	PREDICTED: vitelline membrane outer layer protein 1 homolog [Hyalella azteca]	59.8%	XP_018020559.1		5.049	
61	TRINITY_DN21109_c0_g1	1625	2.49E-136	innexin 7 [Homarus americanus]	67.3%	AIT97138.1		-5.360	-5.713
62	TRINITY_DN21118_c0_g13	362	1.57E-75	hemocyanin subunit L2, partial [Litopenaeus vannamei]	99.2%	AHY86472.1			6.101
63	TRINITY_DN21465_c0_g13	701	1.22E-23	proteoliaisin, partial [Lytechinus variegatus]	55.7%	AAT01142.1		4.140	5.273
64	TRINITY_DN21485_c0_g1	756	3.19E-63	anti-lipopolysaccharide factor AV-R isoform [Litopenaeus vannamei]	100.0%	ABB22832		-6.641	-6.814
65	TRINITY_DN21565_c0_g1	3675	2.80E-13	NP_571788.2|asporin precursor [Danio rerio]		NP_571788.2	-6.228		5.488
66	TRINITY_DN21575_c1_g12	377	6.94E-51	C-type lectin [Litopenaeus vannamei]	96.4%	AGV68681.1			-5.541
67	TRINITY_DN21576_c0_g2	596	1.05E-21	AAEL012161-PA [Aedes aegypti]	71.6%	XP_001655924.1		4.284	
68	TRINITY_DN21632_c3_g11	282	2.50E-38	kazal-type serine protease inihibitor 3 [Fenneropenaeus chinensis]	84.9%	ACL36282.1			6.198
69	TRINITY_DN21632_c3_g3	418	5.15E-53	kazal-type serine protease inihibitor 2 [Fenneropenaeus chinensis]	86.9%	ACL36281.1			5.231
70	TRINITY_DN21632_c3_g7	395	8.07-E48	kazal-type serine protease inihibitor 4 precursor [Fenneropenaeus chinenesis]	89.0%	ACL36283.1		6.217	7.906
71	TRINITY_DN21632_c4_g1	380	2.94E-08	hepatopancreas kazal-tye ase inhibitor[Penaeus monodon]	78.0%	AAP92780.1	-5.518		
72	TRINITY_DN21690_c0_g1	6572	0	PREDICTED: macrophage mannose receptor 1-like [Hyalella azteca]	40.0%	XP_018012021.1		5.976	5.506
73	TRINITY_DN21835_c0_g1	1703	1.90E-150	CP2L1_PANARRecName: Full=Cytochrome P450 2L1; AltName: Full=CYPIIL1	66.4%	Q27712.1		5.222	5.188
74	TRINITY_DN21893_c0_g2	805	2.51E-146	C-type lectin 3 [Fenneropenaeus merguiensis]	83.9%	AGS42194.1			5.157
75	TRINITY_DN21896_c0_g1	660	3.31E-20	PREDICTED: flocculation protein FLO1-like [Hyalella azteca]	50.8%	XP_018013771.1		5.751	5.405
76	TRINITY_DN21897_c2_g1	1617	0	carboxypeptidase B, partial [Litopenaeus vannamei]	98.3%	ABD65300.1			4.260
77	TRINITY_DN21942_c1_g1	685	9.11E-137	glutathione peroxidase 3 [Penaeus monodon]	97.5%	ALM09356.1			4.684
78	TRINITY_DN22197_c3_g2	1734	3.67E-138	tick legumain [Haemaphysalis longicornis]	66.5%	BAF51711.1			4.021
79	TRINITY_DN22321_c0_g1	1242	2.82E-168	alpha glucosidase [Litopenaeus vannamei]	77.0%	CAB85963.1			4.410
80	TRINITY_DN22504_c0_g1	1945	3.00E-10	PREDICTED: proclotting enzyme-like [Metaseiulus occidentalis], mite	26.0%	XP_003743889.1		-5.960	
81	TRINITY_DN22562_c0_g1	1829	1.40E-120	sphingomyelin phosphodiesterase, putative [Pediculus humanus corporis]	55.6%	XP_002429317.1		5.409	
82	TRINITY_DN22624_c0_g2	1247	0	C-type lectin 5 [Fenneropenaeus chinensis]	81.0%	ACJ06429.1	-6.732		6.170
83	TRINITY_DN22645_c0_g1	1570	1.01E-66	peritrophin [Fenneropenaeus chinensis]	59.8%	AAZ66371.1			4.880
84	TRINITY_DN22814_c0_g1	1967	0	AF148497_1beta 1,4-endoglucanase [Cherax quadricarinatus]	78.7%	AAD38027.1		5.393	4.821
85	TRINITY_DN22836_c0_g1	1244	1.91E-43	ricin-type beta-trefoil lectin domain-like protein [Leptospira wolffii serovar Khorat str. Khorat-H2]	42.1%	EPG66778.1		5.784	
86	TRINITY_DN22859_c0_g2	1673	2.00E-16	PREDICTED: leucine-rich repeats and immunoglobulin-like domains protein 1 [Parasteatoda tepidariorum]	29.0%	XP_015919047.1		4.363	
87	TRINITY_DN23025_c1_g1	2292	1.41E-174	D-3-phosphoglycerate dehydrogenase-like, [Hyalella azteca]	55.0%	XP_018020388.1	-5.612		4.721
88	TRINITY_DN23093_c1_g1	208	3.32E-09	C-type lectin receptor [Scylla paramamosain]	62.5%	AHM25604.1			5.561
89	TRINITY_DN23093_c1_g10	227	7.30E-11	C-type lectin receptor [Scylla paramamosain]	62.3%	AHM25604.1			5.149
90	TRINITY_DN23093_c1_g15	309	4.42E-20	C-type lectin [Procambarus clarkii]	51.0%	AGI92548.1	-5.213		8.077
91	TRINITY_DN23093_c1_g4	220	2.04E-21	C-type lectin [Procambarus clarkii]	70.8%	AGI92548.1			5.625
92	TRINITY_DN23093_c1_g6	709	1.28E-17	C-type lectin [Procambarus clarkii]	67.9%	AGI92548.1		5.332	7.377
93	TRINITY_DN23230_c0_g1	1772	4.29E-95	serine proteinase inhibitor-3 [Eriocheir sinensis]	61.5%	AJR22373.1			-4.692
94	TRINITY_DN23273_c0_g1	3275	0	PREDICTED: lysosomal alpha-mannosidase-like [Hyalella azteca]	53.0%	XP_018008120.1			4.024
95	TRINITY_DN23410_c1_g1	1870	1.04E-54	Peroxisomal N(1)-acetyl-spermine/spermidine oxidase [Camponotus floridanus]	49.8%	EFN70212.1		8.586	5.873
96	TRINITY_DN23476_c0_g1	1195	2.95E-63	predicted protein [Nematostella vectensis]	61.0%	XP_001641427.1			4.405
97	TRINITY_DN23533_c0_g2	1872	5.00E-149	PREDICTED: UDP-glucuronosyltransferase-like isoform X2 [Hyalella azteca]	43.0%	XP_018024138.1			4.077
98	TRINITY_DN23585_c0_g1	1557	1.00E-56	Pancreatic lipase-related protein 2 [Crassostrea gigas]	40.0%	EKC35944.1		-5.291	
99	TRINITY_DN23730_c0_g6	1496	0	amylase [Litopenaeus vannamei]	100.0%	AIJ02079.1		4.156	4.898
100	TRINITY_DN23803_c0_g1	1421	6.85E-88	PREDICTED: alkylglycerol monooxygenase-like [Bombyx mori]	56.6%	XP_004931255.1		6.217	5.287
101	TRINITY_DN24015_c3_g11	316	2.13E-65	hemocyanin subunit L2, partial [Litopenaeus vannamei]	99.0%	AHY86472.1			4.364

	Gene ID	Size (bp)	E-value	Description	Similarity	Accesssion No.	Fold change (log2)*		
							0h/Vp-inf	0h/sur-FKC	Vp-inf/sur-FKC
102	TRINITY_DN24015_c3_g2	332	1.50E-29	hemocyanin subunit L1, partial [Litopenaeus vannamei]	98.2%	AHY86471.1		4.122	
103	TRINITY_DN24015_c3_g29	272	5.45E-54	hemocyanin subunit L2, partial [Litopenaeus vannamei]	100.0%	AHY86476.1		4.875	5.984
104	TRINITY_DN24015_c3_g30	240	1.03E-33	hemocyanin subunit L2, partial [Litopenaeus vannamei]	98.4%	AHY86472.1			4.965
105	TRINITY_DN24015_c3_g32	266	5.29E-51	hemocyanin, partial [Penaeus monodon]	98.9%	AAL27460.1			4.342
106	TRINITY_DN24015_c3_g36	418	6.80E-92	hemocyanin subunit L2, partial [Litopenaeus vannamei]	98.6%	AHY86472.1		4.501	4.106
107	TRINITY_DN24015_c3_g5	250	5.58E-37	hemocyanin subunit L2, partial [Litopenaeus vannamei]	100.0%	AHY86472.1			5.821
108	TRINITY_DN24015_c3_g8	1973	0	hemocyanin subunit L1, partial [Litopenaeus vannamei]	99.5%	AHY86471.1			4.387
109	TRINITY_DN24197_c0_g2	1583	1.21E-100	CBPB_ASTASRecName: Full=Carboxypeptidase B [Astacus astacus]	70.3%	P04069.1			4.083
110	TRINITY_DN24265_c0_g33	440	8.09E-55	lecdysteroid regulated-like protein [Litopenaeus vannamei]	74.1%	AEX59149.1		5.680	6.560
111	TRINITY_DN24334_c0_g1	2363	2.51E-57	AGAP000535-PA-like protein [Anopheles sinensis]	48.8%	KFB42535.1			4.328
112	TRINITY_DN24416_c0_g1	3577	0	PREDICTED: hemocyte protein-glutamine gamma-glutamyltransferase [Tribolium castaneum]	68.5%	XP_015836063.1			-4.176
113	TRINITY_DN24478_c3_g1	1158	6.21E-84	Poly(U)-specific endoribonuclease [Daphnia magna]	61.6%	JAN57222.1		4.177	4.806
114	TRINITY_DN24585_c0_g1	1264	0	delta6 fatty acyl desaturase [Litopenaeus vannamei]	100.0%	ALS03812.1		4.879	
115	TRINITY_DN24617_c0_g2	1226	0	serine proteinase inhibitor [Litopenaeus vannamei]	100.0%	AGL39540.1			-4.784
116	TRINITY_DN24643_c0_g1	1896	2.00E-123	PREDICTED: steryl-sulfatase-like [Lingula anatina]	40.0%	XP_013392419.1		4.440	
117	TRINITY_DN24651_c0_g1	2365	0	amylase [Litopenaeus vannamei]	9970.0%	AIJ02081.1		4.443	4.736
118	TRINITY_DN24735_c1_g2	3284	1.28902E-68	PI-PLC X domain-containing protein 1 [Daphnia magna]	54.5%	KZS17294.1		7.571	5.645
119	TRINITY_DN24899_c0_g2	1813	4.00E-156	juvenile hormone esterase-like carboxylesterase 1 [Eriocheir sinensis]	47.0%	APO14259.1		5.619	7.069
120	TRINITY_DN25042_c2_g8	1417	3.47E-84	UDP-glycosyltransferase 208B2 [Daphnia pulex]	55.4%	AHX56922.1			4.756
121	TRINITY_DN25192_c0_g1	2435	0	juvenile hormone esterase-like protein [Portunus trituberculatus]	73.7%	AKN79605.1		4.820	
122	TRINITY_DN25259_c2_g4	406	1.10E-14	cytochrome P450 4C [Portunus trituberculatus]	80.4%	AFH35031.1			5.801
123	TRINITY_DN25259_c2_g7	1869	0	cytochrome P450 [Litopenaeus vannamei]	73.6%	ADD63783.1		6.486	7.780
124	TRINITY_DN25279_c0_g1	2877	0.00E+00	alpha glucosidase [Litopenaeus vannamei]	96.0%	CAB85963.1			4.289
125	TRINITY_DN25389_c1_g1	1775	0	alpha glucosidase [Litopenaeus vannamei]	78.1%	CAB85963.1			4.518
126	TRINITY_DN25407_c0_g1	1951	0	cytochrome P450 CYP2 [Eriocheir sinensis]	72.1%	ALA09303.1		5.024	5.877
127	TRINITY_DN25473_c0_g6	1132	0	vascular endothelial growth factor 2 [Litopenaeus vannamei]	99.4%	ALL27072.1			-5.607
128	TRINITY_DN25519_c0_g3	1864	3.20E-76	Ectonucleotide pyrophosphatase/phosphodiesterase family member [Daphnia magna]	55.1%	JAN58096.1		4.725	
129	TRINITY_DN2553_c0_g1	475	7.65E-19	hepatopancreas kazal-type proteinase inhibitor [Penaeus monodon]	84.3%	AAP92780.1			5.438
130	TRINITY_DN25576_c0_g1	2071	1.62E-120	Beta,beta-carotene 9',10'-oxygenase [Daphnia magna]	56.4%	JAN33623.1			4.543
131	TRINITY_DN25649_c0_g2	1863	5.04E-123	Glycerol-3-phosphate transporter [Daphnia magna]	62.0%	JAN41923.1			6.174
132	TRINITY_DN25704_c1_g1	2186	0	myosin heavy chain type1 [Litopenaeus vannamei]	97.4%	BAM65721.1	5.514	7.089	
133	TRINITY_DN25815_c4_g2	530	1.95E-58	chitinase [Charybdis japonica]	87.3%	AFF59213.1			6.130
134	TRINITY_DN25815_c4_g3	4427	0	chitinase 1 precursor [Litopenaeus vannamei]	98.9%	ACG60513.1		4.903	4.208
135	TRINITY_DN26069_c0_g4	4062	7.00E-19	PREDICTED: keratin, type I cytoskeletal 9-like [Hyalella azteca]	42.0%	XP_018012554.1|		-4.900	-4.739
136	TRINITY_DN26077_c0_g1	4006	0.00E+00	PREDICTED: alpha-mannosidase 2C1-like [Hyalella azteca]	59.0%	XP_018024939.1		6.874	6.601
137	TRINITY_DN26101_c1_g1	4080	0	Indole-3-acetaldehyde oxidase [Daphnia magna]	56.9%	JAN53536.1		6.025	5.200
138	TRINITY_DN26222_c0_g4	2427	0	N-acetylgalactosamine-6-sulfatase precursor [Danio rerio]	75.7%	NP_001074110.2		4.504	5.273
139	TRINITY_DN26236_c0_g1	4854	0	centaurin-alpha 1-like protein [Marsupenaeus japonicus]	99.0%	ACN32215.1		-5.268	
140	TRINITY_DN26294_c0_g36	988	6.25E-106	PREDICTED: UDP-glucuronosyltransferase 2B14-like [Hyalella azteca]	68.9%	XP_018027163.1			5.364
141	TRINITY_DN26490_c2_g5	788	1.53E-43	cytochrome P450 CYP6BQ22 [Dastarcus helophoroides]	58.3%	AGJ51945.1		5.412	
142	TRINITY_DN26534_c1_g7	9291	0	beta-1,3-glucan-binding protein precursor [Astacus astacus]	45.0%	AHK23026.1			6.487
143	TRINITY_DN2664_c0_g1	1792	5.81E-134	serine proteinase inhibitor B3 [Penaeus monodon]	78.7%	ADC42877.1			-6.269
144	TRINITY_DN29375_c0_g1	591	1.36E-26	trypsin [Marsupenaeus japonicus]	90.2%	ACE80257.1			5.465
145	TRINITY_DN2976_c0_g1	1869	3.20E-69	clip domain serine proteinase 2 [Penaeus monodon]	57.1%	ACP19561.1			-5.956
146	TRINITY_DN300_c0_g1	772	4.42E-31	Apolipoprotein D [Daphnia magna]	52.6%	JAN90051.1	4.967		
147	TRINITY_DN35830_c0_g1	1292	1.13E-148	Betaine--homocysteine S-methyltransferase 1, partial [Stegodyphus mimosarum]	78.3%	KFM67443.1		6.647	7.422
148	TRINITY_DN42332_c0_g1	509	1.06E-18	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	65.2%	ABD65299.1		-4.908	
149	TRINITY_DN4578_c0_g1	513	5.68E-104	amylase I, partial [Litopenaeus vannamei]	98.2%	CAB65552.2		4.456	5.141

	Gene ID	Size (bp)	E-value	Description	Similarity	Accesssion No.	Fold change (log2)*		
							0h/Vp-inf	0h/sur-FKC	Vp-inf/sur-FKC
150	TRINITY_DN48925_c0_g1	1747	2.00E-60	Vascular non-inflammatory molecule [Daphnia magna]	45.1%	JAN78270.1		5.457	4.575
151	TRINITY_DN49777_c0_g1	437	2.26E-38	C-type lectin [Litopenaeus vannamei]	81.3%	AGV68681.1	-6.276		6.171
152	TRINITY_DN5233_c0_g1	639	6.28E-28	ecdysteroid regulated-like protein [Litopenaeus vannamei]	58.6%	AEX59149.1			5.069
153	TRINITY_DN5285_c0_g2	2814	0	Neutral endopeptidase [Daphnia magna]	63.8%	JAN68083.1			4.229
154	TRINITY_DN5297_c0_g1	480	6.35E-107	amylase I, partial [Litopenaeus vannamei]	98.7%	CAB65552.2			4.565
155	TRINITY_DN5608_c0_g1	2171	0	serine proteinase inhibitor B3 [Litopenaeus vannamei]	99.8%	ALG75875.1		-6.487	-5.559
156	TRINITY_DN5958_c0_g1	1770	4.09E-127	JHE-like carboxylesterase 1 [Pandalopsis japonica]	60.4%	ADZ96217.1			4.275
157	TRINITY_DN6348_c0_g1	1522	1.31E-132	TSPAN8 [Litopenaeus vannamei]	100.0%	ALI93849.1	-5.700	-6.548	
158	TRINITY_DN8066_c1_g1	305	1.04E-28	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	98.1%	ABD65299.1		7.418	
159	TRINITY_DN8066_c1_g4	305	1.04E-28	ENSANGP00000021035-like, partial [Litopenaeus vannamei]	98.1%	ABD65299.1		6.485	6.126
160	TRINITY_DN8268_c0_g1	4477	0	PREDICTED: xanthine dehydrogenase/oxidase-like [Hyalella azteca]	51.0%	XP_018012291.1			4.180
161	TRINITY_DN9334_c0_g1	1299	1.65E-17	membrane glycoprotein LIG-1, putative [Ixodes scapularis]	47.6%	XP_002399763.1		5.000	5.438

* E-value cutoff 1e-5 and Fold-change log2 >2

Supplementary Table 2 Annotated differentially expressed genes in the stomach among control, Vp-inf and sur-FKC groups of *Litopenaeus vannmaei* .

