



# Comparative Transcriptome Analysis Reveals the Adaptation Mechanism to High Salinity in *Litopenaeus vannamei*

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As an euryhaline commercial species, the Pacific white shrimp *Litopenaeus vannamei* can be cultured under a wide range of salinities. However, different families showed various tolerance to high salinity. In order to elucidate the underlying mechanisms, a comparative transcriptome approach was used to find the differences between tolerant families and susceptible families at the transcriptional level. There were 98 and 58 differentially expressed genes (DEGs) between tolerant and susceptible families under normal and high-salinity conditions, respectively. The DEGs upregulated in tolerant families were mostly enriched in the biological process of response to a stimulus. Nine crustacyanin (CRCN)-encoding genes in this Gene Ontology (GO) term showed significantly higher expression levels in the tolerant families than those in susceptible families, suggesting that crustacyanins might play important roles in shrimp tolerance to high salinity. After high-salinity treatment, a total of 233 and 109 DEGs were identified from tolerant and susceptible families, respectively. Serine-type endopeptidase activity, serine-type peptidase activity, and serine hydrolase activity were the top three GO terms in the tolerant families after high-salinity treatment. Further analysis illustrated that these three GO terms were also the top three enriched GO terms for the DEGs specifically upregulated in the tolerant families after high-salinity stress. Meanwhile, four genes annotated in serine proteases families in these three GO terms were upregulated in the tolerant families but not changed in the susceptible families after high-salinity stress. These results indicated that serine proteases should play key roles in shrimp tolerance to high salinity. This study provides important information for understanding the mechanism of shrimp tolerance to high-salinity stress and would be useful for further molecular breeding of shrimp varieties with high-salinity tolerance.

**Keywords:** high-salinity, comparative transcriptome, tolerant families, susceptible families, *Litopenaeus vannamei*

## INTRODUCTION

The Pacific white shrimp *Litopenaeus vannamei* is one of the most widely cultured and profitable penaeid shrimp species in the world. Due to its fast growth under high density, high resistance to disease, and ability to live under a wide range of salinity (0–40 ppt), its production increased rapidly, which accounts for more than 80% of the output for penaeid shrimp aquaculture in China (Chang et al., 2020). It is now cultured both in inland freshwater areas and in high-salinity coastal areas. However, the survival and growth rates under extremely high-salinity seawater are generally lower than those cultured around optimal salinity seawater (20–25 ppt) (Li et al., 2018; Shen et al., 2020).

Compared with the normal 35 ppt salinity in the ocean, there are many high-salinity seawater bodies in the world. These areas are poorly exploited for aquaculture since no suitable farming species are available. Recently, a good example of taking advantage of high-salinity water for shrimp aquaculture was established in Shandong Province of China. This new practice makes use of the big water surface of solar salt production to culture Pacific white shrimp, which improves the utilization of high-salinity water areas. The salinity of these water bodies ranges from 45 to 60 ppt. Under such high salinity, the shrimp grow slowly, and the survival rate is very low (Chong-Robles et al., 2014). Breeding shrimp varieties with tolerance to high salinity is an optimal way to improve shrimp production in these areas. Based on the fact that different families of shrimp present various abilities to adapt to high-salinity stress, breeding shrimp varieties tolerant to high salinity is an optimal way to improve the utilization of high-salinity water. Understanding the molecular basis of shrimp tolerance to high salinity is regarded as a key step to accelerate the breeding of new shrimp varieties and provides guidance for elucidating the adaptation mechanism of aquatic animals to high-salinity stress.

With the development of high-throughput sequencing (HTS) techniques and decoding of the *L. vannamei* genome (Zhang et al., 2019), transcriptome sequencing has become an effective approach to discover genes related to economic traits (Yang et al., 2020; Santos et al., 2021). Several transcriptomes were conducted to analyze the genes' response to salinity change in *L. vannamei*. The genes related to signal transduction, metabolism, and oxidative pathways were activated after acute low salinity stress (Xu et al., 2015; Gao et al., 2016; Li et al., 2017). It was also reported that long-term high-salinity stress could lead to differential expression of genes related to osmoregulation, ion

transport, antioxidation, immune defense, bacterial lysis, and nutrient metabolism in adult *L. vannamei* (Li et al., 2020; Shen et al., 2020). Since previous studies are mainly focused on the response of shrimp to low or high-salinity stress, the molecular mechanism of shrimp tolerance to high salinity is not well understood yet.

In the present study, comparative transcriptome analysis was performed between tolerant and susceptible shrimp families under normal and high-salinity conditions to elucidate the mechanism of how shrimp tolerate high-salinity stress. This study not only provides important information for understanding the molecular basis of shrimp tolerance to high salinity but also gives guidance for molecular breeding of shrimp varieties tolerant to high salinity.

## MATERIALS AND METHODS

### Selection of Tolerant and Susceptible Families

Juveniles from 140 shrimp families were evaluated for their tolerance to high-salinity stress. Briefly, a total of 100 juveniles from each family were raised in separate tanks with seawater at a salinity of 25 ppt. In order to reduce the tank effect during the experiment, all the families were cultured in the same room, and the culturing seawater in each tank was the same. Moreover, the increase of the salinity in each tank was checked every 6 h per day. The salinity of seawater in each tank was raised from 25 to 55 ppt gradually over 3 days by adding seawater with a salinity of 100 ppt. Briefly, the seawater salinity was adjusted 4 times at 6-h intervals each day, and around 2.5 ppt was raised each time. Dead shrimp were collected every 4 h. The final survival rates of these 140 families after high-salinity treatment were calculated and presented in **Supplementary Figure 1**. Based on the survival rate of each family during high-salinity stress, two families (fam1 and fam2) were chosen as tolerant families (designated as tFam1 and tFam2, respectively), and another two families (fam103 and fam139) were selected as susceptible families (designated as sFam1 and sFam2, respectively). More information about these four families is shown in **Table 1**. In order to exclude the effects of shrimp size, two paired comparison groups (Groups I and II) were chosen for further experiments and sampling. Group I included tFam1 and sFam1, and Group II included tFam2 and sFam2. Shrimp in each group had similar body lengths but different tolerance.

**TABLE 1** | Information of four families used for transcriptome sequencing.

Comparison group	Sample name	Salinity condition	Survival rate (%)	Average body weight (g)
Group I	tFam1-25	Normal (25 ppt)	90.69	0.12 ± 0.01
	tFam1-45	High (45 ppt)		
	sFam1-25	Normal (25 ppt)	13.79	0.21 ± 0.01
	sFam1-45	High (45 ppt)		
Group II	tFam2-25	Normal (25 ppt)	68.75	1.13 ± 0.06
	tFam2-45	High (45 ppt)		
	sFam2-25	Normal (25 ppt)	5.47	0.97 ± 0.05
	sFam2-45	High (45 ppt)		

## Sampling for Transcriptome Sequencing

Another 60 healthy juveniles from above four selected families cultured under normal salinity of 25 ppt were collected and put into four separate tanks. They were kept in seawater with a salinity of 25 ppt at a temperature of  $26^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for 12 h before further high-salinity treatment. The cephalothoraces of nine individuals were collected from each family as normal salinity samples. The entire cephalothoraces of three individuals were put together as one sample, and three samples were taken for each family and preserved in liquid nitrogen for further RNA extraction. The samples of tolerant families (tFam1 and tFam2) were named tFam1-25 and tFam2-25, and the samples of susceptible families (sFam1 and sFam2) were named sFam1-25 and sFam2-25. After the above samples were collected, seawater with a salinity of 100 ppt was added into each tank gradually to raise the salinity of seawater to 45 ppt during the next 48 h. The procedure to raise the salinity of seawater was the same as described in Section 2.1. When the salinity of seawater reached 45 ppt, shrimp were collected from each family. The sampling method was the same as described above. The samples were designated as tFam1-45, tFam2-45, sFam1-45, and sFam2-45 and preserved in liquid nitrogen for further use.

