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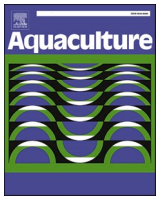

Comparative transcriptome and gene co-expression network analysis identifies key candidate genes associated with resistance to summer mortality in the Pacific oyster (Crassostrea gigas)

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ABSTRACT

The global oyster industry is severely hampered by frequent outbreaks of summer massive mortalities. Selective breeding of disease-resistant broodstock is considered as a key strategy to address this problem, and marker-assisted selection programs would help develop oyster lines that are resistance to summer mortality. However, there is currently a need to identify candidate genes associated with disease resistance and to develop molecular markers based on these genes. To identify candidate genes associated with summer mortality resistance, we selected three most resistant and three most susceptible oyster families and compared their basal transcriptomes. A total of 59 families were tested in two sites, and there were significant differences in summer survival among families of different genetic backgrounds. Principal component analysis showed that there was significant clustering of different resistant families, suggesting that the molecular mechanisms underlying the development of resistance characteristics in different resistant families may be similar. Interestingly, transcriptome results showed that the highly expressed genes in resistant families were mainly associated with innate immunity, especially some pattern recognition receptors, including C-type lectins, fibrinogen-related proteins and scavenger receptors. Some transient receptor potential family genes showed differential expression in the both resistant and susceptible families, which may be related to the thermal tolerance of oysters. Notably, some genes related to antioxidant responses and detoxification were highly expressed in resistant families, such as glutathione S-transferase and Cytochrome P450, suggesting that oysters with summer mortality resistance may have greater antioxidant and detoxification capacities. In addition, weighted gene co-expression network analysis (WGCNA) identified three modules that were significantly positively associated with summer survival. The network map of key modules allows us to identify a series of hub genes, such as *PEAR1, MCT12, RPL23, TRPM2, MFAP4, TLN1, SCARF1, GAL9* and *ACOD*. Overall, our study provides new insight into summer mortality resistance by employing comparative transcriptome and WGCNA, and the genes identified in this study should be further investigated for use in marker-assisted selection breeding programs.

1. Introduction

The Pacific oyster (*Crassostrea gigas*) is an important aquaculture bivalve, currently suffering from summer massive mortality events with significant economic losses worldwide. The first mass mortality event was reported in the 1940s in Japan, the native habitat of the *C. gigas*, affecting both wild populations and harvested beds of adult oysters (Imai et al., 1965). Since the early 1960s, 'abnormal' episodes of *C. gigas* mortality have increased worldwide (Cheney et al., 2000). To date, no single pathogen or environmental factor has been identified as the sole cause of summer mortality. Instead, there is growing evidence that mortality is caused by opportunistic pathogenic infections, as high temperatures and high energy costs associated with reproduction have weakened oysters (Samain and McCombie, 2008; Wendling and Wegner, 2013). While elevated water temperatures are considered as a necessary precursor to oyster summer mortality, it is a complex phenomenon and pathogen infection is thought to be a key driver of mortality (Romalde et al., 2014). In addition, deeper causes may stem from a more general stress response of the oyster itself rather than a pathogen-specific induced response (Taris et al., 2009).

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Due to the current lack of effective options to prevent or control disease outbreaks, improving oyster resistance to summer mortality through selective breeding has become a major objective (Dégremont et al., 2015). The development of selective breeding in aquaculture will also provide increasing opportunities to obtain material showing contrasting phenotypes and thus better understand the molecular basis of complex traits. Advances in molecular genetics and genomics have improved our ability to identify disease-resistance genes and understand the genetics of disease resistance (Houston et al., 2020). Host resistance to disease depends not only on the detection and induction response to pathogen invasion, but also on barriers to avoid invasion and active resistance mechanisms constituted in the absence of pathogens (Ramírez-Tejero et al., 2021). Genetic differences can lead to differences in immune gene expression among individuals, affecting host survival during disease outbreaks. It is widely recognized that integrated analysis of genetic findings with genomics is needed to reveal the molecular mechanisms behind complex traits, such as resistance to summer mortality in *C. gigas*.

Transcriptome sequencing allows the identification of gene expression signatures that can then be used as markers for physiological status and health. These expression signatures may represent ideal candidates for trait selection programs (Rosa et al., 2012). Indeed, the prediction of a disease-resistant phenotype from gene expression data has been widely used in many studies. In human, variations in the basal mRNA abundance of some key genes have been reported as an important genetic basis for resistance (or susceptibility) to many known infectious diseases (Van't Veer et al., 2002). In rice, Bsr-d1 RNA levels were 2-fold higher in susceptible lines than in resistant lines, and it was further demonstrated that inhibition of Bsr-d1 expression could improve resistance to plague infection (Li et al., 2017). For aquaculture species, the gene expression variation of *BIRC7* is heritable and the expression of this gene is associated with the viability of *Meretrix petechialis* after vibrio infection (Jiang et al., 2018). Understanding the genetic basis of resistance to summer mortality in oysters should be deeply helpful in determining the causes of massive mortalities and the traits that characterize enhanced survival capacities in oyster populations. Therefore, more attention should be given to the development of specific resistance-related markers in oysters to improve selective breeding and cultivation practices.

In China, oysters are produced by commercial hatcheries during late winter and early spring, then grown under intensive nursery conditions before being deployed in the field at 3–4 months old. Summer mortality events are usually observed in the first summer (spat stage) and these mortalities can cause serious economic implications (Chi et al., 2021). In previous work, we performed systematic family selection for the summer survival of *C. gigas* (Chi et al., 2022). Here we report RNA-seq data obtained from *C. gigas* that were subjected to family selection and exhibited differential summer survival performances. We aimed to verify the differences in gene expression profile between oyster families with the higher and lower summer survival and identify key genes associated with summer mortality resistance. Furthermore, weighted gene co-expression network analysis (WGCNA) was performed to construct gene co-expression networks, and identified hub genes which may play vital roles in resistance to summer mortality in *C. gigas*.

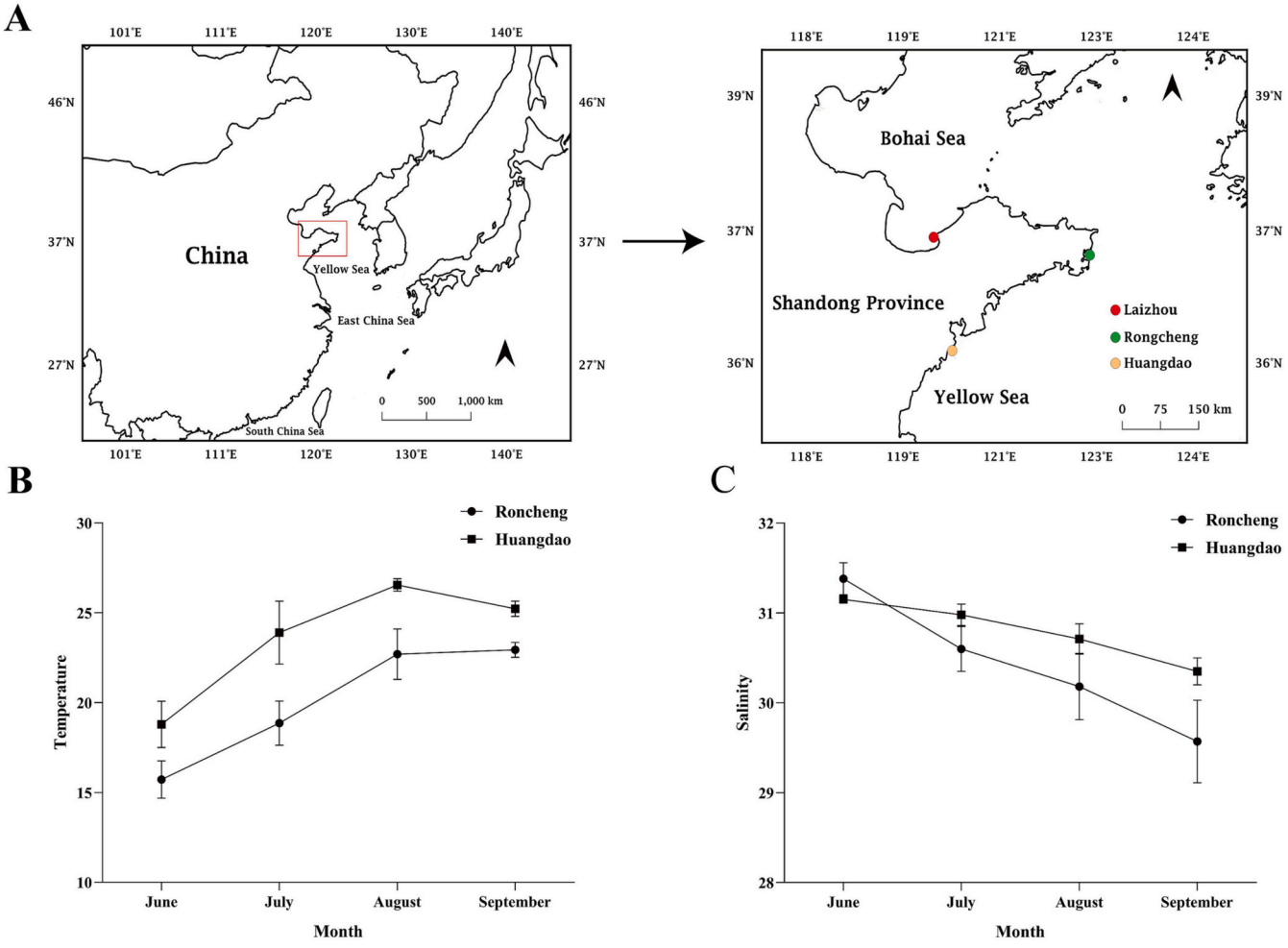

Fig. 1. Location of the broodstock's origin and oyster test sites (A). The average monthly temperature and salinity of the test sites (B & C).

