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Article

Two Independently Comparative Transcriptome Analyses of Hemocytes Provide New Insights into Understanding the Disease-Resistant Characteristics of Shrimp against *Vibrio* Infection

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Abstract: *Vibrio parahaemolyticus* carrying a plasmid encoding toxins PirA and PirB is one of the causative agents leading to the severe disease of acute hepatopancreatic necrosis disease (AHPND) in shrimp aquaculture. However, there is a lack of deep understanding of the host resistant characteristics against *V. parahaemolyticus* infection. Here, we performed comparative transcriptome analysis on the expression profiles at the background level of hemocytes from shrimp with different resistant abilities against *V. parahaemolyticus* infection in two independent populations. Principal component analysis and sample clustering results showed that samples from the same population had a closer relationship than that from shrimp with similar disease-resistant abilities. Differentially expressed genes (DEGs) analysis revealed that the number of DEGs between two populations was much more than that between *V. parahaemolyticus*-resistant and susceptible shrimp. A total of 31 DEGs and 5 DEGs were identified from the comparison between *V. parahaemolyticus*-resistant and susceptible shrimp from population 1 and 2, respectively. There was no DEGs overlap between two comparisons. DEGs from population 1 were mainly cytoskeleton related genes, metabolic related genes, and immune related genes. DEGs from population 2 included tubulin alpha-3, dihydropyrimidinase-like gene, diacylglycerol kinase 1, prohibitin and iroquois-class homeodomain protein IRX-2-like. The data suggest that shrimp from different populations might adopt distinct disease-resistant mechanisms, which provided new insights into understanding disease-resistant mechanisms at population level against pathogen infection. A new strategy for screening disease resistant genes and disease resistant breeding was proposed based on the present study.

Keywords: penaeid shrimp; hemocyte; disease-resistant; *Vibrio parahaemolyticus*

1. Introduction

Shrimp is one of the most important aquatic animals in aquaculture. However, frequent diseases caused by bacteria, viruses and parasites always lead to severe economic losses, which greatly hinder the healthy development of shrimp aquaculture. Acute hepatopancreatic necrosis disease (AHPND), previously called early mortality syndrome (EMS), is one of the most serious diseases caused by bacteria [1]. The agents causing AHPND are *Vibrio* species that contain a plasmid encoding the lethal toxins PirA and PirB [2]. *Vibrio parahaemolyticus* (*Vp*_{AHPND}) carrying the plasmid is one of the causative agents leading to AHPND in shrimp aquaculture [3].

Although hepatopancreas is the main target tissue of the bacteria, *V. parahaemolyticus* tends to cause systemic responses in the host. In the stomach of *Penaeus monodon*, many immune related genes including antimicrobial peptides, proPO system, proteinase/proteinase inhibitors, and signal

transduction pathways were responsive to *V. parahaemolyticus* infection [4]. In the hemocytes of *L. vannamei*, genes related to carbohydrate metabolism, lipid metabolism, amino acid metabolism, and cell growth and anti-apoptosis were significantly changed after *V. parahaemolyticus* infection [5]. Another study target to the hemocytes of *L. vannamei* identified many immune related genes such as crustin, anti-lipopolysaccharide factor, serpin 3, C-type lectin responsive to *V. parahaemolyticus* infection [6]. In the hepatopancreas of *Litopenaeus vannamei*, genes related to programmed cell death, carbohydrate metabolism, and biological adhesion were up-regulated after *V. parahaemolyticus* infection [7]. Another study revealed that the hepatopancreas of *L. vannamei* responded to *V. parahaemolyticus* by activating glucose metabolism, energy metabolism, amino acid metabolism, nucleic acid synthesis, as well as HIF-1 signaling pathway, PI3K-Akt signaling pathway and NF-KappaB signaling pathway [8]. These data provide useful information for our understanding of the overall responses of the host against *V. parahaemolyticus* infection.

Due to the different genetic backgrounds, shrimp exhibit distinct disease-resistant abilities to *V. parahaemolyticus*. The variation of disease-resistant ability could be reflected by differential expression of genes at the background level. In *P. vannamei*, several genes including chymotrypsin A, serine protease, crustin-P and prophenol oxidase activation system 2 showed differential expression levels between *Vp*_{AHPND} tolerant and susceptible populations [9]. A transcriptomic comparison between three *Vp*_{AHPND} resistant and three susceptible families identified 489 differentially expressed genes (DEGs) in *L. vannamei* and 19 tested genes also showed different expression in their offspring [10]. Although few common genes were reported in different studies due to the distinct genetic background, these identified genes could be used as potential biomarkers for understanding *Vp*_{AHPND} resistant of shrimp. However, there is still lack of effective strategies to find out key genes and regulatory processes that used for host defense again *Vp*_{AHPND} infection.