## RNA Extraction and cDNA Synthesis

Total RNA was extracted using RNAiso reagent (Takara, Maebashi, Japan) following the manufacturer's protocol. The quality and integrity of RNA were evaluated by electrophoresis on 1% agarose gel. RNA content was quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was synthesized by PrimeScript<sup>TM</sup> RT reagent Kit with gDNA Eraser kit (Takara, Japan) following the manufacturer's protocol.

## RNA Sequencing and Data Preprocessing

The mRNA was enriched from the total RNA using Oligo (dT) beads and Ribo-Zero<sup>TM</sup> Magnetic Kit (Epicentre, Madison, WI, USA); then the enriched mRNA was fragmented into short fragments, which were reverse transcribed into cDNA. After purification with QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end reparation, and poly(A) tailing, the cDNA fragments were ligated to Illumina sequencing adapters. The ligation products at suitable sizes were sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology Co. (Guangzhou, China). The raw reads from the sequencing platform were filtered by fastp (Chen et al., 2018) with the following parameters: Q quality score  $\leq 20$ , reads with undetermined nucleotides larger than 10%, and reads containing adapters. The rRNA-mapped reads were removed, and the remaining reads were mapped to the genome sequence of *L. vannamei* (<http://www.shrimpbase.net/lva.download.html>) using Bowtie 2 (Langmead and Salzberg, 2012) and TopHat2.2.4 (Kim et al., 2015). The mapped reads were assembled with StringTie v1.3.1, and the gene expression levels were calculated using the FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method (Pertea et al., 2015; Pertea et al., 2016).

## Identification of Differentially Expressed Genes

Differential expression analyses of unigenes were carried out using DESeq2 (Love et al., 2014) software. The differentially expressed genes (DEGs) were selected by the false discovery rate (FDR)  $< 0.05$  and the expression fold change (FC)  $> 2$  ( $\log_2|\text{FC}| > 1$ ). Firstly, the transcriptional differences between tolerant and susceptible families were focused on; therefore, the DEGs in comparison groups (Group I and II) under both normal and high salinity were identified. Secondly, the response of shrimp to salinity change at the transcriptional level was analyzed, and the DEGs for each family were analyzed when they were cultured under normal or high-salinity seawater. In order to reduce the false positives, the DEGs with similar expression patterns in two comparison groups or similar tolerance families were selected for further analysis.

## Functional Enrichment Analysis of Differentially Expressed Genes

All selected DEGs were used for Gene Ontology (GO) functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Briefly, DEGs were mapped to GO terms in the GO database (<http://geneontology.org>) and pathways in the KEGG database (<http://www.genome.jp/kegg/>). Then, significantly enriched GO terms and KEGG pathways were recognized by a hypergeometric test-taking  $\text{FDR} \leq 0.05$  as a threshold.

## Quantitative Real-Time PCR Validation

The samples used for qPCR detection were the same as those used for transcriptome sequencing. Four DEGs were quantified with qPCR to validate the transcriptome analysis result. The Primer3plus (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>) was used for the design of gene-specific primers based on their sequences. Primers used for qPCR detection are shown in **Supplementary Table 1**, and 18S rRNA was used as the reference gene. The qPCR was conducted as follows: denaturation at  $94^{\circ}\text{C}$  for 2 min, 40 cycles of  $94^{\circ}\text{C}$  for 30 s and annealing at temperature  $58^{\circ}\text{C}$  for 20 s, and a final extension of 30 s at  $72^{\circ}\text{C}$ . The melting curve was used to check the specificity of primers, and the relative expression level was calculated with the  $2^{-\Delta\Delta\text{Ct}}$  method (Livak and Schmittgen, 2001). The two-way ANOVA (factor: salinity and family) was utilized to determine the statistically significant differences between different factors, and a one-way ANOVA was used to reveal the significant differences among different samples. These statistical analyses were performed in R.

## RESULTS

### Summary of RNA Sequencing Data

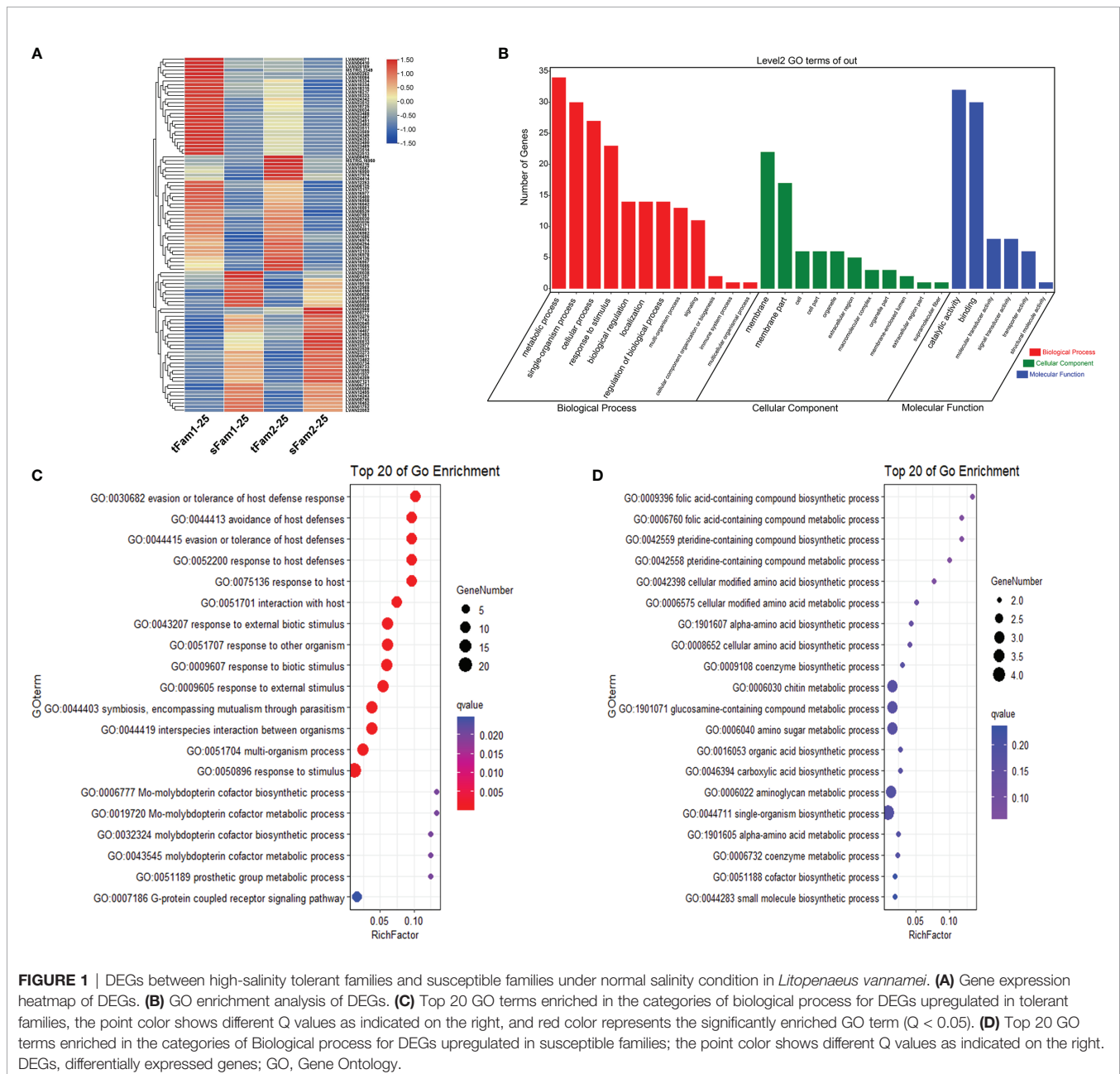
A total of 24 RNA-seq libraries were constructed for two tolerant families and two susceptible families cultured under two salinity conditions. All transcriptome sequencing data were uploaded to National Center for Biotechnology Information (NCBI) with the accession number PRJNA799461. The sequencing information was summarized in **Supplementary Table 2**. After filtering, 99.68%–

99.85% of raw reads were taken as clean reads, and 85.82%–89.70% of the clean reads were mapped to the reference genome of *L. vannamei* (Zhang et al., 2019). A total of 31,753 unigenes were annotated, and each library contained 60.74%–78.57% of total genes.