	Gene ID	Size (bp)	E-value	Description	Similarity	Accesssion No.	Fold change (log2)*		
							0h/Vp-inf	0h/sur-FKC	Vp-inf/sur-FKC
1	TRINITY_DN10293_c0_g1	405	1.85E-45	calcified cuticle protein CP14.1 [Callinectes sapidus]	84.8%	ABB91676.1	-5.9		
2	TRINITY_DN11240_c0_g1	533	5.16E-19	Cuticular protein 56F, partial [Daphnia magna]	63.5%	JAN72312.1			4.9
3	TRINITY_DN11503_c0_g1	811	1.34E-13	cuticular protein hypothetical 28 precursor [Bombyx mori]	62.4%	FAA00495.1, NP_001166749.1	-5.0		
4	TRINITY_DN11518_c0_g1	529	1.28E-27	CUPA2_CANPGRecName: Full=Cuticle protein AM1159; Short=CPAM1159 [Cancer pagurus]	73.3%	P81576.1	-5.7	-4.6	
5	TRINITY_DN11885_c0_g1	777	3.81E-29	PREDICTED: fibroncetin-binding protein A-like [Hyalella azteca]	77.1%	XP_018021772.1	-6.7	-5.7	
6	TRINITY_DN12115_c0_g1	1583	7.06E-102	cyclophilin A [Litopenaeus vannamei]	100.0%	AEP83534.1	-4.6		
7	TRINITY_DN12406_c0_g1	369	3.51E-11	Cuticle protein [Daphnia magna]	80.5%	AN65546.1	-5.6		
8	TRINITY_DN13089_c0_g1	690	7.63E-23	Cuticle protein 19.8 [Lepeophtheirus salmonis]	63.3%	ACO12877.1	-4.7		
9	TRINITY_DN13403_c0_g1	1092	3.78E-22	Cuticle protein 7 [Lepeophtheirus salmonis]	61.3%	ACO12949.1	-5.9		
10	TRINITY_DN13533_c0_g1	1064	3.03E-85	PREDICTED: probable pathogenesis-related protein ARB_02861 [Hyalella azteca]	81.3%	XP_018009280.1			6.0
11	TRINITY_DN13725_c0_g1	2186	1.29E-21	PREDICTED:alpha cabonic anhydrase 8-like [Hyalella azteca]	87.3%	XP_018016017.1	-8.7	-6.0	
12	TRINITY_DN13839_c1_g1	712	6.29E-31	arthrodial cuticle protein AMP9.3 [Callinectes sapidus]	72.3%	ABB91673.1		-11.0	-10.9
13	TRINITY_DN13839_c1_g3	712	6.29E-31	arthrodial cuticle protein AMP9.3 [Callinectes sapidus]	72.3%	ABB91673.1		6.8	6.0
14	TRINITY_DN13910_c0_g1	726	1.98E-52	anti-lipopolysaccharide factor isoform 6 [Penaeus monodon]	95.10%	AER45468.1		-5.8	-6.8
15	TRINITY_DN14358_c0_g1	6867	3.04E-16	neuroparsin [Scylla paramamosain]	69.6%	ALQ28570.1	-4.7		
16	TRINITY_DN15439_c0_g1	936	1.30E-58	AAEL011897-PA [Aedes aegypti]	66.5%	XP_001662027.1	-6.1		
17	TRINITY_DN15442_c0_g1	382	2.77E-18	CU04_HOMAMRecName: Full=Cuticle protein AMP4; AltName: Full=HA-AMP4 [Homarus americanus]	84.5%	P81388.1	-6.0		
18	TRINITY_DN15442_c0_g5	382	2.77E-18	CU04_HOMAMRecName: Full=Cuticle protein AMP4; AltName: Full=HA-AMP4 [Homarus americanus]	84.5%	P81388.1		-7.1	
19	TRINITY_DN15918_c0_g1	1302	9.33E-61	PREDICTED: trypsin-1 isoform X1 [Cerapachys biroi]	59.0%	XP_01134101		-6.0	-6.9
20	TRINITY_DN16205_c1_g1	326	8.03E-34	DD9B, partial [Marsupenaeus japonicus]	89.9%	BAA90876.1	-4.4		
21	TRINITY_DN16205_c3_g1	672	7.63E-26	arthrodial cuticle protein AMP8.1 [Callinectes sapidus]	71.4%	AAV28476.1	-4.5		
22	TRINITY_DN16670_c0_g1	1185	1.08E-30	PREDICTED: cytochrome b5-like [Dendroctonus ponderosae]	79.8%	XP_019761362.1	5.6		-5.1
23	TRINITY_DN16792_c0_g1	1009	7.00E-33	PREDICTED: protein FAM98A-like [Hyalella azteca]	72.4%	XP_018024394.1			6.0
24	TRINITY_DN17304_c0_g1	756	2.20E-39	C-type lectin [Marsupenaeus japonicus]	63.7%	AHA83582.1	-7.1		
25	TRINITY_DN17639_c0_g1	897	9.23E-16	cuticle protein precursor [Acyrthosiphon pisum]	63.6%	BAH72338.1, NP_001156673.1		-5.2	-7.5
26	TRINITY_DN17695_c0_g1	3448	0	PREDICTED: periostin-like [Hyalella azteca]	66.4%	XP_018019143.1	-4.1		
27	TRINITY_DN17950_c1_g1	608	3.81E-20	arthrodial cuticle protein AMP16.3 [Callinectes sapidus]	72.6%	ABC26005.1	-9.0	-7.6	
28	TRINITY_DN18026_c0_g1	3731	0	PREDICTED: agrin-like [Hyalella azteca]	71.2%	XP_018009698.1	-5.1		5.3
29	TRINITY_DN18340_c0_g1	2315	5.33E-21	growth and transformation-dependent protein [Culex quinquefasciatus]	67.7%	EDS41561.1, XP_001845741.1	-6.2		
30	TRINITY_DN18468_c0_g1	1981	5.66E-60	serine proteinase inhibitor-3 [Eriocheir sinensis]	58.1%	AJR22373.1	-8.4		6.2
31	TRINITY_DN18515_c1_g1	1198	9.97E-81	glycosyl-phosphatidylinositol-linked carbonic anhydrase [Carcinus maenas]	59.8%	ABX71209.1	-5.7		
32	TRINITY_DN18887_c1_g4	1379	3.12E-39	thymosin beta [Penaeus monodon]	68.0%	AIW64741.1			4.2
33	TRINITY_DN18896_c0_g1	2980	2.12E-173	PREDICTED: SEC14 domain and spectrin repeat-containing protein 1-like [Hyalella azteca]	74.7%	XP_018026615.1	-5.2		
34	TRINITY_DN19375_c0_g1	678	2.72E-14	antimicrobial peptide type 2 precursor IIb [Pandalopsis japonica]	75.0%	AGU01544.1		-5.1	
35	TRINITY_DN19472_c0_g1	911	5.57E-30	putative cuticle protein [Lepeophtheirus salmonis]	73.5%	ABU41025.1	-4.8		
36	TRINITY_DN19593_c0_g1	1563	2.65E-26	Chitin binding Peritrophin-A domain [Daphnia magna]	49.3%	JAN64265.1	-4.4		
37	TRINITY_DN19886_c0_g5	1825	0	clip domain serine proteinase 2 [Penaeus monodon]	84.8%	ACP19561.1	-4.7		4.8
38	TRINITY_DN20350_c0_g1	1644	2.77E-53	thrombospondin II [Penaeus monodon]	50.9%	AGI56293.1	-7.9		
39	TRINITY_DN20405_c0_g1	2426	5.41E-153	Beta-hexosaminidase fdl [Daphnia magna]	56.0%	JAN68536.1		5.4	5.3
40	TRINITY_DN20537_c0_g2	866	2.32E-39	PREDICTED: apolipoprotein D [Tribolium castaneum]	58.5%	XP_971031.1	-6.2		
41	TRINITY_DN20757_c0_g15	322	5.60E-37	early cuticle protein 6 [Callinectes sapidus]	81.4%	ADI59754.1	-4.8		
42	TRINITY_DN20952_c0_g1	1850	2.59E-63	PREDICTED: heparan sulfate 2-O-sulfotransferase pipe [Acyrthosiphon pisum]	55.4%	XP_001950548.2	-5.2		
43	TRINITY_DN21030_c0_g1	3928	5.39E-55	Transmembrane protease serine 6 [Bactrocera cucurbitae]	61.8%	JAD00537.1			6.5
44	TRINITY_DN21223_c0_g13	303	1.17E-40	anti-lipopolysaccharide factor isoform 2 [Penaeus monodon]	100.0%	ABP73291.1		-5.3	
45	TRINITY_DN21248_c1_g1	1175	5.69E-54	farnesoic acid O-methyltransferase [Nilaparvata lugens]	56.9%	ACL26692.1		-5.0	-7.0
46	TRINITY_DN21251_c0_g1	1310	3.46E-23	PREDICTED: protein FAM98A-like [Hyalella azteca]	71.5%	XP_018024394.1			5.1
47	TRINITY_DN21435_c0_g1	2221	1.57E-95	Chitin deacetylase 9-like protein [Daphnia magna]	61.6%	KZS21268.1			-7.5
48	TRINITY_DN21768_c1_g1	4645	0	PREDICTED: serine-rich adhesin for platelets-like isoform X4 [Hyalella azteca]	80.6%	XP_018027100.1	-4.5		
49	TRINITY_DN21823_c0_g1	1442	0	interferon regulatory factor [Litopenaeus vannamei]	99.2%	AKG54423.1		-5.4	
50	TRINITY_DN22229_c0_g6	513	1.52E-36	strongly chitin-binding protein-1 [Procambarus clarkii]	81.6%	BAM99303.1	-6.7	-5.3	
51	TRINITY_DN22265_c0_g1	1985	1.64E-90	trypsin, partial [Homarus americanus]	88.8%	ABL75452.1		-7.7	
52	TRINITY_DN22666_c3_g5	937	0	trypsinogen 1 [Litopenaeus vannamei]	99.2%	AEZ67461.1		-5.7	-7.9
53	TRINITY_DN22804_c0_g1	1430	5.93E-92	innexin 3, partial [Homarus americanus]	62.0%	AIT97135.1		-5.4	-7.5

	Gene ID	Size (bp)	E-value	Description	Similarity	Accesssion No.	Fold change (log2)*		
							0h/Vp-inf	0h/sur-FKC	Vp-inf/sur-FKC
54	TRINITY_DN22833_c1_g32	335	8.31E-35	crustacyanin subunit C [Fenneropenaeus merguiensis]	94.9%	AEA06587.1		-5.9	
55	TRINITY_DN23128_c0_g1	2221	0	moesin ezrin radixin 1-like protein isoform 1 [Melanoplus sanguinipes]	87.0%	ALX00076.1			-5.8
56	TRINITY_DN23191_c2_g1	413	2.98E-19	strongly chitin-binding protein-1 [Procambarus clarkii]	82.1%	BAM99303.1	-6.6	-6.0	
57	TRINITY_DN23431_c0_g2	2821	0	Chorion peroxidase precursor [Daphnia magna]	74.4%	JAN48285.1	-6.5		6.0
58	TRINITY_DN23488_c0_g1	1572	3.46E-56	PREDICTED: ceramide synthase 6 [Harpegnathos saltator]	52.4%	XP_011151355.1			-5.1
59	TRINITY_DN23651_c7_g3	1004	1.91E-163	Chymotrypsin BI, partial [Litopenaeus vannamei]	97.1%	CAA71672.1		-6.0	-6.3
60	TRINITY_DN23892_c0_g1	6784	0	I-connectin [Procambarus clarkii]	82.1%	BAB64297.1		4.8	
61	TRINITY_DN23894_c0_g1	775	3.44E-15	techylectin-5B isoform [Tachypleus tridentatus]	76.9%	BAA84190.1			-7.2
62	TRINITY_DN23894_c0_g2	946	1.07E-62	fibrinogen-like protein [Fenneropenaeus merguiensis]	67.3%	AKR15662.1			-6.3
63	TRINITY_DN23894_c0_g3	1005	2.23E-70	fibrinogen-like protein [Fenneropenaeus merguiensis]	65.7%	AKR15662.1			-6.7
64	TRINITY_DN23987_c5_g2	286	1.19E-11	arthrodial cuticle protein AMP8.1 [Callinectes sapidus]	84.1%	AAV28476.1	-9.0	-8.3	
65	TRINITY_DN23987_c5_g4	326	1.05E-11	CUPA2_CANPGRecName: Full=Cuticle protein AM1159; Short=CPAM1159 [Cancer pagurus]	79.7%	P81576.1	-6.6	-5.7	
66	TRINITY_DN24298_c0_g1	2961	2.00E-131	PREDICTED: heparan sulfate 2-O-sulfotransferase pipe isoform X2 [Fopius arisanus]	70%	XP_011313801.1			4.8
67	TRINITY_DN24329_c0_g1	2007	1.37E-56	40S ribosomal protein S12, putative [Pediculus humanus corporis]	56.2%	XP_002427381.1 , EEB14643.1	-5.0		
68	TRINITY_DN24432_c4_g1	592	7.60E-33	PREDICTED: hyphally regulated cell wall protein 3-like isoform X1 [Hyalella azteca]	81.2%	XP_018010978.1	-9.1	-6.3	
69	TRINITY_DN24874_c0_g1	2027	1.30E-61	Group 3 secretory phospholipase A2 [Daphnia magna]	56.1%	JAN56610.1			-4.8
70	TRINITY_DN24908_c1_g1	2824	0	Procollagen-lysine,2-oxoglutarate 5-dioxygenase [Daphnia magna]	75.1%	JAN62576.1	-6.4		
71	TRINITY_DN25169_c3_g1	1363	1.75E-53	PREDICTED: carbohydrate sulfotransferase 11 [Nasonia vitripennis]	60.7%	XP_001603452.1	-5.8		
72	TRINITY_DN25169_c5_g16	220	3.39E-19	Cuticle protein 19.8 [Lepeophtheirus salmonis]	66.2%	ACO12877.1			5.2
73	TRINITY_DN25334_c0_g1	3454	9.21E-148	Solute carrier family 28 member 3 [Daphnia magna]	69.2%	KZS13463.1			-5.1
74	TRINITY_DN25601_c0_g1	15142	5.00E-103	PREDICTED: mucin-17-like [Hyalella azteca]	40.0%	XP_018028113.1			5.0
75	TRINITY_DN25712_c0_g4	1739	5.93E-125	JHE-like carboxylesterase 1 [Pandalopsis japonica]	58.4%	ADZ96217.1			-6.0
76	TRINITY_DN25727_c2_g1	3492	0	chitinase 7 [Spodoptera exigua]	82.6%	AFM38213.1	-4.5		
77	TRINITY_DN25854_c0_g1	2959	1.58E-81	CTX_SEPESRecName: Full=SE-cephalotoxin; Short=SE-ctx; Flags: Precursor [Sepia esculenta]	49.5%	B2DCR8.1	5.4		-6.0
78	TRINITY_DN25995_c0_g1	4814	5.68E-09	PREDICTE: mucin-19-like isoform X1 [Hyalella azteca]	66.2%	XP_018009833.1			5.1
79	TRINITY_DN26346_c0_g7	2952	0	Villin protein quail [Daphnia magna]	72.3%	JAN72017.1			-5.9
80	TRINITY_DN26504_c0_g13	4951	0	PREDICTED: protein mesh-like isoform X1 [Hyalella azteca]	72.0%	XP_018022334.1			-5.0
81	TRINITY_DN27290_c0_g2	345	1.01E-24	CU04_HOMAMRecName: Full=Cuticle protein AMP4; AltName: Full=HA-AMP4	77.9%	P81388.1	-4.6		
82	TRINITY_DN29399_c0_g1	1651	3.40E-64	ovarian peritrophin [Fenneropenaeus merguiensis]	51.9%	AAV83539.2	-8.8	-5.5	
83	TRINITY_DN29724_c0_g1	1023	3.92E-149	PREDICTED: aminopeptidase Ey-like [Nasonia vitripennis]	78.3%	XP_001608209.2	-5.4		
84	TRINITY_DN3091_c0_g1	2603	3.79E-80	C-type lectin 4 [Danaus plexippus]	75.5%	EHJ70690.1	-7.3		4.3
85	TRINITY_DN3431_c0_g1	1398	2.57E-22	cuticular protein [Artemia franciscana]	60.9%	ACB05778.1	-8.6	-7.7	
86	TRINITY_DN49451_c0_g1	778	2.68E-25	cuticular protein [Artemia franciscana]	61.0%	ACB05778.1	-5.3		
87	TRINITY_DN54305_c0_g1	2163	1.98E-63	Secreted protein [Daphnia magna]	61.6%	JAN44460.1			4.8
88	TRINITY_DN8417_c0_g16	836	1.37E-06	PREDICTED: muscle-specific protein 20-like [Hyalella azteca]	83.8%	XP_018016767	5.1		
89	TRINITY_DN9216_c0_g1	388	3.91E-26	AMRecName: Full=Cuticle protein AMP1B; [Homarus americanus]	78.6%	P81385.1	-4.5		
90	TRINITY_DN9228_c0_g1	1243	1.08E-42	PREDICTED: putative defense protein 3 [Athalia rosae]	60.4%	XP_012260417.1	-5.9		
91	TRINITY_DN9509_c0_g3	625	1.97E-24	PREDICTED: adhesive plaque matrix protein-like [Hyalella azteca]	71.1%	XP_018008917.1	-6.9		5.5

* E-value cutoff 1e-5 and Fold-change log2 >2

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Chapter 3

Association of *Lv*ALF AV-R and Vp_PirAB-like toxin resistant shrimp

Abstract

The RNA sequencing was previously performed from the hepatopancreas and stomach of surviving *L. vannamei* (sur-FKC) after dietary FKC-VpD6 (*V. parahaemolyticus* AHPND causing strain) or Vp_PirAB-like toxin. The differentially expressed genes (DEGs) between sur-FKC, AHPND-infected shrimp, and normal shrimp were identified. Of the ten selected genes for qPCR validation, only one gene which is homologous to *L. vannamei* anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R) was expressed significantly more strongly in the hepatopancreas of sur-FKC than in the other groups. In this chapter, *Lv*ALF AV-R was further investigated, to clarify the association of *Lv*ALF AV-R and shrimp resistant to Vp_PirAB-like toxin. In the hepatopancreas, expression of *Lv*ALF AV-R mRNA was not affected by VpD6-immersion or dietary of FKC-VpN7 (*V. parahaemolyticus* non-AHPND causing strain) but was highly induced during FKC-VpD6 feeding. Moreover, significantly higher expression of *Lv*ALF AV-R was also observed in shrimp that survived in three other trials of FKC-VpD6 selection. Histological analysis of sur-FKC shrimps showed that three of four observed shrimp exhibited normal characteristic of hepatopancreas cells without AHPND signs.

Recombinant protein of ALF AV-R (rALF AV-R) was produced for *in vitro* and *in vivo* studies. rALF AV-R bound to LPS, PGN, Gram-negative bacteria, and some Gram-positive bacteria in ELISAs. But rALF AV-R did not interact with native Vp_PirAB-like toxin in an ELISA or a Far-Western blot analysis. For *L. vannamei* orally fed rALF AV-R for 3 days, the

survival rate following challenge with VpD6-immersion was not significantly different from that of shrimp fed two control diets (rGFP and PBS). These results suggest that mRNA expression of *Lv*ALF AV-R is induced in the hepatopancreas of shrimp in specific response to the presence of Vp_PirAB-like toxin. *Lv*ALF AV-R plays an indirect role in shrimp resistant to Vp_PirAB-like toxin by promoting other shrimp molecules to directly inhibit virulence of toxin.

[A part of Chapter 3 has been published under title "Identification of an anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R) related to Vp_PirAB-like toxin resistance in *Litopenaeus vannamei*" in *Fish & Shellfish Immunology*, Vol. 84, p 178-188, 2019.]

1. Introduction

Anti-lipopolysaccharide factor (ALF) is a small polypeptide (114-124 amino acid residues) that belongs to an antimicrobial peptide family [Tassanakajon *et al*., 2015] and only found in crustaceans. ALFs was initially characterized in hemocytes of horseshoe crabs *Limulus polyphemus* [Tanaka *et al*., 1982; Ohashi *et al*., 1984; Morita *et al*.,1985]. *Limulus* ALF bound to lipopolysaccharide (LPS) and exhibited strong anti-bacterial activity. cDNA clones homologous to *Limulus* ALF were subsequently identified in other crustaceans, e.g. crab [Li *et al*., 2008; Yedery and Reddy, 2009; Yue *et al*., 2010], crayfish [Liu *et al*., 2006; Sun *et al*., 2011], lobster [Beale *et al*., 2008], and shrimp [Gross *et al*., 2001; Supungul *et al*., 2004; Liu *et al*., 2005; Rosa *et al*., 2008; Mekata *et al*., 2010]. Typically, ALFs contain a hydrophobic N-terminal region and a conserved LPS-binding domain, which is flanked by two conserved cysteine residues that form a disulfide bond [Aketagawa *et al*., 1986; Hoess *et al*., 1993; Yang *et al*., 2009]. Tassanakajon *et al*. (2011) have characterized penaeid shrimp's ALFs into three groups (A, B, and C), based on the sequence similarities and phylogenetic analysis. ALFs in Group A, B, and C are cationic proteins which could be find in one shrimp species. In 2013, a new group of shrimps ALFs was identified and named as Group D, which are the anionic polypeptides and lack many residues of LPS binding site [Rosa *et al*., 2013]. Additionally, Group E ALFs was recently identified in *Marsupenaeus japonicus* and showed protecting function in bacterial response [Jiang *et al*., 2015].

The ALFs of penaeid shrimp have a wide-range of functions in shrimp immunity, being involved in anti-bacterial, anti-fungal, and anti-viral activities. Recombinant protein of *Penaeus monodon* ALF 3 (rALF*Pm*3) has a broad spectrum of anti-fungal properties, anti-bacterial activities (Gram-negative and Gram-positive) and exhibited high efficiency against various *Vibrio* species [Somboonwiwat *et al*., 2005]. An injection of rALF*Pm*3 to *P. monodon* could induce mRNA expression of immune genes in the hemocytes and reduce the mortality after *V. harveyi* infection [Pornprateep *et al*., 2009]. Also, an injection of WSSV pre-treated

rALF*Pm*3 to *P. monodon* showed reduction of WSSV propagation and delayed shrimp mortality [Tharntada *et al*., 2009]. The *in vivo* role of ALF in protecting shrimp from bacterial and fungal infections is supported in the *L. vannamei* ALF1 (*Lv*ALF1) gene knockdown [De la Vega *et al*., 2008].

In previous study, *L. vannamei* resistant to Vp_PirAB-like toxin were selected by oral administration with FKC-VpD6 supplemented diet. The surviving shrimp after 2 weeks feeding was collected and assumed as Vp_PirAB-like toxin resistance (sur-FKC). The hepatopancreas and stomach of sur-FKC were subjected to RNA-Seq analysis. mRNA expression of genes in sur-FKC group were compared to Vp-AHPND infected shrimp (Vp-inf) and non-treated shrimp (control), to identify differentially expressed genes (DEGs). A total of 10 selected DEGs in qPCR validation, only gene which is homologous to *L. vannamei* anti-lipopolysaccharide factor (*Lv*ALF AV-R) showed significantly high expression in the hepatopancreas of sur-FKC group. *Lv*ALF AV-R isoform was firstly isolated from cDNA library of *L. vannamei* hemocytes [Jimenez-Vega and Vargas-Albores, 2007], however its functions neither *in vitro* nor *in vivo* has been studied. Interestingly, *Lv*ALF AV-R mRNA might be induced by Vp_PirAB-like toxin, resulting in an increasing of ALF AV-R polypeptides, which directly react to toxin in the hepatopancreas and reduce toxin virulence.

To clarify the association between *Lv*ALF AV-R and Vp_PirAB-like toxin, the FKC-VpD6 selection were repeated with juvenile *L. vannamei*. Moreover, functions of *Lv*ALF AV-R protein have been demonstrated for the first time in this study.

2. Materials and Methods

2.1 Sequence analyses and 3D structure of *Lv*ALF AV-R

The coding sequence of *Lv*ALF AV-R gene was predicted by https://www.ncbi.nlm.nih.gov/orffinder/, and the molecular weight and pl were computed with https://web.expasy.org. Signal peptide was predicted with the SMART analysis service (http://smart.embl-heidelberg.de/). The 3D structure prediction of *Lv*ALF AV-R mature peptide was constructed by web-server I-TASSER [Zhang, 2008] and viewed with UCSF Chimera software [Pettersen *et al*., 2004].

2.2 mRNA expression of *Lv*ALF AV-R

2.2.1) Tissue distribution

*Lv*ALF AV-R gene, which was highly expressed in the survived shrimp, was examined in three apparently healthy *L. vannamei* (body weight about 7 g). Total RNA was extracted from hemocytes and 9 other tissues (gills, muscle, heart, hepatopancreas, stomach, intestine, eye, lymphoid organ and nerve).

2.2.2) Bacterial stimulations

L. vannamei (body weight about 3 g) were divided into two groups (15 shrimp per group) and reared in 15-L plastic tanks with aerated artificial seawater (28 ppt) at 28 °C. One group was daily fed a commercial diet containing 5% (w/w) of FKCs of *V. parahaemolyticus* non-AHPND strain N7 (FKC-VpN7) [Tinwongger *et al*., 2014], whereas the other group was immersed with *V. parahaemolyticus* AHPND-causing strain D6 (VpD6) at a bacterial concentration of $2x10^5$ CFU/ml and fed with a commercial pellet. The hepatopancreas was collected at 6 h, 24 h, 3 d and 7 d from three shrimps individual and the total RNA was extracted.

2.2.3) FKC-VpD6 feeding

Seventeen *L. vannamei* (approximately 10 g body weight) were reared in 15-L tank and fed daily with diet supplemented with FKC-VpD6. To observe *Lv*ALF AV-R expression while feeding test, the hepatopancreas was collected from three shrimps individually at each time point of 12 h, 24 h, 3 d and 7 d and the total RNA was extracted.