As the main immune related tissue, hemocytes play important roles in host defense against pathogen infection. However, the identification of genes in hemocytes related to *Vp*_{AHPND} resistance has not been reported. In the present study, the comparative transcriptome analysis was performed on two independent populations. The transcriptional expression profiles were analyzed at the background level of hemocytes from shrimp with different resistant abilities against *Vp*_{AHPND} infection in each population. Some genes related to *Vp*_{AHPND} resistance were identified in shrimp hemocytes while no common DEG existed between two comparisons. A strategy was proposed based on the present study for further identification of key genes and regulatory processes related to disease resistance.

2. Materials and methods

2.1. Experimental animals

Two batches of *Litopenaeus vannamei* purchased from Rizhao breeding farm in Shandong were used for two independent experiments. Before experiments, the shrimp were cultured in aerated seawater laboratory aquaculture tank at 26 °C for seven days. The shrimp weights were 10 g ± 0.5 g in the first batch (population 1) and 19.5 g ± 1 g in the second batch (population 2).

2.2. Visible Implant Elastomer (VIE) fluorescent labeling and hemolymph collection

The VIE distinguished with eight different colors (red, pink, orange, brown, white, purple, green, yellow) was injected into each shrimp in the third segment with different color fluorescence. After one week, 200 µl hemolymph was exsanguinated from each shrimp with a sterile syringe containing an equal volume of ice-cold anticoagulant (27 mM trisodium citrate, 336 mM sodium chloride, 115 mM glucose, 9mM EDTA•Na₂·2H₂O, pH 7.4). The hemocytes were separated by centrifugation at 1000 g for 10 min and stored in -80 °C. Then, all the shrimp were returned to the laboratory tanks.

2.3. *V. parahaemolyticus* immersion and hepatopancreas collection

One week after exsanguination, the shrimp were infected with *V. parahaemolyticus*. The bacteria strain was cultured in tryptic soy broth with 2% sodium chloride liquid media. The cultured bacteria were further checked by positive polymerase chain reaction (PCR) amplification of *PirA^{vp}* and *PirB^{vp}* genes. Bacterial titer was counted with a hemocytometer under a light microscope. The soak infection dose in two batches of shrimp were set at the medial lethal doses, 2.5×10^6 CFU/ml and 5×10^6 CFU/ml respectively. The mortality was continuously recorded for two days. The hepatopancreas of moribund shrimp and survival shrimp after infection for 48 h were collected and stored at -80°C .

2.4. DNA extraction and bacteria load detection

The DNA from the hepatopancreas was extracted using TIANGEN Plant Genomic DNA Extraction Kit according to the manufacture's instruction. The concentration and purity of DNA were determined using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) by coagulation and 1% gel electrophoresis. *PirA* copy number was estimated by Ependorf MasterCycler EP Realplex (Ependorff, Germany) using TaqMan Assay. A forward primer 5'-GGTGACCAGACCTGCTTTGAG-3' and reverse primer 5'-CCAGCTAGGATAACCGTAACATG-3' were used to amplify a 284 bp fragment of *PirA* gene, with a TaqMan hydrolysis probe 5'-FAM-CCGCCAGCCATAAATGGCGCACC-BHQ1-3'). The amplification program consisted of one cycle of contamination digestion at 37°C for 2 min, one cycle of pre- denaturation at 95°C for 5 min, and 45 cycle of denaturation at 95°C for 10 s and extension at 60°C for 30 s. A standard curve was obtained using serial dilutions of plasmid *PirA* (full-length ORF of *PirA* gene of *V. parahaemolyticus* was cloned into pUC57 vector), which was used to quantify the *V. parahaemolyticus* copy number. Each assay was carried out in four parallel. Therefore, all shrimp were divided into resistant group and sensitive group (9hpi, 12hpi and the surviving individual) according to the time of death and vibrio load.

2.5. RNA extraction and transcriptome sequencing

Based on the death and survival data and *Vibrio* load data, all shrimp were divided into *V. parahaemolyticus* resistant and susceptible individuals. The collected hemocytes of *V. parahaemolyticus* resistant and susceptible shrimp were mixed respectively. For population 1, three biological replicates were prepared for resistant and susceptible shrimp. The samples were designated as HcS1-1, HcS1-2 and HcS1-3 for resistant shrimp and designated as HcD1-1, HcD1-2 and HcD1-3 for susceptible shrimp. For population 2, four replicates were prepared for resistant and susceptible shrimp. The samples were designated as HcS2-1, HcS2-2, HcS2-3 and HcS2-4 for resistant shrimp and designated as HcD2-1, HcD2-2, HcD2-3 and HcD2-4 for susceptible shrimp. Total RNA of hemocytes was then extracted by RNAiso Plus (TaKaRa, Kyoto, Japan) according to the manufacturers' protocols. The total RNA was qualified by 1% agarose gel electrophoresis and quantified by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Fragmentation buffer was used to break the mRNA into short fragments. The first cDNA strand was synthesized using random hexamers, followed by the addition of buffer, dNTPs, RNase H, and DNA polymerase I to synthesize the second cDNA strand. Poly(A) was added to connect to the sequencing adaptor. Finally, the Illumina HiSeqTM platform was used to sequence the library at Guangzhou Sagene Biotech Co., Ltd. (Guangzhou, China).