## Differentially Expressed Genes Between Tolerant and Susceptible Families Cultured Under Normal Salinity

A total of 963 DEGs were identified in Group I between tFam1-25 and sFam1-25, while 1,242 DEGs were identified in Group II between tFam2-25 and sFam2-25. In order to reduce the false-positive results caused by the family itself, the DEGs with similar

expression patterns within two comparison groups were chosen for further analysis. A total of 98 DEGs presented similar expression profiles in these two groups. Among them, 58 DEGs were upregulated and 40 DEGs were downregulated in two tolerant families (Figure 1A). GO enrichment analysis on these DEGs showed that they were enriched in metabolic process, single-organism process, cellular process and response to a stimulus for the biological process, membrane and membrane part for cellular component, and binding and catalytic activity for molecular function (Figure 1B). Further analysis of the GO terms for the biological process showed that DEGs enriched in response to stimulus were upregulated in tolerant families (Figure 1C). Nine genes encoding



**FIGURE 1** | DEGs between high-salinity tolerant families and susceptible families under normal salinity condition in *Litopenaeus vannamei*. **(A)** Gene expression heatmap of DEGs. **(B)** GO enrichment analysis of DEGs. **(C)** Top 20 GO terms enriched in the categories of biological process for DEGs upregulated in tolerant families, the point color shows different Q values as indicated on the right, and red color represents the significantly enriched GO term ( $Q < 0.05$ ). **(D)** Top 20 GO terms enriched in the categories of Biological process for DEGs upregulated in susceptible families; the point color shows different Q values as indicated on the right. DEGs, differentially expressed genes; GO, Gene Ontology.

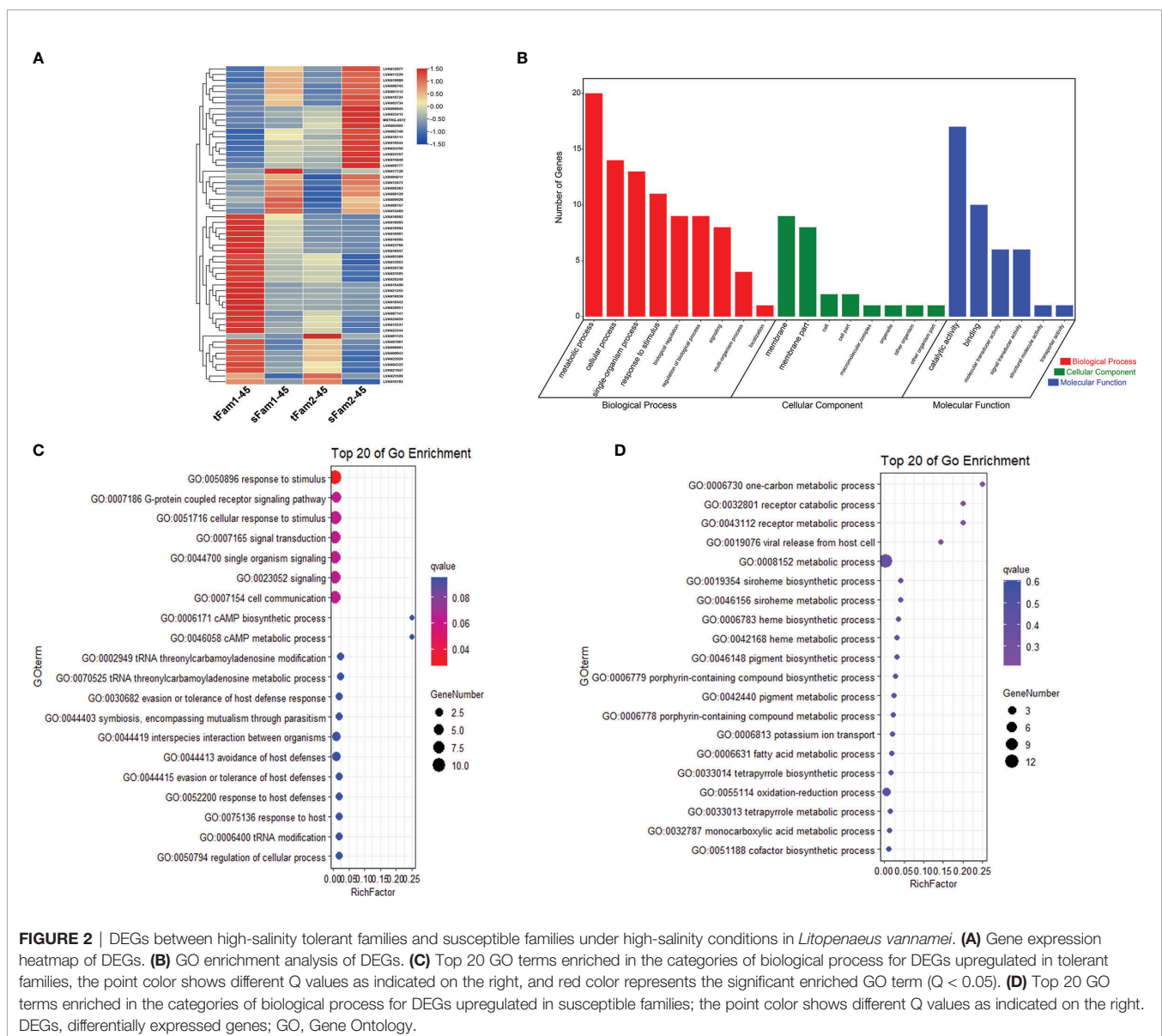


crustacyanins were found among these DEGs. In contrast, there were no significant enriched GO terms for DEGs, which were highly expressed in susceptible families (**Figure 1D**).

## Differentially Expressed Genes Between Tolerant and Susceptible Families After High-Salinity Treatment

A total of 865 DEGs were identified in the comparison group tFam1-45 and sFam1-45 and 1,660 DEGs in the other comparison group tFam2 and sFam2-45. In order to reduce the false positive caused by the family itself, 56 DEGs with the same expression pattern in the two comparison groups were chosen for further analysis. Among these DEGs, 30 DEGs showed higher expression levels in tolerant families, and 26

DEGs presented higher expressions in susceptible families (**Figure 2A**). Further analysis of these DEGs showed that metabolic process, cellular process, single-organism process, and response to stimulus were the top four enriched GO terms in the category of the biological process. Binding and catalytic activity were the top two enriched GO terms in the category of molecular functions, which were very similar to those under normal salinity (**Figure 2B**). Statistical analysis showed that genes highly expressed in tolerant families were enriched significantly ( $Q$  value  $< 0.05$ ) in terms of response to a stimulus for the biological process. Two genes encoding crustacyanins were found in the enriched response to the stimulus GO term. As for the DEGs upregulated in susceptible families, no significant enrichment term was found (**Figures 2C, D**).