One μg of total RNA of each sample was used for cDNA synthesis with High-Capacity cDNA Reverse Transcription kits (Applied Biosystems, USA). The mRNA level of *Lv*ALF AV-R was examined with specific primer set of ALF AV-R (Table 1) by qPCR. qPCR conditions were 95 °C for 60 s followed by 40 cycles of 95 °C for 15 s, 67 °C for 60 s and additional of dissociation curve analysis. The annealing temperature used was optimized to discriminate an expression of ALF AV-R and other ALF isoforms.

2.3 Repetitions of surviving shrimp selection after dietary toxin

To confirm the relation between *Lv*ALF AV-R and toxin resistance, three other trials (trials 2-4) of FKC-VpD6 feeding were performed. In the second and third trials, 500 *L. vannamei* (body weight 3-5 g) were reared in two 500-L plastic tanks (250 shrimps each) with aerated seawater (20 ppt) at 28 °C. In the fourth trial, 540 *L. vannamei* (body weight 2-3 g) were reared in two 500-L plastic tanks (270 shrimps each). All groups were fed the diet supplemented with FKC-VpD6 equivalent to approximately 5% of body weight per day for one week. In addition, while performing the fourth trial, shrimp were collected at 24, 48, and 72 h for histological analysis. After one week feeding, all surviving shrimps from the second and third trials and a part of surviving shrimp from the fourth trial were collected. The hepatopancreases was dissected and the total RNA was extracted. One μg of total RNA from individual shrimp was used for cDNA synthesis. The expression of *Lv*ALF AV-R was quantified by qPCR as described above.

2.4 Protein expression of *Lv*ALF AV-R in *L. vannamei* tissues

Anti-ALF AV-R antibody was generated in rabbits by Eurofins Scientific Company (Japan) using synthetic peptides matching region (Figure 1). An apparently healthy *L. vannamei* was collected hemocytes and 9 other tissues (lymphoid organ, heart, gills, muscle, hepatopancreas, stomach, intestine, nerve, and eye). The tissue samples were homogenized in chilled 1xPBS containing protease inhibitor (complete Protease Inhibitor Cocktail, Roche, Germany) which placed on ice. The homogenized tissues were centrifuged at 15,000 rpm/4 °C for 15 min. The supernatant was collected as total protein sample and stored at -30 °C prior use.

Total protein samples were mixed with an equal volume of 2xSDS sample buffer (0.125 M Tris-HCL pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose and 0.01% bromophenol blue), boiled for 5 min and cool down on ice. The total protein samples were separated on 15% SDS-PAGE gel and blotted on to PVDF membrane (Atto, Japan) for Western blot analysis. The blotted membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20 (TBS-t) for 1 h, incubated with anti-ALF AV-R serum as primary antibody (1:5000 dilution in blocking buffer) for 1 h, washed 4 times with TBS-t, incubated with goat anti-rabbit IgG (Fc) AP conjugate (Promega, Japan) as secondary antibody (1:5000 dilution in blocking buffer) for 30 min, washed 3 times with TBS-t, and stained with BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich, Japan).

Additionally, the peptide blocking assay was performed to determine whether ALF AV-R antiserum specifically detect *Lv*ALF AV-R native protein. The peptide blocking was done following the standard protocol of Western blotting as described above. But an ALF AV-R antiserum was pre-incubated with 100 µg of ALF AV-R peptides for 30 min with shaking, then used as primary antibody. The positive signal will be presented on normal Western blotting membrane only and disappeared in peptide blocking membrane. In contrast, the false-positive signal will be shown in peptide blocking membrane and normal Western blotting membrane.

2.5 Histopathology of *L. vannamei* hepatopancreas

2.5.1) Hematoxylin and eosin staining (H&E)

L. vannamei from the fourth trial were sampled after feeding FKC-VpD6 diet for 24, 48, and 72 h and the hepatopancreas were collected. Moreover, some surviving shrimp (sur-FKC 4) after feeding for one week were also sampled and collected hepatopancreas tissue for histological examination. The hepatopancreases were fixed in Davidson's fixative solution (10% filtrated seawater, 30% water, 30% ethanol (99%), 20% formalin, and 10% glacial acetic acid) for 24 h, transferred to 70% ethanol, dehydrated with 70% - 100% ethanol, embedded in paraffin, and sectioned at 5 µm thickness. The sections were stained with H&E following the standard methods [Lightner *et al*., 1996].

2.5.2) Immunohistochemistry

The hepatopancreas sections of normal shrimp and sur-FKC 4 shrimp from 2.5.1 were used. The selected sections were deparaffinized with xylene, washed in serial dilutions of ethanol, tap water, and distilled water (D.W.). Sections were blocked an endogenous peroxidase activity by incubation with 3% H_2O_2 solution for 10 min and rinsed with D.W. and 1xPBS. For immunodetection, sections were incubated with PBS containing 5% BSA (blocking buffer) for 1 h, then incubated with primary antibody; an anti-ALF AV-R rabbit antiserum (1:2,000 dilution in blocking buffer) which was pre-incubated with crude proteins of *L. vannamei* hepatopancreas (2.5 µg/ml) to reduce non-specific binding or normal rabbit serum (Rabbit IgG-UNLB, SouthernBiotech, USA) at concentration of 5 µg/ml as negative control. After incubation with primary antibody for 1 h, sections were washed 4 times with PBS - 0.05% Tween 20 (PBS-t), incubated with Goat Anti-Rabbit IgG H&L (HRP) (Abcam, UK) for 30 min as secondary antibody (1:5,000 dilution in blocking buffer), washed 3 times with PBS-t and stained with DAB Substrate Kit (Abcam, UK) to visualize the antibody

reactions. Sections then were counterstained with hematoxylin for 1 min, rinsed in tap water for 5 min, immersed in D.W. for 10 min, dehydrated through 5 times of alcohol (90%, 95%, 100% and 100%), cleared with xylene. Lastly, tissue was mounted with Malinol mounting medium and covered with coverslip. The developed signal was observed under a microscope (Nikon DS-Ri1).

2.6 Production of *Lv*ALF AV-R recombinant protein

2.6.1) Constructing of stable cell lines

*Lv*ALF AV-R without its signal peptide was amplified from the hepatopancreas of sur-FKC shrimp using the rALF AV-R/no SP primer set, where *EcoR*I and *Not*I were added to the forward (5′ end) and reverse (3′ end) primers, respectively (Table 1). The *Lv*ALF AV-R amplicon was cloned into pGEM-T easy vector, which was then transformed into *Escherichia coli* JM109 (Nippon gene, Japan). A plasmid carrying the correct sequence was digested with *EcoR*I and *Not*I and ligated to pMT/BiP/V5-His C Drosophila Expression Vectors (Invitrogen, USA). The ligated vector and pCoBlast vector were co-transfected into *Drosophila* S2 cells with Effectene Transfection Reagent (Qiagen, Germany) following the manufacturer's instructions. Stable cell lines were selected by passaging the cells several times in Schneider *Drosophila* medium (SDM) containing 25 µg/ml blasticidin.

2.6.2) Purification of recombinant protein

Following large-scale culture of the stable cell lines, protein expression was induced with 500 mM $CuSO_4$. Five days after induction, the cultured cells were centrifuged at 300 x g and the supernatant was collected. The supernatant was filtered through a 0.22 µm filter (Merck Millipore Ltd., Ireland). The recombinant protein was then purified with a polypropylene column containing Ni-NTA agarose (Qiagen, Germany), following the manufacturer's protocol. The concentration of recombinant protein was measured with a Qubit™ Protein

Assay Kit (Thermo Fisher Scientific, USA). A GFP recombinant protein (rGFP) was also constructed as a negative control in further experiments.

2.6.3) SDS-PAGE and Western blot analysis of recombinant protein

The purified recombinant proteins of *Lv*ALF AV-R (rALF AV-R) and GFP (rGFP) were mixed with an equal volume of 2xSDS sample buffer and boiled for 5 min. Recombinant proteins were separated by 15% SDS-PAGE. The electrophoresed gel was stained with CGP (Yasumitsu *et al*. 2010) or blotted onto 0.2 µm PVDF membrane (Atto, Japan) for Western blotting. The blotted membrane was blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20 (TBS-t) for 1 h, incubated with V5 tag monoclonal antibody (Invitrogen, USA) as primary antibody (1:5000 dilution in blocking buffer) for 1 h, washed 4 times with TBS-t, incubated with anti-mouse IgG (H+L), AP conjugate (Promega, Japan) as secondary antibody (1:5000 dilution in blocking buffer) for 30 min, washed 3 times with TBS-t, and stained with BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich, Japan) to visualize the detected proteins.

2.7 Partially purification of Vp_PirAB-like toxin

2.7.1) Ammonium sulfate precipitation

V. parahaemolyticus AHPND strain D6 (VpD6) was cultured in 200 ml of heart infusion (HI) broth at 30 °C for 16-20 h with aggregation. Cells and debris were removed by centrifugation at 6,000 rpm for 10 min and supernatant was filtrated through 0.22 µm filter (Merck Millipore Ltd.). Vp_PirAB-like toxin was precipitated from cell-free supernatant with 60-65% ammonium sulfate (modified from Sirikharin *et al*. 2015). The precipitated proteins were suspended in PBS and dialyzed against cold-PBS at 4 °C for at least 16 h with one replacement of PBS. The protein concentration was measured using Qubit™ Protein Assay Kit

(ThermoFisher Scientific, United states). In addition, the cell-free supernatant protein of *V. parahaemolyticus* non-AHPND strain N7 (VpN7) was prepared for using as control.

2.7.2) SDS-PAGE and Western blot analysis of partially purified Vp_PirAB-like toxin

The expression of Vp_PirA- and B-like toxins in partially purified toxin from VpD6 and cell-free supernatant of VpN7 were examined by SDS-PAGE and Western blot analysis. The protein samples were applied to 15% SDS-PAGE gel. One set of electrophoresed gel was stained in CGP staining and another gel was continually done the Western blot analysis. Proteins were transferred onto 0.2 µm PVDF membrane (Atto, Japan), blocked with blocking buffer for 1 h, incubated with anti-Vp_PirA-like or anti-Vp_PirB-like rabbit antiserum as primary antibodies (1:10,000 dilution in blocking buffer) for 1 h, washed 4 times with TBS-t, incubated with goat anti-rabbit IgG (Fc) AP conjugate (Promega, Japan) for 30 min as secondary antibody (1:5,000 dilution in blocking buffer), washed 3 times with TBS-t and stained with BCIP/NBT alkaline phosphatase substrate (Sigma-Aldrich, Japan) to visualize the antibody reactions.

2.8 Bacterial polysaccharides binding of rALF AV-R

Bacterial polysaccharides, including lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (SIGMA, Germany) and peptidoglycan (PGN) derived from *Bifidobacterium thermophilum* were used in enzyme-linked immunosorbent assay (ELISA). LPS and PGN were dissolved in phosphate-buffered saline (PBS) at a concentration of 50 µg/ml. One hundred µl (5 µg) of polysaccharide solution was used to coat each well of a Clear Flat-Bottom Immuno Nonsterile 96-Well Plate (Thermo Scientific, USA). The plate was incubated at 4 °C for 12-16 h, washed with 100 µl of PBS with 0.5% tween 20 (washing buffer) three times, and blocked with 1% bovine serum albumin (BSA) in PBS (blocking buffer) at room temperature (RT) for 2 h. The rALF AV-R or rGFP (control) was diluted with PBS to four different concentrations

(2.5, 5, 10, and 20 µg/ml). After blocking, the plate was washed three times, incubated with 100 µl of diluted recombinants (0.25, 0.5, 1, and 2 µg) at RT for 1 h, washed three times, incubated with 100 µl of monoclonal anti-V5 antibody (1:5000 dilution in PBS) at RT for 1 h, washed three times, incubated with 100 µl of anti-mouse IgG (H+L), AP conjugate at RT for 1 h, washed three times, mixed with 100 µl of phosphatase substrate solution (Sigma-Aldrich, Japan), incubated in the dark for 15 min, mixed with 50 µl of 3 N NaOH solution to stop reaction. The absorbance was read at 405 nm. The average test values were deducted with average value of negative control wells, which coated with PBS.

2.9 Bacterial binding activity of rALF AV-R

Four bacterial species including two Gram-negative bacteria (VpD6 and *E. coli*) and two Gram-positive bacteria (*Streptococcus agalactiae* and *Bacillus amyloliquefaciens*) were used in the ELISAs. *V. parahaemolyticus* was cultured in HI broth at 30 °C, *S. agalactiae* was cultured in Todd Hewitt broth at 37 °C, and *E. coli* and *B. amyloliquefaciens* was cultured in LB broth at 37 °C for 12-16 h. The cells were pelleted and resuspended in 1xPBS. The ELISA 96-well plate was coated with 100 µl of bacterial suspension (10^7 to 10^8 CFU/ml), incubated at 4 °C for 12-16 h and blocked with blocking buffer at RT. rALF AV-R or rGFP was then added to each well as described in bacterial polysaccharides binding assay.

2.10 Binding property of rALF AV-R and Vp_PirAB-like toxin

2.10.1) ELISA

Recombinant Vp_PirA-like and Vp_PirB-like proteins were prepared as previously described [Tinwongger *et al*., 2016]. Two µg of recombinant toxin was coated on an ELISA 96-well plate. The plate was incubated at 4 °C for 12-16 h and blocked with blocking buffer. rALF AV-R or rGFP was added to each well to determine the binding as described above. In addition, twenty-five µg of cell-free supernatant (suspended in PBS) of VpD6 was used in the

binding assay with rALF AV-R, whereas, the cell-free supernatant protein of VpN7 was used as control.

2.10.2) Far-Western blotting

The cell extract or cell-free supernatant of VpD6 and VpN7 and recombinant Vp_PirA- and B-like proteins were used as bait protein. The cell extract was prepared by sonication in cold 1xPBS. The soluble fraction was filtered through a 0.22 µm filter (Merck Millipore Ltd., Ireland) and its concentration was measured with a Qubit™ Protein Assay Kit (ThermoFisher Scientific, USA). Six µg of cell extract, 12 µg of supernatant, and 500 ng of recombinant toxin proteins were separated by 18% SDS-PAGE and transferred onto PVDF membranes. In addition, 6 µg of BSA was loaded as a negative control. The blotted membranes were blocked and probed with rALF AV-R or rGFP (control) for 1 h. The binding complex was detected with V5 tag antibody and anti-mouse IgG (H+L), AP conjugate as primary and secondary antibodies, respectively. The interaction was visualized by adding BCIP/NBT alkaline phosphatase substrate.

A Western blot analysis was performed to detect the Vp_PirA- and B-like proteins in the samples. Specific antisera against Vp_PirA- and B-like peptides [Tinwongger *et al*., 2016] were used as primary antibodies (1:5000), followed by anti-rabbit IgG (Fc), AP conjugate (Promega, Japan) as secondary antibody and BCIP/NBT alkaline phosphatase substrate.

2.11 Bactericidal activity of rALF AV-R

A single colony of *V. parahaemolyticus* strain D6 was cultured overnight in HI broth (3% NaCl). The cell culture was diluted with HI broth to 1/100 dilution. The diluted bacterial was mixed with melted HI top agar, poured on HI agar plate, and left at RT to solidify. To test bactericidal activity, 25 µg of rALF AV-R was dropped on sterile filter paper which is put on top of solidified top agar. Plate was inverted and incubated overnight at 37 °C. An equal

concentration of rGFP and kanamycin were used as negative control and positive control, respectively.

2.12 Oral administration of rALF AV-R and challenge test

The rALF AV-R supplemented diet was prepared by mixing recombinant protein (suspended in PBS) in commercial feed powder (about 65 µg/g). The mixture was pelleted, and the pellets were dried at 60 °C for 2-3 h. rGFP- and PBS-supplemented diets were also prepared for controls.

In addition, the degradation of rALF AV-R while drying pellets at 60 °C was determined. An equal volume of rALF AV-R protein (suspended in PBS) was incubated at 60 °C or 4 °C for 3 h and used in ELISA binding with bacterial cells of VpD6. The binding assay was performed as described above.

For challenge test, *L. vannamei* (body weight 2-3 g) were reared in three 50-L tanks (50 shrimps each) with artificial seawater (28-32 ppt.) at 28 °C. Shrimp were fed the diets at a rate of 10 µg/shrimp/day (corresponding to 5% of body weight per day). After three days of feeding, shrimp from each group were divided into two groups (18 shrimps each) for immersion challenge with VpD6 at final concentrations of $2.5x10^5$ and $5x10^5$ CFU/ml. The survival rates were observed at 12, 24, 36, 48, 60 and 72 h post challenge. During the challenge tests, shrimp were continually fed the supplemented diets until the end of the experiment.

2.13 Statistical analysis

mRNA expressions (log-values) as determined by qPCR were analyzed by one-way ANOVA with Dunnett's multiple comparison test at a P value < 0.05. The survival rate was analyzed using the Kaplan-Meier method and compared using the log-rank test at a P value < 0.05. Statistical analyses and graphs were performed using GraphPad Prism 7.04 (GraphPad Software Inc., USA).

Table 1. List of primers used in this study.

Target gene		Sequences (5′-3′)	Length (bp)
ALF AV-R	Fw	TGACAAGCCTGGTGGTGGC	19
	Rv	GTGTCCTGGCTTCCCCTC	18
EF-1α	Fw	TGGCTACTCACCTGTGCTTG	20
	Rv	CCAGCTCCTTACCAGTACGC	20
rALF AV-R/no SP	Fw	GCGGCCGCGTTCAGCCACCGCTTAG	25
	Rv	GAATTCGCAAGGATGGCAGGCTGTGG	26

3. Results

3.1 Sequence analysis

*Lv*ALF AV-R gene consists of 369 bp, which be translated to 122 amino acid residues. The predicted molecular mass of *Lv*ALF AV-R protein is 13.75 kDa and theoretical pl is 10.03. The predicted signal peptide sequence is located in the first 25 nucleotide bases which is underlined by single line in Figure 1. A mature peptide size is about 11 kDa. The predicted 3D structure model of *Lv*ALF AV-R peptide was shown as ribbon diagrams in Figure 2.

3.2 Expression of *Lv*ALF AV-R mRNA

In healthy *L. vannamei*, *Lv*ALF AV-R was highly expressed in hemocytes, heart and lymphoid organ but very weakly expressed in nerve, muscle, eye, and hepatopancreas (Figure 3A). In *L. vannamei* immersed with VpD6, *Lv*ALF AV-R mRNA in the hepatopancreas was increased at 3 d post immersion. After shrimp were fed FKC-N7 supplemented diet, an expression of *Lv*ALF AV-R slightly increased from 6 h to 7 d. Nevertheless, the two treated groups did not significantly differ from the control in all sampling times (Figure 3B).

On the other hand, mRNA expression of *Lv*ALF AV-R was highly expressed in the hepatopancreas of shrimp fed FKC-VpD6 at 6 h and 24 h (Figure 3C), but an expression was only significant difference at 6 h (compared to control). All treated shrimp died after 24 h of feeding.

FKC-VpD6 feeding was repeated in the second, third, and fourth trials to obtain AHPND-toxin resistant shrimp. After one week feeding, 16 shrimps (3.2%) survived in the second trial, 5 shrimps (1%) survived in the third trial, and 32 shrimps (6.4%) survived in the fourth trial. All surviving shrimp were collected from the second to fourth trials and named as sur-FKC_2, sur-FKC_3, and sur-FKC_4, respectively. In case of sur-FKC_4, mRNA expression was examined in 24 of 32 shrimps. *Lv*ALF AV-R mRNA was highly expressed in

the hepatopancreas of surviving shrimp in each of all trials. Its expression in the surviving shrimp was also significantly higher than it was in the control and Vp-inf shrimp (Figure 3D).

3.3 Expression of *Lv*ALF AV-R native protein

The SDS-PAGE gel stained with CGP showed that total protein of *L. vannamei* in each tissue was applied with similar concentrations (Figure 4A). Western blot analysis showed the detection of ALF AV-R native protein at predicted size of 11 kDa in hemocytes, whereas in the other 5 tissues (lymphoid organ, hepatopancreas, intestine, nerve, and eye) showed the detection signal at size about 10 kDa (Figure 4B). The positive control rALF AV-R was detected at predicted size of 16.4 kDa. On the other hand, using peptide blocking to inhibit the binding of antibody and target protein showed that only a detection signal in hemocytes was disappeared as the positive control (rALF), while the signals in other tissues were remained (Figure 4C). These results suggest that ALF AV-R antiserum could detect ALF AV-R protein in hemocytes only, whereas the detection in other tissues were false-positive detection.

3.4 Histological changes in the hepatopancreas

The hepatopancreas of *L. vannamei* after being fed with FKC-VpD6 for 24 h (Figure 5b), 48 h (Figure 5c), and 72 h (Figure 5d) showed signs of AHPND such as sloughing of tubule epithelial cells, hemocytic infiltration surrounding the hepatopancreas tubules, and enlargement of some nuclei in hepatopancreas tubule epithelial cells. One of the four surviving shrimps examined showed some hemocytic infiltration and enlarged nuclei, but without the sloughing of epithelial tubule cells (Figure 5e). The hepatopancreases of the other three did not show any signs of AHPND (Figure 5f-5h) and were similar to the hepatopancreas in a non-treated shrimp (Figure 5a).

3.5 Detection of *Lv*ALF AV-R protein in *L. vannamei* hepatopancreas

Immunohistochemical analysis revealed that positive signal detection (brown color) of *Lv*ALF AV-R protein was observed in the hepatopancreas of both normal shrimp and sur-FKC shrimp. In normal shrimp, signals were presented in tubular lumen (TB) of the hepatopancreas as secreted granular material (Figure 6A). For two surviving shrimps of the fourth trial (sur-FKC_4), signals were observed surrounding hemocytes (he) and F-cells (F) in sur-FKC_4-1 (Fig. 6B), whereas, sur-FKC_4-2 was observed in B-cells (B) and intertubular space (IS) (Figure 6C). On the other hand, the positive signal did not exhibit in the negative controls (Figure 6 D-F).

3.6 Purified recombinant protein expression

Recombinant r*Lv*ALF AV-R was successfully expressed in insect cells. rALF AV-R is composed of 163 amino acid residues with addition of a V5 epitope and His-tagged at the C-terminus. Purified rALF AV-R and rGFP were observed in a gel at the predicted sizes of 16.4 and 33.7 kDa, respectively (Figure 7A). A Western blot analysis using an anti-V5 monoclonal antibody detected both recombinant proteins at the predicted size (Figure 7B).