2.6. De novo Assembly and Annotations

In order to ensure the data quality, the clean reads obtained from the preliminary filtering were subjected to more stringent filtering. High-quality clean reads were obtained by eliminating all the reads containing the A base and more than 10% of the reads containing N. The clean reads were mapped to the reference genome of *L. vannamei* by HISAT2.2.4 [11]. The mapped reads were assembled using StringTie v 1.3.1 [12] in a reference-based approach. The reconstructed transcripts were aligned to the reference genome. Annotation was carried out by blast against Nr (NCBI non-redundant proteins), KEGG (genomic encyclopedia database), COG (homologous protein cluster), and Swiss-Prot databases.

2.7. Differential Expression and Enrichment Analysis

The differentially expressed genes (DEGs) in hemocytes between *V. parahaemolyticus* resistant and susceptible were identified by calculating the FPKM (fragment per kilobase of transcript per million mapped reads) value. DEGs were analyzed using the edgeR package (<http://www.rproject.org/>), with the parameter of false discovery rate (FDR) < 0.05 and the absolute fold change ≥ 2 . The correlation analysis between samples was carried out among samples to ensure the reliability of experimental data.

The Gene Ontology (GO) function and KEGG pathway enrichment analyses of DEGs were performed using the online OmicShare tools (<http://www.omicshare.com/tools>). The Q value < 0.05 were considered statistically significant.

3. Results

3.1. The loads of *V. parahaemolyticus* in hepatopancreas of infected shrimp

In order to distinguish the resistant ability of shrimp against *V. parahaemolyticus* infection, the load of *V. parahaemolyticus* in hepatopancreas of infected shrimp was detected. A standard curve equation of PirA^{VP} was established as $y = -3.2029x + 40.884$, where x represented the log value of *Vibrio* copy number per μL standard plasmid PirA and y represented the Ct value. The correlation coefficient was 0.9652, which showed a good linear relationship between the concentration of standard substance and the Ct value obtained (Figure 1A).

The Ct value of each shrimp detected by Taqman fluorescence quantitative PCR was substituted into the standard curve of PirA to calculate the number of *Vibrio* copies per ng DNA. As shown in Figure 1B, the loads of *V. parahaemolyticus* in hepatopancreas of shrimp died at 9 hpi and 12 hpi were very low, which were 5.07 and 59.99 copies per ng hepatopancreas DNA. The loads of *V. parahaemolyticus* were high in hepatopancreas of shrimp died at 15 hpi, 18 hpi, 24 hpi and 36 hpi, all of which were more than 1000 copies per ng hepatopancreas DNA. The load of *V. parahaemolyticus* in hepatopancreas of survival shrimp (still alive at 48 hpi) was 10.57 copies per ng hepatopancreas DNA. According to the death time and the load of *V. parahaemolyticus* in hepatopancreas, shrimp that died at 9 hpi and 12 hpi were deemed as susceptible individuals and those survival after 48 hpi were deemed as resistant individuals. Hemocytes from *V. parahaemolyticus* resistant and susceptible shrimp in two populations were used for independent comparative transcriptome analysis.

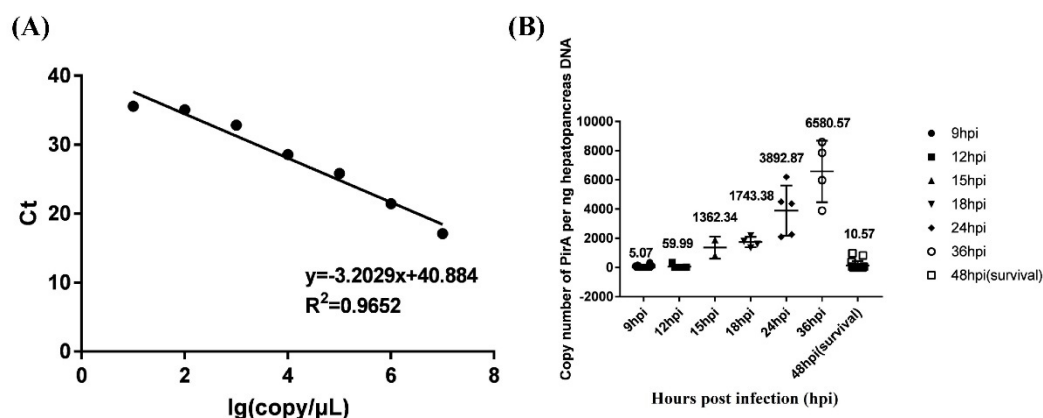


Figure 1. The load of *V. parahaemolyticus* in hepatopancreas of infected shrimp from two populations. (A) showed the standard curve equation of PirA^{VP}. (B) showed the copy number of *V. parahaemolyticus* per ng hepatopancreas DNA at different hours post infection (hpi). Survival shrimp after 48 hpi was expressed by 48hpi(survival).