2. Materials and method

2.1. Production of oyster families

In October 2020, wild *C. gigas* were collected from non-farming areas in Rongcheng, Shandong Province, China, and these oysters were used to produce 15 full-sib families (Fig. 1A). Thirty-three "Top selection" and 11 "Down selection" families were produced from broodstocks that originating from a family selection program (Chi et al., 2022). The breeding design of "Top selection" and "Down selection" was shown in Fig. 2. These full-sib families were produced in March 2021 at the Laizhou breeding center (Shandong Province, China) (Fig. 1A). The rearing conditions for full sibling families are described in detail by Chi et al. (2022). Briefly, the larvae of each family were reared separately in 100–L buckets at 23–25 °C and filtered seawater was changed once a day. The larvae were fed three times a day with a mixture of phytoplankton (*Isochrysis galbana* and *Platymonas* sp). Attached spat were settled in tanks and reared under controlled hatchery conditions.

2.2. Field test and sample collection

To determine if families were resistant or susceptible to summer mortality, field tests were conducted with 59 different full-sib families. Two hundred oysters from each family were retained at the Laizhou breeding center while other individuals from the same families were tested in the field. In June 2021, these 59 families were deployed to two sites along the Shandong coast: Rongcheng and Huangdao (Fig. 1A). Families were placed in lantern nets and cultured on suspended long lines according to local practices. Each family was replicated three times, and each replicate lantern nets contained 40 oysters from each family. The Rongcheng field test began on June 6, 2021 and ended on September 18, 2021, while the Huangdao field test began on June 10, 2021, and ended on September 25, 2021. Water temperature and salinity data for Rongcheng and Huangdao were obtained from the National Marine Data Center, National Science & Technology Resource Sharing Service Platform of China (http://mds.nmdis.org.cn/) (Fig. 1B & C).

After the field test (September 28, 2021), we collected samples at the Laizhou breeding center. Considering the survival rate at both sites, the top three families from the upper tail of the survival rate distribution were considered as resistant families, and the bottom three families from the lower survival rate were considered as susceptible families. Three

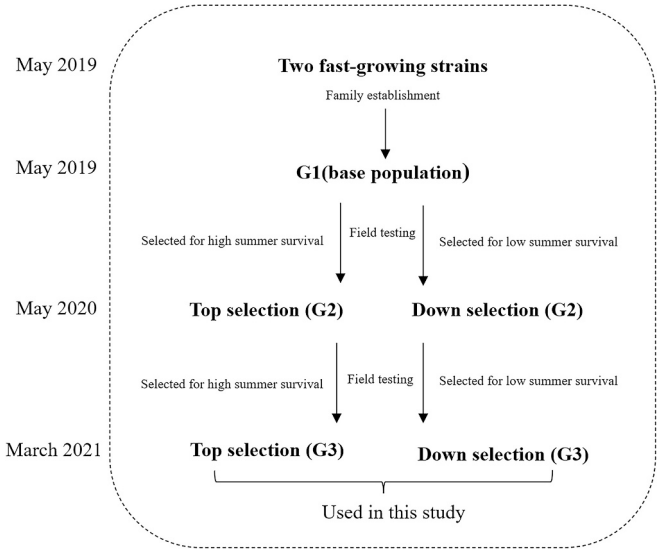

Fig. 2. Schematic diagram of the selection design used in oyster selective breeding program.

individuals were sampled from each family, resulting in a total of 18 oysters randomly sampled from the six families. After dissection, gill tissue of each sample was immediately placed in liquid nitrogen and then stored in an ultralow temperature freezer at −80 °C until RNA extraction.

These sampled individual oysters were not deployed to the field for mortality assessment during the summer, but were left in breeding center conditions and therefore were not affected by summer mortality. Under these breeding center conditions, water quality, food ration and temperature were controlled and oyster mortality was monitored daily. The disinfected seawater was filtered through a sand filter and a non-woven polypropylene fabric. The water temperature was 19–20 °C, salinity was 30–32%, PH was 8.1–8.2 and dissolved oxygen was 8–9 mg/L.

2.3. RNA extraction and library construction for transcriptome sequencing

Total RNA of each sample was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Poly-T oligo-attached magnetic beads were used to purify total RNA. First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse. After degrading RNA via RNase H, DNA Polymerase I system and dNTPs were added to synthesize the second strand cDNA. cDNA fragments range from 250 bp to 300 bp were selected by AMPure XP system and enriched by PCR, which were subsequently purified again. PCR was then performed with Phusion High-Fidelity DNA polymerase, universal PCR primers and Index (X) primers. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. Finally, library preparations were sequenced by the Illumina NovaSeq 6000 platform to generate 150 bp paired-end reads.

2.4. Differential expression analysis

Clean reads were obtained after removing adapter, ploy-N and low-quality reads. Then, Q20, Q30 and GC content for clean reads were calculated in the Fastp v0.19.7. The reference *C. gigas* genome (cgigas_uk_roslin_v1) was downloaded from NCBI. Subsequently, the clean reads were mapped to the *C. gigas* reference genome using Hisat2 v2.2.1 to assemble the mapping reads into the transcript. The number of reads mapped to each gene was counted by Feature Counts v2.0.3. Differential expression analysis of resistant and susceptible families was performed with the R package DESeq2 v1.38.1 with $|\log2(\text{fold change})| > 1$ and a false discovery rate (FDR) < 0.05. R package clusterProfiler v4.6.0 was used to process the Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. $P_{adj} < 0.05$ and qvalue <0.2 was regarded as significantly enriched in the pathway.

2.5. WGCNA and hub gene mining

A weighted co-expression network was constructed using WGCNA packages v1.71 in R 4.2.0 to obtain specific co-expressed gene modules associated with summer survival. A suitable soft threshold was determined based on the correlation between genes to make the constructed network more consistent with the characteristics of a scale-free network. The gene expression values were then imported into "WGCNA" through the automatic network construction function to construct co-expression modules. The hierarchical clustering tree was cut using a dynamic hybrid tree-cut algorithm, and each module was defined as a branch after tree cutting. The merging threshold for similar modules was 0.25 and the minimum module gene number was 200. Biologically significant modules were identified by correlation coefficients between module signature genes and summer survival. Modules with module-trait relationship (MTR) > 0.7 were selected as the key modules (Fig. 5B). Cytoscape v3.9.1 was then used to visualize the gene networks in

modules and to show biological interaction of key genes.

2.6. Quantitative real-time PCR (qRT-PCR) validation

To validate the results of RNA-seq, six DEGs (common DEGS in two pairwise comparisons) were selected for qRT-PCR analysis, and the gene-specific primers were designed using Primer Premier 5.0 (Table S2). The elongation factor 1-α (*EF1-α*) gene was used as an internal control to normalize the expression of target genes (Renault et al., 2011; Li et al., 2022; Fang et al., 2022). After the assessment of integrity and purity, total RNA was reverse-transcribed into cDNA. The 20 μL reaction system containing 2 μL of diluted cDNA was configured with ChamQ SYBR Colour qPCR Master Mix (High ROX Premixed) as described in the manual. qRT-PCR was performed on a LightCycler 480 II detection system (Roche, Switzerland). The amplification efficiency was determined for each primer pair by constructing a standard curve from serial dilutions of the original cDNA. Expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.7. Statistical analysis

Before analysis of variance, data were checked for normality and homogeneity of variance. Differences in summer survival rate among families were analyzed with one-way analysis of variance followed by multiple comparison Tukey test using SPSS 22.0 software. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Survival rate of the oyster families

High variability in survival rates, ranging from 5.00% to 93.33%, was observed among families (Fig. S1A & B). Survival rates observed for the 59 families in Rongcheng (59.84%, ranging from 15.00% to 93.33%) were significantly higher than the Huangdao (47.20%, ranging from 5.00% to 83.33%) ($P < 0.05$). As expected, the survival rate of offspring from "Top selection" was the highest (74.95% in Rongcheng and 61.39% in Huangdao), whereas in contrast, the lowest survival rate (34.17% in Rongcheng and 23.18% in Huangdao) was in the offspring of "Down selection" (Fig. 3B). The survival rates of the offspring belonging to "Wild" in Rongcheng and Huangdao were 45.44% and 33.61%, respectively (Fig. 3B). No abnormal mortality (survival rate > 90%) was observed in any of the oyster families at the Laizhou breeding center prior to sampling.

Considering the survival rate (averaged across the two-field test), families TS8, TS23 and TS33 were chosen as the resistant families (mean survival rate 86.53%) and families W3, DS9 and DS4 were chosen as the susceptible families (mean survival rate 11.67%). The survival rates of three resistant families TS8, TS23 and TS33 were 86.67%, 86.67% and 86.25%, and those of three susceptible families W3, DS9 and DS4 were 10.83%, 11.67% and 12.50%, respectively (Fig. 3A). The parents of the three resistant families were survivors from full-sib families, which were selected based on their high summer survival rates. Two susceptible families (DS9 and DS4) came from broodstock that were selected based on their low summer survival rates, and one susceptible family (W3) came from wild broodstock recruited in a no-farming area. These families were divided into three groups according to their genetic backgrounds, resistant group (RG), selected susceptible group (SG_S) and wild susceptible group (SG_W).

3.2. Transcriptome sequencing quality assessment

To obtain the *C. gigas* gill transcriptome expression profiles of different oysters, 18 cDNA libraries were constructed. A total of 907,147,474 raw paired-end reads were obtained (Table S1). After filtering, 867,834,454 clean reads (Q30: 91.86%–94.00%) were retained and used for transcriptome assembly. The average alignment rate of clean data mapped to the *C. gigas* reference genome for each sample was 77.87% (Table S1). Because of the huge quantity of sequencing data, we used principal component analysis (PCA) to assess the reliability of the samples (Fig. 4A). The three groups were effectively separated, indicating significant differences in gene expression between the resistant and susceptible families. Notably, the RG group samples showed significant clustering, indicating minor gene expression differences among different resistant families.