## Common Differentially Expressed Genes Between Tolerant and Susceptible Families Under Both Normal and High-Salinity Conditions

Further analysis of the DEGs between tolerant and susceptible families under both normal and high salinities showed that four DEGs had higher expression levels in tolerant families, and six DEGs presented higher expression levels in susceptible families (Table 2). Four highly expressed genes in tolerant families were crustacyanin subunit C, 26S protease regulatory subunit 6A-B, actin, and toll-like receptor. Among the six DEGs highly expressed in susceptible families, four genes had annotations, including trypsin-1-like, compound eye opsin BCRH2-like, triose-phosphate isomerase, and mucin-2-like.

## Transcriptomic Response to High-Salinity Treatment in Tolerant and Susceptible Families

A total of 891, 409, 1,649, and 1,149 DEGs were identified from tFam1, sFam1, tFam2, and sFam2 families, respectively, after high-salinity treatment. Further analysis showed that 26 DEGs presented similar expression patterns in these four families. Among them, 21 DEGs showed upregulated expression levels and five DEGs showed downregulated expression levels after high-salinity treatment (Figure 3A). GO enrichment analysis on these DEGs showed that they were enriched in the items including single-organism process, metabolic process, cellular process for biological process, membrane and membrane part for cellular components, catalytic activity, and binding for molecular functions (Figure 3B). Further analysis showed that oxidation–reduction process was the most enriched item for the biological process (Figure 3C). Among the 21 upregulated DEGs, six unigenes were related to the oxidation–reduction process, including cytochrome P450 epoxidase-like protein, D-aspartate oxidase, alkylglycerol monooxygenase-like, spermine oxidase-like isoform X2, type I iodothyronine deiodinase-like isoform X1, and Ia-related protein 6-like.

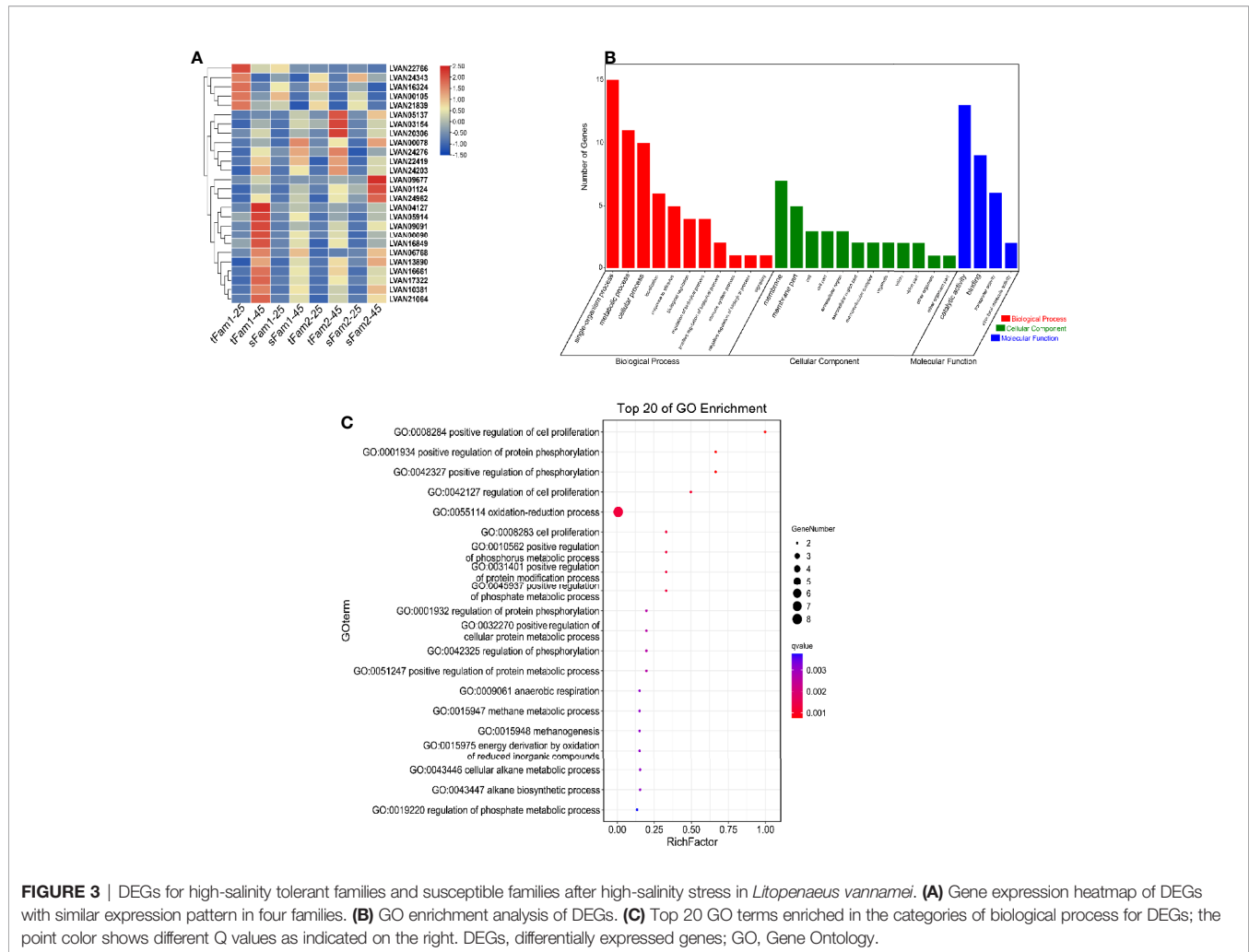
In order to elucidate how the tolerant family adapts to high-salinity treatment, the DEGs for the two tolerant families under normal and high salinity were further analyzed. A total of 233 DEGs presented similar expression profiles in these two families (Figure 4A). Among these DEGs, 160 DEGs were upregulated and 73 DEGs were downregulated after high-salinity treatment in comparison with their controls at a salinity of 25 ppt (Figure 4B). GO enrichment analysis indicated that metabolic process was the most enriched GO term for the biological process, membrane was the most enriched one for cellular component, and catalytic activity was the most enriched GO term for molecular function (Figure 4C). Among them, serine-type endopeptidase activity, serine-type peptidase activity, and serine hydrolase activity were the top three enriched GO terms (Figure 4D). Amino acid metabolic process including tyrosine metabolism pathway and beta-alanine metabolism pathway were the top two KEGG enrichment pathways (Figure 4E). Genes encoding serine proteases, prophenoloxidase, and some osmoregulation genes, such as solute carriers and sodium/glucose cotransporter, showed significant upregulations in these two tolerant families after high-salinity treatment. These data indicated that the amino acid metabolism pathway and osmoregulation process might play a key role in shrimp to handle with high-salinity stress. Interestingly, five rhodopsin genes were found to be upregulated in tolerant families under high-salinity treatment, and they were also DEGs between tolerant families and susceptible families under high-salinity conditions.