3.2. The correlation of the transcriptome data from all samples

The relationship of all samples from two populations were analyzed by PCA and sample clustering methods. The results showed that samples from the same population had a close

relationship (Figure 2A and 2B). However, the difference between *V. parahaemolyticus* resistant and susceptible shrimp was small in each population (Figure 2C and 2D). The results suggest that shrimp from the same population, even with different *V. parahaemolyticus* resistant abilities, has more similar transcriptome profiles than those from different populations.

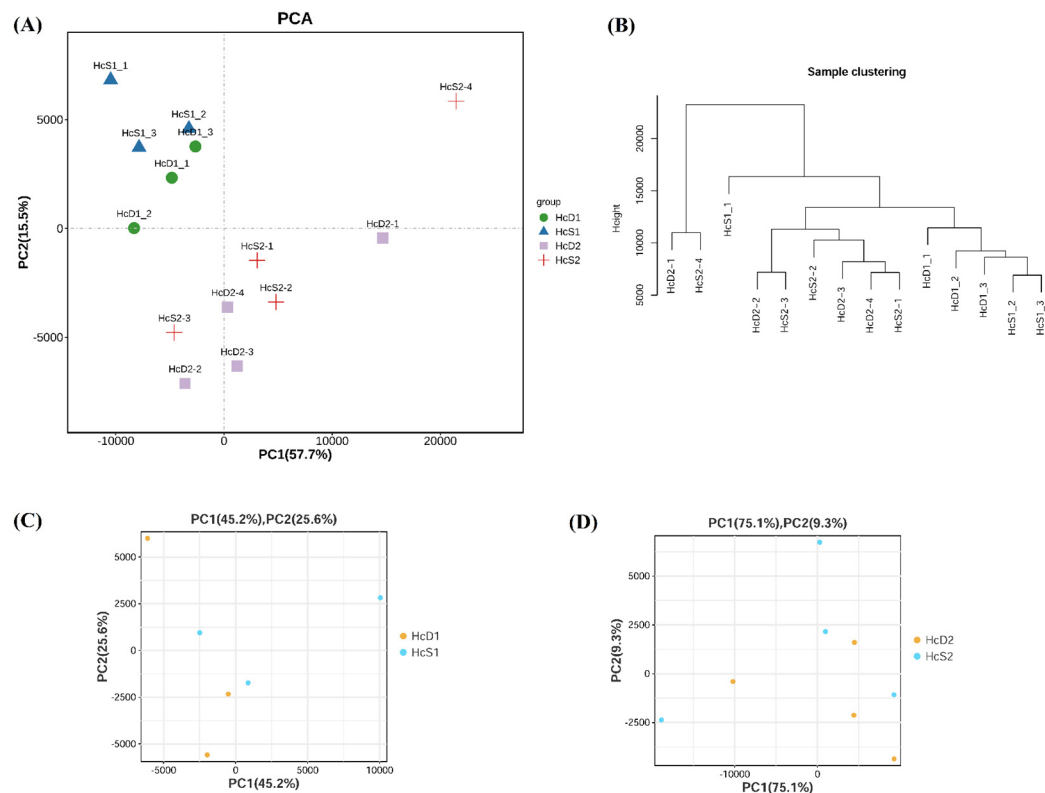


Figure 2. Sample relationship analyzed by principal component analysis (PCA) and sample clustering. (A) and (B) showed the results of PCA and clustering analysis for all samples from two populations. (C) and (D) showed the results of PCA for samples from population 1 and 2, respectively.

3.3. Differentially expressed genes (DEGs) between two populations and between *V. parahaemolyticus* resistant and susceptible shrimp

Corresponding to the sample relationship, much more DEGs were identified between two populations than those between *V. parahaemolyticus* resistant and susceptible shrimp in each population. Between two populations, there were a total of 984 DEGs, including 496 up-regulated DEGs and 488 down-regulated DEGs in the hemocytes from population 2 (Figure 3A). There were 678 DEGs between the *V. parahaemolyticus* resistant shrimp between population 1 and 2 (Figure 3B, HcS1-vs-HcS2). There were 342 DEGs the *V. parahaemolyticus* susceptible shrimp between population 1 and 2 (Figure 3B, HcD1-vs-HcD2).