3.3. Differential expression analysis and functional enrichment analysis

Two pairwise comparisons of different groups (RG vs SG_S, RG vs SG_W) were generated to identify differential expression genes (DEGs) between resistant families and susceptible families. A total of 2408 genes showed differential expression patterns between RG and SG_S, in which 1209 genes were highly expressed in RG and 1199 genes were highly expressed in SG_S (Fig. 4B & C). To examine the observed expression patterns in more detail, GO and KEGG enrichment analyses were conducted for significantly up- and down-regulated DEGs. In GO functional enrichment analyses, the up-regulated DEGs of RG were mainly related to metabolism processes and binding. Top 20 significant enriched GO

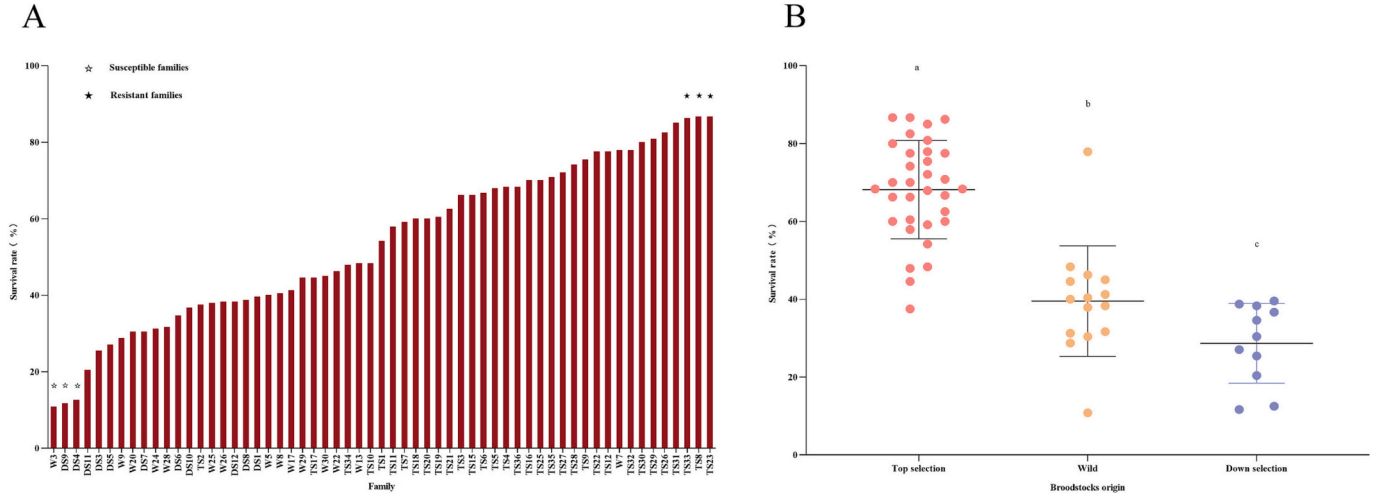

Fig. 3. The average survival rate of oyster families in two test sites (A). The average survival rates of three populations with different genetic backgrounds at two test sites (B).

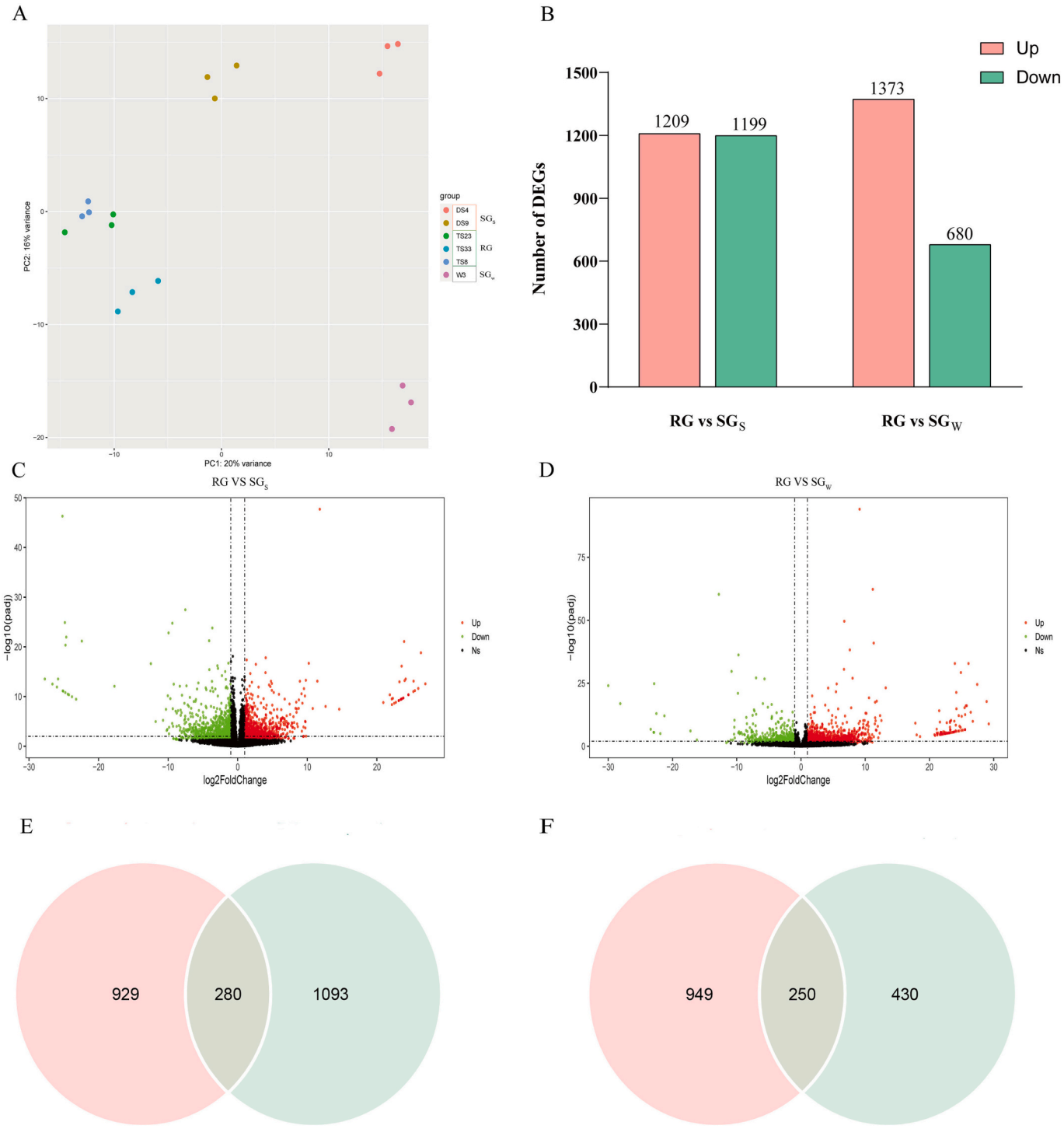

Fig. 4. Principal component analysis (PCA) for the correlation among samples (A). Numbers of differential expression genes (DEGs) in resistant group (RG) vs selected susceptible group (SG_S) and RG vs wild susceptible group (SG_W) comparisons. Red and green indicate up-regulated and down-regulated DEGs, respectively (B). The bubble chart showed the DEGs in the RG vs SG_S and RG vs SG_W comparisons (C & D). Venn diagram analysis of up-regulated DEGs in RG vs SG_S and RG vs SG_W comparisons (E). Venn diagram analysis of down-regulated DEGs in RG vs SG_S and RG vs SG_W comparisons (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

terms were shown in Fig. 5A. The most significantly enriched KEGG pathway in RG was "Lectins [BR:ko04091]", and followed by "Butanoate metabolism" and "Ribosome [BR:ko03011]" (Fig. 5B). The DEGs enriched in these pathways were mainly associated with innate immunity, metabolism, ion transport, ribosome and oxidoreductase activity (Table S3). In GO functional enrichment analyses, the up-regulated DEGs of SG_S were mainly related to response to stimulus and cell communication. The top 20 significant enriched GO terms were shown in Fig. 6A. The most significantly enriched KEGG pathways of SG_S was "Cell adhesion molecules" followed by "Hematopoietic cell lineage", "Adherens junction", "CD molecules", and "Toll and Imd signaling pathway" (Fig. 6B).

A total of 2053 genes were differentially expressed between RG and SG_W, including 1373 genes highly expressed in RG and 680 genes highly