In order to know whether the DEGs shared by two tolerant families showed different expression patterns in susceptible families after high-salinity treatment, the expressions of those DEGs in susceptible families were further analyzed. A total of 63 upregulated DEGs and 45 downregulated DEGs were found to be specifically recognized in tolerant families after high-salinity treatment (Tables 3, 4). Serine-type peptidase activity, serine hydrolase activity, and serine-type endopeptidase activity were the top three enriched GO terms in the DEGs specifically upregulated in the tolerant families. Four genes belonging to serine proteases families, including clip domain serine proteases, serine proteases, chymotrypsin-like protein, and prophenoloxidase-activating enzyme

**TABLE 2** | DEGs between tolerant families and susceptible families under both normal and high-salinity conditions.

Group	Gene ID	Gene annotation	Relative expression folds (log2 transformed)			
			tFam1-25 and sFam1-25	tFam2-25 and sFam2-25	tFam1-45 and sFam1-45	tFam2-45 and sFam2-45
<b>Upregulated</b>	LVAN07881	Actin, alpha skeletal muscle	1.01	1.58	1.65	1.01
	LVAN23509	Crustacyanin subunit C	4.10	2.09	5.12	2.19
	LVAN06601	Toll-like receptor	3.03	6.80	4.21	3.49
	LVAN15400	26S protease regulatory subunit 6A-B	12.03	6.51	7.90	9.90
<b>Downregulated</b>	LVAN00777	Uncharacterized protein LOC108675418	-2.57	-1.96	-1.86	-1.93
	LVAN12977	Unknown	-1.58	-2.05	-1.79	-1.57
	LVAN08745	Trypsin-1-like	-15.28	-10.75	-9.91	-7.53
	LVAN03734	Compound eye opsin BCRH2-like	-11.36	-11.66	-7.50	-13.09
	LVAN04611	Triose-phosphate isomerase	-1.66	-8.04	-1.01	-6.79
	LVAN09928	Mucin-2-like	-2.79	-2.36	-1.08	-2.16

DEGs, differentially expressed genes.



III, were shared by the above three GO terms (Figures 5A, B), which showed higher expressions in tolerant families after high-salinity treatment. These data suggested that serine protease activity should play key roles in shrimp tolerance to high salinity. As for the DEGs specifically downregulated in tolerant families, no significant enriched GO term was found (Figures 5C, D).

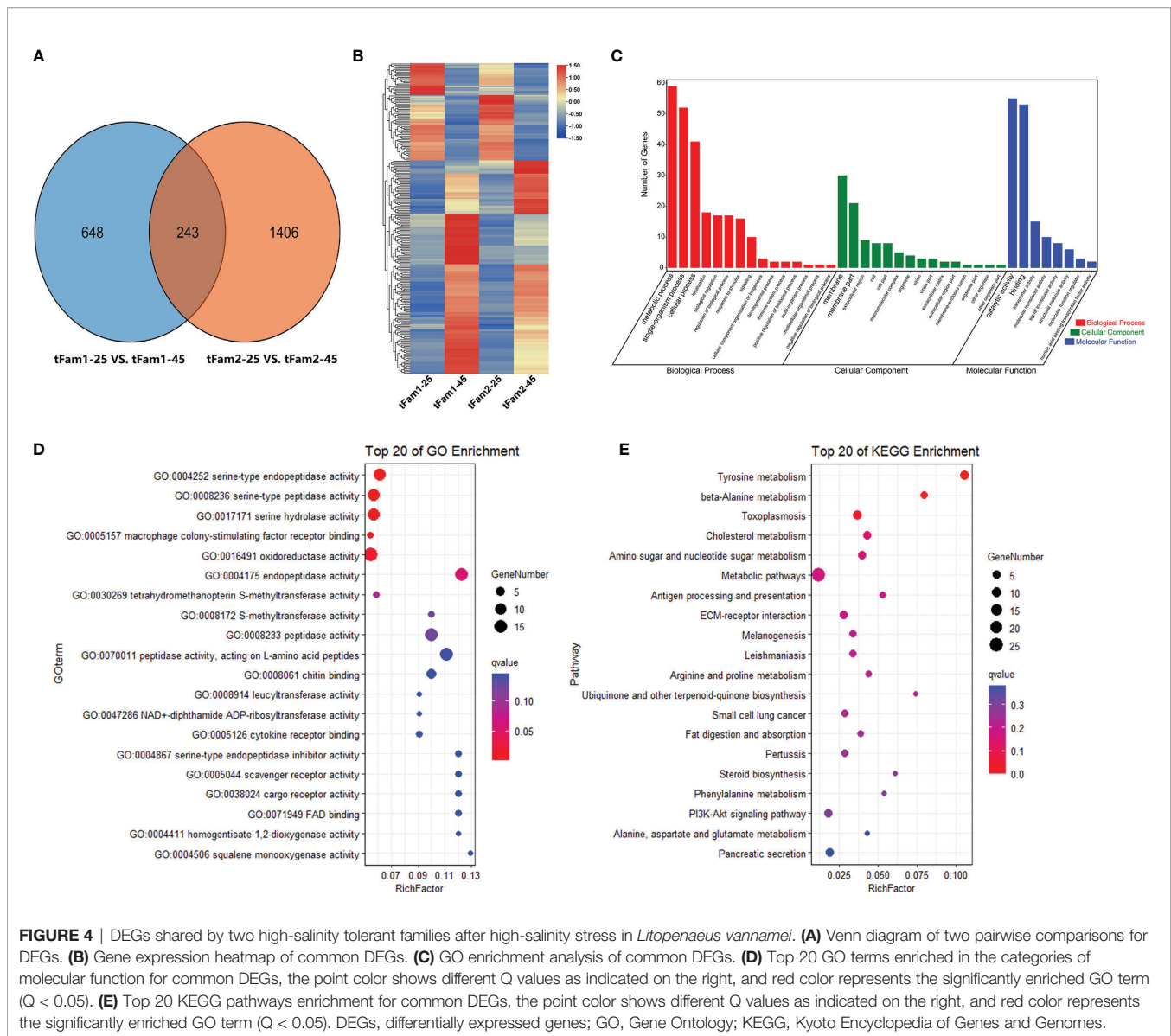
## Confirmation of Transcriptome Sequencing Data

The qPCR detection of four representative DEGs, including LVAN11695, LVAN07881, LVAN23509, and LVAN04611, are shown in Figure 6. The expression tendency of these four genes was consistent with the transcriptomic sequencing results, suggesting that the transcriptome analysis data were reliable.

## DISCUSSION

Comparative transcriptome analysis showed that response to stimulus was one of the most enriched GO terms for the biological process among the DEGs upregulated in tolerant

families in comparison with susceptible families. There were 20 genes enriched in response to stimulus GO term, which was annotated as crustacyanin, 26S protease regulatory subunit 6A-B, rhodopsin, and myosin heavy chain type 2, etc. It is interesting to note that multiple crustacyanin-encoding genes were present in GO terms of response to a stimulus with higher expressions in tolerant families. Crustacyanins are members of the lipocalin family of proteins, which can bind to astaxanthin to contribute to antioxidation in crustaceans (Ferrari et al., 2012; Gao et al., 2021). Their high expressions in tolerant families might indicate that more astaxanthin could be stored in shrimp however, the content of astaxanthin in shrimps has not been measured yet. Astaxanthin was reported to scavenge reactive oxygen free radicals in cells and reduce oxidative damage (Stanier et al., 1971; Ando & Hatano, 1988). High-salinity stress can cause oxidative stress and stimulate the production of pro-inflammatory and pro-apoptotic factors in aquaculture species, but the antioxidant system can resist oxidative stress and protect the body from high-salinity damage (Caxico Vieira et al., 2018; Wang et al., 2022). In the present study, we found that the oxidation-reduction process was the most enriched term for the biological process in both tolerant and susceptible families after



high-salinity treatment. This result also supports that oxidation–reduction process is a main physiological response in shrimp exposed to high-salinity stress. Therefore, higher expressions of crustacyanins in tolerant families should contribute to the shrimp’s tolerance to high salinity.

