Fish and Shellfish Immunology 151 (2024) 109705

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Whole-genome DNA methylation profiling revealed epigenetic regulation of NF-κB signaling pathway involved in response to *Vibrio alginolyticus* infection in the Pacific oyster, *Crassostrea gigas*

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ARTICLE INFO

Keywords:
Crassostrea gigas
Vibrio alginolyticus
DNA methylation
Immune response
NF-Kappa B pathway

ABSTRACT

DNA methylation, an essential epigenetic alteration, is tightly linked to a variety of biological processes, such as immune response. To identify the epigenetic regulatory mechanism in Pacific oyster (*Crassostrea gigas*), whole-genome bisulfite sequencing (WGBS) was conducted on *C. gigas* at 0 h, 6 h, and 48 h after infection with *Vibrio alginolyticus*. At 6 h and 48 h, a total of 11,502 and 14,196 differentially methylated regions (DMRs) were identified ($p<0.05$, FDR<0.001) compared to 0 h, respectively. Gene ontology (GO) analysis showed that differentially methylated genes (DMGs) were significantly enriched in various biological pathways including immunity, cytoskeleton, epigenetic modification, and metabolic processes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that transcription machinery (ko03021) is one of the most important pathways. Integrated transcriptome and methylome analyses allowed the identification of 167 and 379 DMG-related DEGs at 6 h and 48 h, respectively. These genes were significantly enriched in immune-related pathways, including nuclear factor kappa B (NF-κB) signaling pathway (ko04064) and tumor necrosis factor (TNF) signaling pathway (ko04668). Interestingly, it's observed that the NF-κB pathway could be activated jointly by TNF Receptor Associated Factor 2 (*TRAF2*) and Baculoviral IAP Repeat Containing 3 (*BIRC3*, the homolog of human *BIRC2*) which were regulated by DNA methylation in response to the challenge posed by *V. alginolyticus* infection. Through this study, we provided insightful information about the epigenetic regulation of immunity-related genes in the *C. gigas*, which will be valuable for the understanding of the innate immune system modulation and defense mechanism against bacterial infection in invertebrates.

1. Introduction

The Pacific oyster (*Crassostrea gigas*) is a marine organism known for its excellent adaptation to different challenges that may be encountered in the aquatic environment, such as variations in intertidal zone [1,2]. Because of their adaptability to different environments, oysters are widely distributed around the world and become important aquatic economic species [3]. Recently, oyster mortality has increased remarkably during summer season, led to a significant negative economic impact on production [4–7]. Studies have shown that high mortality rates during summer have been linked to several variables, including pathogen infections, genetic variation, and environmental stress [8,9]. Currently, this problem has become a serious threat to farmers in China. In this respect, a screening of the pathogenic factors allowed the identification of several *Vibrio* species including *Vibrio alginolyticus* as the primary pathogen involved in summer mass mortality [10,11]. In several recent studies, RNA-seq analyses were carried out to identify differentially expressed genes and immune signaling pathways in the *C. gigas* after infection with *V. alginolyticus* [12–14]. A set of immune-related genes involved in the immune response against *V. alginolyticus*, including C1qDC-7 [15], Toll-3 [16], IAPs [17], CytA [18], and LRFN [19], were characterized in the target species. It has also

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https://doi.org/10.1016/j.fsi.2024.109705
Received 25 March 2024; Received in revised form 8 June 2024; Accepted 14 June 2024
Available online 15 June 2024

been recognized that environmental stressors such as high temperature [20] and low salinity [21] may contribute to the *V. alginolyticus* infection of Pacific oysters. However, due to the intricate molecular control, the immunological mechanism of the *V. alginolyticus* infection in this species still requires further investigation.

Expression of genes in eukaryotes is regulated by multifaceted mechanisms [22]. From this, the epigenetic modification is considered an important process that interferes in regulating gene expression, which could be linked to many biological processes [23–25]. From these modifications, DNA methylation has been widely studied and investigated [26]. Previous studies in vertebrates reported that DNA was highly methylated in the promoter region compared to other regions owing to the transcriptional silencing and subsequent repression of downstream genes [27]. Genes with greater body methylation levels exhibit higher and more stable expression in plants and invertebrates, whereas genes with lower body methylation levels exhibit lower but inducible expression [28–31]. Nowadays, high-throughput sequencing technologies have also been applied to characterize DNA methylation in aquatic species. In this regard, elucidating how the methylome responds to biotic and abiotic factors causing stress is critical in invertebrates [32,33]. In oysters, the majority of studies dealt with DNA methylation analysis have concentrated on embryo development [34], germ cells (male gametes) [35], ploidy [36], growth [37], and several environmental stress factors including salinity [38], oxygen exposure [39], ocean acidification [40], copper contamination [41] and herbicide exposure [42]. Besides, DNA methylation also plays a crucial role in the immune response of oysters, including methylation-related enzymes such as *Cg-DNMT3* [43] and *Cg-LSD1* [44] and their protential role in regulating immune response in the *C. gigas*. In this respect, Gawra et al. proposed that the epigenetic mechanism control of immune response can greatly promote the rapid adaptation of the host to Pacific oyster mortality syndrome (POMS) caused by ostreid herpesvirus 1 microvariant (OsHV-1 μVar) [45]. However, the mass mortality of oysters is not caused by a single pathogenic factor, and the role of epigenetic regulation involved in responses to *Vibrio* infection has not been reported.