3.7 Expression of native Vp_PirA- and B-like toxins

The native Vp_PirA- and B-like proteins (toxin A and toxin B) were isolated from culture supernatant of VpD6. The Vp_PirA- and B-like proteins were observed in CGP stained gel at predicted sizes of 13 kDa and 50 kDa, respectively (Figure 8A). The Vp_PirA- and B-like proteins were detected with specific polyclonal antibodies in AHPND strain D6 only, which E1 strain showed higher expression of toxin than D6 strain (Figure 8B).

3.8 Binding activities of rALF AV-R

LPS and PGN were used as the representatives of cell wall components of Gram-negative and Gram-positive bacteria, respectively. In ELISAs, rALF AV-R bound to LPS and PGN (Figure 9A) and Gram-negative bacteria (VpD6 and *E. coli*) (Figure 9B). With respect to Gram-positive bacteria, rALF AV-R bound to *B. amyloliquefaciens*, but not *S. agalactiae*, while rGFP also bound to *B. amyloliquefaciens* but only at the highest concentration (Figure 9C).

3.9 Interaction of rALF AV-R and Vp_PirAB-like toxin

In ELISAs, rALF AV-R strongly bound to Vp_PirB-like recombinant protein (toxB), but not to native toxin in the VpD6 supernatant or Vp_PirA-like recombinant (toxA) (Figure 10A). rGFP also bound to Vp_PirB-like recombinant protein (Figure 10A).

Far-Western blot analysis was performed to confirm the interaction of rALF AV-R and native Vp_PirAB-like toxin. In the left panel of Figure 11B, rALF AV-R-binding proteins were detected, either in VpD6 or VpN7 at approximately size of 10 kDa in the cell extract and 10-15 kDa in the supernatant. In the right panel of Figure 11B, rGFP-binding proteins were detected in Vp_PirA-like recombinant (toxA) at size of 15 kDa and in Vp_PirB-like recombinant (toxB) at size of 50 kDa.

3.10 Bactericidal activity of rALF AV-R

rALF AV-R did not exhibit the bactericidal activity to the VpD6 strain or no clear zone was observed around the filter paper (Figure 11A), as the result of negative control, rGFP (Figure 11B). Unlike using of an antibiotic (kanamycin), the clear zone was observed around the filter paper (Figure 11C).

3.11 *In vivo* effect of *Lv*ALF AV-R recombinant protein

ELISA binding of rALF AV-R and bacterial cells of VpD6 showed that the binding ability of 60 °C - rALF AV-R was reduced about 40%, compared with recombinant that kept at 4 °C (Figure 12A).

L. vannamei treated with rALF AV-R, rGFP, and PBS supplemented diets were challenged with VpD6-immersion at final concentrations of $2.5x10^5$ and $5x10^5$ CFU/ml. The survival rate of shrimp treated with rALF AV-R was not significantly different from that of shrimp treated with PBS or rGFP groups at 72 h post challenge with either dose (Figure 12B, C). However, the survival rate of the rALF AV-R group was higher than that of PBS group at most other observation times for both challenges.

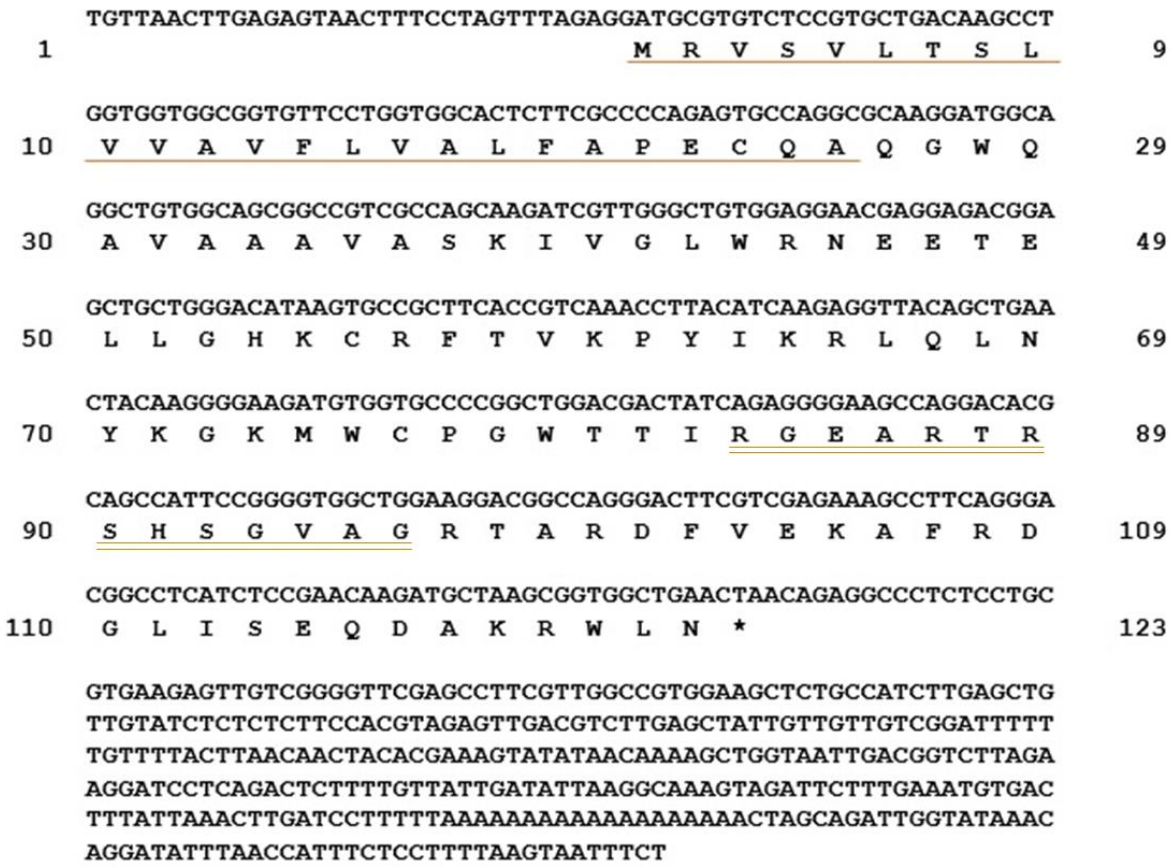

Figure 1. Nucleotide and deduced amino acid sequences of *Lv*ALF AV-R gene. Single line is the predicted signal peptide sequence. Double line is the selected peptide sequences for antibody generation.

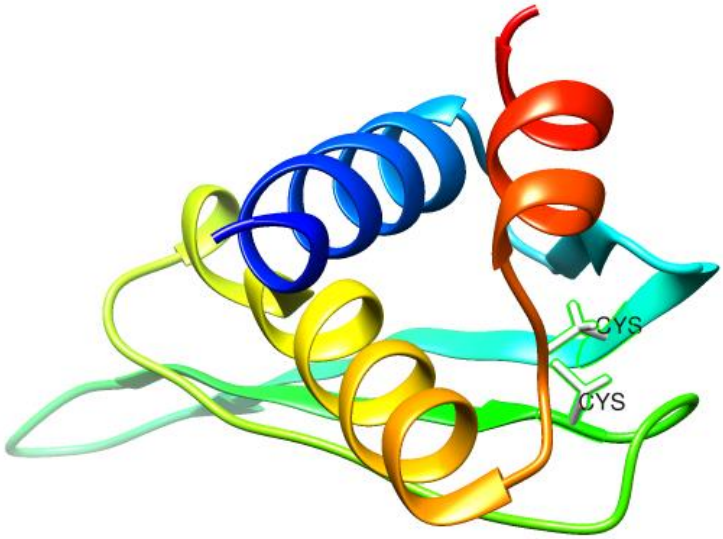

Figure 2. The 3D structure prediction of *Lv*ALF AV-R mature peptide. Spring-shaped ribbons represent the alpha helices and flat arrow represent the beta strands. The position of cystine (Cys) was labeled (Cys 31 and Cys 51).

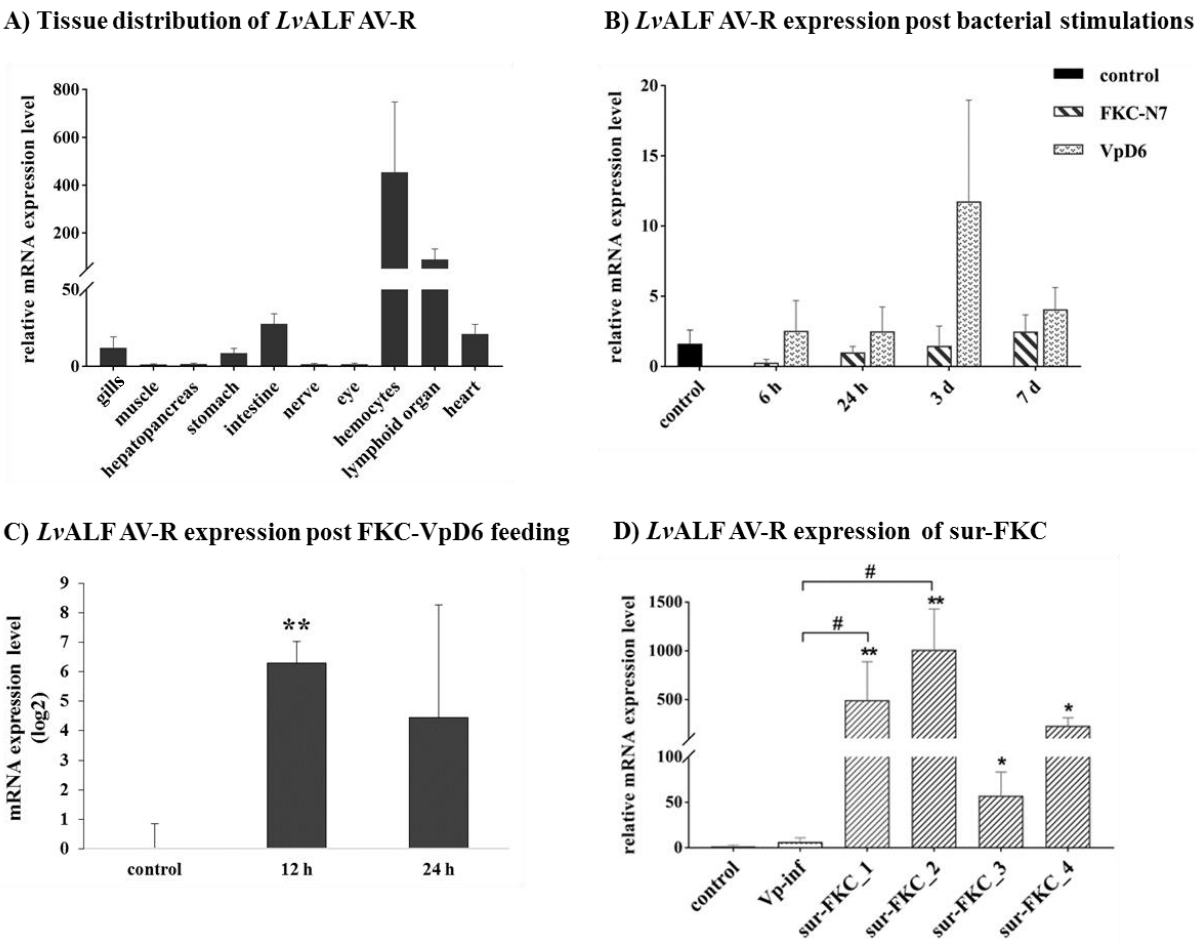

Figure 3. The mRNA expression of *Lv*ALF AV-R in *L. vannamei* hepatopancreas measured by qPCR. **A)** Expression in different tissues of healthy shrimp (N=3) normalized to expression in the nerve. **B)** Expression after feeding with FKC-VpN7 (N=3) and immersion in VpD6 (N=3). **C)** Expression after feeding with FKC-VpD6 (N=3) **D)** Expression in surviving shrimps after feeding with FKC-VpD6 from three trials of sur-FKC_1 (N=4), sur-FKC_2 (N=16), sur-FKC_3 (N=5), and sur-FKC_4 (N=24), compared with control (* significant difference) and Vp-inf (# significant difference). Data are presented as mean and vertical bars represent ± SEM. Statistical analysis was analyzed by one-way ANOVA with Dunnett's multiple comparison test at *P* value < 0.05.

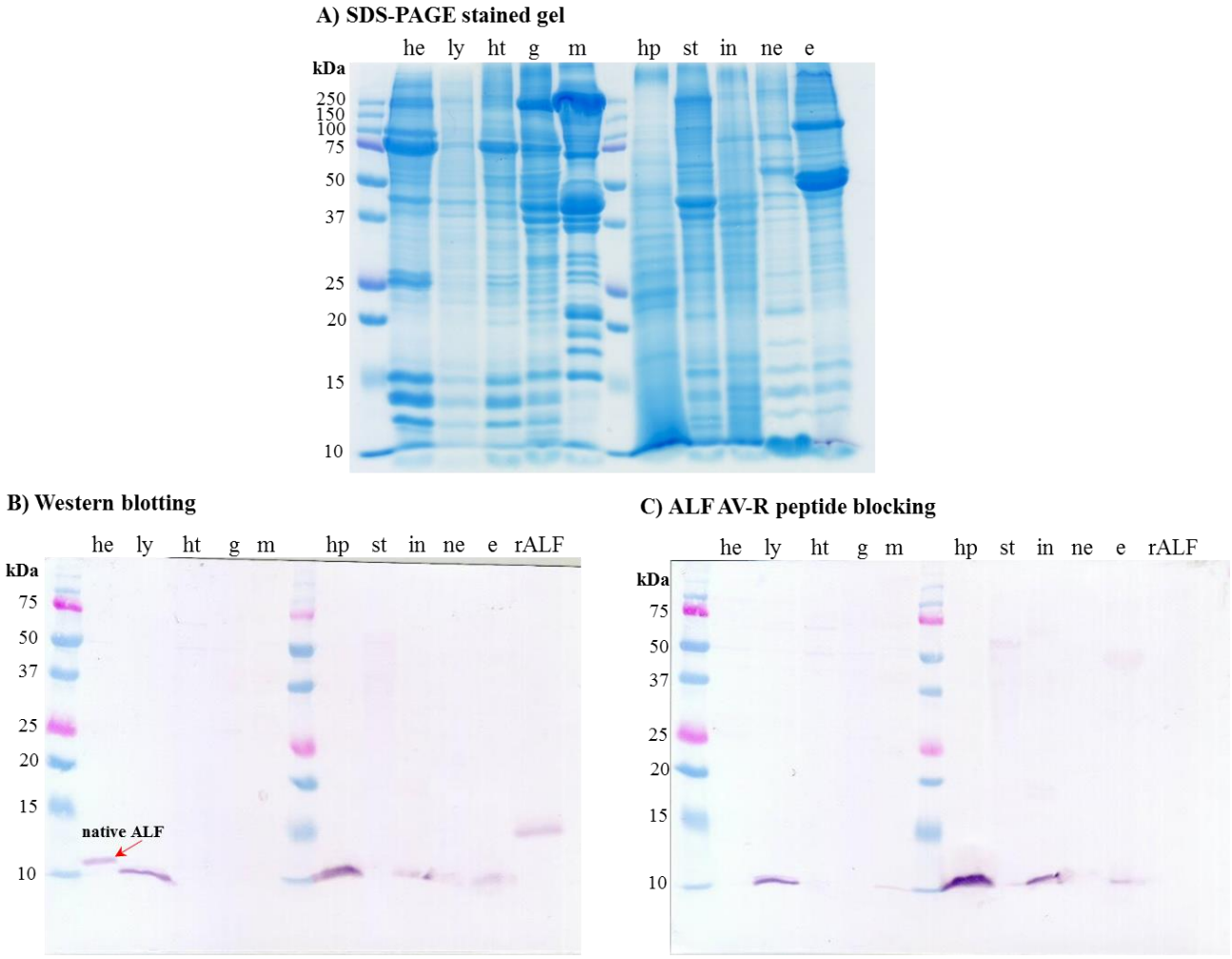

Figure 4. *Lv*ALF AV-R protein expression in *L. vannamei* tissues. Ten tissues were used including hemocytes (he), lymphoid organ (ly), heart (ht), gills (g), muscle (m), hepatopancreas (hp), stomach (st), intestine (in), nerve (ne), and eye (e). **A)** CGP-stained gel of total proteins from shrimp tissues. **B)** Western blotting analysis of *Lv*ALF AV-R native protein. The primary antibody was ALF AV-R peptide antiserum. **C)** Peptide blocking assay of ALF AV-R antiserum. The primary antibody was ALF AV-R antiserum pre-incubated with ALF AV-R peptides. rALF AV-R recombinant protein (rALF) was used as positive control.

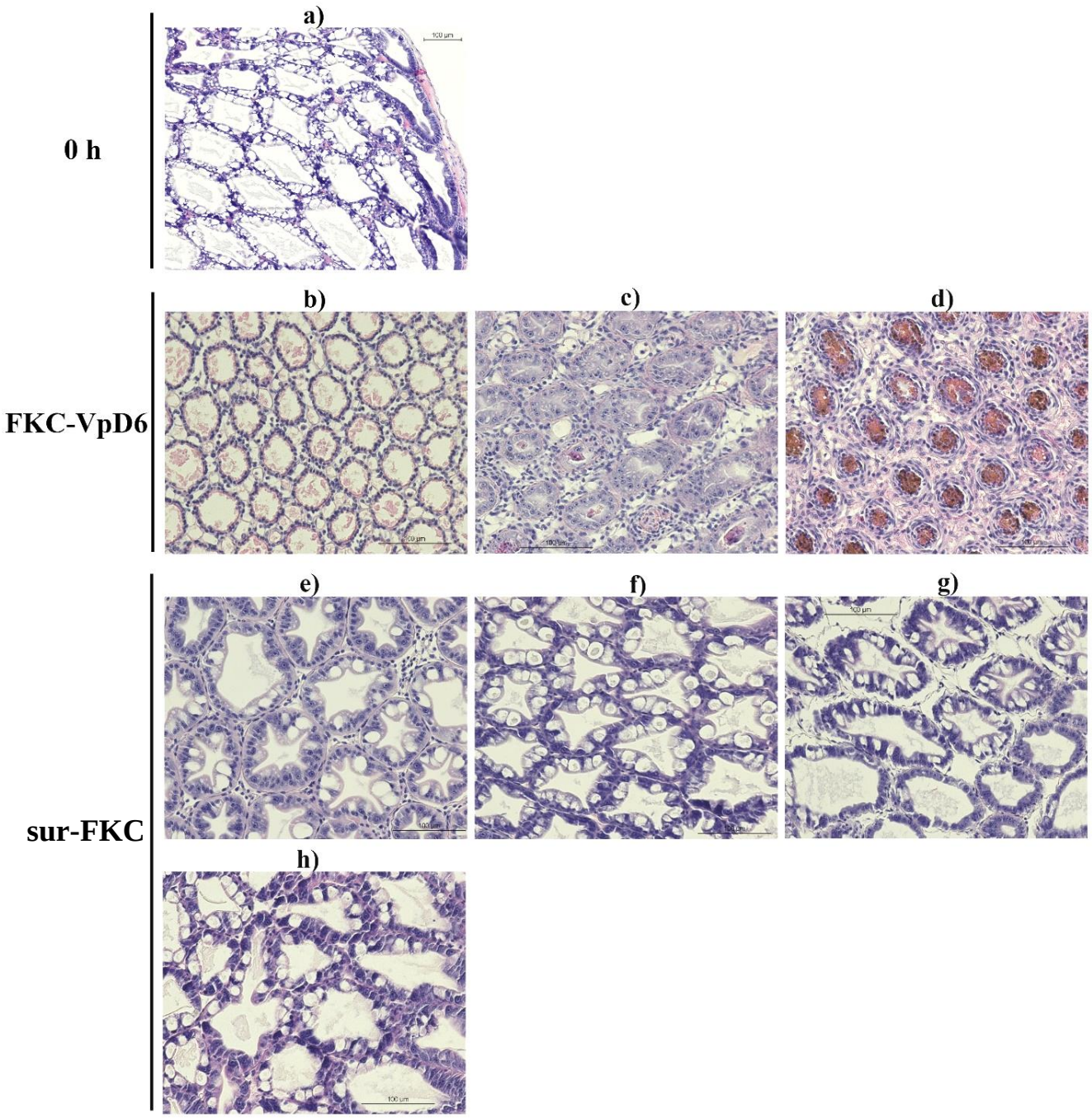

Figure 5. Histological sections of *L. vannamei* hepatopancreas with H&E staining. **a)** Normal shrimp without treatment. **b-d)** Shrimp after feeding with FKC-VpD6 for 24 h, 48 h, and 72 h, respectively. **e-h)** Four individual surviving shrimps after one week feeding with FKC-VpD6. Scale bars = 100 µm.