Aside from crustacyanins, we found that 26S protease regulatory subunit 6A-B, alpha actin, and toll-like receptors also showed higher expressions in tolerant families. 26S proteasome regulatory subunit plays a central role in protein degradation (Fan et al., 2016); the upregulation of the 26S proteasome regulatory subunit gene in tolerant families may help to remove the damaged proteins caused by high-salinity stress and improve oxidative stress tolerance (Kurepa et al., 2008). Alpha actins are often found in muscle tissue cells, which was reported to be related to cytoskeleton remodeling (Beck et al., 2014); the high expression of alpha actin gene in

tolerant families may help to reduce the high-salinity damage on muscle tissue. Toll-like receptor, as a pattern recognition receptor, plays key roles in the humoral immunity of shrimp (Li and Xiang, 2013). The upregulation of toll-like receptor gene in tolerant families might produce more immune effectors, such as antimicrobial peptides (AMPs), to stimulate innate immunity and enhance shrimp health, although the health status of shrimps was not assessed in this study. In addition, trypsin-1-like, triose-phosphate isomerase, etc., showed lower expressions in the tolerant families. Trypsin is a digestive protease that mainly participates in food digestion (Huang et al., 2020), and triose-phosphate isomerase also plays important roles in metabolism (Liao et al., 2020). The downregulations of trypsin and triose-phosphate isomerase genes in tolerant families might influence energy supply and substance metabolism. The variance of energy and metabolites may change the salinity tolerance of shrimp.



**TABLE 3** | Genes upregulated significantly in tolerant families but not significant in susceptible families under high-salinity stress.

Gene ID	Gene annotation	Relative expression folds (log2 transformed)			
		tFam1-25 and tFam1-45	tFam2-25 and tFam2-45	sFam1-25 and sFam1-45	sFam2-25 and sFam2-45
LVAN00047	Unknown	1.34	1.36	0.95	0.49
LVAN00985	Unknown	2.08	3.32	0.44	0.84
LVAN01466	Prophenoloxidase-2	1.66	1.01	0.43	0.99
LVAN01467	Prophenoloxidase 2	1.90	1.06	0.39	0.90
LVAN02645	Low-density lipoprotein receptor-related protein 2-like	1.10	1.22	-0.49	0.25
LVAN02996	Unknown	1.24	1.01	0.36	0.19
LVAN03211	Sphingosine-1-phosphate lyase	1.29	1.26	0.21	0.89
LVAN03903	Kynureninase-like	1.05	1.04	-0.71	0.82
LVAN04215	Unknown	3.49	2.87	0.39	0.84
LVAN04611	Triosephosphate isomerase	1.03	1.51	0.37	0.28
LVAN05407	C type lectin containing domain protein	3.66	3.06	0.32	0.65
LVAN05534	X-linked interleukin-1 receptor accessory protein-like 1	1.64	1.11	0.31	0.44
LVAN05704	Unknown	1.28	1.44	0.67	0.61
LVAN06624	Uncharacterized protein	1.23	1.05	0.95	0.64
LVAN07651	Chitinase 1 precursor	1.90	1.41	0.93	0.86
LVAN07652	Chitinase 1 precursor	1.96	1.29	0.96	0.83
LVAN08270	Hemocyanin subunit L2, partial	1.29	1.42	0.26	0.44
LVAN08427	Unknown	1.14	1.05	0.32	0.90
LVAN08851	Dopamine beta hydroxylase, partial	2.19	1.34	0.86	0.96
LVAN09584	C-type lectin	2.27	3.88	-0.74	-0.42
LVAN09603	Chitinase	1.50	1.33	0.67	0.77
LVAN09726	Uncharacterized protein	1.44	1.62	0.03	0.71
LVAN11238	Unknown	1.16	1.78	0.15	0.52
LVAN11239	Clip domain serine proteinase 1	1.13	1.26	0.41	0.94
LVAN11549	Organic cation transporter protein-like	1.61	1.07	0.42	0.99
LVAN12159	Pacifastin light chain-like serine proteinase inhibitor	1.66	1.01	0.41	0.77
LVAN12348	Serine proteinase	1.62	1.44	0.89	0.95
LVAN12835	Alpha 2 macroglobulin	1.29	1.07	0.67	0.69
LVAN13031	Unknown	1.09	1.12	0.99	0.73
LVAN13150	Unknown	1.35	1.38	0.77	0.65
LVAN13456	Coagulation factor IX-like	1.29	1.12	-0.01	0.89
LVAN13458	Coagulation factor IX-like	1.85	1.26	-0.27	0.90
LVAN13911	Pretinoid-inducible serine carboxypeptidase-like	1.06	1.09	0.32	0.84
LVAN14321	Unknown	1.01	1.09	0.98	0.77
LVAN15786	Facilitated trehalose transporter Tret1-2 homolog isoform X1	1.43	1.03	0.86	0.53
LVAN16003	Apolipoprotein D-like	1.08	2.42	0.93	0.39
LVAN16370	Sorbitol dehydrogenase	1.03	2.91	0.10	0.96
LVAN16437	Unknown	1.04	5.16	-0.17	0.33
LVAN16516	Pacifastin light chain-like serine proteinase inhibitor	1.51	1.12	0.45	0.80
LVAN16825	Unknown	3.02	1.20	0.31	0.70
LVAN17679	Kazal-type serine proteinase inhibitor 2	1.32	1.10	0.63	0.97
LVAN17854	Pentraxin-related protein PTX3-like	1.25	1.01	-0.15	0.92
LVAN18146	Chitinase 1 precursor	1.69	2.41	0.53	0.44
LVAN19285	Unknown	1.30	1.53	0.26	0.26
LVAN20738	Fibrocystin-L	1.10	1.29	0.86	-0.62
LVAN21999	Chitinase 5	1.10	1.04	0.25	0.75
LVAN22263	Serine proteinase 1	1.67	1.18	0.76	0.60
LVAN22382	Asialoglycoprotein receptor 2	1.45	2.26	-0.43	-0.84
LVAN24399	Prophenoloxidase-activating enzyme III	2.29	1.36	0.42	0.63
LVAN24765	Cubilin-like	1.14	1.08	0.39	0.90
LVAN25326	Tick legumain	1.46	1.50	0.81	0.48
MSTRG.10509	Cyclin-dependent kinases regulatory subunit-like	1.73	1.05	0.58	0.29
MSTRG.12484	Juvenile hormone esterase-like carboxylesterase 1	1.85	3.82	0.66	-0.86
MSTRG.17515	Hypothetical protein L798_07968	2.97	3.20	-0.14	-0.07
MSTRG.19342	Renin receptor-like	1.32	1.36	0.26	0.18
MSTRG.20671	Molybdenum cofactor biosynthesis protein 1 isoform X3	1.82	1.73	0.91	-0.47
MSTRG.21499	Unknown	1.60	1.23	0.87	-0.56

(Continued)

**TABLE 3** | Continued

Gene ID	Gene annotation	Relative expression folds (log2 transformed)			
		tFam1-25 and tFam1-45	tFam2-25 and tFam2-45	sFam1-25 and sFam1-45	sFam2-25 and sFam2-45
MSTRG.21587	Spermine oxidase isoform X3	1.88	2.70	0.99	0.68
MSTRG.22578	Unknown	1.20	3.01	-0.10	0.38
MSTRG.22625	Battenin-like	1.58	1.34	0.37	0.15
MSTRG.22790	Zinc fingers and homeoboxes protein 1, isoform 2-like	3.10	2.02	0.60	0.00
MSTRG.3538	Formin-like protein CG32138	1.33	1.76	0.71	0.16