Very few DEGs were identified between *V. parahaemolyticus* resistant and susceptible shrimp. There were 31 DEGs and 5 DEGs between *V. parahaemolyticus* resistant and susceptible shrimp in population 1 and population 2, respectively (Figure 3B, HcS1-vs-HcD1, HcS2-vs-HcD2).

A total of 121 DEGs were overlapping between HcS1-vs-HcS2 and HcD1-vs-HcD2 (Figure 3C). The expression trends of these DEGs showed distinct differences between two populations (Figure 3D). However, there was no overlapping DEGs between HcS1-vs-HcD1 and HcS2-vs-HcD2 (Figure 3C), indicating that the disease resistant mechanism of shrimp against *V. parahaemolyticus* infection was different in two populations.

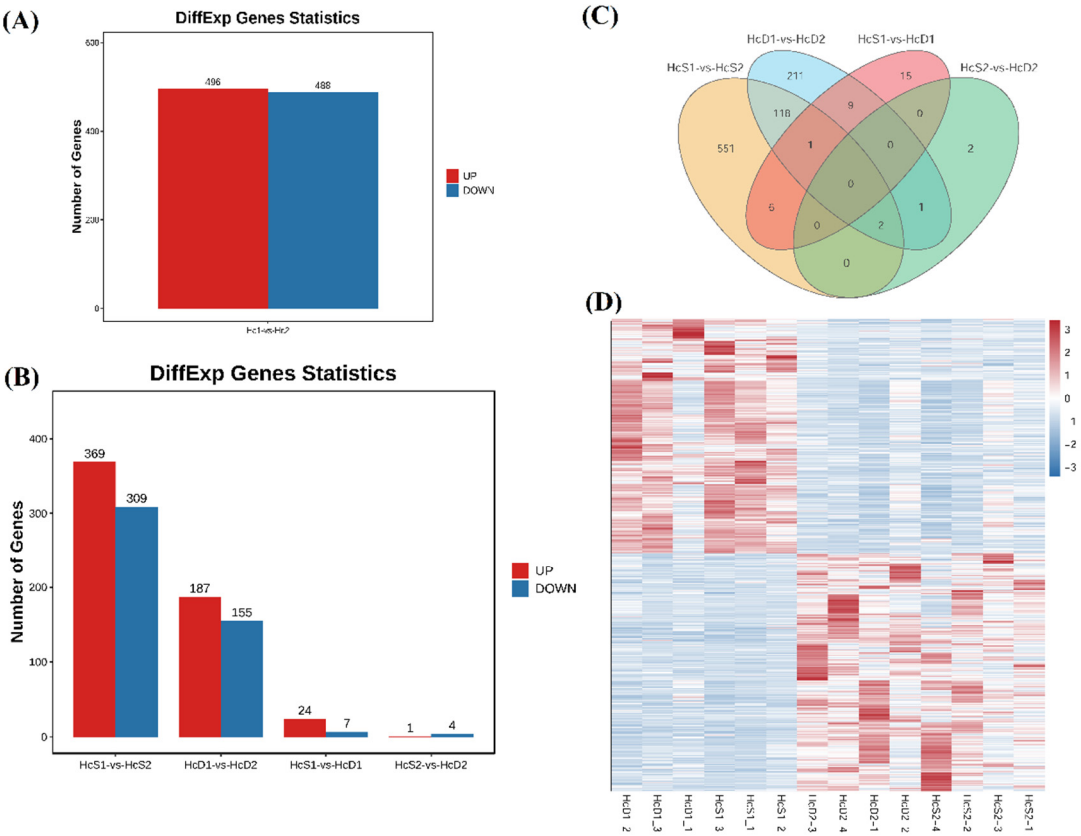


Figure 3. Identification of differentially expressed genes (DEGs) between different comparisons. (A) showed DEGs between population 1 and 2. (B) showed DEGs from comparisons HcS1-vs-HcS2, HcD1-vs-HcD2, HcS1-vs-HcD1 and HcS2-vs-HcD2. (C) showed the result of Venn analysis of DEGs from different comparisons. (D) showed the heatmap of DEGs between two populations.

3.4. Enriched GO items and KEGG pathways between *V. parahaemolyticus* resistant and susceptible shrimp

GO and KEGG enrichment results showed many DEGs in population 1 were enriched in GO items and KEGG pathways. The enrichment items in cell composition were mainly “actin cytoskeleton”, “myosin complex”, “cytoskeleton”, etc. The enriched items in molecular function were “motor activity”, “nucleoside-triphosphatase activity”, “pyrophosphatase activity”, etc. (Figure 4A). The enriched KEGG pathways included “viral myocarditis”, “hypertrophic cardiomyopathy”, “dilated cardiomyopathy” and “thyroid hormone signaling pathway” (Figure 4B). There was no GO item and KEGG pathway enriched for DEGs in population 2 because only five DEGs were identified.