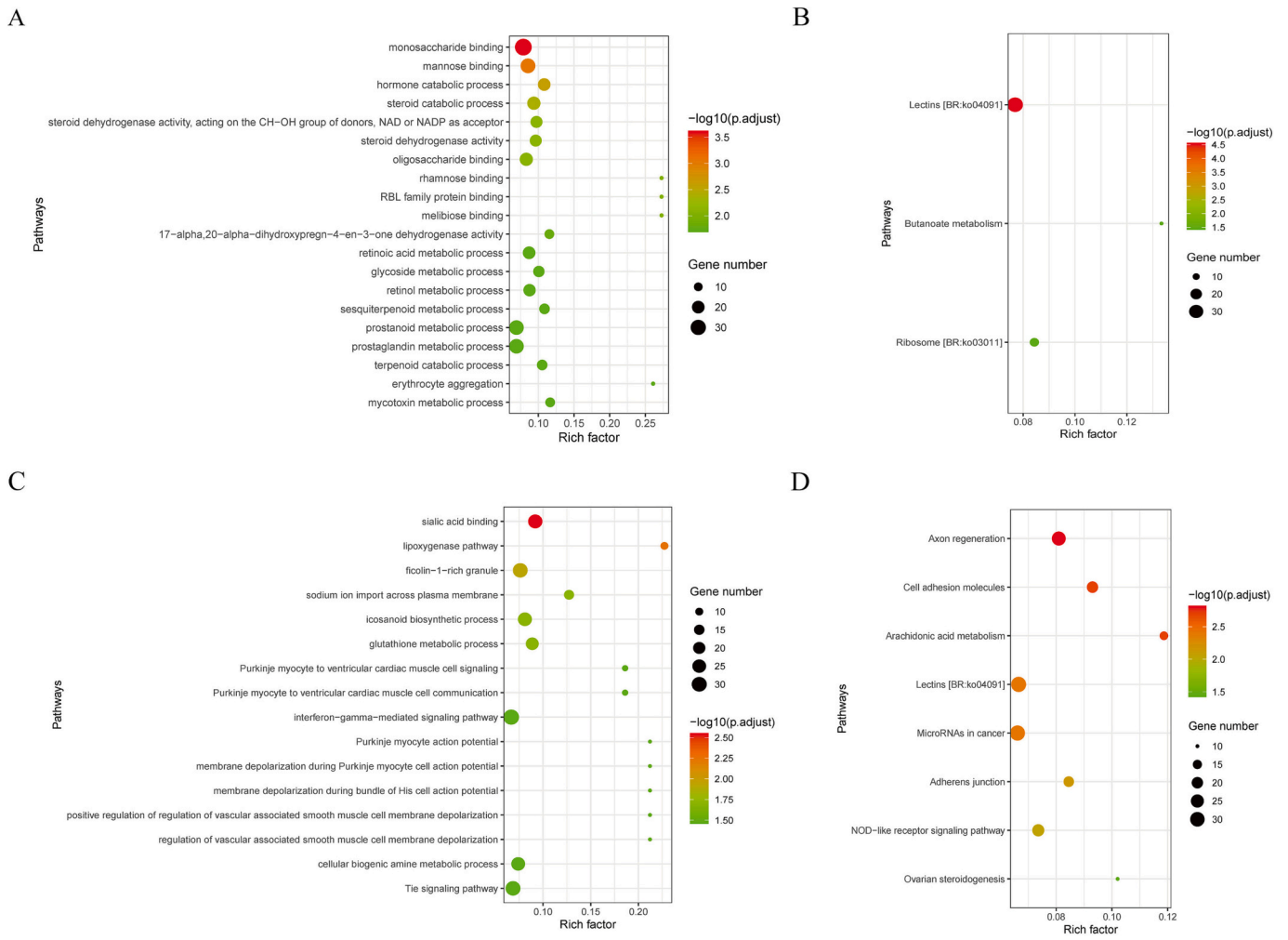

Fig. 5. Top 20 Enriched GO pathways for up-regulated DEGs (A) and enriched KEGG terms for up-regulated DEGs in the resistant group (RG) vs selected susceptible group (SG_S) comparison (B). Enriched GO pathways for up-regulated DEGs (C) and enriched KEGG terms for up-regulated DEGs in the RG vs wild susceptible group (SG_W) comparison (D).

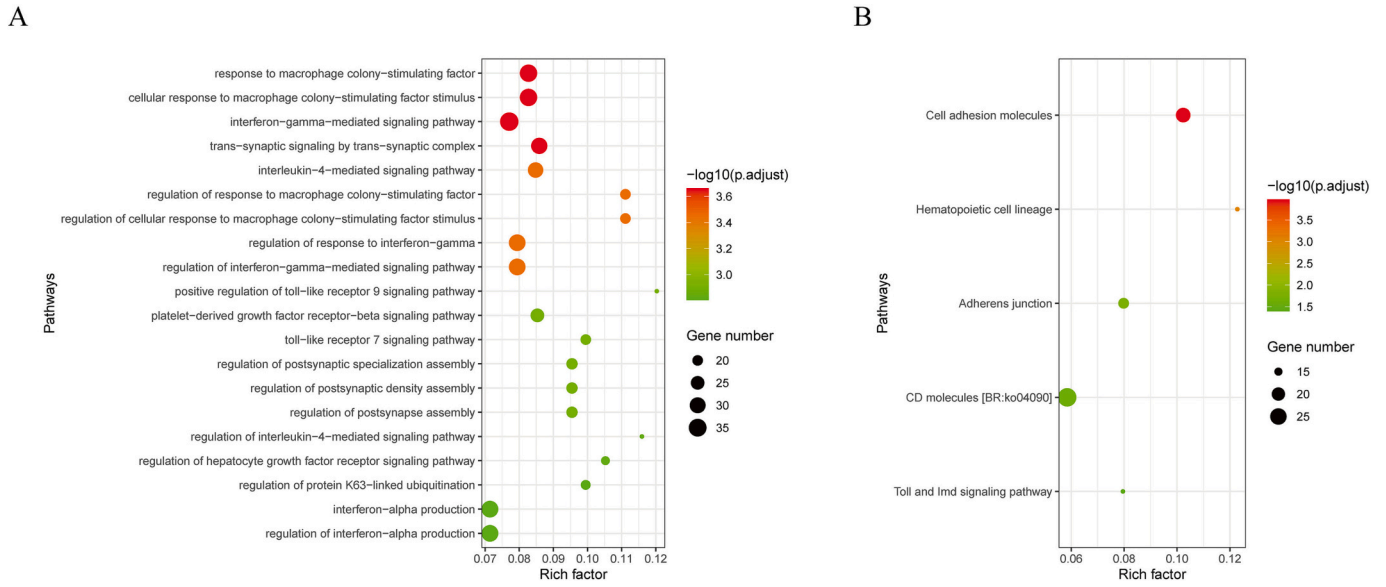

Fig. 6. Top 20 Enriched GO pathways for down-regulated differential expression genes (DEGs) (A) and enriched KEGG terms for down-regulated DEGs in the resistant group (RG) vs selected susceptible group (SG_S) comparison (B).

expressed in SG_W (Fig. 4D). In GO functional enrichment analyses, "sialic acid binding", "lipoxygenase pathway" and "ficolin-1-rich granule" were the three most enriched pathways (Fig. 5C). In KEGG enrichment analysis, the most significantly enriched pathways for up-regulated DEGs in RG were as follows: "Axon regeneration", "Cell adhesion molecules", "Arachidonic acid metabolism", "Lectins [BR: ko04091]", "MicroRNAs in cancer", "Adherens junction", "NOD-like receptor signaling pathway" and "Ovarian steroidogenesis" (Fig. 5D). The DEGs enriched in these pathways are mainly associated with innate immunity, metabolism and ion transport (Table S4). Interestingly, the up-regulated DEGs of SG_W were not significantly enriched in any pathways ($P_{adj} > 0.05$).

3.4. Analysis of DEGs associated with summer mortality resistance

In two pairwise comparisons of RG vs SG_S and RG vs SG_W, we identified the common DEGs. A total of 528 DEGs were shared in two comparisons, including 279 DEGs highly expressed in the resistant families and 249 DEGs highly expressed in susceptible families (Fig. 4E & F). Since upregulated genes may have a positive impact on resistance, we focused on upregulated DEGs related to immunity and stress related processes that were shared in the two comparisons (Table 1). Some genes related to innate immunity were highly expressed in resistant families, especially some immune-related genes involved in pattern-recognition receptors (PPRs), such as *CD209*, *PLC*, *PLP, ASGR*, *MFAP4*, *SCARF1* and *FCN2* (Table 1). In addition to immune-related genes, we found that some transient receptor potential family genes (TRPs) were highly expressed in resistant families. Two ribosomal protein genes were highly expressed in the resistant families, including the *RPS3A* and *RPL23*. Some genes related to detoxification and antioxidation were highly expressed in resistant families, such as Cytochrome P450 (CYP450) and glutathione S-transferase theta-1 (GSTT1). DEGs highly expressed in susceptible families are mainly associated with cell adhesion, cell communications and immune, some genes related to pathogen-binding were identified, including *NECTIN4*, *TSPAN9* and *FNDC2* (Table 1).

3.5. WGCNA and functional annotation of module genes

To identify specific genes highly associated with summer survival, WGCNA of 25,925 genes was carried out. Based on the scale-free topology criterion, we chose a power of $\beta = 9$ to generate the hierarchical tree (Fig. 7A & B). All genes were divided into 18 different modules based on the similarity of expression patterns (Fig. 7C). The number of genes per module was highly variable, ranging from 92 (grey module) to 4479 (Table S5). Three modules with MTR > 0.7 are considered key modules, including the lightgreen module, magenta module and royalblue module ($P < 0.001$) (Fig. 8). To further explore the biological functions of the modules, KEGG enrichment analysis was performed on the genes of the three key modules. The results showed that enriched pathways were mainly related to metabolism, immunity and apoptosis (Fig. 9).

3.6. Hub genes

To identify key genes associated with summer survival, gene network analysis (the first 3000 edges) was performed by Cytoscape software on three key modules (Tables S6, S7 and S8). The top 30 genes with the highest correlations with others were considered as "hub genes" and were represented as nodes (Fig. 10). According to our hub gene screening method (removing the unknown genes), 12 genes (*PEAR1*, *MCT12*, *RPL23*, *TSPN9*, *MFAP4*, etc.) were identified as hub genes in the lightgreen module (Table 2); 8 genes, such as *TRPM2*, *TLN1*, *SCARF1*, were identified as hub genes in the magenta module (Table 3); 17 genes, such as *ACOD*, *GAL9* and *MFN2*, were identified as hub genes in the royalblue module (Table 4).

Table 1
The key candidate DEGs shared in two comparisons. The focus was on genes related to immunity and stress related processes.