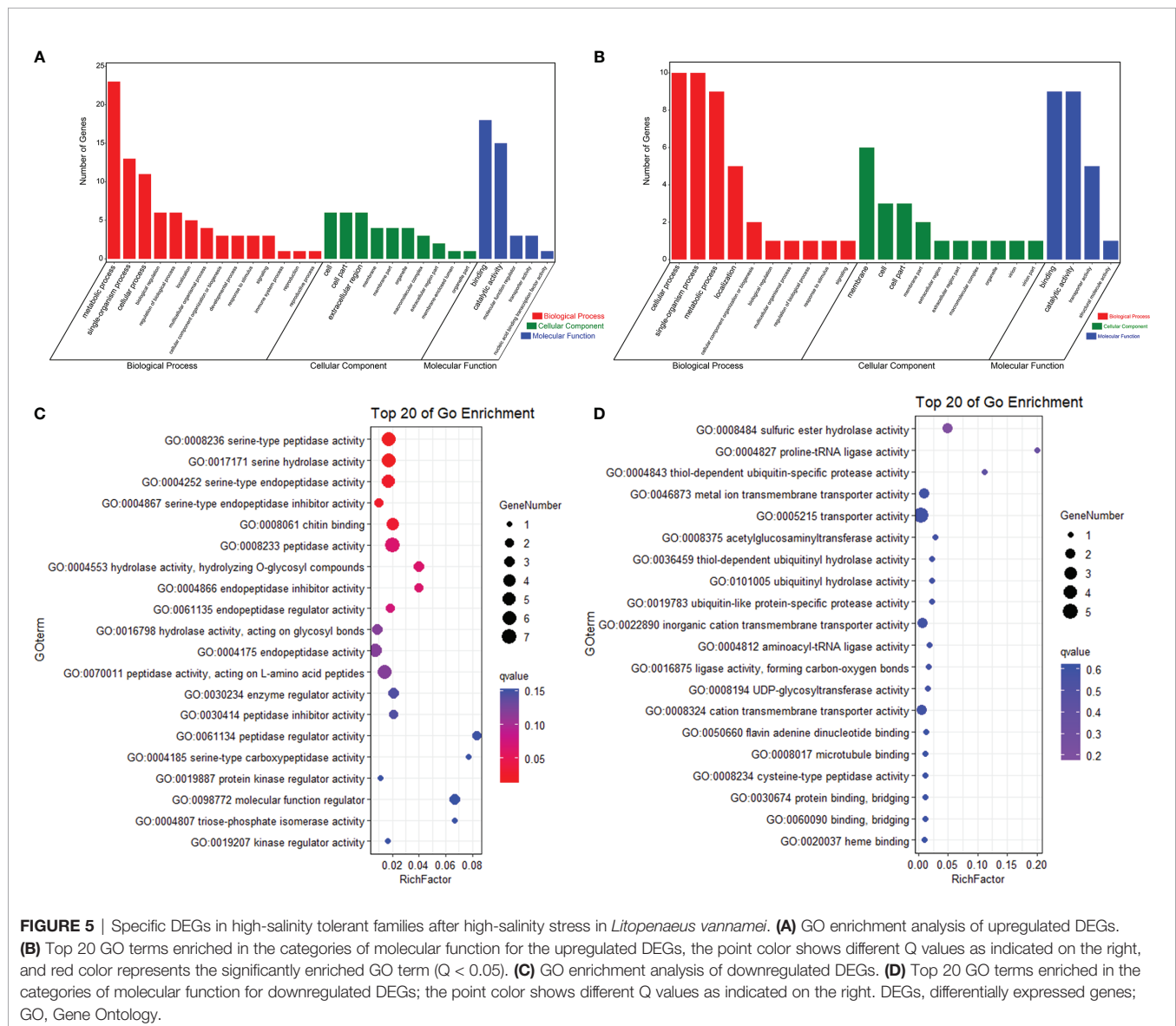
**TABLE 4** | Genes downregulated significantly in tolerant families but not significant in susceptible families under high-salinity stress.

Gene ID	Gene annotation	Relative expression folds (log2 transformed)			
		tFam1-25 and tFam1-45	tFam2-25 and tFam2-45	sFam1-25 and sFam1-45	sFam2-25 and sFam2-45
LVAN00066	Unknown	-4.06	-1.15	-0.92	-0.45
LVAN00215	Brain chitinase and chia	-1.51	-1.60	-0.97	0.98
LVAN01161	KDEL motif-containing protein 1-like isoform X5	-1.36	-1.18	-0.64	-0.49
LVAN01227	Arylsulfatase B-like	-1.06	-1.22	-0.49	-0.04
LVAN01269	Protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase 1-like	-1.32	-1.25	-0.89	0.25
LVAN01895	Calcified cuticle protein CP19.0 isoform A	-4.06	-1.36	-0.79	-0.04
LVAN02171	Delta(24)-sterol reductase	-2.18	-1.64	-0.69	-0.97
LVAN02610	Conserved Plasmodium protein, unknown function	-1.03	-1.89	-0.30	0.04
LVAN03543	Glycerol-3-phosphate phosphatase-like	-1.22	-2.12	-0.84	-0.76
LVAN04921	AAEL012429-PA, partial	-1.03	-1.43	-0.56	-0.47
LVAN06319	Ubiquitin carboxyl-terminal hydrolase isozyme L5	-1.25	-1.60	0.63	-0.76
LVAN06834	LOW QUALITY PROTEIN: arylsulfatase B-like	-2.47	-1.00	-0.76	-0.71
LVAN08005	Outer dense fiber protein 3	-1.79	-2.12	-0.71	-0.84
LVAN10260	Uncharacterized protein	-4.64	-1.09	-0.94	-0.81
LVAN10397	Major facilitator superfamily transporter 17	-1.18	-1.89	-0.84	0.23
LVAN10944	Unknown	-4.06	-1.15	-0.64	-0.74
LVAN11539	Pollen-specific leucine-rich repeat extensin-like protein 2 isoform X1	-1.51	-2.00	-0.92	-0.69
LVAN12029	CYP18A1, partial	-1.12	-1.89	-0.74	0.18
LVAN12711	Ecdysteroid regulated-like protein	-1.18	-2.74	-0.54	-0.14
LVAN13778	Protein ecdysoneless homolog	-1.36	-1.74	-0.32	0.14
LVAN13933	Hemolin-like protein	-3.06	-1.06	-0.60	-0.49
LVAN15320	Fed tick salivary protein 6	-1.64	-1.47	0.16	-0.62
LVAN15326	Fed tick salivary protein 6	-1.64	-1.47	0.16	-0.62
LVAN17523	M28	-3.64	-2.40	0.21	0.55
LVAN18229	Nocturnin-like	-1.06	-1.25	-0.03	0.45
LVAN18465	Sphingomyelin synthase-related protein 1-like	-1.15	-1.18	-0.03	-0.67
LVAN18686	Ecdysteroid-regulated protein	-2.56	-1.32	-0.97	-0.69
LVAN19805	Unknown	-1.43	-1.94	-0.76	-0.47
LVAN20503	Sodium/myo-inositol cotransporter, partial	-1.12	-1.74	-0.14	-0.14
LVAN20947	Keratin, type I cytoskeletal 9-like	-1.15	-1.12	-0.64	0.07
LVAN24085	RING finger protein nhl-1	-1.22	-1.09	-0.67	0.01
LVAN24136	Heat shock protein 70 kDa, partial	-1.43	-1.84	0.00	-0.36
LVAN24492	Bifunctional proline-tRNA ligase	-1.64	-1.03	-0.92	0.24
LVAN25261	Sodium/myo-inositol cotransporter, partial	-1.22	-1.56	-0.45	0.55
MSTRG.11278	Unknown	-2.94	-1.94	0.73	-0.56
MSTRG.12970	Unknown	-1.64	-6.64	0.57	0.94
MSTRG.1859	Unknown	-2.74	-6.64	0.70	-0.62
MSTRG.19734	Transmembrane protein 268 isoform X3	-1.32	-5.06	-0.23	-0.60
MSTRG.19845	Transmembrane protein 53-A-like isoform X2	-1.29	-1.36	0.32	-0.38
MSTRG.20148	Unknown	-3.84	-1.40	0.74	0.00
MSTRG.22155	Unknown	-1.64	-3.32	-0.49	0.21
MSTRG.22225	Unknown	-1.89	-1.47	-0.94	0.06
MSTRG.22404	Deoxynucleotidyltransferase terminal-interacting protein 2, partial	-2.47	-4.64	-0.14	0.40