In this study, we used whole genome bisulfite sequencing (WGBS) to profile the genome-wide methylation in gills at 6 h and 48 h after *V. alginolyticus* infection to investigate the potential role of DNA methylation modification in the immune response of *C. gigas*. Furthermore, integrative analysis of transcriptome and DNA methylome was performed to explore the relationship between DNA methylation and gene expression for a better understanding of the genome methylation alteration in the regulation of the key genes and signaling pathways involved in the immune response. Through this study, we sought to provide further theoretical references for preventing the occurrence of mass death of oysters from the perspective of epigenetic mechanisms.

2. Material and methods

2.1. Samples collection and bacterial infection

The animals used for this study were two-year-old Pacific oysters which were healthy without infection after diagnosis (Table S1). A total of 50 oysters were used for the bacterial infection experiment. Before the experiment, the oysters were acclimatized in a 50 L glass tank with filtered seawater (23 °C, salinity 30‰) for one week. During this period, the seawater was continuously aerated and daily changed. The oysters were fed with Chlorella ad libitum. The bacterial pathogen used for the challenge was *V. alginolyticus* Cg5 strain that was previously isolated [11]. Before the injection process, the centrifuge tubes were slowly turned over at regular intervals so that the bacterial suspensions were well-mixed. The purified strain was first inoculated into tryptone soy broth media and incubated at 23 °C for 24 h. Then the bacterial pellets were harvested by centrifuging at 8000 × g for 5 min at 4 °C and re-suspended in saline solution. Finally, the concentration of bacterial suspension was adjusted to 5×10^7 CFU/mL for use. Thereafter, the animals were anesthetized in magnesium chloride ($MgCl_2$, 50 g/L) solution followed by injection with each of 100 μL *V. alginolyticus* suspension (5×10^7 CFU/mL) into the adductor muscles using the microinjector (100 ± 0.5 μL). After injection, the oysters were randomly divided into three replicate groups and cultured in the filtered seawater (23 °C, salinity 30‰) with the same above-mentioned procedure. Furthermore, gill tissues were collected from six oysters (two individuals from each group) at 0 h, 6 h, and 48 h post-infection, respectively. The samples collected at 0 h (hereinafter referred to as G0h) were used as the control group. The samples at the stages of 6 h and 48 h were hereinafter referred to as the two time points (G6h and G48h), respectively. An equal amount of the tissues from the two individuals were pooled together as one sample to generate three biological replicates at each time point. Each sample was divided into two parts, one for RNA extraction and sequencing (RNA-Seq), whereas, the other part was used for DNA extraction of whole-genome bisulfite methylation sequencing (WGBS). All samples were stored in - 80 °C freezer until further use.

2.2. DNA extraction, WGBS, and data analysis

The Phenol-Chloroform method was used to extract the total DNA from the samples [46]. The DNA quality and concentration were measured by running 1% agarose gel electrophoresis and Nanodrop (Thermo Fisher Scientific). The three DNA samples (0 h, 6 h, and 48 h) were fragmented using sonication and subjected to bisulfite conversion. Adapters were ligated to the single-stranded DNA fragments using the Accel-NGS Methyl-Seq DNA Library kit (Swift, MI, USA). In addition to changing the composition of the enzymatic buffer, oligonucleotides and tiny fragments were removed using SPRI-bead clean-ups. The high-throughput sequencing was performed on Illumina NovaSeq 6000 for paired-end reads (2 × 150 bp).