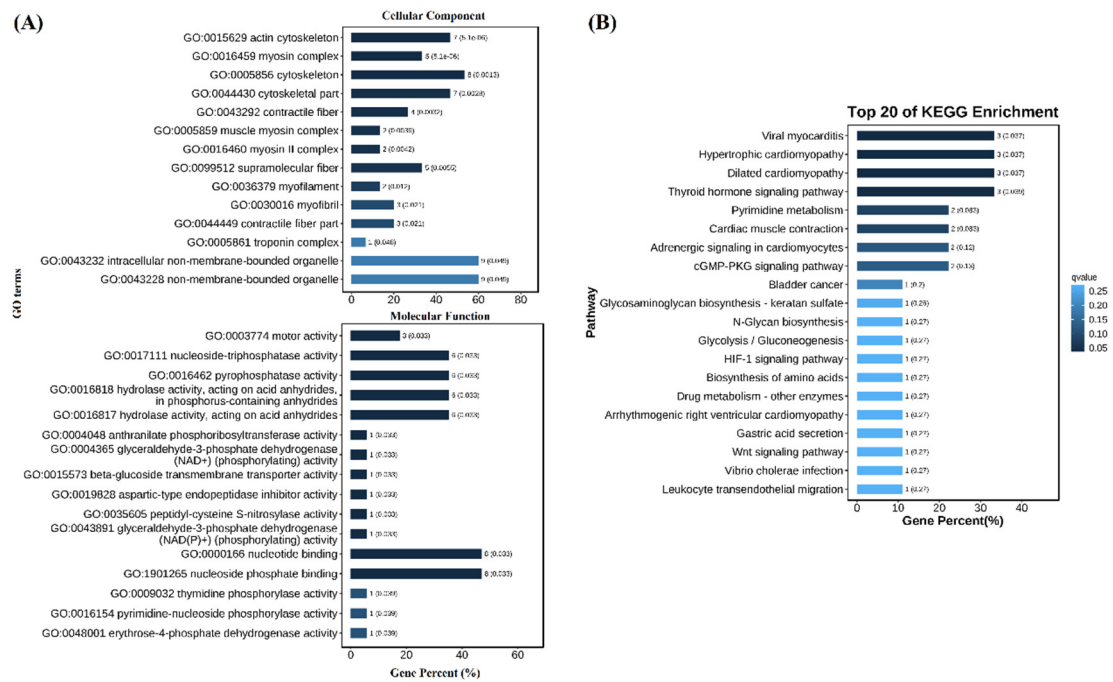


Figure 4. GO (A) and KEGG (B) enrichment of DEGs between *V. parahaemolyticus* resistant and susceptible shrimp in population 1.

3.5. Detailed analysis of DEGs between *V. parahaemolyticus* resistant and susceptible shrimp

Among the 31 DEGs in population 1, 19 had functional annotations (Table 1). Most of these DEGs exhibited higher expression levels in *V. parahaemolyticus* susceptible shrimp. DEGs encoded cytoskeleton related proteins, including troponin, myosin, septin-4, and actin, as well as immune and signal transduction-related genes, such as immune-associated nucleotide-binding protein 13-like, E3 ubiquitin-protein ligase TRIM32, and MAM and LDL-receptor class A domain-containing protein 2-like, had higher expression levels in *V. parahaemolyticus* susceptible shrimp. Sugar metabolism related genes including glyceraldehyde-3-phosphate-dehydrogenase and alpha-(1,6)-fucosyltransferase-like were also highly expressed in *V. parahaemolyticus* susceptible shrimp.

Table 1. DEGs with functional annotations between *V. parahaemolyticus* resistant and susceptible shrimp in population 1.

Gene ID	HcS1_fpkms	HcD1_fpkms	log2(HcD1/HcS1)	FDR	Description
ncbi_113820920	0.40	0.00	-8.63	0.0376	structural maintenance of chromosomes protein 2-like
ncbi_113815624	0.71	0.08	-3.13	0.0154	vang-like protein 2
MSTRG.1662	5.77	1.38	-2.07	0.0017	APC membrane recruitment protein 1-like
ncbi_113812130	67.07	109.46	0.71	0.0499	4-coumarate--CoA ligase 1 isoform X1
ncbi_113824615	186.25	373.56	1.00	0.0001	spermatogonial stem-cell renewal factor
MSTRG.4583	11.36	42.55	1.91	0.0154	arasin-like protein
ncbi_113807541	51.35	197.85	1.95	0.0397	immune-associated nucleotide-binding protein 13-like
ncbi_113817613	5.39	25.35	2.23	0.0027	E3 ubiquitin-protein ligase TRIM32
ncbi_113824399	82.06	388.92	2.24	0.0023	Septin-4-like protein
ncbi_113813611	0.34	3.21	3.24	0.0373	troponin I
ncbi_113805465	0.66	6.27	3.25	0.0017	myosin light chain 2
ncbi_113822686	0.65	7.58	3.55	0.0001	myosin light chain
ncbi_113820123	5.27	68.89	3.71	0.0000	glyceraldehyde-3-phosphate-dehydrogenase
ncbi_113819252	0.12	2.74	4.50	0.0154	actin 2
ncbi_113816511	0.04	0.95	4.64	0.0035	myosin heavy chain, muscle-like isoform X4
MSTRG.15220	0.18	7.35	5.34	0.0002	Retrovirus-related Pol polyprotein from transposon 297
ncbi_113807016	0.01	0.43	6.44	0.0411	myosin heavy chain, muscle-like isoform X8
ncbi_113823028	0.00	2.97	11.53	0.0000	alpha-(1,6)-fucosyltransferase-like