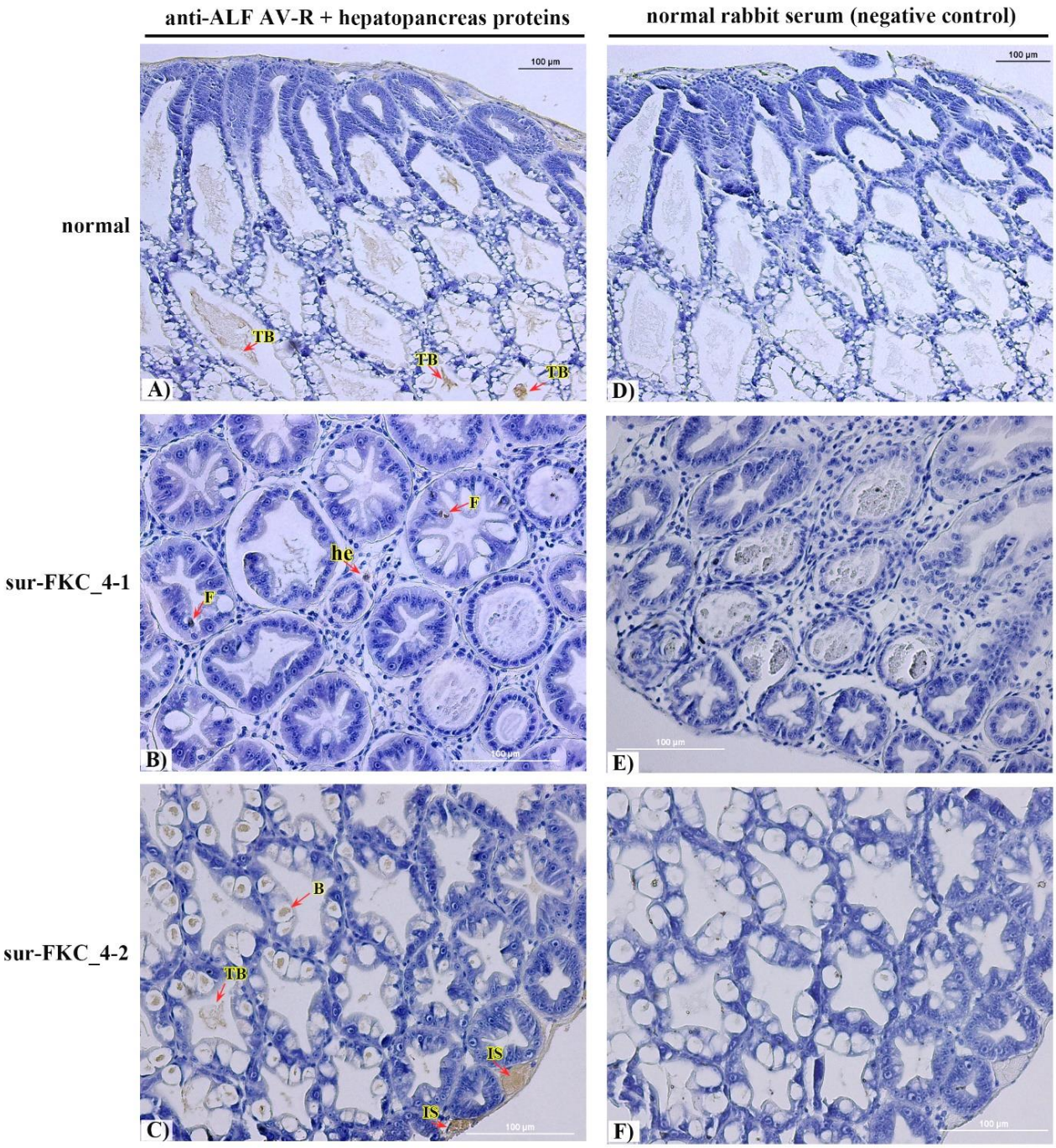

Figure 6. Immunohistochemistry detection of *Lv*ALF AV-R protein in the hepatopancreas of *L. vannamei*. The hepatopancreas sections of one normal shrimp and two resistant shrimps (sur-FKC_ 4-1 and -2) were incubated with anti-ALF AV-R peptide antiserum (**A-C**) or normal rabbit serum (**D-F**). Red arrows represent some positive signal detections (brown color) in tubule lumen (TB), F-cells (F), B-cells (B), intertubular space (IS), and hemocytes (he).

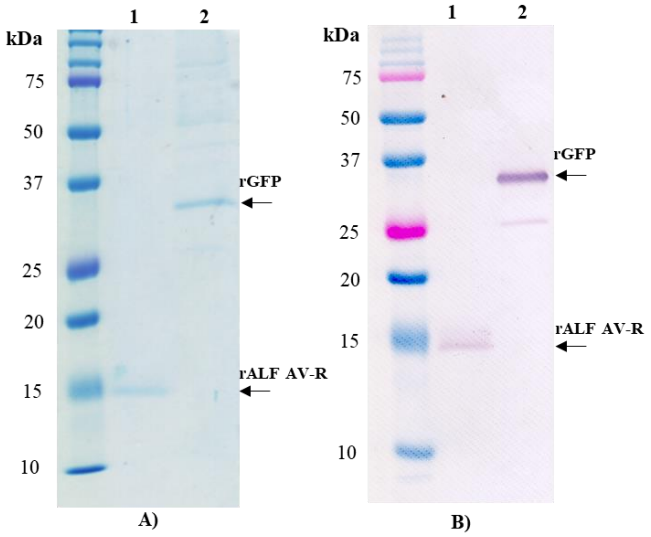

Figure 7. Expression of purified recombinant proteins rALF AV-R (lane 1) and rGFP (lane 2). **A)** CGP-stained gel and **B)** Western blot analysis using anti-V5 monoclonal antibody.

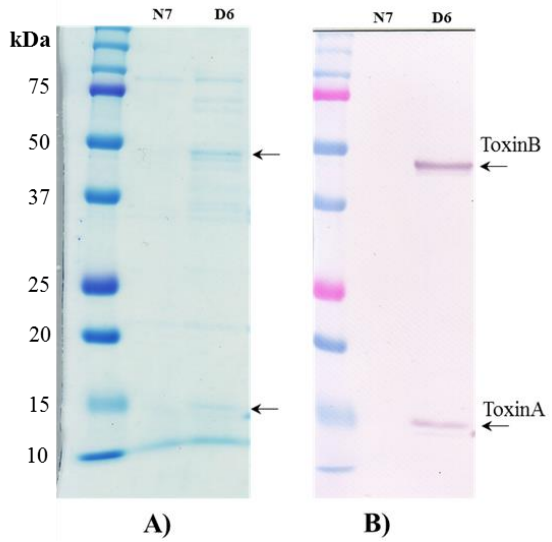

Figure 8. Expression of partially purified native Vp_PirA- and B-like proteins. **A)** CGP stained gel of cell-free supernatant of VpN7 and VpD6. **B)** Western blot analysis using specific polyclonal antibodies against Vp_PirA- and B-like toxin.

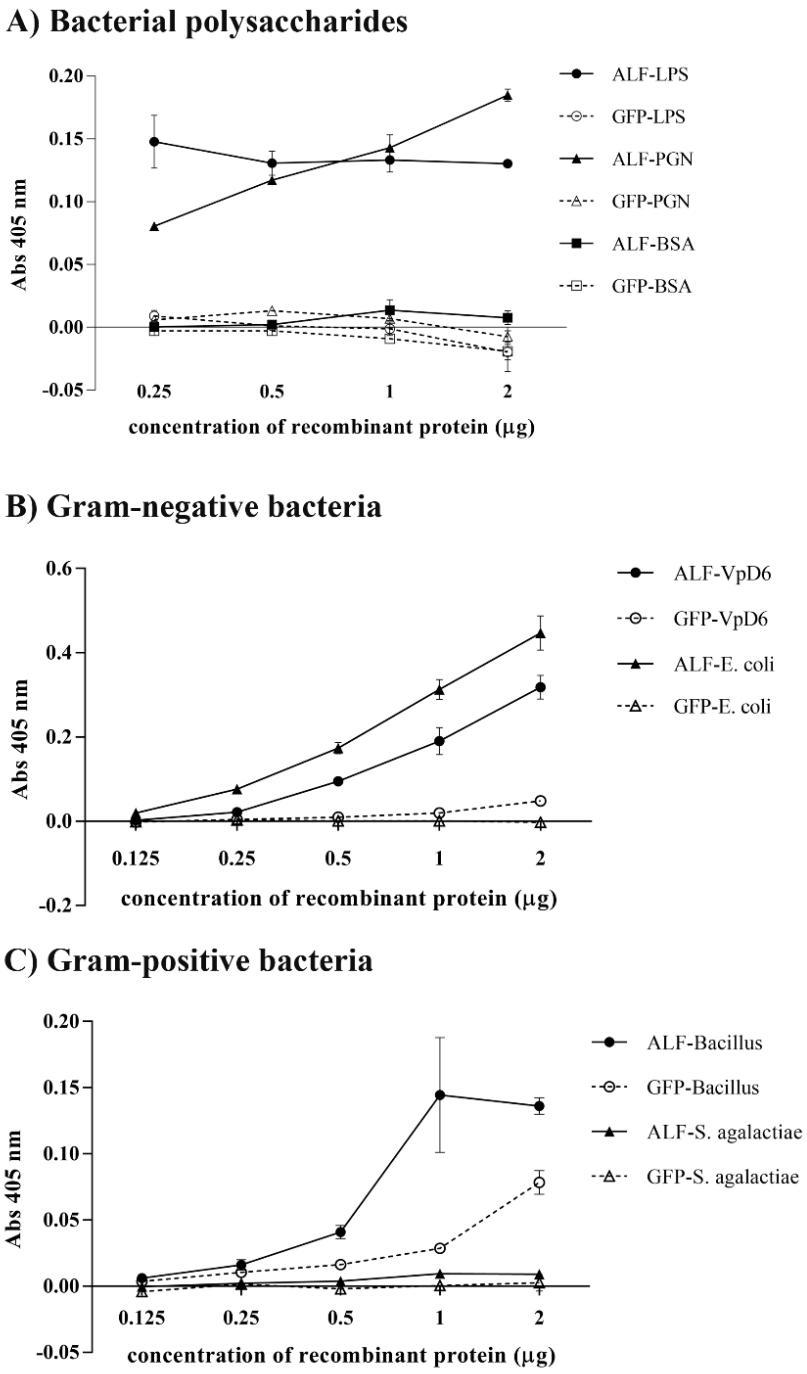

Figure 9. Binding activity of rALF AV-R to bacterial components. **A)** LPS and PGN polysaccharides. **B)** Gram-negative bacteria. **C)** Gram-positive bacteria. The GFP recombinant protein (rGFP) was added in all experiments for using as negative recombinant protein. These data represent the mean ± SEM of at least two independent experiments.

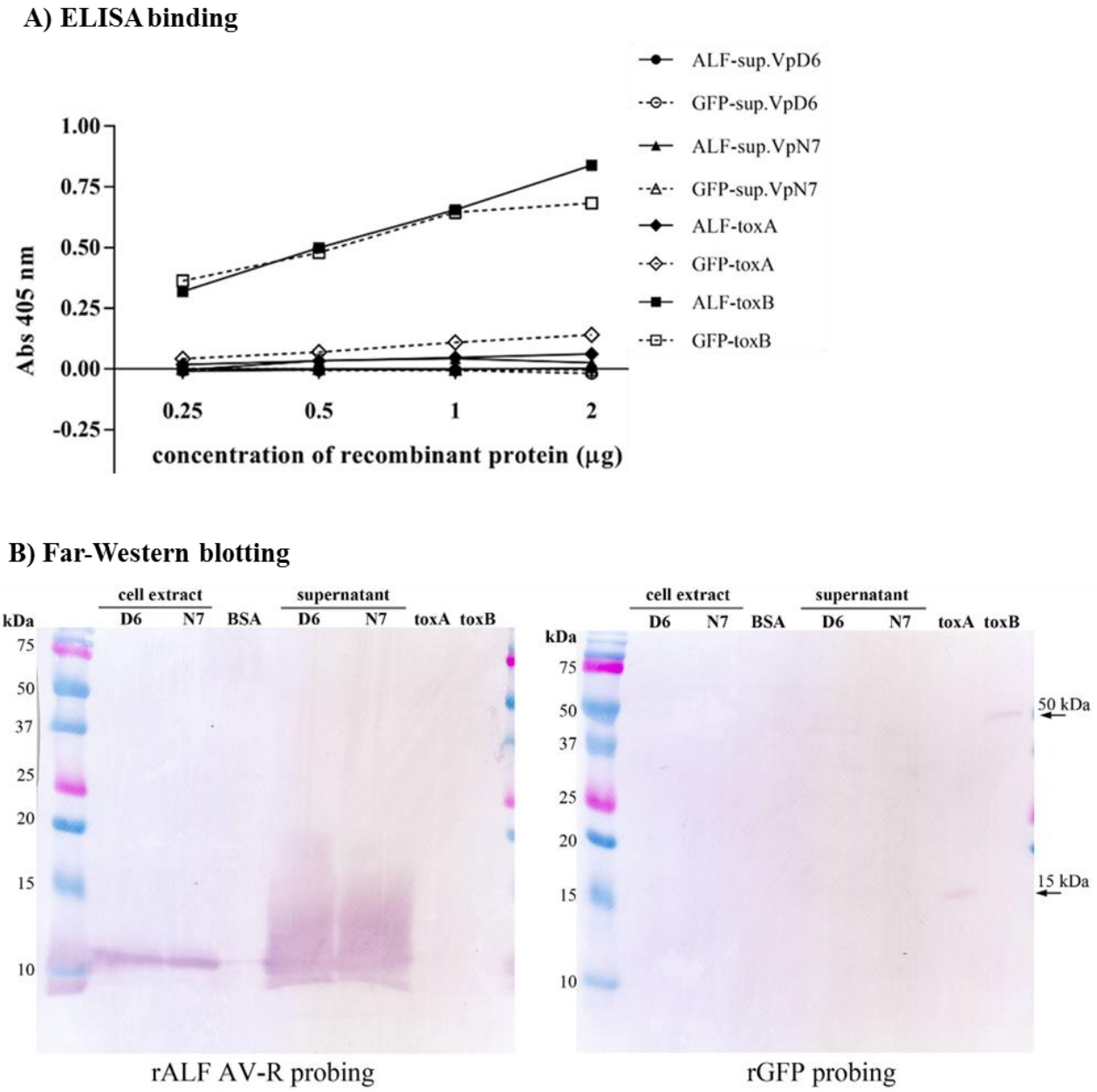

Figure 10. The interaction of rALF AV-R and the Vp_PirAB-like toxin. **A)** ELISA binding assay of native toxin from VpD6's supernatant (sup.VpD6) and the Vp_PirA- and B-like toxin recombinants (toxA and toxB), whereas the supernatant protein of VpN7 (sup.VpN7) was used as negative supernatant protein. These data represent the mean ± SEM of at least two independent experiments. **B)** Far-Western blot analysis of cell extract and supernatant from VpD6 or VpN7, and recombinant Vp_PirA- and -B-like (toxA and toxB), probed with rALF AV-R or rGFP. BSA was loaded as negative control.

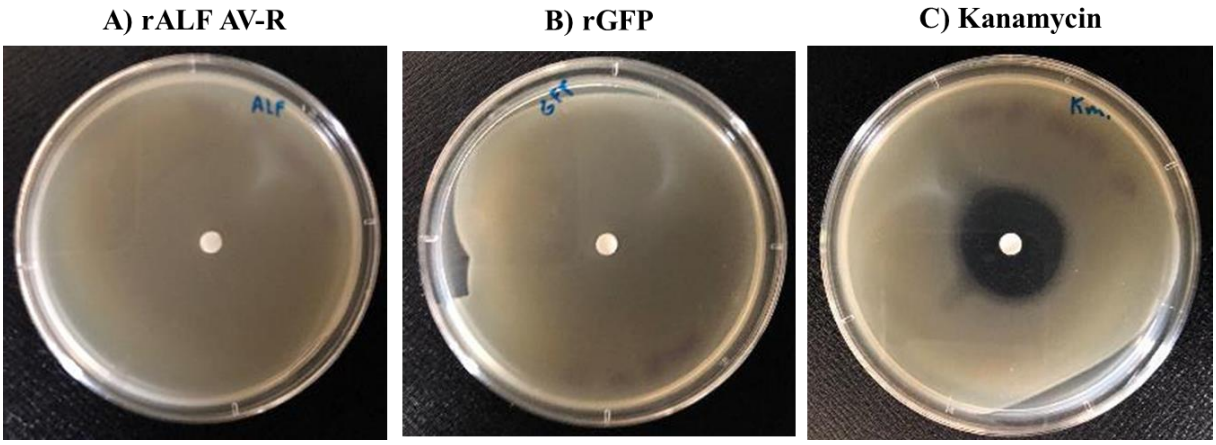

Figure 11. Bactericidal activity of rALF AV-R to *V. parahaemolyticus* strain D6. The filter paper was treated with 25 µg of **A)** rALF AV-R, **B)** rGFP (negative control), and **C)** Kanamycin (positive control).

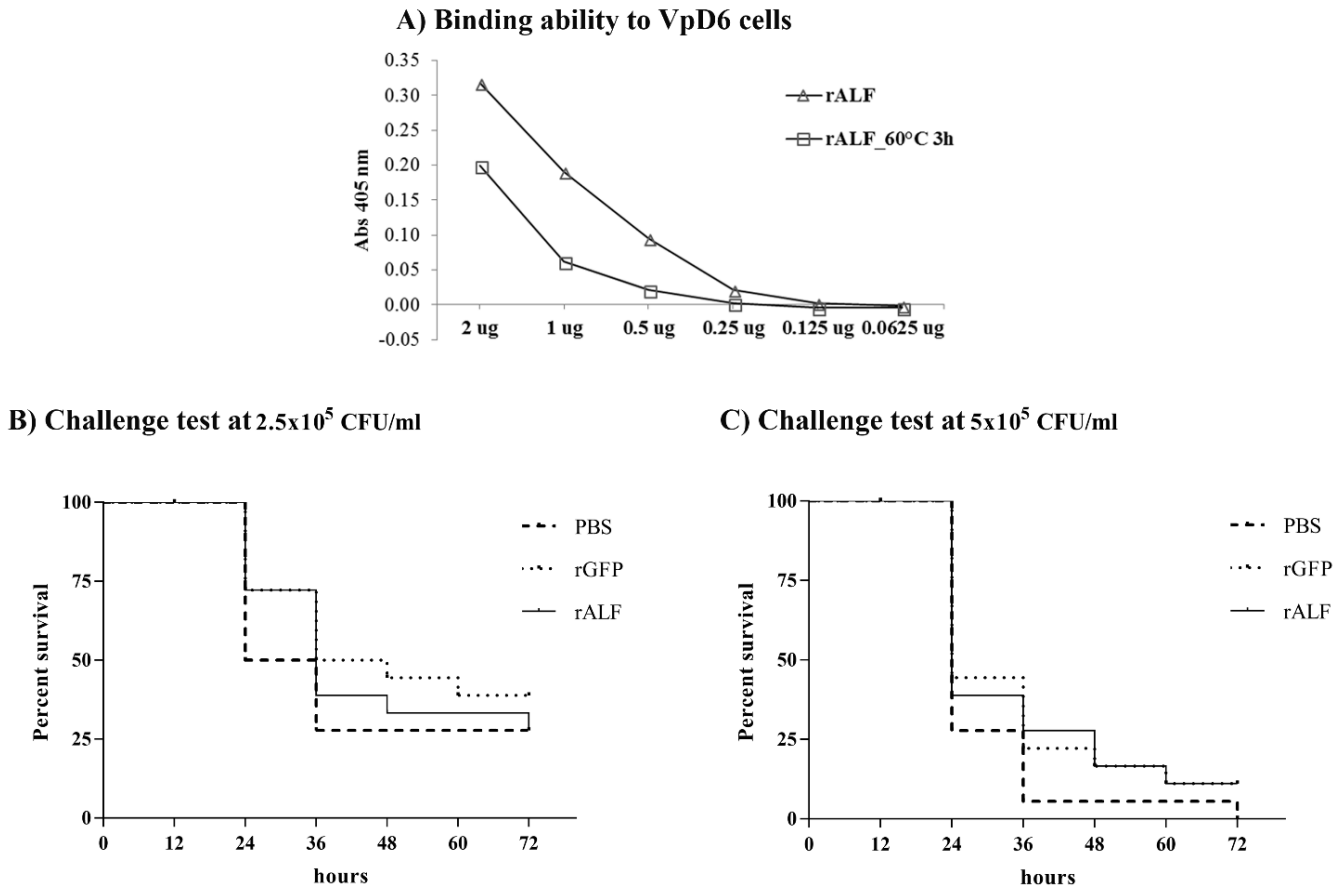

Figure 12. The effect of heat incubation to rALF AV-R and challenge tests of *L. vannamei* treated with rALF AV-R. **A)** Bacterial binding ability of rALF AV-R post incubation at 60 °C (rALF_60 °C 3h), compared with control (rALF) which stored at 4 °C. **B)** and **C)** Survival rates of rALF AV-R treated-*L. vannamei* (rALF) challenged with two doses of VpD6 at $2.5x10^5$ CFU/ml and $5x10^5$ CFU/ml, respectively. rGFP and PBS treated shrimp were used as control groups.

4. Discussion

ALFs have been broadly characterized in the penaeid shrimp because of an advantage in the potent antimicrobial activities. Especially, ALF*Pm*3 has been greatly studied and indicated its potential use in therapeutic and pharmaceutical applications in aquaculture [Pornprateep *et al*., 2012; Methatham *et al*., 2017].

The homologous transcript of *Lv*ALF AV-R that identified from the hepatopancreas/ stomach transcriptome in this study, does not show 100% identity of nucleotide sequence to the previously identified (accession number DQ208702) by Jimenez-Vega and Vargas-Albores (2007), although their amino acid sequences are identical. Nucleotide sequence and amino acid sequence of *Lv*ALF AV-R show very high identity (99%) to other three isoforms (*Lv*ALF VV-R, *Lv*ALF AV-K and *Lv*ALF AA-K) with differences of one or two amino acid residues only. It suggests that these four ALF isoforms might be the result of base substitutions in gene-level mutation while replication [Hartl *et al*., 2002].

Of all four trials of Vp_PirAB-like toxin-resistance selection, shrimp survival rates were ranged between 1-6.4%. The used shrimp in each trial were bought from several sources that use different broodstocks and nursery conditions. The qPCR results in the three more trials of Vp_PirAB-like toxin-resistant selection affirmed that *Lv*ALF AV-R was significantly more strongly expressed in the hepatopancreas of surviving *L. vannamei* (Figure 3D). Shrimp was fed FKC-VpD6 diet for 1 week in the second to fourth trials, not 2 weeks as first trial because over 90% of shrimp died in 36 h post feeding, therefore 1 week is suitable to select resistant shrimp.

Transcriptome of mitten crab, *Eriocheir sinensis* showed that ALF was highly expressed in the hepatopancreas after injection with three pathogens (*Micrococcus luteus, Vibrio alginolyticus and Pichia pastoris*) [Li *et al*., 2013]. Besides, ALFs of *Macrobrachium rosenbergii* (*MrALF5*, *MrALF6*, and *MrALF7)* were up-regulated in the hepatopancreas after

V. anguillarum and WSSV challenges [Ren *et al*., 2012] and some *Mr*ALF isoforms were up-regulated in response to *V. parahaemolyticus* infection [Rao *et al*., 2015].

To determine whether the high expression of *Lv*ALF AV-R in the hepatopancreas of the resistant shrimp was due to the typical cell components of *V. parahaemolyticus* in the diet, I fed other shrimp with FKC of non-AHPND-causing strain (VpN7). These shrimp did not show a significant increase in the expression of *Lv*ALF AV-R in the hepatopancreas (Figure 3B). Similarly, a previous report showed that inoculation of heat-killed *V. alginolyticus* did not upregulate *Lv*ALF AV-R in shrimp hemocytes as other two isoforms (*Lv*ALF VV-R and *Lv*ALF AA-K) [Jimenez-Vega and Vargas-Albores, 2007]. On the other hand, immersing shrimp in a non-lethal dose of VpD6 for one week, also did not significantly induce the expression of *Lv*ALF AV-R in the hepatopancreas (Figure 3B). These results suggest that upregulation of *Lv*ALF AV-R mRNA expression in the shrimp hepatopancreas is a specific response to the Vp_PirAB-like toxin at a certain amount, which is a lethal dose to juvenile shrimp.