After sequencing, the raw reads obtained by WGBS were filtered using FastQC [47] to obtain high-quality clean reads. Then, we used the Trimmomatic software with main parameters (SLIDINGWINDOW: 4:15; LEADING:3; TRAILING:3; ILLUMINACLIP: adapter.fa: 2:30:10; MINLEN:36) to filter the adapters and low-quality data [48]. The clean reads were mapped to the *C. gigas* reference genome (Assembly: GCA_902806645.1, cgigas_uk_roslin_v1) using Bismark software [49]. The Mmint program (https://github.com/lijiacd985/Mmint) was used to calculate the average CpG methylation ratio and the number of CpG sites of the samples were examined to assess the accuracy of the sequencing data and the reliability of the experiment.

2.3. Genome-wide methylation profiling and comparison

Specifically, two factors were used to estimate the DNA methylation level across the oyster whole genome: (1) Using bismark_methylation_extractor to extract and calculate different methylation types based on the genome alignment analysis; (2) Computing the average CpG methylation level in the transcription unit, 2 kb upstream and 2 kb downstream of the transcription start site (TSS) and transcription end sites (TES), respectively. The differential methylation levels between the samples at two time-points and the control group were calculated and presented across the whole genome in a circos plot.

2.4. Annotation and enrichment analysis of differentially methylated genes

Differentially methylated regions (DMRs) between two time points and the control group were calculated using the MCOMP module in the MOABS software [50] with default parameters. DMRs were annotated and analyzed using the BEDTools software [51] and the genomation package [52] to identify differentially methylated genes (DMGs). DMGs are identified as genes whose gene body region or promoter region overlaps with DMRs by at least 1 bp. Enrichment analysis of DMGs was conducted using Gene Ontology (GO) (https://www.geneontology.org/)

and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (https://www.genome.jp/kegg/) using the R package clusterProfiler [53]. The results with a *p*-adjust <0.05 were considered significantly enriched. The interaction network of selected DMGs was constructed using the String database (http://string-db.org/) [54]. The levels of both gene expression and DNA methylation of the key genes were visualized by using an integrative genomics viewer (IGV).

3. Results

3.1. Whole-genome bisulfite sequencing and mapping

Whole-genome bisulfite sequencing generated a total of 237.81 Gb raw reads from all samples with 212.84 Gb clean reads obtained after quality control (Table S2). The proportion of Q30 for each library was above 89.5%, and the GC base content was between 17.24% and 17.38% (Table S2). The clean reads were aligned to the reference genome of *C. gigas* with an average alignment rate of 35.7% (Table S2). The analysis conducted using Mmint software revealed that as sequencing depth increased, the average CpG methylation rate of each group marginally decreased, indicating the high quality and reliability of the sequencing data for further analysis. Additionally, the number of CpG sites at different depths in each group was also evaluated (Fig. S1).

3.2. Genome-wide methylation profiling

We calculated genome-wide methylation levels with an average sequencing depth of 15 × (Table S3) to analyze the variations in overall DNA methylation levels between the two time points and the control group. The results revealed that methylated CpG dinucleotides accounted for the highest proportion (11.197%) of all cytosine (C) sites, with the other types being less than 1% (Table S3). Among the genomic cytosines, approximately 1% were methylated cytosines, and approximately 83.78–84.58% of the methylated cytosines were CpG dinucleotides, followed by CHH (13.56–14.21%) and CHG (1.86–2.01%) (Table S3, Fig. 1A, B, and C). The comparison of the methylation levels of all samples from 2 kb upstream of the transcription start site (TSS) to 2 kb downstream of the transcription end sites (TES) indicated that the methylation levels were higher in the gene body region than those at TSS and TES regions (Fig. 1D). In addition, the control group appeared with slightly higher methylation levels compared to two time points (Fig. 1D). We created the whole genome methylation circos plot at the chromosomal scale to further compare the methylation levels and overall changes between the two time points and the control group. The results demonstrated that all chromosomal locations had significantly differential methylation between the groups (Fig. 2A and B). The two time points and the control group showed drastic differences in the methylation levels at the genome-wide scale ($p<0.01$) (Fig. 2C and D).

3.3. Characterization of differentially methylated regions

Differentially methylated regions (DMRs) are significant indicators of epigenetic alterations after *Vibrio* infection. A total of 11,502 and 14,196 DMRs were identified at 6 h and 48 h after infection, respectively. The number of DMRs within introns was much higher than that within the promoter regions (Fig. 3A). Notably, the number of hypo-methylated regions at each functional element was higher than the number of hyper-methylated regions in both infection groups, suggesting a potential role of DNA methylation in response to infection with *V. alginolyticus*. From DMRs, a total of 5280 and 6343 differentially methylated genes (DMGs) were identified at 6 h and 48 h after infection, respectively. The overall methylation level of two time points was significantly higher than that of the control group in both promoter and genebody ($p<0.01$). The number of hypo-methylated genes was also higher than that of hyper-methylated genes at two time points (Fig. 3B).