(Continued)

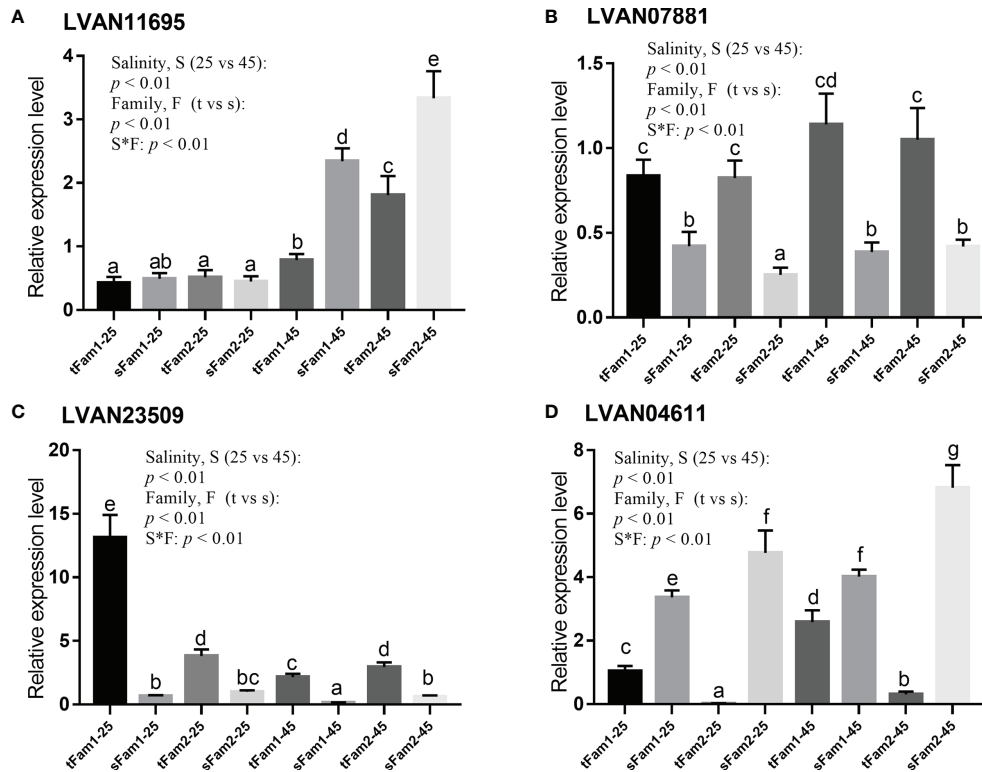
TABLE 4 | Continued

Gene ID	Gene annotation	Relative expression folds (log2 transformed)			
		tFam1-25 and tFam1-45	tFam2-25 and tFam2-45	sFam1-25 and sFam1-45	sFam2-25 and sFam2-45
MSTRG.5916	Unknown	-1.06	-1.03	-0.54	0.65
MSTRG.6710	ATP-binding cassette sub-family B member 6, mitochondrial	-3.06	-4.64	0.80	0.31



In addition, the serine-type endopeptidase activity, serine-type peptidase activity, and serine hydrolase activity were the top three GO terms for DEGs, which were only upregulated in two tolerant families after high-salinity stress. These three GO terms were mostly related to the innate immunity of aquatic animals (Sun et al., 2018; Xu et al., 2018; Lyu et al., 2019). Serine proteases

regulate several invertebrate defense responses, including hemolymph coagulation, antimicrobial peptide synthesis, and melanization of pathogen surfaces (Gorman and Paskewitz, 2001), which were reported to play an important role in the innate immune response of honeybee and shrimp (Zou et al., 2006; Dong and Xiang, 2007). It was reported that the



**FIGURE 6** | Comparison of the relative expression levels of four DEGs between tolerant families and susceptible families under salinity of 25 and 45 ppt detected by qPCR. Two-way ANOVA (factor: salinity and family) analysis was utilized to determine the statistical differences, and one-way ANOVA was used to reveal the significant differences among different samples,  $p$  represents the  $p$ -value of two-way ANOVA, S\*F represents the interaction between salinity and family, t represents tolerant family, and s represents susceptible family. Columns with the same letters show no significant difference, while columns with different letters indicate significant differences. **(A)** LVAN11695: betaine-homocysteine S-methyltransferase, **(B)** LVAN07881: actin, alpha skeletal muscle, **(C)** LVAN23509: crustacyanin subunit C, and **(D)** LVAN04611: triose-phosphate isomerase. DEGs, differentially expressed genes.

susceptibility of shrimp to pathogens increased when they were exposed to salinity stress (Wang and Chen, 2005; Joseph and Philip, 2020). Genes related to immune defense and apoptosis were upregulated in the high-salinity environment in *L. vannamei* (Li et al., 2020). Therefore, we hypothesized that tolerant families might have enhanced immunity to protect them from pathogen infection under high salinity.

It was reported that osmoregulation-related genes, such as solute carriers, sodium/glucose cotransporter, and sodium- and chloride-dependent glycine transporter, were responsive to high-salinity stress (Li et al., 2020; Shen et al., 2020). In the present study, we found that the above genes did not show any difference between tolerant families and susceptible families. These data indicated that although the abovementioned osmoregulation-related genes were responsive to high-salinity stress, they are not the key genes related to the tolerance of shrimp to high salinity. It is very surprising to note that five rhodopsin genes were not only upregulated in tolerant families under high-salinity stress but also the DEGs between tolerant families and susceptible families cultured under high-salinity conditions. Rhodopsins are usually known as primary photoreceptor molecules for the vision of animals (Papatsenko

et al., 1997). Recent studies indicated that rhodopsins in bacteria participate in chloride pumps and help them to adapt to salinity changes (Mous et al., 2022). Therefore, we hypothesized that rhodopsins might play important roles in shrimp to adapt to high salinity. As for the functioning mechanism of rhodopsins in the high-salinity tolerance of shrimp, further work needs to be done in the future.

## CONCLUSION

In this study, we performed comparative transcriptome analyses between high-salinity tolerant and susceptible families to explore the adaptation mechanism of *L. vannamei* to high salinity. Our study identified some pathways or genes associated with shrimp tolerance to high salinity. The identified genes in the present study will not only provide clues for dissecting the molecular mechanism of shrimp tolerance to high salinity but also have the potential to be used as gene markers for assisting the genetic breeding of shrimp by distinguishing tolerant and susceptible families at gene expression levels.



## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, PRJNA799461.

## AUTHOR CONTRIBUTIONS

ZL, YY, and FL contributed to the conception and design of the study. ZL performed the statistical analysis and wrote the first draft of the manuscript. QZ and ZB prepared the material. YY, JX, and FL wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.864338/full#supplementary-material>

**Supplementary Figure 1** | Survival rate of different families under high-salinity stress in *L. vannamei*. The black columns represent families selected for comparative transcriptome analysis.

**Supplementary Table 1** | Primer sequences and annealing temperature used for RT-qPCR.

**Supplementary Table 2** | Summary of RNA sequencing data information.

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