ncbi_113829244	0.00	10.32	13.33	0.0000	MAM and LDL-receptor class A domain-containing protein 2-like
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In population 2, one cytoskeleton related genes encoding tubulin α -3 was also highly expressed in *V. parahaemolyticus* susceptible shrimp. The other four DEGs, including dihydropyrimidinase-like isoform X3, prohibitin, iroquois-class homeodomain protein IRX-2-like, and diacylglycerol kinase 1, were all highly expressed in *V. parahaemolyticus* resistant shrimp (Table 2).

Table 2. DEGs with functional annotations between *V. parahaemolyticus* resistant and susceptible shrimp in population 2.

Gene ID	HcS2_fpkm	HcD2_fpkm	log2(HcD2/HcS2)	FDR	Description
ncbi_113815990	11.16	3.46	-1.69	0.0184	dihydropyrimidinase-like isoform X3
ncbi_113825784	13.80	5.06	-1.45	0.0473	prohibitin
ncbi_113824882	5.22	2.06	-1.34	0.0184	iroquois-class homeodomain protein IRX-2-like
ncbi_113829145	6.34	2.66	-1.25	0.0402	diacylglycerol kinase 1
ncbi_113813376	1.43	6.81	2.26	0.0142	tubulin alpha-3 chain-like

4. Discussion

In order to screen genes in shrimp hemocytes related to *V. parahaemolyticus* resistance, the present study established a method to collect hemocytes from shrimp with different resistant abilities against *V. parahaemolyticus* infection. The results showed that shrimp more susceptible to *V. parahaemolyticus* infection died early with low pathogen load. When shrimp exhibited higher resistant ability against *V. parahaemolyticus* infection, they could suffer from higher pathogen load and stayed alive for longer time. Shrimp with the highest resistant ability against *V. parahaemolyticus* infection could clear the pathogen in hepatopancreas after a longer time (48 h) post infection. Some other studies constructed families with distinct resistances against specific pathogen, while individuals in the same family usually exhibited different disease resistant abilities. The method used in the present study could collect hemocytes from shrimp before pathogen infection with clear pathogen resistant information.

Two independent transcriptome analyses on hemocytes of shrimp with different *V. parahaemolyticus* resistant abilities were carried out, which aimed to identify common genes related to the biological trait. However, the identified DEGs in two populations shared no overlap. Some transcriptome studies also identified genes related to *V. parahaemolyticus* resistance in shrimp hepatopancreas, but the identified genes were rarely overlap [9,10]. The results were comparable with the data from the present study. It might be caused by the different genetic backgrounds of shrimp used in different studies. In other words, different populations might employ distinct molecular mechanisms to resist *V. parahaemolyticus* infection. This brings difficulties for identification of key genes or regulatory processes related to specific pathogen.

Although there was no overlap between DEGs identified from two populations, many DEGs were reported relevant to immunity or pathogen infection. The cytoskeleton has four main components including actin, microtubules, intermediate filaments and septins, which all have important functions in cell-autonomous immunity [13]. An E3 ubiquitin-protein ligase TRIM32 gene was crucial under oxidase stress and during *Vibrio* infection in *L. vannamei* [14]. In intestine, the MAM and LDL-receptor class A domain-containing protein could regulate enterohepatic bile acid signaling, while bile acids are vital factors in mucosal immunity and inflammation [15,16]. Glyceraldehyde-3-phosphate-dehydrogenase is the key enzyme in the glycolytic pathway, which benefits viral replication during white spot syndrome virus (WSSV) in shrimp [17]. These genes were differentially expressed in hemocytes between *V. parahaemolyticus* resistant and susceptible shrimp in population 1, indicating that these genes might contribute to the resistant ability of shrimp against *V. parahaemolyticus* infection.