ID	Description	Abbreviation	Log2foldchange	
			RG vs SG_S	RG vs SG_W
Immune related genes				
LOC105326002	asialoglycoprotein receptor 1-like	*ASGR1*	3.79	3.96
LOC105327658	ryncolin-1	*RCN1*	4.11	6.38
LOC105326729	complement C1q-like protein 3	*C1QL4*	2.35	2.97
LOC117686528	complement C1q-like protein 4	*C1QL3*	3.81	4.86
LOC117691729	scavenger receptor class F member 1-like	*SCARF1*	5.03	5.22
LOC117688044	scavenger receptor class F member 1-like	*SCARF1*	7.09	7.60
LOC117687969	CD209 antigen-like protein E	*CD209*	10.79	6.86
LOC117686831	perlucin-like protein	*PLP*	7.79	4.97
LOC117685219	perlucin-like protein	*PLP*	8.18	7.47
LOC105348603	perlucin-like protein	*PLP*	5.48	3.37
LOC105332177	perlucin	*PLC*	4.68	5.86
LOC117682384	multiple epidermal growth factor-like domains protein 10	*MEGF10*	3.65	5.15
LOC117687348	multiple epidermal growth factor-like domains protein 10	*MEGF10*	23.44	22.98
LOC117687494	multiple epidermal growth factor-like domains protein 10	*MEGF10*	2.66	2.41
LOC117691056	multiple epidermal growth factor-like domains protein 10	*MEGF10*	2.15	6.76
LOC117690149	E3 ubiquitin-protein ligase TRIM71-like	*TRIM71*	1.02	1.36
LOC117685399	E3 ubiquitin-protein ligase TRIM71-like	*TRIM71*	8.76	5.37
LOC105320289	E3 ubiquitin-protein ligase TRIM71-like	*TRIM71*	5.63	4.69
LOC117691877	E3 ubiquitin-protein ligase XIAP-like	*XIAP*	3.22	7.49
LOC117684351	B-cell receptor CD22-like	*CD22*	6.81	7.33
LOC105326592	ficolin-2-like	*FCN2*	1.66	1.50
LOC117684971	tripartite motif-containing protein 3-like	*TRIM3*	1.07	1.13
LOC117691528	tripartite motif-containing protein 55-like	*TRIM55*	1.90	1.88
LOC105324668	peptidoglycan-recognition protein SC2	*PGSC2*	1.34	4.36
LOC105342748	microfibril-associated glycoprotein 4	*MFAP4*	1.21	2.82
LOC105327045	microfibril-associated glycoprotein 4-like	*MFAP4*	1.25	1.64
LOC105337737	toll-like receptor 4	*TLR4*	1.37	1.42
LOC105344743	tumor necrosis factor ligand superfamily member 11	*TNFSF11*	1.18	1.96
LOC105337761	CUB and sushi domain-containing protein 1	*CSMD1*	1.43	2.96
Antioxidant and detoxification related genes				
LOC105335381	cytochrome P450 3A24	*CYP3A24*	6.07	6.59
LOC105326181	probable cytochrome P450 49a1	*CYP49A1*	1.46	2.11
LOC105332013	cytochrome P450 4A2	*CYP4A2*	1.09	2.42
LOC105325143	cytochrome P450 3A24	*CYP3A24*	1.61	2.34
LOC105338129	glutathione S-transferase theta-1	*GSTT1*	1.69	3.43
Ion channel				
LOC105327005	transient receptor potential cation channel	*TRPM2*	12.63	11.34

(*continued on next page*)

Table 1 (*continued*)

ID	Description	Abbreviation	Log2foldchange	
			RG vs SG_S	RG vs SG_W
	subfamily M member 2-like			
LOC117683174	transient receptor potential cation channel subfamily M member-like 2	*TRPM2*	2.68	3.08
LOC105325948	transient receptor potential cation channel subfamily M member-like 2	*TRPM2*	1.31	2.08
LOC105326899	transient receptor potential cation channel subfamily M member-like 2	*TRPM2*	1.10	2.23
Ribosomal protein				
LOC105324366	40S ribosomal protein S3a	*RPS3A*	3.00	3.31
LOC117682815	60S ribosomal protein L23	*RPL23*	2.39	4.52
pathogen-binding related genes				
LOC117684916	nectin-4-like	*NETCIN4*	−2.56	−2.48
LOC105338012	tetraspanin-9-like	*TSPN9*	−3.24	−3.72
LOC105318603	fibronectin type III domain-containing protein 2	*FNDC2*	−6.06	−6.34
LOC105318604	fibronectin type III domain-containing protein 2	*FNDC2*	−6.04	−5.52

3.7. qPCR validation

The fold change of the selected DEGs detected by qPCR was compared with the results of RNA-seq expression analysis (Fig. 11). The amplification efficiencies of these primers ranged from 0.92 to 1.08. Although the fold changes of DEGs calculated by qPCR were not completely consistent with those calculated by RNA-seq analysis, the direction of genetic expression change detected by qPCR was similar to that of RNA-seq analysis, which confirmed the accuracy and reliability of the RNA-seq method.

4. Discussion

The presence of a major genetic component of oyster resistance to summer mortality has been widely documented, offering the possibility of developing lines that were “resistant” or “susceptible” to summer mortality (Dégremont et al., 2007; Samain et al., 2007; Chi et al., 2022). In fact, oyster summer mortality is caused by complex interactions between the host, the environment and opportunistic pathogens (Samain and McCombie, 2008). Therefore, understanding the susceptibility of oyster to mass mortalities requires field studies that take into account the response of oysters to abiotic factors and pathogens (De Lorgeril et al., 2018b). Our working hypothesis is that the higher summer survival of resistant families is due to higher basal defense systems and that differences in gene expression profiles can explain the different phenotypes. Therefore, we selected three most resistant and three most susceptible oyster families and compared their basal transcriptomes under the same control conditions and without exposure to field. A greater understanding of the gene expression character of resistant and susceptible families could provide valuable insight into the functional basis of summer mortality resistance and contribute to more effective genetic improvement of resistance traits.

Improving the disease resistance in economically important species through selective breeding has long been considered as feasible (Satterlee and Johnson, 1988). This study found that there were significantly differences in summer survival among families derived from different genetic backgrounds. Mean survival rate of “Top selection” line was higher than that of the “Down selection” line and “Wild” population ($P < 0.05$). Notably, a large difference in survival rate was observed between the “Top selection” and “Down selection” lines. This is in consistent with the results found by Dégremont et al. (2010) and confirms the positive response of bi-directional selection. Interestingly, wild oysters from non-farming areas also showed high susceptibility to summer mortality, suggesting disease pressure may be much lower in non-farm areas than in the test areas. No abnormal mortality was observed in any oyster families under breeding center conditions. Therefore, the mortality differences observed in the field between families were likely due to summer mortality rather than other factors, such as the effects of lethal genes, carryover effects of inbreeding or poor hatching conditions. In previous studies, inbreeding has been shown to have no significant effect on field survival of *C. gigas* (Chi et al., 2023). As oysters are produced during the winter season when favorable environmental conditions allow oysters to experience their first gametogenesis at three months of age. In fact, the reproductive characteristics (such as gametogenic intensity and spawning behavior) of oysters are highly dependent on the rearing site (Huvet et al., 2008). Oysters cultured in Huangdao showed higher mortality compared to genotypic replicates from Rongcheng. Due to the higher seawater temperature of Huangdao, the proportion of mature juvenile oysters may be greater than that of Rongcheng oysters. At the end of the experiment, we found that some oysters were fully sexually mature, which could lead to additional stress. Gametogenesis is thought to be a period of intense physiological transformation because most of the acquired energy is utilized for the production of gametes (Soletchnik et al., 1997). The high energetic demand associated with gametogenesis could result in an energetic imbalance that leads to reduced defenses against pathogens during the reproductive season (Huvet et al., 2010). In addition, pathogens may vary across environments and future studies will investigate this specific issue.

The gene expression profiles of different resistant families were more similar than those of susceptible families, suggesting that resistant families may develop resistance mechanism and genetic architecture for summer survival after multiple generations of family selection. Two pairwise comparisons of different groups (RG vs SG_S, RG vs SG_W) were performed to identify DEGs between resistant and susceptible families. Not surprisingly, a series of new findings became evident. We found that DEGs highly expressed in the resistant oyster families were mainly associated with innate immunity, especially some pattern recognition receptors (PRRs). Due to the lack of an adaptive immune system, oysters rely primarily on the innate immune system to against pathogens invasion (Song et al., 2010). Through the innate immune response, immune cells recognize invading pathogens through PRRs, which then trigger various downstream effector cells to mediate the immune response (Iwasaki and Medzhitov, 2015). Resistant families may be highly sensitive to pathogen-associated molecular patterns (PAMPs) which often result in a series of rapid responses to pathogen infection. C-type lectins (CTLs) are the most abundant lectin family with many functions in innate immunity, including removal of bacterial, cell adhesion and aggregation (Zhan et al., 2016; Odintsova et al., 2001; Wang et al., 2011; Bi et al., 2020). A number of genes belong to the CTLs were found to be significantly upregulated in the resistant families, such as *CD209*, *PLC*, *PLP* and *ASGR1*. In addition, we found some genes belonging to fibrinogen-related proteins (FREPs) and complement C1q domain containing proteins (C1qDCs) were also highly expressed in resistant families, included *MFAP4*, *FCN2*, *C1QL4* and *C1QL3*. Indeed, both FREPs and C1qDCs are known to activate the complement pathway of the innate immune system, but they act in slightly different ways, with C1qDCs triggering the classical pathway and FREPs activating the lectin pathway (Fujita et al., 2004). The classical complement pathway requires C1q activation via antigen: antibody complexes. Although