Generally, invertebrate's AMPs are constitutively expressed and stored in circulating hemocytes [Bachère *et al.*, 2004; Iwanaga and Lee, 2005]. In the apparently healthy *L. vannamei*, *Lv*ALF AV-R mRNA was highly expressed in hemocytes but weakly expressed in the hepatopancreas (Fig. 3A), in agreement with previous reports [Mekata *et al*., 2010; Zhan *et al*., 2015; Jiang *et al*., 2015]. Expression of *Lv*ALF AV-R transcript has been observed in hemocytes of Vp_{AHPND} toxin (Vp_PirAB-like toxin) challenged shrimp which showed upregulation after toxin injection [Maralit *et al*., 2018]. Histological sections of FKC-VpD6-fed shrimps showed that epithelial cells were infiltrated with hemocytes surrounding the hepatopancreas tubules (Figure 5b-d), raising the possibility that these hemocytes may have increased *Lv*ALF AV-R expression. However, this seems to be unlikely in the case of the resistant shrimp, whose hepatopancreases appeared normal, without hemocytic infiltration

(Figure 5g-h). This result indicates that the high expression of *Lv*ALF AV-R in the hepatopancreas of resistant shrimp is barely correlated with the hemocytes infiltration.

Expression of *Lv*ALF AV-R protein in shrimp tissues were determined using Western blotting. Protein expression of *Lv*ALF AV-R was only observed in hemocytes (Figure 4B), which showed the highest mRNA expression. The polyclonal antibody against *Lv*ALF AV-R peptides used in this study showed false-positive detection in several shrimp tissues. In contrast, the pre-immunized serum did not show any detection signals in all observed tissues (data not shown). The polyclonal antibodies recognizes multiple epitopes of the same antigen, unlike monoclonal antibodies which bind to one unique epitope [Voskuil, 2014]. The *Lv*ALF AV-R peptide antibody may cross-react to similar peptides that located in another shrimp protein. In immunodetection, an expression of *Lv*ALF AV-R protein was detected in the hepatopancreas tubule lumens of normal shrimp, but no signal was observed by Western blotting. Shrimp hepatopancreas is fulling with digestive enzymes that can degrade other proteins while collecting or preparing sample before applying to SDS-PAGE. In the hepatopancreas of sur-FKC_4-2, *Lv*ALF AV-R was observed mostly in the vacuole of B-cells. Hepatopancreas B cell is the primary secretory cell that functions in digestive enzymes production, nutrient accumulation and transport of digested material etc. [Vogt, 1993; Franceschini-Vicentini *et al*., 2009].

The function of *Lv*ALF AV-R protein was examined by producing the recombinant protein for *in vitro* and *in vivo* assays. The recombinant protein of *Lv*ALF AV-R (rALF AV-R) was firstly expressed in *E. coli* cells but the protein expression was very low and could not be purified. *Lv*ALF AV-R is an antimicrobial peptide, its recombinant protein might be toxic to *E. coli* either before or after induction [Rosano and Ceccarelli, 2014].

*Lv*ALF AV-R is classified in the Group B of ALF family, which includes highly cationic peptides. In general, ALFs Group B could neutralize LPS and exhibited more potent antimicrobial activity than those with lower isoelectric point [Rosa *et al*., 2013; Jiang *et al*.,

2015]. Results of ELISAs showed that rALF AV-R bound to LPS and PGN (Figure 9A), which are the major cell wall component of Gram-negative and Gram-positive bacteria, respectively. Moreover, rALF AV-R more strongly bound to Gram-negative bacteria than to Gram-positive bacteria (Figure 9B, C) as expected because it contains an LPS-binding domain. The unexpected finding that rALF AV-R didn't bind to the Gram-positive *S. agalactiae* (Fig. 6C) might be due to the presence of different cell wall components in this species, compared to *Bacillus* spp. Most Gram-positive bacteria incorporate peptidoglycan, anchored with cell-wall glycopolymers, which have highly variable structures among species [Weidenmaier and Peschel, 2008].

The finding that rALF AV-R bound to recombinant Vp_PirB-like toxin but not to native toxin (Figure 10A) is a false-positive result, because recombinant Vp_PirB-like also bound to rGFP (Figure 10A). This was supported by the Far-Western blotting, which showed that rALF AV-R interacted with ~10 kDa *V. parahaemolyticus* proteins but not with the toxin proteins (Figure 10B). These rALF AV-R-binding proteins may be lipopolysaccharide-related proteins that are mainly found in Gram-negative bacteria. Also, in the Far-Western blotting, unlike the ELISA, rALF AV-R did not bind to Vp_PirB-like recombinant protein, while rGFP bound to both Vp_PirA- and B-like recombinant proteins. These results suggest that the recombinant proteins can differ in structures and functions from the native forms [Zhou *et al*., 2016]. Moreover, the structures of Vp_PirA- and B-like toxins under non-denaturing (ELISA) and denaturing (Far-Western) conditions were different and may have affected their interactions.

Previous studies have reported that ALF isoform 3 of *Penaeus monodon* (ALF*Pm*3) [Supungul *et al*., 2017] and a single WAP domain-containing protein of *L. vannamei* (*Lv*SWD) [Visetnan *et al*., 2017], exhibited antimicrobial activities against *V. parahaemolyticus* AHPND-causing strains and promoted survival of *L. vannamei* after infection, but rALF AV-R did not exhibit bactericidal activity. Present study showed that the survival rate of rALF AV-R-fed shrimp after challenge was not significantly different from that of the control groups (Figure

12B, C). However, the mortality of the rALF AV-R-fed shrimp was not as sudden as it was for the PBS group at both doses of VpD6. Role of *Lv*ALF AV-R in shrimp defense mechanism is supported with silencing of *Lv*ALF isoforms significantly increased mortality by VP_{AHPND} toxin injection [Maralit *et al*., 2018]. Similarly, M-ALF of *M. japonicus* did not exhibit direct antimicrobial activity against *V. penaeicida* but mortality was significantly increased in silenced M-ALF shrimp after *V. penaeicida* challenge [Kadowaki *et al*., 2011].

The efficiency of pre-heated rALF AV-R was examined by bacterial binding assay to assess the remained function after supplemented in diet. Pre-heated rALF AV-R showed lower binding efficiency than non-heated protein (Figure 12A), suggesting rALF AV-R was degraded by high temperature incubation, during preparing diet. Administration of recombinant protein to shrimp by oral route, need to be optimized the potential amount because of denaturation and fragmentation by gut environment. Hence, increasing of rALF AV-R concentration in the diet or the feeding time prior to an infection may increase shrimpsurvival. Also, the finding that the survival of rGFP group has higher than that of the PBS group (Figure 12B, C) might be due to non-self molecule recognition either by innate effector molecules or phagocytosis [Medzhitov and Janeway, 2002; Buchmann K., 2014].

In conclusion, these results demonstrate that *Lv*ALF AV-R transcription is upregulated in the hepatopancreas of toxin-resistant *L. vannamei* in response to Vp_PirAB-like toxin. *Lv*ALF AV-R mRNA can be used as a biomarker for selection of *L. vannamei* resistant to AHPND or Vp_PirAB-like toxin in shrimp aquaculture. In addition, *Lv*ALF AV-R protein did not show direct effect against the toxin. It raises the possibility that *Lv*ALF AV-R may not be the only factor involved in the defense against Vp_PirAB-like toxin virulence.

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Chapter 4

Genetic variation of Vp_PirAB-like toxin receptor candidates in resistant *Litopenaeus vannamei*

Abstract

In previous result, a total thirteen homologous genes of Cry toxin receptors were identified from the transcriptome of normal shrimp (control/Vp-inf). These homologous genes were assumed as the Vp_PirAB-like toxin receptor candidates.

In this cahpter, the Vp_PirAB-like toxin receptor candidates were identified from *L. vannamei* sur-FKC transcriptome by using previously identified receptor candidates of normal shrimp as queries in local blasting. Four genes (cadherin-like 1, aminopeptidase N-like 1 and ATP-binding cassette C 2 -like 1 and 2) were selected for pairwise alignment of amino acid sequence. Based on the RNA-seq data, genetic variations between normal and resistant shrimp were detected in all selected genes. Validation of gene variants in cDNAs by direct sequencing showed that only cadherin-like 1 gene showed a nonsynonymous SNP (single nucleotide polymorphism) at locus 4,326 C/A, same as in the RNA-seq result.

The association of SNP 4,326 C/A with Vp_PirAB-like toxin resistance was elucidated by analysis of SNP genotyping in both cDNA and genomic DNA levels. At cDNAs level, the frequency distributions of genotype AA, AC and CC in resistance group were 64%, 36%, and 0%, respectively in resistance group, while those of control/Vp-inf group were 17%, 0%, and 83%, respectively. For genomic DNA level, SNP was genotyped in susceptible and resistance groups. Association analysis indicated that the genotype frequency distribution between two groups was significantly different ($P < 0.05$). In the susceptible group, the AA, AC and CC genotypes were 45%, 34% and 21%, respectively. The frequency distributions in resistance

group were 64%, 36% and 0%, respectively. Interestingly, the genotype CC was not observed in the resistance group.

Lastly, to determine the stability of resistance phenotype, two-rounded selection of toxin resistant shrimp was performed. The first-round survival shrimp after 1-week dietary of FKC-VpD6, was re-fed FKC-VpD6 to observe mortality. The result showed that all first-round surviving shrimp died in 3 days after re-challenging.

These results indicate that mutation of Vp_PirAB-like receptor candidates is not directly related to the resistance mechanism because this resistant characteristic was not permanently expressed. However, the SNP 4,326 C/A in cadherin-like 1 gene might be used as a genetic marker to select AHPND-resistant *L. vannamei*.

1. Introduction

Genetic variation is the difference in DNA sequences between individuals within a population that occurs either in germ cells or somatic cells. Commonly, genetic variation is divided into three main types of single base-pair substitution or known as single nucleotide polymorphism (SNP), insertion or deletion (indel), and structural variation [https://www.ebi.ac.uk/]. Genetic variation can influence the level of gene expression, which may reside within the regulatory sequences, promoters, enhancers, and splice sites etc. [Williams *et al*., 2007; Francesconi and Lehner, 2014; Garieri *et al*., 2017]. On the other hand, the changes of amino acid sequence also influence the shape, function, or binding properties of proteins [Wang and Moult, 2001; Nakagawa *et al*., 2012; Bhattacharya *et al*., 2017]. Mutation is one of the primary sources of genetic variation that correlates with either increasing or decreasing the susceptibility to pathogens. Several studies in human, plants, and aquatic animals have reported the association of genetic variation with resistance to pathogens [Gjedrem and Gjøen, 1995; Gjøen *et al*., 1997; Hofmann *et al*., 1997; Zhang *et al*., 2003; Yáñez *et al*., 2014; Withrock *et al*., 2015; Vandeputte *et al*., 2017; Liao *et al*., 2018].

Vp_PirAB-like toxin is the virulent factor of shrimp disease named AHPND. Vp_PirAB-like toxin compose of two proteins, Vp_PirA-like and Vp_PirB-like. Up-to-date, the mode of action of Vp_PirAB-like toxin is not fully clarified. Lee *et al*. (2015) have reported the structural topology of Vp_PirA- and B-like toxins, which are similar to that of *Bacillus thuringiensis* delta-endotoxin or Cry toxins, the insecticides in sprays and in transgenic crops [de Maagd *et al*., 2001; James, 2011]. The Vp_PirA-like corresponding to Cry domain III, whereas Vp_PirB-like N-terminal corresponding to Cry domain I and Vp_PirB-like C-terminal corresponding to Cry domain II [Lin *et al*., 2017]. Previous study has reported the localization of Vp_PirA-like and Vp_PirB-like toxins after AHPND infection, toxins were detected in the stomach, hemolymph, and hepatopancreas [Lai *et al*., 2015]. However, only hepatopancreas tissue displayed severe damages, suggesting that toxins need to interact with specific molecule

on cell membrane of the hepatopancreas, which is lacking in stomach tissue, then insert into the hepatopancreas cells and cause sloughing of tubule epithelium cells. These raise the high possibility that Vp_PirAB-like toxins use similar mechanism of Cry toxins to invade shrimp hepatopancreas cells that lead cell death.

Generally, the activated Cry toxins compose of 3 domains in structure. Domain I is implicated in oligomerization and pore-formation, domain II and domain III are involved in receptor binding [de Maagd *et al*., 2003; Soberon *et al*., 2009]. The mode of action of Cry toxins has been characterized in Lepidotera [de Maagd *et al*., 2001; Bravo *et al*., 2007]. After ingestion by the susceptible insect, protoxin is solubilized and cleaved by midgut proteases to be an active toxin. The activated toxin then sequentially binds to the specific receptors on the brush border membrane of the midgut epithelium columnar cells before insertion into membrane. Toxin insertion leads the pore-forming in columnar cells that cause ion leakage and cell lysis.

Resistance mechanisms to Cry toxins in different lepidopteran insects have been extensively studied and shown that resistance can be developed by different mechanisms including alterations in toxin activation, inducing an elevated immune response [Rahman *et al*., 2004; Hernandez-Martinez *et al*., 2010], and alteration of receptor binding [Jurat-Fuentes and Adang, 2006; Pardo-Lopez *et al*., 2013]. Reduction of Cry toxin-receptor binding is the most common resistance mechanism which is linked to mutation of toxin receptors such as cadherin [Xie *et al*., 2005], alkaline phosphatase [Jurat-Fuentes *et al*., 2011], aminopeptidase N [Zhang *et al*., 2009], and ABCC transporter [Gahan *et al*., 2010]. These mutations such as amino acid substitution and insertion of a retrotransposon, may affect functional domain of receptor or transcription of receptor genes.

In the previous results, the Cry toxin-receptor homologs were identified from the RNA-seq (hepatopancreas/stomach) of normal *L. vannamei*. These receptor homologs were assumed as the Vp_PirAB-like toxin receptor candidates. In order to test the hypothesis that receptor

gene of Vp_PirAB-like toxin in resistant shrimp may be mutated from the susceptible and affecting the action of toxin which is resulted in the toxin-resistance. In the present study, the receptor candidates of resistant shrimp were identified for comparing sequences with the non-resistant shrimp. The putative SNP was observed in a cadherin-like gene and its association with resistant shrimp was analyzed.

2. Materials and methods

2.1 Identification of Vp_PirAB-like toxin-receptor homologs in surviving *L. vannamei*

RNA-sequence of surviving shrimp (sur-FKC) was reassembled alone to obtain sur-FKC transcriptome. Thirteen Vp_PirAB-like toxin-receptor candidates which were previously identified from control/Vp-inf transcriptome (assumed as normal group), were used as query sequence for searching homologous gene in sur-FKC transcriptome by BLAST+ program with TBLASTN mode. The coding sequences of all receptor homologs were predicted by the open reading frame finder (https://www.ncbi.nlm.nih. gov/orffinder/).

2.2 RT-PCR detection of toxin receptor candidates

Total RNA from the hepatopancreas or stomach of individual shrimp from control (500 ng each), Vp-inf (500 ng each), and sur-FKC (250 ng each) were pooled and separated by tissue and group. The pooled total RNAs (totally 6 samples) were used to construct cDNA with M-MLV Reverse Transcriptase (Invitrogen, USA) and oligo (dT) primer (5′-TTTTTTT TTTTTTTTTTTTTTTTTT-3′). cDNAs were used as templates for reverse transcriptase PCR (RT-PCR) with specific primer sets of each receptor gene (Table 1). The primer sequences were designed based on the transcriptome data. The house keeping gene, EF1-alpha was used as control. PCR conditions were pre-heating at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 1 min.

2.3 Sequence comparison of receptor homologs

From BLAST results, four genes which showed similarity in size and high identity (but not 100%) between normal and sur-FKC, were selected for further analysis. The coding sequence of selected genes (cadherin-like 1, APN-like 1, ABCC-like 1, and ABCC-like 2) from normal and sur-FKC were aligned using ClustalW by GENETYX software.

To validate the detected differentiations in each pairwise, pooled cDNAs of the hepatopancreas from control and sur-FKC shrimp were used as template for partially sequencing of receptor candidates. The regions that showed differences in amino acid residue between normal and sur-FKC groups were amplified with specific primer sets (Table 1). PCR conditions were pre-heating at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 1 min.

Amplified products were purified using ExoSAP-IT PCR product cleanup (Affymetrix, Inc., USA) following the manufacturer's instruction and used as template for DNA sequencing with a 3130 XL Genetic Analyzer (Applied Biosystems, USA). DNA sequence of each gene was compared by pairwise alignment with BLASTN (http://blast.ncbi.nlm.nih.gov/).

2.4 Identification of single nucleotide polymorphisms (SNPs) in cadherin-like 1

Cadherin-like 1 gene which showed one amino acid residue change in sur-FKC, was further analyzed SNPs based on the hepatopancreas transcriptome of control and sur-FKC groups. Initially, Trimmomatic [Bolger *et al*., 2014] was performed to remove adapter sequences and filter quality of raw reads. The nucleotide sequence of DN14090 from control/Vp-inf transcriptome was used as reference sequence for aligning (Figure 1). Short reads from two groups (normal and sur-FKC) were separately aligned onto the reference sequence with Bowtie2 version 2.3.0 and then the SAM format alignments were indexed and converted to BAM format by SAMtools. A BAM format alignment files that consist of reference transcript and the mapped normal and sur-FKC Illumina reads were visualized in Integrative Genomics Viewer v.2.4.14 (Robinson *et al*., 2011). The detected variants that found at same position in each read with read depth higher than four were recorded.

2.5 SNP validation and genotyping by sanger sequencing

The SNP 4,326 C/A of cadherin-like 1 gene, which showed difference of allelic distribution between normal and sur-FKC groups, was further analyzed in the cDNA and genomic DNA (gDNA) samples.

2.5.1) cDNAs of control, Vp-inf, and sur-FKC shrimp

Cadherin-like 1 gene region (nt 3748-4621) was amplified from the hepatopancreas cDNA of individual shrimp from control, Vp-inf, and sur-FKC shrimp (trial 1-3), with specific primer. All cDNA samples were obtained from the studies in Chapter 2 and 3. PCR conditions were pre-heating at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 1 min. Amplified products were purified with ExoSAP-IT and performed DNA sequencing as described above. All sequences were aligned to a reference sequence DN14090.

2.5.2) Genomic DNAs of susceptible and sur-FKC shrimp

Five-hundred juvenile *L. vannamei* (1-2 g) were reared in 100-L plastic tank, with aerated, recirculating artificial seawater (28 ppt) at 28 °C. Shrimp were fed commercial pellets containing 5% (w/w) of formalin-killed cells (FKCs) of *V. parahaemolyticus* AHPND-causing strain D6 (FKC-VpD6). The FKC diet was prepared as previously reported [Tinwongger *et al.*, 2016]. Shrimp were fed twice per day (a total of 5% of body weight) and daily checked the mortality. The dead shrimps were collected as Vp_PirAB-like toxin-susceptible shrimp, muscles were sampled from individual shrimp and stored in TNES-urea buffer (10 mM Tris-HCl pH 7.5, 125 mM NaCl, 10 mM EDTA pH 7.5, 0.5% SDS, 5 M Urea) at 4 °C prior isolation of gDNA.

For gDNAs of sur-FKC shrimp, the muscle or intestine tissues from the survival shrimp of trials 1-3 were used. Tissues were stored in TNES-urea buffer or RNA preservation buffer prior gDNA isolation.

Genomic DNA extraction was slightly modified from Asahida *et al*. (1996). Briefly, tissue was homogenized in TNES-urea buffer, treated with proteinase K, then the mixture was extracted with phenol-chloroform (1:1), treated with RNase, and precipitated with ethanol. Genomic extraction was suspended with 1xTE buffer and kept at -30 °C for further analysis.

All genotyping data were obtained from sequencing chromatograms. The chi-square test was used to analyze the allele and genotype frequencies between surviving and susceptible groups (https://www.icalcu.com/stat/chisqtest.html).

2.6 Re-challenging of dietary FKC-VpD6 to resistant *L. vannamei*

Two hundred Juvenile *L. vannamei* shrimp (body weight 1-2 g) were reared in a 100-L plastic tank with aerated, recirculating artificial seawater (28 ppt) at 28 °C. Shrimp were fed FKC-VpD6 supplemented diet (5% w/w), twice per day (a total of 5% of body weight) and checked daily to remove dead shrimp from the tank. After 1 week of feeding, all surviving shrimp were collected and transferred to new aquarium 15-L tank, with aerated. For recovery period, surviving shrimp were daily fed with normal commercial diet for 1 week. Then the second-round feeding challenge was started by daily feeding the survival shrimp with FKC-VpD6 diet (5% of body weight per day) and the mortality was observed every day post feeding.

3. Results

3.1 Cry toxin receptor homologs in sur-FKC transcriptome

The Vp-PirAB-like toxin receptor candidates were identified from sur-FKC library (Table 2). From a total of identified 13 genes in sur-FKC, three genes are 100% identical to normal group (ALP-like1, APN-like3, and APN-like 4). One gene (cadherin-like 2) showed 100% identity, but the length of gene was different.

3.2 Detection of receptor candidate transcripts

All thirteen receptor candidates were detected in all shrimp groups (control, Vp-inf, and sur-FKC) either in the hepatopancreas or stomach (Figure 2A-D). Some genes were higher expressed in the hepatopancreas (cadherin-like 1, ALP-like 1, and APN-like-1) and some genes were higher expressed in the stomach (cadherin-like 2, APN-like 2, ABCC2-like 1, and ABCC2-like 3).

3.3 Pairwise alignment and partially sequencing

Four receptor candidates which showed high identity (> 95%) and similar in size between normal and sur-FKC were selected for sequence alignment including cadherin-like 1, APN-like 1, ABCC-like 1, and ABCC-like 2. The amino acid sequence alignment of four selected candidates are shown in Figure 3. In predicted cadherin-like 1, 46 amino acid residues in 5′ region were lacked in sur-FKC and one amino acid residue was unidentical, which is a result of a substitution of one nucleotide (Figure 3A). In predicted APN-like 1, 19 amino acid residues were unidentical and one amino acid residue was deleted in normal shrimp at locus 915 (Figure 3B). In predicted ABCC2-like 1, 78 amino acid residues were deleted in sur-FKC and 7 amino acid residues were unidentical (Figure 3C). In predicted ABCC2-like 3, 43 amino acid residues were unidentical (Figure 3D).