Although the DEGs in population 2 were totally different with those in population 1, they were also related to immunity and pathogen infection. In the central nervous system, dihydropyrimidinase-like 3 could regulate the inflammatory response of activated immune cells

microglia, which respond to infection and inflammation by producing cytokines and phagocytosing cell debris and pathogens [18]. In *Fenneropenaeus chinensis*, two prohibitin genes played important roles in hemocytes during WSSV infection [19]. Diacylglycerol kinase 1 is responsible for the ATP-dependent phosphorylation of diacylglycerol to phosphatidic acid, both of which are crucial second messengers involved in many immune processes such as regulating Toll-like receptor-induced cytokine production [20]. Tubulin alpha-3 is the major constituent of microtubules, one component of cytoskeleton. These genes might be responsible for variable resistant abilities of shrimp against *V. parahaemolyticus* infection in population 2.

Although no common gene related to *V. parahaemolyticus* resistance were identified in hemocytes from two populations, genes in specific GO item like cytoskeleton could be found in DEGs from both transcriptome data. It means that the key processes or genes related to *V. parahaemolyticus* resistance could be obtained by comprehensive analysis of multiple omics data information. A strategy was proposed here to approach this purpose (Figure 5). Individuals from the same family, which have relatively consistent genetic background, will be used to construct *V. parahaemolyticus* resistant and susceptible hemocytes samples based on the method established in the present study. Differentially expressed genes (Gene Set, GS) as well as genetic makers (GM) will be identified after comparative analysis between *V. parahaemolyticus* resistant and susceptible samples from each family. The GS and GM information from each family will be pooled to obtain the GS union and GM union. The GS union will be put for GO and KEGG enrichment analysis to identify key processes and genes related to *V. parahaemolyticus* resistance. Further association analysis between these identified key processes and genes and GM union will help to obtain useful genetic markers. The information will provide basis for understanding regulatory mechanisms of shrimp defense *V. parahaemolyticus* infection and for disease-resistant breeding.

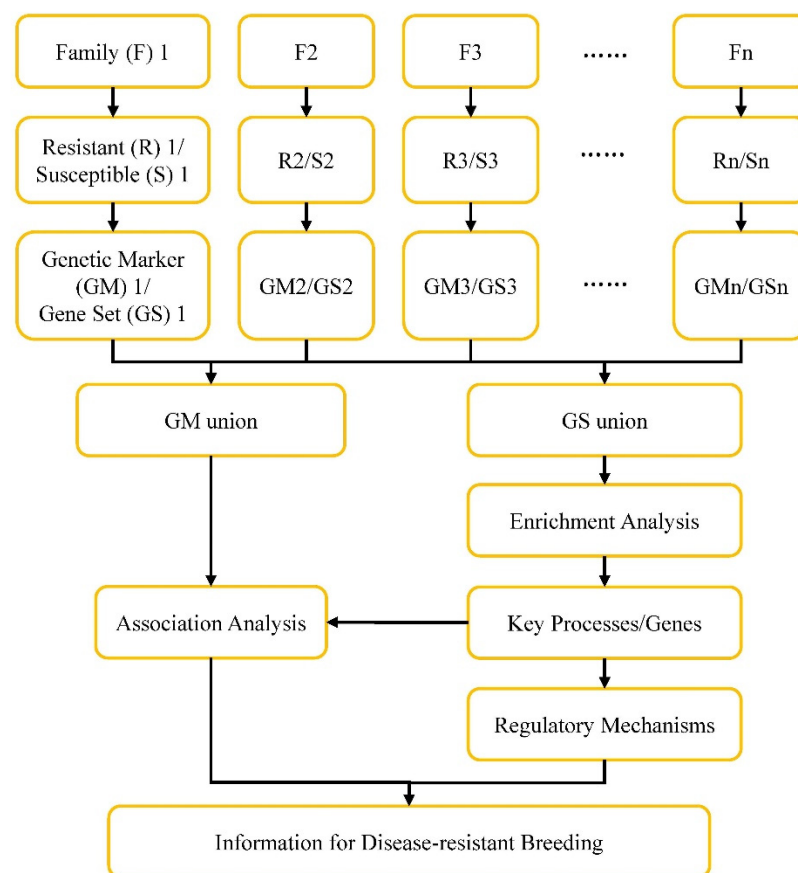


Figure 5. Framework for identification of disease-resistant processes/genes and disease-resistance breeding of shrimp.

Author Contributions: Conceptualization, S.L. and F.L.; methodology, K.Z. and W.D.; statistical analysis, S.L. and K.Z.; writing—original draft preparation, S.L. and K.Z.; writing—review and editing, S.L.; All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study used shrimp as experimental animals, which are not endangered invertebrates. In addition, there is no genetically modified organism used in the study. According to the national regulation (Fisheries Law of the People's Republic of China), no permission is required to collect the animals and no formal ethics approval is required for this study.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

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Conflicts of Interest: The authors declare no conflict of interest.

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