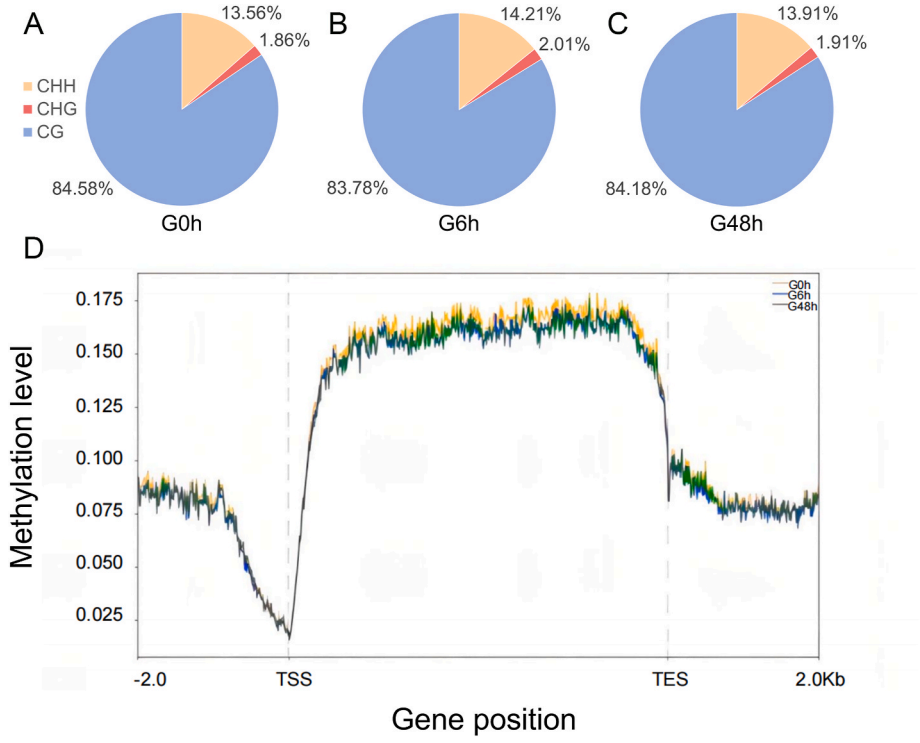

Fig. 1. Genome-wide DNA methylation profile of Pacific oyster after *Vibrio* infection. A, B, C: Proportion of different types of methylated C in total methylated Cytosine in the control group (G0h) and two time points (G6h and G48h) after *Vibrio* infection, CG(CpG), CHG, and CHH represented three types of methylated C, H corresponds to A, T, or C; D: The methylation levels within regions from 2 kb upstream of the transcription start site (TSS) to 2 kb downstream of transcription end sites (TES) in different groups of oysters.