Of the four partially sequenced genes, only cadherin-like 1 gene showed one amino acid residue change at residue 1442 (nucleotide 4,326), according to the sequence alignment result of RNA-seq. This amino acid substitution was a result of single nucleotide polymorphism (SNP) C/A at locus 4,326. Whereas, the other three genes (APN-like 1, ABCC2-like 1, and ABCC2-like 2) showed identical sequence between control and sur-FKC.

Moreover, the sequence chromatograms of cadherin-like 1 gene at locus 4,326 presented three types of genotype, that are homozygotes CC, AA and heterozygote CA (Figure 4).

3.4 Identified SNPs and SNP genotyping in cadherin-like 1

Total of 12 potential SNPs were detected in cadherin-like 1 gene (Table 3). The allelic distribution of SNP 4,326 C/A was different between normal and sur-FKC groups. Therefore, only SNP 4,326 C/A was analyzed the frequencies of genotype and allele at both cDNA and gDNA levels.

At cDNA level, three genotypes AA, CC, and AC were detected in normal group, whereas only AA and AC genotypes were detected in resistance group (Table 4). The frequencies of genotype AA, AC, and CC in resistance group were 64%, 36%, and 0%, respectively, while the frequency distributions of normal group were 17%, 0%, and 83%, respectively. The chi-square test revealed that SNP 4,326 C/A significantly associated with the resistance of Vp_PirAB-like toxin in *L. vannamei* at both levels of genotype ($P < 0.001$) and allele ($P < 0.001$) (Table 4).

At gDNA level, SNP 4,326 C/A was genotyped in susceptible and resistance groups. Association analysis indicated that the genotype frequency distributions between two groups was significantly different ($P < 0.05$). In the susceptible group, the AA, AC, and CC genotypes were 45%, 34%, and 21%, respectively, while those in the resistance group were 64%, 36%

and 0%, respectively (Table 4). Interestingly, the genotype CC was not observed in resistant group.

3.5 Stability of resistance phenotype

After 1 week of feeding with FKC-VpD6 diet, six shrimp survived in the first-round challenge. During the recovery period (1 week later), one shrimp died. Five shrimp were continually re-fed with FKC-VpD6 for second-round challenge and the result showed that all shrimp died in 3 days after re-challenging. These results indicate that the resistant characteristic of these collected shrimp is not permanently expressed.

Table 1. List of primers used in this study.

Name		Sequences (5′-3′)	Length (bp)
RT-PCR			
Cadherin-like 1	Fw	CCGAGGCAATAGACCCAGAC	20
	Rv	CTAGTGGTGGTGGTGGTGGTGGAGGCTTCGTGTTC	35
Cadherin-like 2	Fw	CCGAAGTCGCCTACGTAGAA	20
	Rv	CTAGTGGTGGTGGTGGTGGTGCTTGAGGCGTCTTC	35
Cadherin-like 3	Fw	AGAACCGCACCATCACCTAC	20
	Rv	CATAGGCTTCCACACGAATCTC	22
ALP-like 1	Fw	GACACGTGTCCACCATGAAG	20
	Rv	GATAGCGACGCACTCAGTGC	20
ALP-like 2	Fw	AGAATGAGAAGGTCGCCAGA	20
	Rv	GTGTAAGGCATCCCGTCTGT	20
APN-like 1	Fw	CAACTACGACGCCGAGAACT	20
	Rv	CGCATTCAGATTTCACAGACTC	22
APN-like 2	Fw	CTGCAGCAGACGGGCTACTA	20
	Rv	ACGCAGATGACCCTAAGCGA	20
APN-like 3	Fw	GAACATCAACCAAAAGGGCTAC	22
	Rv	TCATCACTGTTTCTGCGAGTTC	22
APN-like 4	Fw	GTCACGCTCCAGCCCAATCT	20
	Rv	GCCCACAGGCACTTCAATTGG	21
APN-like 5	Fw	CGGTTCCTCCTCCTACAACA	20
	Rv	TTCGTCTTCCTCAGCAACCT	20
ABCC-like 1	Fw	ATGCTGGTGTGCGAGAACTG	20
	Rv	ACAGTGCTGCTGACAAATGC	20
ABCC-like 2	Fw	GCCTTTGGCAGATCTCATCA	20
	Rv	ACCTGAAGGGTGTTCTTCACTG	22
ABCC-like 3	Fw	CCAGTTGGCCAAGTTCTTAACA	22
	Rv	TTTGAGCGTATGGTCTGCTG	20
Lv EF1	Fw	GCGAGTTCGAAGCTGGTATC	20
	Rv	CTTCACCGACACGTTCTTCA	20

Name		Sequences (5′-3′)	Length (bp)
Sequencing			
cad-1_SQ	Fw	CCGAGGCAATAGACCCAGAC	20
(aa. 1249-1541)	Rv	CTGTGATGACACCCACGTTC	20
APN-1_SQ	Fw	CAGTACATCAACTCGAACGTCG	22
(aa. 834-998)	Rv	CGCATTCAGATTTCACAGACTC	22
ABCC2-1_SQ	Fw	CCAGTGCATCTTAGGTGCTCT	21
(aa. 727-1081)	Rv	CCTTGTCAAAGGCACGAATG	20
ABCC2-2_SQ	Fw	CATCAGGGCCATCGGTATCA	20
(aa. 967-1300)	Rv	GAACTCGACTACCCCGTGCT	20

Table 2. List of *Litopenaeus vannamei* genes homologous to the Cry toxin receptors.

Genes	Cry toxin receptor homologs in *L. vannamei* transcriptomes					
	normal (control/Vp-inf)		sur-FKC		BLAST result of control/Vp-inf against sur-FKC	
	gene id.	length	gene id.	length	% identities	E value
1. Cadherin						
1.1 cadherin-like 1	DN14090_c0_g1_i1	1,760	DN10817_c0_g1_i1	1,714	99.94	0
1.2 cadherin-like 2	DN23271_c0_g1_i1	1,825	DN25800_c0_g1_i1	232	100	0
1.3 cadherin-like 3	DN22247_c0_g1_i2	991	DN14129_c0_g1_i2	584	99.51	0
2. Alkaline phosphatase (ALP)						
2.1 ALP-like 1	DN17552_c0_g1_i1	570	DN12795_c0_g1_i1	570	100	0
2.2 ALP-like 2	DN8155_c0_g1_i1 [p]	542	DN14140_c0_g1_i1 [p]	568	98.83	0
3. Aminopeptidase N (APN)						
3.1 APN-like 1	DN20883_c0_g1_i1	993	DN15224_c0_g2_i1	994	97.99	0
3.2 APN-like 2	DN19107_c0_g1_i4	938	DN11412_c0_g1_i1	592	68.78	0
3.3 APN-like 3	DN18022_c0_g1_i1	917	DN12807_c0_g1_i1	917	100	0
3.4 APN-like 4	DN23067_c0_g1_i1	867	DN14213_c0_g1_i1	867	100	0
3.5 APN-like 5	DN21738_c0_g1_i1[p]	821	DN15148_c0_g1_i1	625	38.31	3E-128
4. ATP Binding Cassette Subfamily C Member 2 (ABCC2)						
4.1 ABCC2-like 1	DN21605_c0_g2_i3	1,452	DN15819_c0_g2_i1	1,374	99.27	0
4.2 ABCC2-like 2	DN23110_c0_g1_i1	1,537	DN15209_c0_g2_i2	1,537	97.20	0
4.3 ABCC2-like 3	DN18494_c0_g1_i1	1,436	DN12755_c0_g1_i1	1,245	99.37	0

* Length of predicted coding sequence; p = partial coding sequence

Table 3. Summary of the identified SNPs in the cadherin-like 1 from RNA-sequencing.

No.	locus	SNP	group	SNP number		
				A	B	C
1	123	A > G	nor	2	20	
			res	0	6	
2	214	C > T	nor	1	24	
			res	0	5	
3	236	C > G	nor	30	2	
			res	7	0	
4	237	C > T	nor	31	2	
			res	7	0	
5	262	A > C	nor	0	31	
			res	4	4	
6	283	A > C	nor	31	0	
			res	5	2	
7	332	C > T	nor	41	0	
			res	6	2	
8	2,811	G > T	nor	37	0	
			res	4	2	
9	2,972	A > C > T	nor	0	28	0
			res	2	5	2
10	3,864	C > T	nor	0	47	
			res	9	10	
11	4,326	A > C	nor	16	40	
			res	26	1	
12	5,070	A > G	nor	64	0	
			res	23	6	

nor = normal, res = resistance

Table 4 Distribution of SNP 4,326 C/A in resistant and susceptible *L. vannamei*.

	Group	Genotype number			X^2	*P*-value	Allele number		X^2	*P*-value
		AA	CC	CA			A	C		
cDNA	normal	1	5	0	24.97	< 0.001	2	10	19.44	< 0.001
	resistance	16	0	9			41	9		
gDNA	resistance	49	23	38	6.02	0.049	136	84	0.05	0.820
	susceptible	14	0	8			28	16		

Normal group is the control/Vp-inf shrimp.

P-value < 0.05 is considered as significant difference.

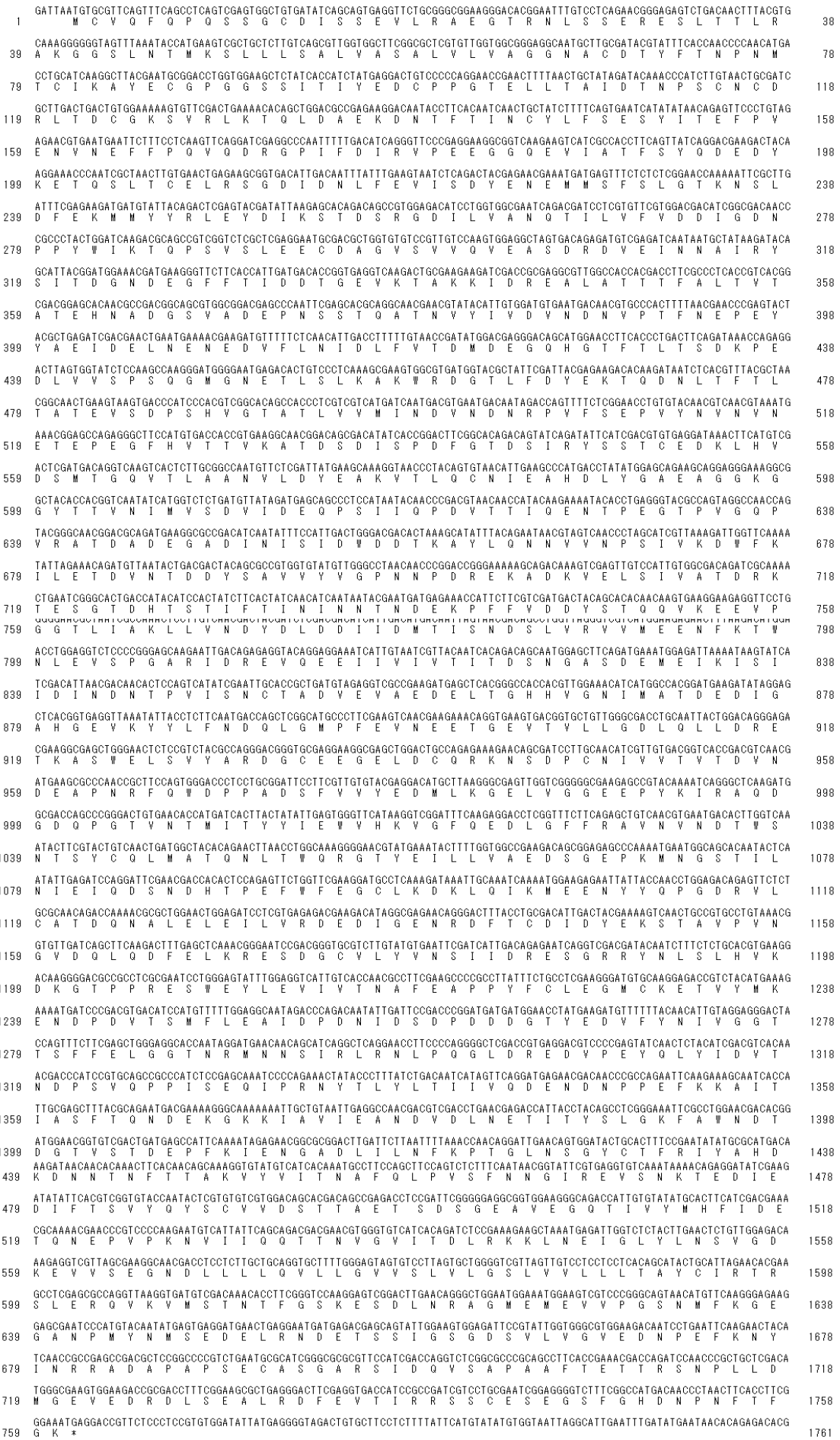

Figure 1. The predicted coding sequence of DN14090; cadherin-like 1 gene (5,403 bp).

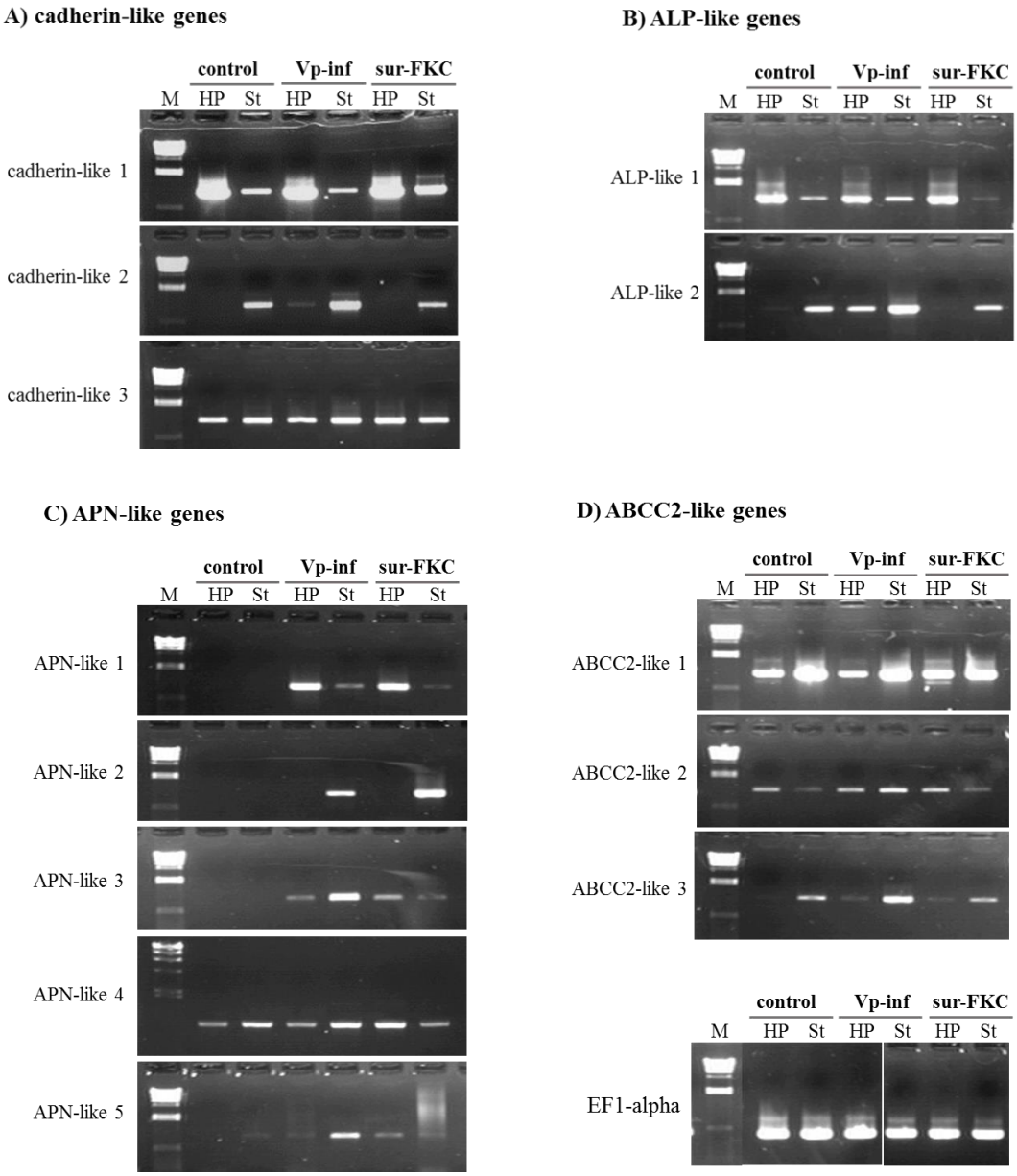

Figure 2. Detection of Vp_PirAB-like toxin receptor candidates by RT-PCR. The expressions of receptor genes were examined in the hepatopancreas (HP) and stomach (St) of control, Vp-inf, and sur-FKC groups. Lane M is lambda Hind III marker. **A)** cadherin-like genes, **B)** ALP-like genes, **C)** APN-like genes, and **D)** ABCC2-like genes. EF-1 alpha was used as internal control.

A) cadherin-like 1

Figure 3. The amino acid sequence alignment of receptor candidates generated by ClustalW. Sequence of sur-FKC shrimp was aligned to normal shrimp. **A)** cadherin-like 1, **B)** APN-like , **C)** ABCC2-like 1, and **D)** ABCC2-like 2. Identical residues are presented in dot (.), different residues are highlighted in black color, and hyphens (-) represent the gap sequence.

B) APN-like 1

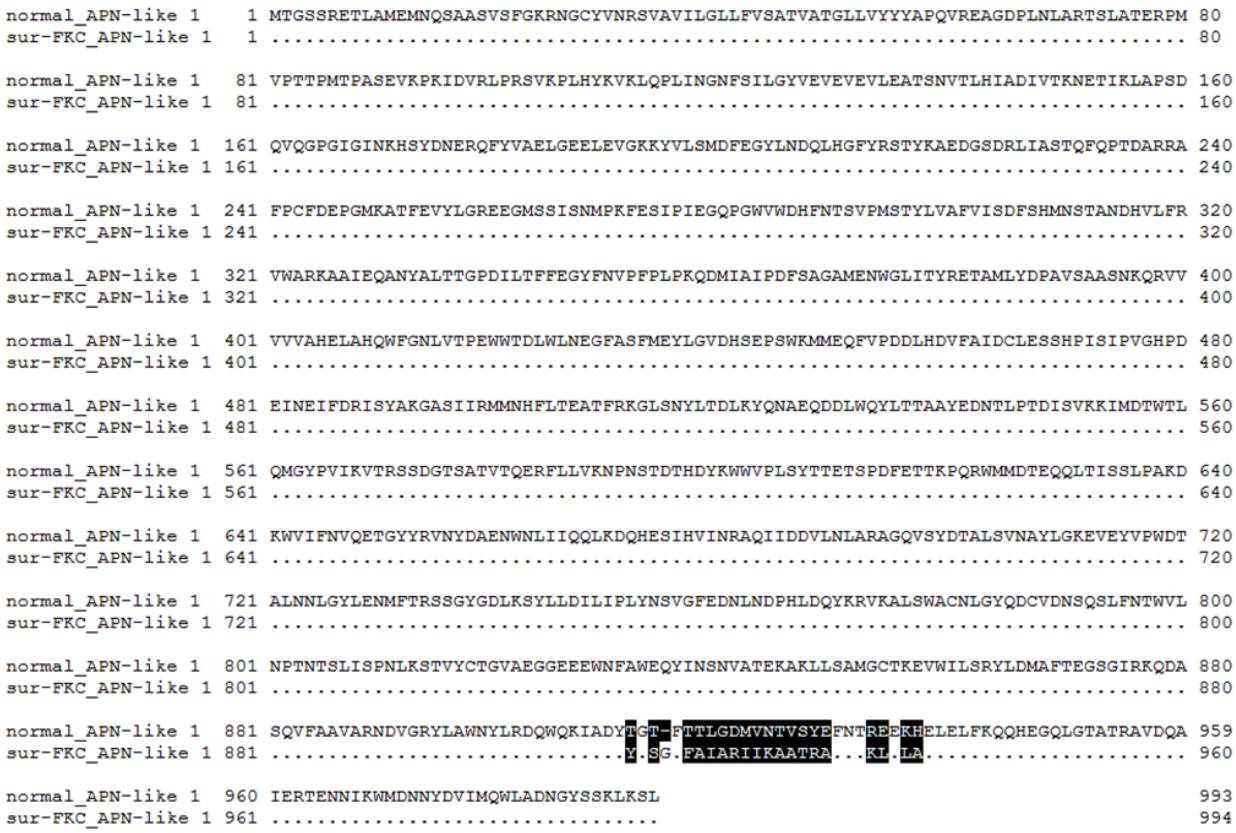

Figure 3 (continued). The amino acid sequence alignment of receptor candidates generated by ClustalW. Sequence of sur-FKC shrimp was aligned to normal shrimp. **A)** cadherin-like 1, **B)** APN-like , **C)** ABCC2-like 1, and **D)** ABCC2-like 2. Identical residues are presented in dot (.), different residues are highlighted in black color, and hyphens (-) represent the gap sequence.