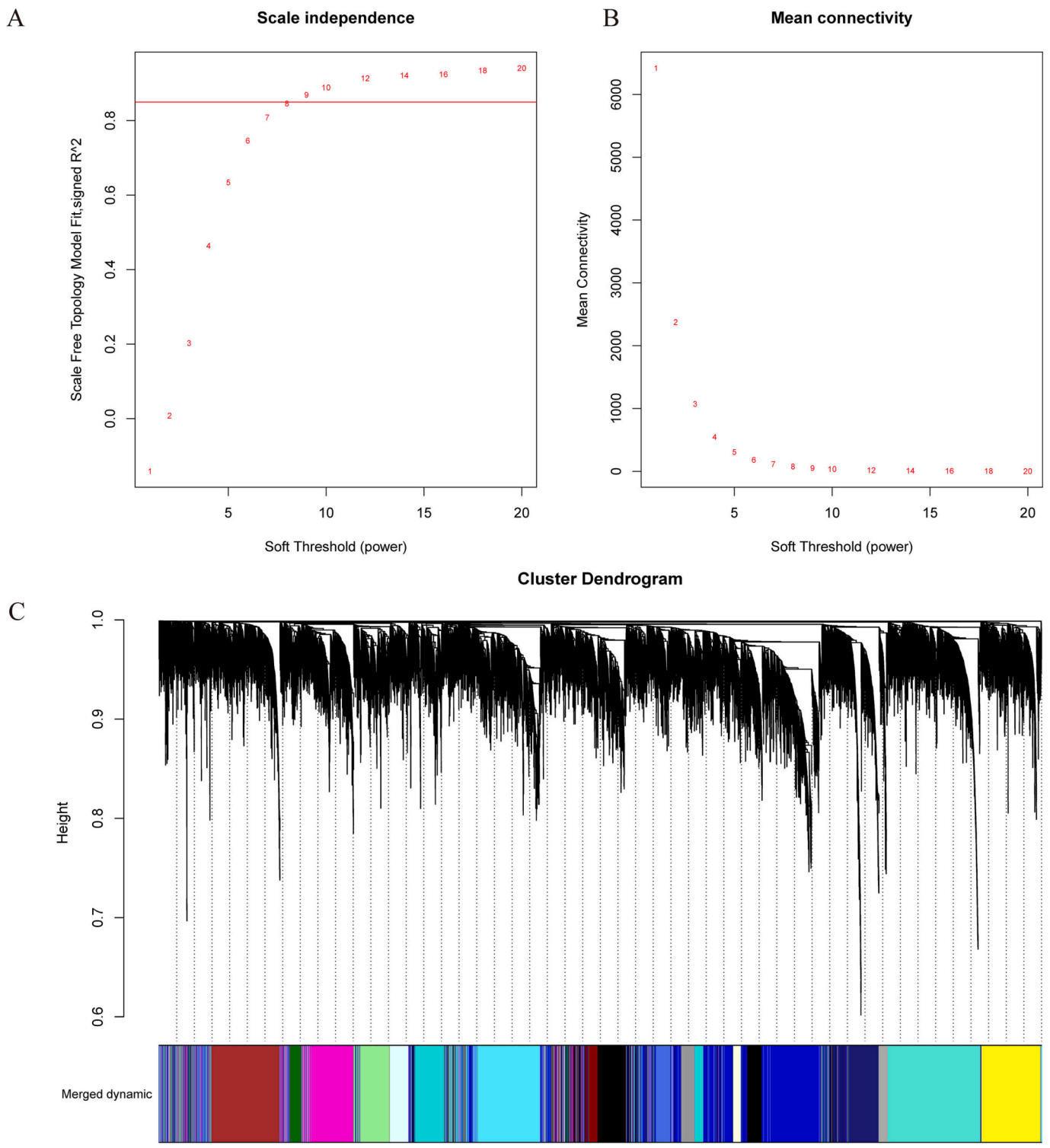

Fig. 7. Analysis of the scale-free fit index and the mean connectivity for different soft-thresholding powers (β). β = 9 was the most fit power value (A & B). Hierarchical cluster tree based on dynamic tree cut of weighted gene correlation network analysis (WGCNA). Each leaf represents a gene, each branch represents a gene module, and the colour below corresponds to that module (C).

oyster do not produce antibodies, 321 C1qDCs have been identified in the genome of *C. gigas* (Zhang et al., 2012). In addition, C1qDCs is also involved in pathogen agglutination and lysis (Guo and Ford, 2016). Since both FREPs and C1qDCs are important components for initiating the complement system. It is hypothesized that primitive complement-like systems capable of providing tailored immune protection against various pathogens are also present in *C. gigas*. In addition, WGCNA results showed that three PRRs were identified as hub gene, including *MFAP4*, *SCARF1* and *GAL9*. These genes are particularly interesting because its function could explain how these resistant oysters detect pathogens infection early, thus enabling them to generate a more rapid and effective immune response.

Oyster haemocytes are a type of immune cells and are one of the target cell types attacked by pathogens. Pathogen infection of oyster

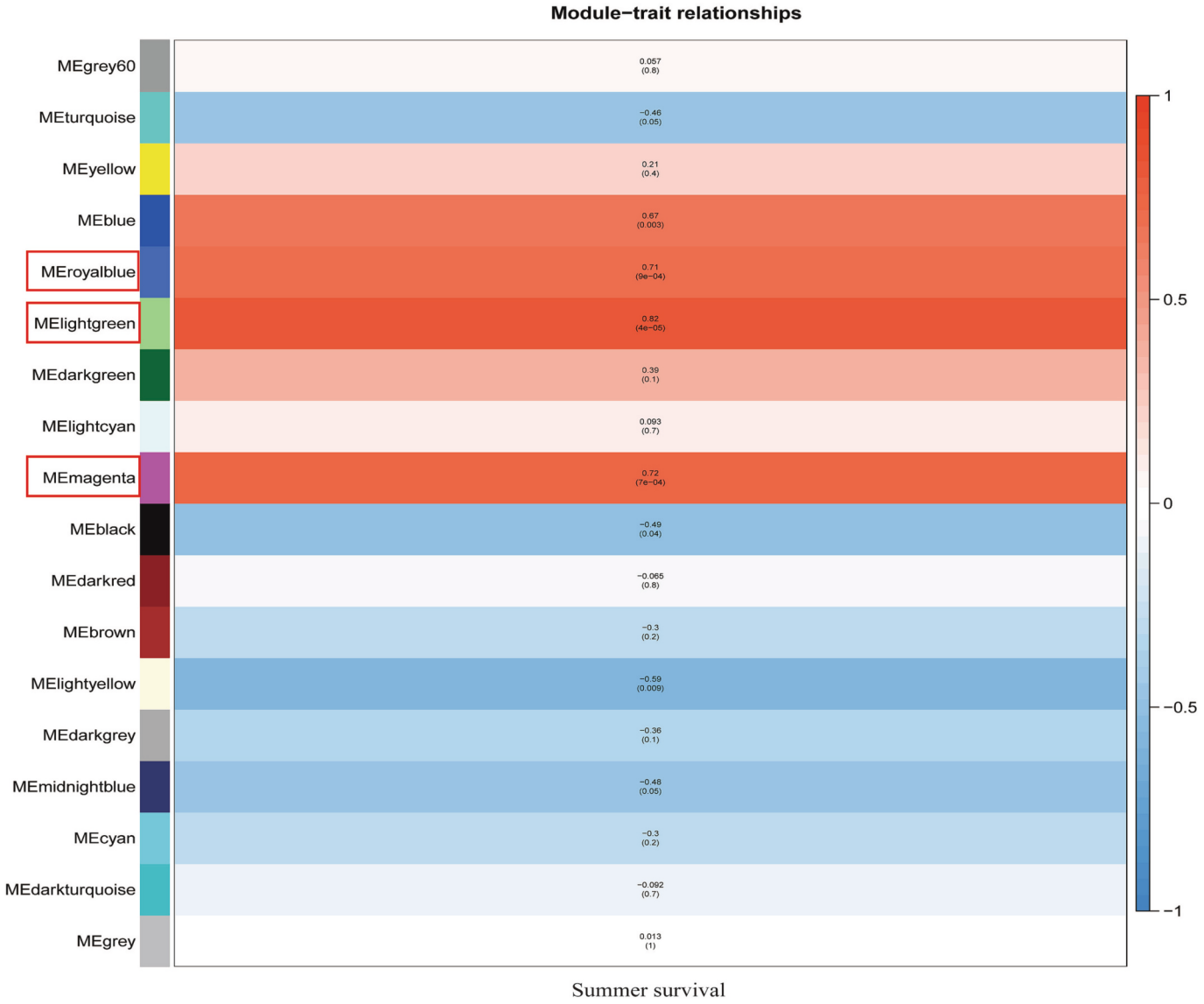

Fig. 8. Heatmap of module-trait relationships. The numbers in the module boxes represent the correlation of the module genes with the traits and the *p*-values.

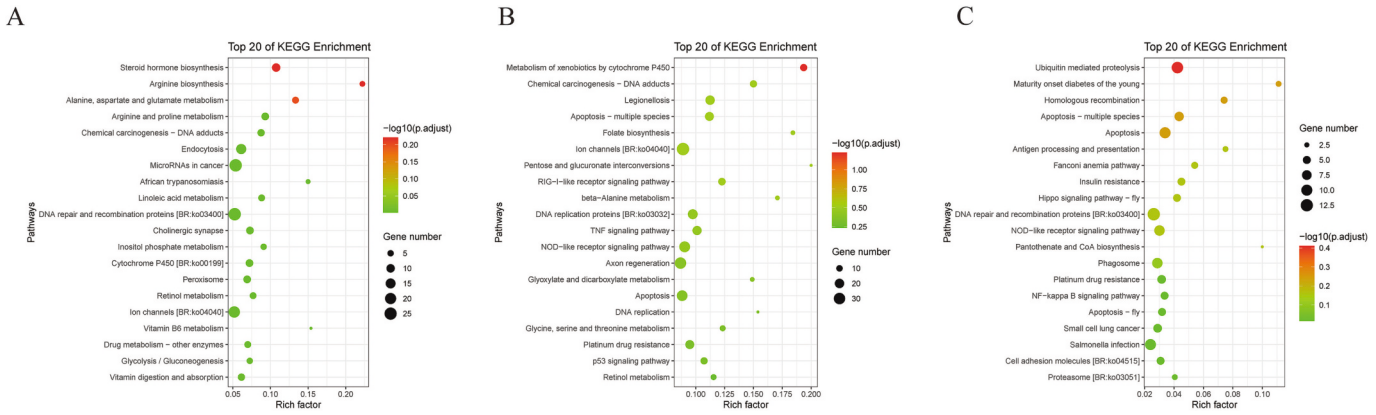

Fig. 9. Top 20 of KEGG enrichment analysis of lightgreen (A), magenta (B) and royalblue (C) modules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

haemocytes has been reported to lead to oyster-associated microbiota dysbiosis, subsequently leading to bacteremia and mortality (De Lorgeril et al., 2018a). We found that some clotting-related genes were up-regulated in resistant families, such as *RCN1* and *PEAR1*. Previous studies predicted that *RCN1* would initiate platelet aggregation and complement activation due to its sequence homology with ficolin