C) ABCC2-like 1

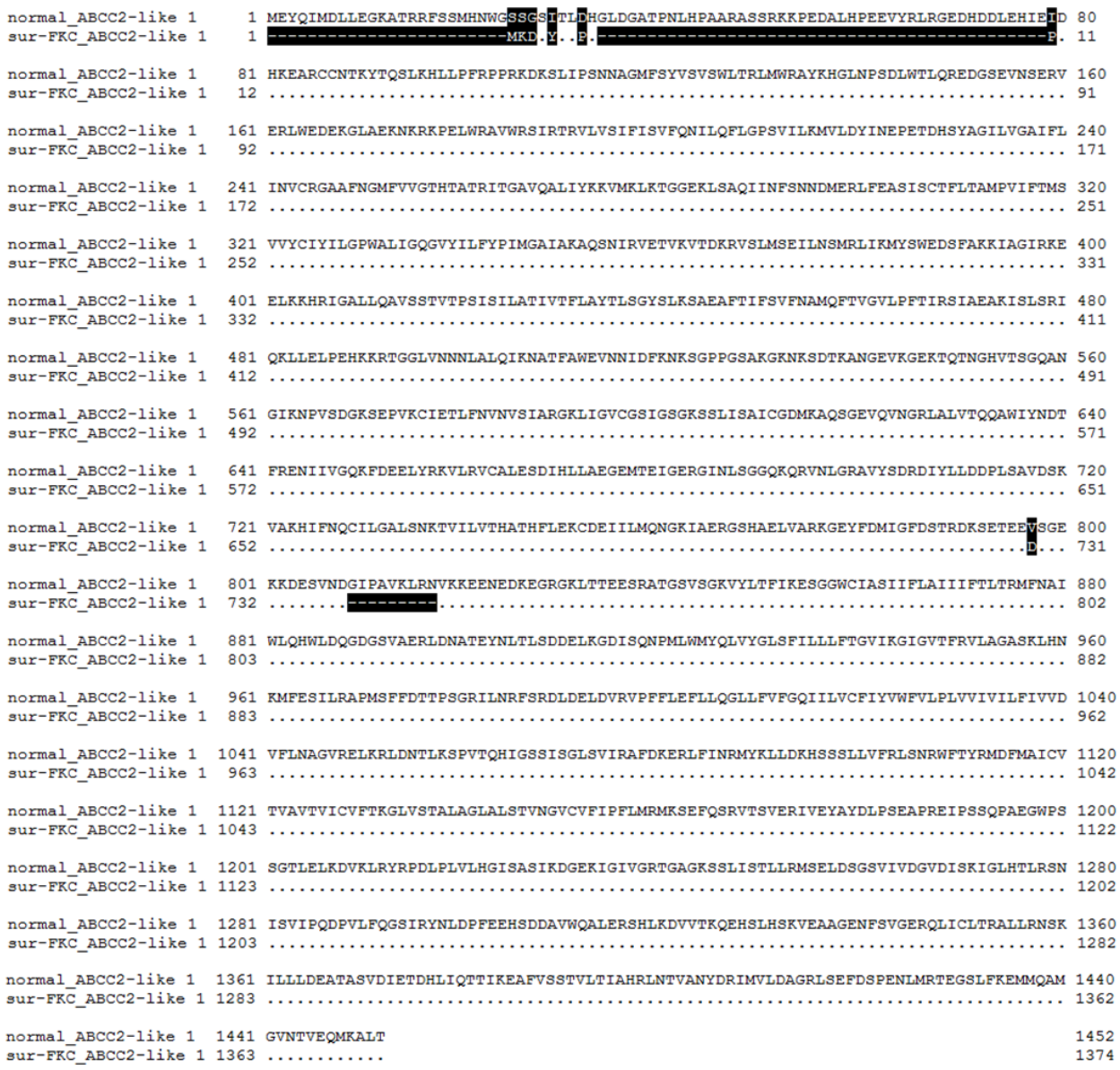

Figure 3 (continued). The amino acid sequence alignment of receptor candidates generated by ClustalW. Sequence of sur-FKC shrimp was aligned to normal shrimp. **A)** cadherin-like 1, **B)** APN-like , **C)** ABCC2-like 1, and **D)** ABCC2-like 2. Identical residues are presented in dot (.), different residues are highlighted in black color, and hyphens (-) represent the gap sequence.

D) ABCC2-like 2

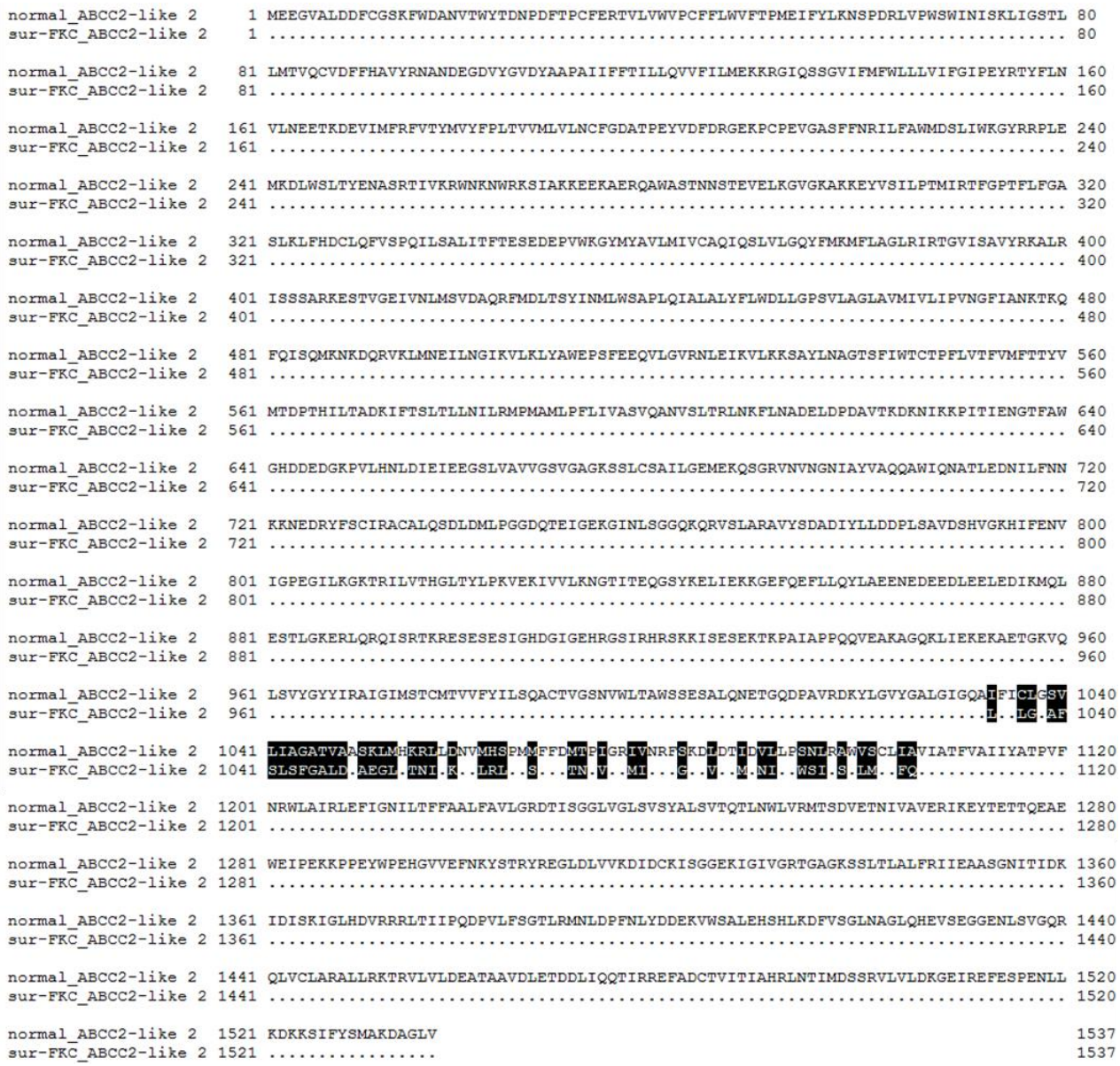

Figure 3 (continued). The amino acid sequence alignment of receptor candidates generated by ClustalW. Sequence of sur-FKC shrimp was aligned to normal shrimp. **A)** cadherin-like 1, **B)** APN-like , **C)** ABCC2-like 1, and **D)** ABCC2-like 2. Identical residues are presented in dot (.), different residues are highlighted in black color, and hyphens (-) represent the gap sequence.

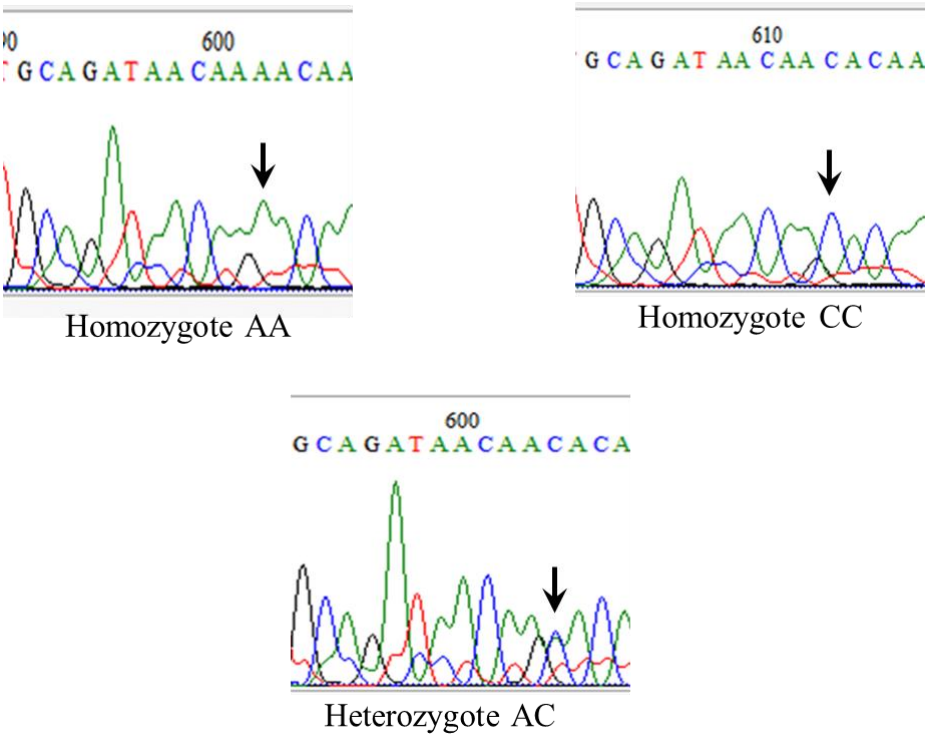

Figure 4. The sequence chromatograms of SNP 4,326 C/A in cadherin-like 1 gene. Arrows indicate the position of locus 4,326 SNP.

Discussion

The thirteen Vp_PirAB-like toxin receptor candidates were previously identified from the co-assembled transcriptome data of hepatopancreas and stomach tissues from control and Vp-inf *L. vannamei* (control/Vp-inf). This transcriptome was assumed as the representative of normal shrimp, which can be either susceptibility or resistance to toxin. In contrast, transcriptome of survival *L. vannamei* (sur-FKC) was separately assembled to identify receptor candidates of toxin-resistant shrimp. Excluding gene expression analysis, RNA-seq is used to identify genomic variants [Piskol *et al*., 2013]. Based on the RNA-seq data, between normal and resistant shrimp, only three candidates are identical with 100% coverage of size and some candidates showed high percentage of identity with low size coverage (Table 2). RNA sequencing, unlike genomic sequencing, highly presents the uneven coverage distribution which due to the gene expression variation. Also, using of different tools for assembling the RNA sequence reads can affect the identified contigs. Bushmanova *et al*. (2018) have demonstrated the comparison of *de novo* assemblies from five different assemblers (Trans-ABySS, IDBA-tran, SOAPdenovo-Trans, Trinity, and SPAdes) on *Mus musculus* RNA-seq dataset. Trinity produced high number of misassembled transcripts, although the database coverage and 50% / 95%-assembled isoforms were higher than the others.

Direct sequencing was used to verify the detected variants of receptor candidates from RNA-seq. Of the four selected candidates, only cadherin-like 1 gene presented a point mutation in nucleotides at locus 4,326 of the predicted coding sequence and it was named as SNP 4,326 C/A. While, the other three candidates showed no differentiations. These may be the results of lower number of raw reads in sur-FKC transcriptome (total raw reads = 18.5 million) than control/Vp-inf transcriptome (total raw reads = 46.4 million), which affects the contig sequence post assembly.

Cadherins are the transmembrane proteins that contain the repeated extracellular cadherin domains (ectodomain). The functions of ectodomains have been characterized and

showed the involvement in recognition and binding to cells that express the same or similar cadherin molecules [Nose *et al*., 1990]. In both vertebrate and *Drosophila* have identified cadherins as molecular components of the adherens junction, also are required for regulation of dynamic morphogenetic processes [Posy *et al*., 2008; Oda and Takeichi, 2011]. The epithelial cadherin proteins (E-cadherins) of several vertebrate species have been shown to mediate internalization through binding of the bacterium *Listeria monocytogenes* [Mengaud *et al*. 1996], the fungus *Candida albicans* [Phan *et al.* 2007], and the infectious pancreatic necrosis virus [Moen *et al*., 2015].

In Lepidopteran insects, cadherin proteins have functions related to the mode of action of Cry toxins by acting as receptor that localized on the midgut membrane [Pigott and Ellar, 2007]. Binding of Cry toxin to cadherin causes toxin oligomerization, which is required for effective pore-formation and ultimately toxicity. Mutations of cadherin genes are genetically linked to Cry toxins resistance in insect larvae have been reported. For examples of Cry1Ac toxin resistance, a single point mutation of cotton bollworm cadherin caused protein mislocalization from midgut cell membrane to the endoplasmic reticulum [Xiao *et al*., 2017] and the retrotransposon insertion mediated disruption of *BtR-4* gene encoding cadherin-like protein, resulted in no protein expression was detected on midgut cells [Jurat-Fuentes *et al*., 2004].

Additionally, the cadherin-like 1 gene was deeply searched for other SNPs from RNA-Seq. The twelve putative SNPs were discovered, including the SNP 4,326 C/A. The results indicated that only SNP 4,326 C/A showed difference distribution of allelic abundance between normal and resistant shrimp (Table 3). Therefore, SNP 4,326 C/A was selected for genotyping analysis using cDNAs and gDNAs of resistant and non-resistant shrimp. Three genotypes of CC, AA, and AC were detected at locus 4,326. Chi-square test of genotype and allelic frequencies between resistant and control/Vp-inf cDNAs showed highly significant difference ($P < 0.001$). However, these results might be unreliable because only few samples were used

and control/Vp-inf samples can be either resistance or susceptible to toxin. Also, the nucleotide sequences of cDNA samples may be changed during cDNA synthesis, which used reverse transcriptase to create the complementary DNA of mRNA. In contrast, gDNA samples were collected directly from the susceptible and resistant shrimp. Between resistance and susceptible, the distribution of genotype frequency was significant difference ($P < 0.05$) (Table 3). Interestingly, genotype CC at locus 4,326 SNP was not detected in the resistant shrimp, suggesting the high possibility of correlation between SNP 4,326 C/A genotype and Vp_PirAB-like toxin susceptibility.

SNPs located in the exons may change the encoded amino acids (nonsynonymous) or can be silent (synonymous), whereas SNPs in the noncoding regions may influence the promoter activity (i.e. gene expression), messenger RNA (mRNA), conformation (stability), and subcellular localization of mRNAs and/or proteins and hence may produce disease [Shasty, 2009]. The SNP 4,326 C/A of cadherin-like 1 is a nonsynonymous mutation, in which an amino acid residue is changed from asparagine (N) to lysine (K) in resistant shrimp. Both asparagine and lysine are the polar amino acids that quite frequently involved in protein active or binding sites, though lysine is a positively-charged amino acid [Betts and Russell, 2003]. It is possible that this amino acid substitution has effect on protein structure of cadherin-like 1, resulting in disruption of Vp_PirAB-like toxin binding on the hepatopancreas cells membrane. However, the result of two-rounded feeding of FKC-VpD6 diet, which showed no shrimp survival after second feeding, does not support this hypothesis. Because, if this SNP is a mutation associated with resistance mechanism by disrupting of toxin binding, the resistant phenotype should be permanently expressed.

Prediction of SNPs in the two transcriptome of *L. vannamei* larvae revealed high number of detected SNPs, about one SNP per 476 bp but only half of SNPs were identical [Yu *et al*., 2014]. This result suggested the low genetic diversity in the *L. vannamei* population. Moreover, SNPs also were detected in immune-related genes of penaeid shrimps and showed

relation to shrimp resistance to different pathogens such as SNPs of the heat shock protein 70 and hemocyanin related to susceptibility of TSV [Zeng *et al*., 2008; Zhao *et al*., 2012] and SNPs of an anti-lipopolysaccharide factor related to WSSV-resistance [Liu *et al*., 2014]. In addition, Yu *et al*. (2017) used the genes set based association analysis of SNPs in immune-related genes to identify markers associated with WSSV resistance in *L. vannamei* and revealed that the combination SNPs locus in TRAF6, Cu/Zn SOD and *nLv*ALF2 exhibited a significant effect in resistant shrimp.

This study demonstrates the association between genotype CC at locus 4,326 in cadherin-like 1 gene and the Vp_PirAB-like toxin resistant phenotype. This genotype may be used as a genetic marker to select *L. vannamei* resistance to AHPND in breeding program.

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Chapter 5

Summary and General Conclusion

Shrimp farming is one of important industry in world aquaculture to support an increasingly demand of people around the world. Through the decades, shrimp industry has frequently plagued with a variety of diseases that affect the shrimp production. To maintain the sustainable shrimp aquaculture, the farm managements have been developed and the broodstock and breeding program have been improved. Aside from this, farmers also use several chemical agents including antibiotics to control infectious diseases caused by bacteria. Recently, a newly emerging disease named AHPND which caused by unique bacterial strains, has been distributed in several countries in Asia and Americas. To date, AHPND still has affected in shrimp farming, this might raise the antibiotic usage in the shrimp farms. Therefore, the alternative solution for prevention and controlling of AHPND should be considered and studied. Numerous studies in invertebrates have reported the importance of host immune system in giving the protection against invading pathogens, also the genetic variation in some genes showed the relevance to the disease resistance. Because the virulent factor of AHPND; Vp_PirAB-like toxin can be found in several bacterial species, this study focused on the toxin.

In the present study, the novel genes that may involve in the resistance mechanism to Vp_PirAB-like toxin in *L. vannamei* were identified using RNA sequencing of the hepatopancreas and stomach of resistant shrimp (sur-FKC), 24h-infected AHPND shrimp (Vp-inf), and non-treated shrimp (control). The differentially expressed genes (DEGs) among three groups were identified at $P < 10^{-5}$ and fold-change greater than 4-fold. In the hepatopancreas and stomach, 224 DEGs and 194 DEGs were identified, respectively. Ten immune-related genes which showed either high or low expression in the resistant shrimp transcriptome were

validated mRNA expression by qPCR. Of the ten selected genes, only gene homologous to *L. vannamei* anti-lipopolysaccharide factor AV-R isoform (*Lv*ALF AV-R) showed significantly high expression in the hepatopancreas of resistant shrimp than the other groups.

The association of *Lv*ALF AV-R to Vp_PirAB-like toxin resistance mechanism was investigated with further experiments. The mRNA expression of *Lv*ALF AV-R in the hepatopancreas was determined under different stimulations including FKC of VpAHPND (*V. parahaemolyticus* AHPND-causing strain) feeding, FKC of non-VpAHPND feeding, and low dose of VpAHPND immersion. qPCR results showed that *Lv*ALF AV-R was up-regulated in shrimp fed with FKC-VpAHPND, not in other stimulations. Also, the three more trials of toxin resistance selection by feeding with FKC-VpAHPND were performed and strongly supported the previous result that *Lv*ALF-AV-R was highly expressed in the hepatopancreas of all resistance groups. Of the ten different tissues in apparently healthy shrimp, *Lv*ALF AV-R mRNA was highly expressed in hemocytes and lymphoid organ, whereas its protein expression was detected only in hemocyte. Moreover, the histopathological analyses of hepatopancreas sections revealed that resistant shrimp did not exhibit the sloughing of epithelium cells as in the moribund shrimp of FKC-VpAHPND feeding. The immunohistochemistry detection of *Lv*ALF AV-R showed positive signals in the hepatopancreas of resistant shrimp. Functions of *Lv*ALF AV-R protein were examined by *in vitro* and *in vivo* using the recombinant protein. In ELISA, *Lv*ALF AV-R bound to bacterial polysaccharides (LPS and PGN) and some Gram-negative and Gram-positive bacteria, although it lacked bactericidal activity to VpAHPND strain. The ability of *Lv*ALF AV-R to neutralize Vp_PirAB-like toxin was determined by ELISA and Far-Western blotting. *Lv*ALF AV-R did not show any interaction to the native toxin which was isolated from culture supernatant and crude cellular extract of VpAHPND. *In vivo* effect of *Lv*ALF AV-R to AHPND infection was investigated using oral administration of the recombinant protein and followed by challenge test. After 72 h of challenge, the survival rate of shrimp fed *Lv*ALF AV-R was not significant different from the control groups.

On the other hand, the mutation of Vp_PirAB-like toxin receptor genes might affect the action of toxin for insertion into host cells and resulted in toxin resistance. Therefore, the receptor candidates were separately identified from the control/Vp-inf transcriptome which was assumed as representative of normal shrimp and the resistance transcriptome. The previously identified receptors of Cry toxins in insects were used as query for homology search against *L. vannamei* transcriptomes. From blast results, four candidates which showed high identity and similar in size were selected for sequence analysis. Pairwise alignment of amino acid sequences revealed the differentiations in their sequences. Direct sequencing was done to validate the detected variations using pooled cDNA of control or resistant shrimp. Of the four observed genes, only cadherin-like 1 gene showed a differentiation in one amino acid residue which was caused by SNP, named SNP 4,326 C/A. The sequence chromatograms of locus 4,326 in cadherin-like 1 gene presented 3 genotypes of CC, AA, and AC. The genotyping of 4,326 C/A SNP was performed in both cDNA (control, Vp-inf, and resistant shrimp) and gDNA levels (resistant and susceptible shrimp). The association analysis showed that the genotype frequency in gDNAs between resistant and susceptible shrimp was significantly different ($P < 0.05$), but not allele frequency. Interestingly, genotype CC was not detected in the resistant shrimp. In addition, the stability of resistance phenotype was investigated by two-rounded feeding shrimp with FKC-VpAHPND diet. In the second round of feeding, all first-rounded survival shrimp died in 3 days after feeding, suggesting that this resistance phenotype is not permanently expressed.

Taken together, this study provides evidence that *Lv*ALF AV-R is associated with the resistance mechanism to Vp_PirAB-like toxin in *L. vannamei*. *Lv*ALF AV-R plays an indirect role in shrimp protection against Vp_PirAB-like toxin by promoting other shrimp molecules that can directly inhibit virulence of toxin. In contrast, the mutation SNP 4,326 C/A which was detected in the receptor candidate cadherin-like 1 gene is not related to the resistance

mechanism. However, the genotype CC of at locus 4,326 might be used as a genetic marker to select Vp_PirAB-like toxin-resistant shrimp.