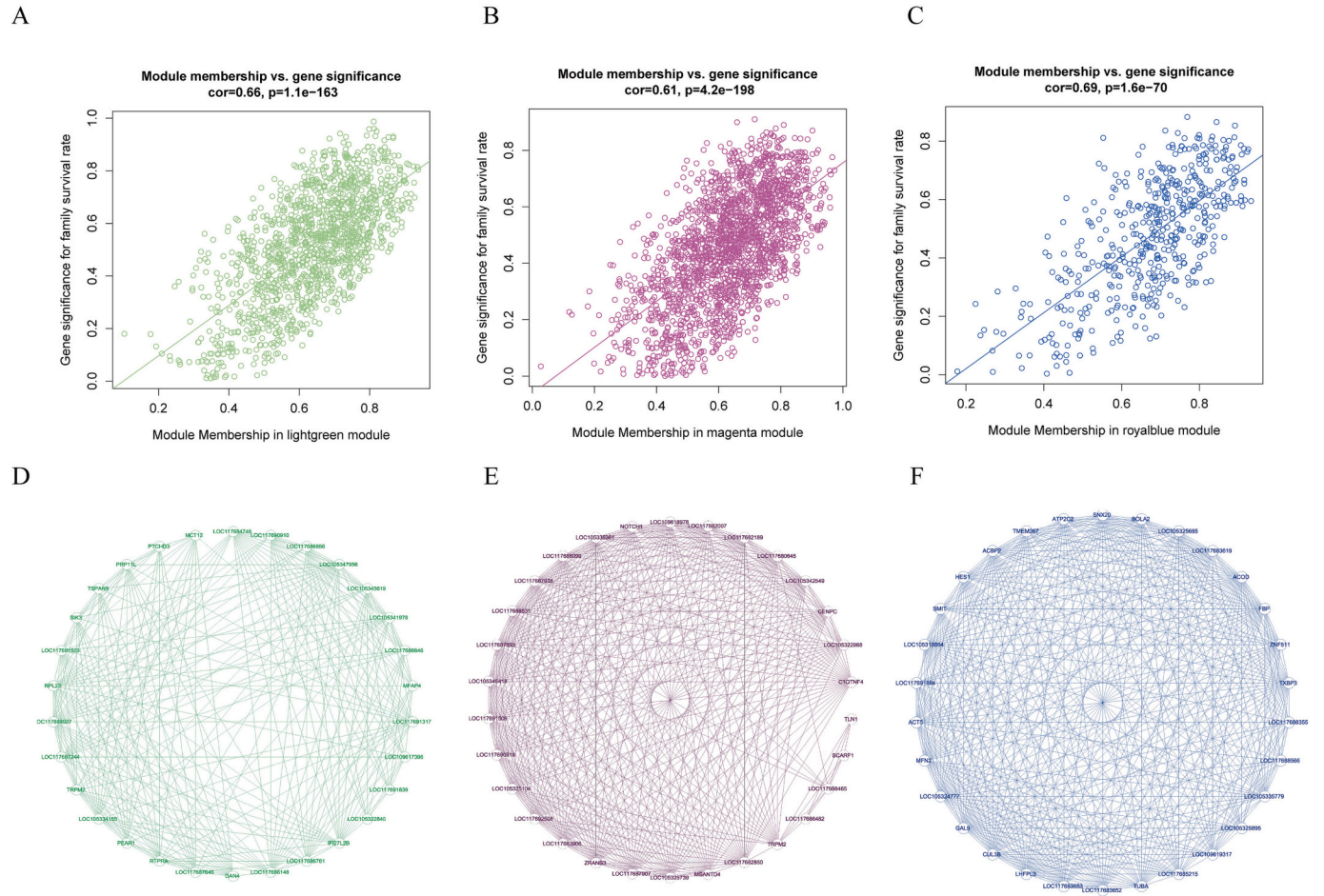

Fig. 10. Linkage of genes to modules and traits in lightgreen (A), magenta (B) and royalblue (C). Each point represents a gene. The horizontal coordinate represents the correlation of the gene with the module, and the vertical coordinate represents the correlation with the trait. Visualization of interactions between Hub genes with high connectivity (degree) within the lightgreen (D), magenta (E) and royalblue (F) modules screened by Cytoscape. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Hub genes in lightgreen module.

ID	Description	Abbreviation	RG vs SG
LOC109620516	interferon alpha-inducible protein 27-like protein 2B	*IFI27L2B*	down
LOC105343959	platelet endothelial aggregation receptor 1	*PEAR1*	up
LOC105318677	proline-rich protein 11-like	*PRP11L*	up
LOC117693062	monocarboxylate transporter 12-like	*MCT12*	up
LOC105335146	receptor-type tyrosine-protein phosphatase alpha	*RTPRA*	down
LOC117682815	60S ribosomal protein L23	*RPL23*	up
LOC117681421	serine/threonine-protein kinase SIK3-like	*SIK3*	down
LOC105343022	cell wall protein DAN4	*DAN4*	down
LOC105317633	transient receptor potential cation channel subfamily M member-like 2	*TRPM2*	down
LOC105339142	patched domain-containing protein 3	*PTCHD3*	down
LOC105338012	tetraspanin-9-like	*TSPAN9*	down
LOC117690829	microfibril-associated glycoprotein 4-like	*MFAP4*	ns

Table 3
Hub genes in magenta module.

ID	Description	Abbreviation	RG vs SG
LOC117682995	neurogenic locus notch homolog protein 1-like	*NOTCH1*	ns
LOC105335669	DNA annealing helicase and endonuclease ZRANB3	*ZRANB3*	up
LOC105327005	transient receptor potential cation channel subfamily M member 2-like	*TRPM2*	up
LOC117687391	talin-1-like	*TLN1*	up
LOC105329128	complement C1q tumor necrosis factor-related protein 3	*C1QTNF3*	up
LOC117684330	centromere protein C-like	*CENPC*	ns
LOC117688044	scavenger receptor class F member 1-like	*SCARF1*	up
LOC105325550	myb/SANT-like DNA-binding domain-containing protein 4	*MSANTD4*	ns

(OmPraba et al., 2010). *PEAR1* was identified as hub gene by WGCNA, which also mediates platelet aggregation induced by platelet-platelet contact (Vandenbriele et al., 2015). Clotting is an important mechanism for animals to defend themselves against microbial infection and maintain homeostasis (Zhu and Su, 2021). Although their exact role in *C. gigas* haemocytes is unknown, further studies of their function may shed light on their role in resistance to summer mortality. In addition, we found that four EGF-like domain containing proteins (*MEGF10*) were significantly up-regulated in resistant families. Interestingly, *MEGF10* basal expression levels were previously suggested to predict the survival of *C. gigas* in response to vibrio infection (Rosa et al., 2012). *TLN1* from

Table 4
Hub genes in royalblue module.

ID	Description	Abbreviation	RG vs SG
LOC117684295	acyl-CoA desaturase-like	*ACOD*	up
LOC105329936	LHFPL tetraspan subfamily member 3 protein	*LHFPL3*	ns
LOC105322757	galectin-9	*GAL9*	ns
LOC105343476	mitofusin-2	*MFN2*	ns
LOC105341431	bolA-like protein 2	*BOLA2*	ns
LOC105342092	cullin-3-B	*CUL3B*	ns
LOC105320637	tax1-binding protein 3 homolog	*TXBP3*	ns
LOC105348556	transcription factor HES-1	*HES1*	ns
LOC105329397	calcium-transporting ATPase type 2C member 1	*ATP2C2*	ns
LOC105331237	acyl-CoA-binding domain-containing protein 5	*ACBP5*	ns
LOC105345343	actin-5	*ACT5*	ns
LOC105320875	fibropellin-1	*FBP1*	up
LOC117686940	transmembrane protein 267-like	*TMEM267*	ns
LOC105331828	sorting nexin-20	*SNX20*	ns
LOC105328729	zinc finger protein 511	*ZNF511*	ns
LOC117684542	tubulin alpha chain	*TUBA*	ns
LOC105333119	sodium/myo-inositol cotransporter	*SMIT*	up

the magenta module was identified as a hub gene by WGCNA. *TLN1* is a key activator that leads to the physical separation of integrin α and β cytoplasmic tails and activation of integrins (Miihkinen et al., 2021). Integrins promote cell adhesion and are involved in various aspects of cellular immunity, activated β-integrin plays an important role in pathogen phagocytosis and immune response of *C gigas* (Terahara et al., 2006; Lv et al., 2019). In conclusion, differences in basal expression levels of immune-related genes suggest that resistant and susceptible oysters may have different basal defense systems.

Temperature is an important abiotic factor that has a strong effect on the immune defense system of marine mollusks. High water temperatures can damage the immune system and make oysters more susceptible to pathogens (de Kantzow et al., 2016). In addition to immune-related genes, some TRPs were deferentially expressed in resistant and susceptible families and these genes are mainly enriched in pathways associated with cardiac contraction (Fig. 5C). Interestingly, two TRPM genes from the lightgreen and the magenta modules was identified as hub genes by WGCNA. TRPs are important transmembrane ion channels that can be activated by temperature and play a key role in thermal tolerance of oyster (Fu et al., 2021). Previous studies have found that TRPM2 plays a crucial role in different physiological processes, not only involving temperature perception, but also immune response and apoptosis (Castillo et al., 2018; Huang et al., 2019; He et al., 2022). Cardiac performance has been used as an indicator of thermal tolerance in some aquaculture species (Chen et al., 2016; Xing et al., 2016; Xing et al., 2019). From a global warming perspective, sustained elevated temperatures may have profound effects on oyster farming around the world in the near future. Cardiac performance may be an important indicator of oyster resistance to summer mortality when seawater temperatures are abnormally higher. We found some ribosomal proteins were highly expressed in the resistant oyster families, such as *RPS3A* and *RPL23*. Interestingly, *RPL23* from the lightgreen module was identified as hub gene by WGCNA. Ribosomal proteins have been reported to be involved in a variety of physiological and pathological processes, many of which act as antimicrobial proteins in anti-infection response (Zhou et al., 2015; Wiesner and Vilcinskas, 2010). Some ribosomal proteins have been found to act as antimicrobial proteins in the innate immune system of aquaculture species. For example, 60S ribosomal proteins, including *C. gigas* L29 and *Silurus asotus* L27, and 40S ribosomal protein S30 from rainbow trout, all showed bactericidal activity (Fernandes and Smith, 2002; Seo et al., 2017; Oh et al., 2020). Although their exact roles in anti-infection response are unknown, further studies on their function may shed light on their role.

Reactive oxygen species (ROS) and xenobiotics pose a constant threat to marine bivalves such as *C. gigas*. Some genes associated with antioxidant responses and detoxification were highly expressed in resistant families, such as GSTT1 and CYP450. Glutathione S-transferases (GSTs) are important detoxification enzymes that catalyzes the binding of glutathione to a variety of endogenous and xenobiotics, including environmental toxins and the oxidative stress products (Salinas and Wong, 1999). De Lorgeril et al. (2011) found that GST was up-regulated in *C. gigas* hemocytes after challenged with a pathogenic vibrio. CYP450 is a diverse superfamily of haemoproteins that plays a central role in oxidative metabolism and detoxification in animals (Snyder, 2000; Nebert et al., 2004). In addition, CYP450 has also been reported to be associated with the host defense responses of *Ostrea edulis* to *Bonamia ostreae* infection (Morga et al., 2011). Lower antioxidant capacity may lead to higher level of ROS, and susceptible families may be more susceptible to damage from oxidative stress. Notably, ROS production has been reported to be a major factor differentiating summer mortality susceptible and resistant oyster lines (Lambert et al., 2007; Delaporte et al., 2007).

Previous studies suggest that pathogens or environmental stress can trigger degradation of metabolic activity in mollusks, potentially leading to mass mortality in infected bivalve populations (Lochmiller and Deerenberg, 2000). The WGCNA results showed that genes in the lightgreen module were mainly enriched in pathways related to energy metabolism, such as alanine, aspartate and glutamate metabolism, arginine and proline metabolism and linoleic acid metabolism. Two genes related to energy metabolism, *ACOD* and *MCT12*, were identified as hub genes by WGCNA. *ACOD* is a fatty acid desaturase that plays a key role in fatty acid metabolism and regulation of the cell membrane fluidity (Macartney et al., 1994). *MCTs* catalyze the transport of lactate, pyruvate and related monocarboxylates across cellular membranes and therefore play a vital role in facilitating nutrient uptake and influencing the dynamic balance of metabolism (Halestrap and Meredith, 2004). In the face of pathogen infection, the host independently regulates metabolic levels to ensure adequate energy to complete immune responses (Thwe et al., 2017). Oysters have the lowest tissue energy reserves in summer due to reproductive activity and are more susceptible to metabolic exhaustion under the combined influence of high temperatures and pathogens (Berthelin et al., 2000; Li et al., 2007). Therefore, high expression of metabolism-related genes increases the energy reserves of resistant oysters, which have enough energy to mount an immune response when infected by pathogens.

In this study, families DF9 and DF4 were generated from a family breeding program based on low summer survival. Therefore, these two families may have developed a genetic architecture with susceptibility to summer mortality that is significantly different from the W3 family, whose parents are from wild populations in non-farming areas. We propose that this could explain the differential gene expression patterns observed in PCA analysis. We found that some genes related to cell adhesion were down-regulated in resistant families and up-regulated in susceptible families. Interestingly, greater adhesion of hemocyte substrates has been reported in susceptible families than in resistant families prior to a massive summer mortality outbreak (Samain et al., 2007). In addition, the expression level of adhesion proteins was higher in the susceptible oyster family than in the resistant oyster family after heat challenge (Lang et al., 2009). Notably, some genes related to pathogen-binding were up-regulated in susceptible families, including *NECTIN4*, *TSPAN9* and *FNDC2*. *NECTIN4* belongs to the immunoglobulin-like family of cell adhesion molecules and was considered a potential cellular receptor for several animal viruses (Pratakpiriya et al., 2012; Birch et al., 2013). It has been reported that overexpression of the *NECTIN4* in teleost enhanced the replication kinetics of the virus, while its silencing leads to attenuated virus-host cell interactions (Krishnan et al., 2019). Tetraspanins (TSPAN) are small transmembrane proteins that play important role in cellular processes such as migration and proliferation (Levy and Shoham, 2005). However, pathogens can also

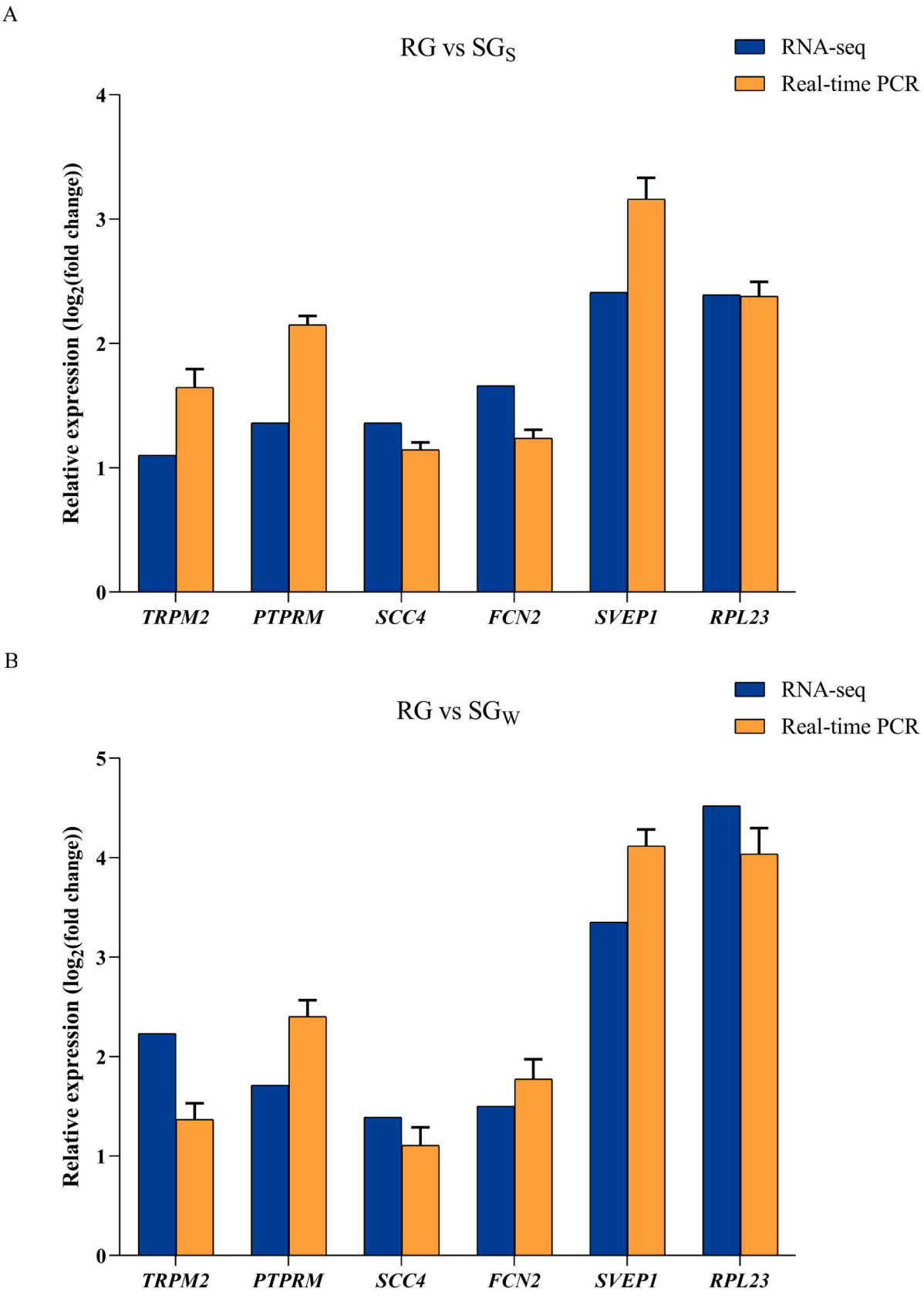

Fig. 11. Comparison of RNA-seq expression levels and qRT-PCR results of DEGs (A & B).

use TSPAN to enter host cells for further colonization and invasion (Van Spriel and Figdor, 2010). Interestingly, this gene was also identified as a hub gene by WGCNA, and high expression of this gene may result in oysters being more susceptible to summer mortality. Fibronectin is known to be a host protein that can be utilized as a receptor by some pathogenic microorganisms (Henderson et al., 2011; Ito et al., 2004). The interaction between fibronectin and bacterial adhesins is thought to play an important role in the initiation of infection (Joh et al., 1999). Therefore, the high basal expression of these genes in susceptible oysters may facilitate the binding entry of pathogens.

It has been reported that stress resistance in *C. gigas* may be mainly due to pre-stress differences rather than differences in induced responses at the transcriptome level (Taris et al., 2009). Differences in gene expression between resistant and susceptible oysters identified prior to stress can be maintained after stress, independent of their physical response to bacterial infection (Fleury et al., 2010). A recent transcriptomic study of several oyster biparental families exhibiting different susceptibilities to POMS showed that resistant oysters exhibit constitutive differences in gene expression involved in some immune and antiviral pathways (De Lorgeril et al., 2020). In contrast, we did not find a significant enrichment in pathways associated with resistance to viruses, but we found that some genes involved in innate immunity, ion transport, antioxidation and metabolic were significantly upregulated in the resistant families. Considering these previous findings, variation in resistance levels may be defined, at least in part, by differences in the basal gene expression patterns in oyster not only in response to the pathogen but also prior to infection. Recently, *Vibrio alginolyticus* has been identified as the main pathogen responsible for massive summer mortality in cultured *C. gigas* in China, with high temperature being another important environmental factor (Yang et al., 2021; Li et al., 2023). The identification of these genes may provide a valuable genetic basis for breeding new strains with high immunity and strong disease resistance.

5. Conclusion

In this study, we investigated the basal gene expression patterns between resistant and susceptible families and found that the higher summer survival rate in the resistant families may be associated with a stronger basal defense system. Notably, innate immunity in oysters plays a key role in determining susceptibility or resistance to summer mortality, particularly genes involved in the lectin and complement systems. In addition, high expression of some genes related to ion channels, metabolism and antioxidant activity may be also associated with oyster summer mortality resistance. Susceptible families may be more susceptible to pathogen infection because of their reduced ability to recognize PAMPs and the high expression of some genes related to pathogen-binding. These results not only expand our understanding of the resistance mechanisms in *C. gigas*, but also generate a new list of resistance genes. In future studies, key genes should be validated and genetic markers should be identified as selection criteria in the breeding of *C. gigas*.

CRediT authorship contribution statement

Yong Chi: Conceptualization, Investigation, Formal analysis, Methodology, Writing-original draft. **Hang Yang**: Investigation, Methodology, Software. **Chenyu Shi**: Methodology, Software. **Ben Yang**: Methodology. **Xianchao Bai**: Investigation. **Qi Li**: Funding acquisition, Supervision, Project administration, Writing-review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739922.

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