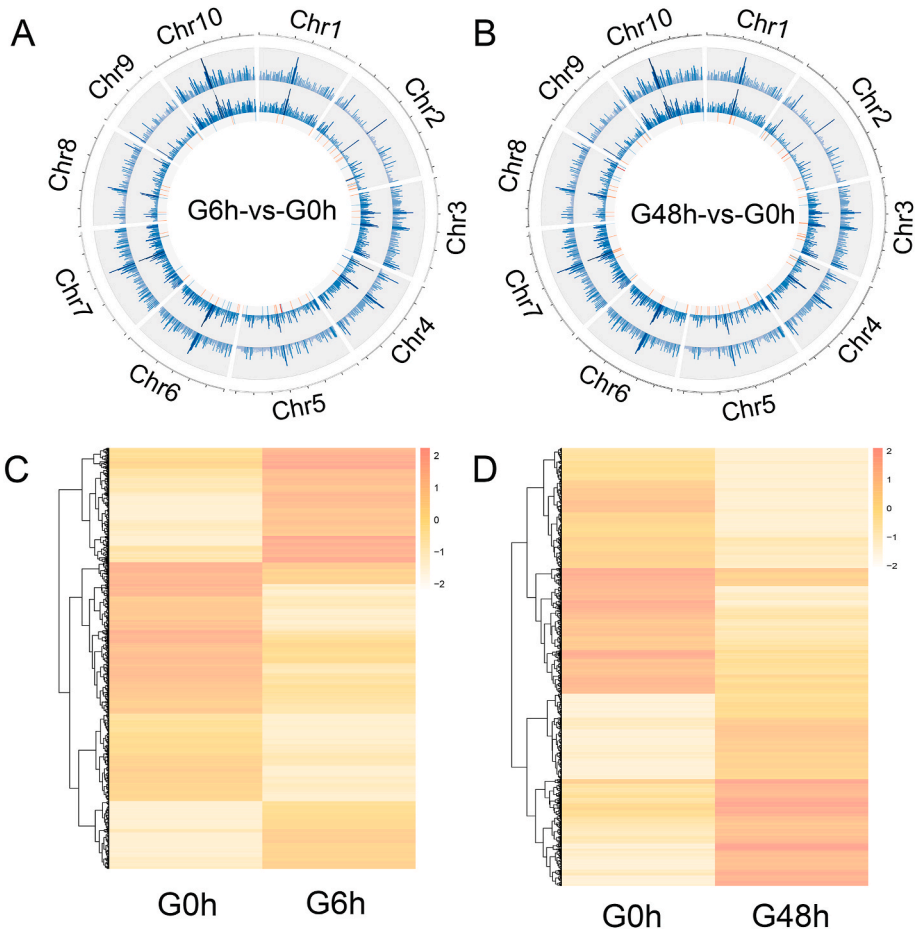

Fig. 2. Genome-wide methylation comparison between the control group and two time points after *Vibrio* infection. A, B: Genome-wide methylation comparison between the control group and infection group at 6 h (A) and 48 h (B) after *Vibrio* infection. From outside to inside, the first circle represents the ten chromosomes of *C. gigas*; the second and third circles represent the methylation level of the challenge group and control group presented with the histogram, respectively. The fourth circle represents the differential methylation level between the two time points and the control group presented with the heatmap. C, D: Cluster analysis of methylated regions at 6 h (C) and 48 h (D). Each row represents a methylated region and each column represents a set of samples. Different colors represent the different levels of methylation. The darker color indicates a higher level of methylation.

3.4. Functional analysis of differentially methylated genes

To investigate the potential functions of DMGs, we performed GO and KEGG pathway enrichment analyses. The results showed that 395 and 552 GO terms were significantly enriched (p-adjust<0.05) in DMGs identified at 6 h and 48 h after *Vibrio* infection, respectively (Table S4 and Table S5). Some immune-related GO terms including somatic diversification of immunoglobulins (GO: 0016445), somatic diversification of immune receptors (GO: 0002200), and somatic diversification of immune receptors via germline recombination within a single locus (GO: 0002562) were significantly enriched at two time points (Table S4 and Table S5).

Moreover, the top 30 GO terms were listed in ascending order of p-adjust (Fig. 4A and B). At 6 h after infection, the most significant functional term was chromosome, centromeric region (GO: 0000775) in the cellular component, meanwhile the most significant functional term was phosphatidylinositol-3,5-bisphosphate binding (GO: 0005547) in molecular function. In terms of the biological process, mitotic spindle organization (GO: 0007052) was the most significant functional term. Likewise, KEGG analysis showed that DMGs were enriched in 378 pathways (Table S4), and the top 30 pathways in ascending order of p-adjust were listed in Fig. 4C, whereby, 23 and 67 DMGs identified at 6 h after infection were significantly enriched in the pathways related to inositol phosphate metabolism (ko00562) and transcription machinery (ko03021), respectively. On the other hand, DMGs identified at 48 h were significantly enriched in chromosome, centromeric region (GO: 0000775) in the cellular component (Fig. 4B). In molecular function, the most significant functional term was GTPase regulator activity (GO: 0030695), and for the biological process, ribonucleoprotein complex subunit organization (GO: 0071826) and meiosis I (GO: 0007127) were the most significant functional terms. Meanwhile, 100 and 118 DMGs were significantly enriched to spliceosome (ko03041) and DNA repair and recombination proteins (ko03400), respectively (Fig. 4D).

3.5. Integrative analysis of DMGs and DEGs

We combined methylome data with the transcriptome data that have been published in our previous study [11] to examine the connection between DNA methylation and gene expression. The integrative analysis allowed the identification of 167 and 379 DMG-related DEGs at 6 h and 48 h after *Vibrio* infection, respectively (Fig. 5A and B). Of which, 23 and 145 genes identified at 6 h post-infection were located within the promoter and gene body regions, respectively. Only one gene (*MCD*, malonyl-CoA decarboxylase, LOC105348931) showed methylation differences in both promoter and gene body regions (Fig. 5C). A set of 51 and 335 genes identified at 48 h post-infection was located within the promoter and gene body regions, respectively. Seven genes including copine-3 (*CPNE3*, LOC105324813), geranylgeranyl transferase type-2 subunit alpha (*RABGGTA*, LOC105330888), autophagy-related protein 2 homolog B (*ATG2B*, LOC105338878), sacsin-like X2 (*SACS*,

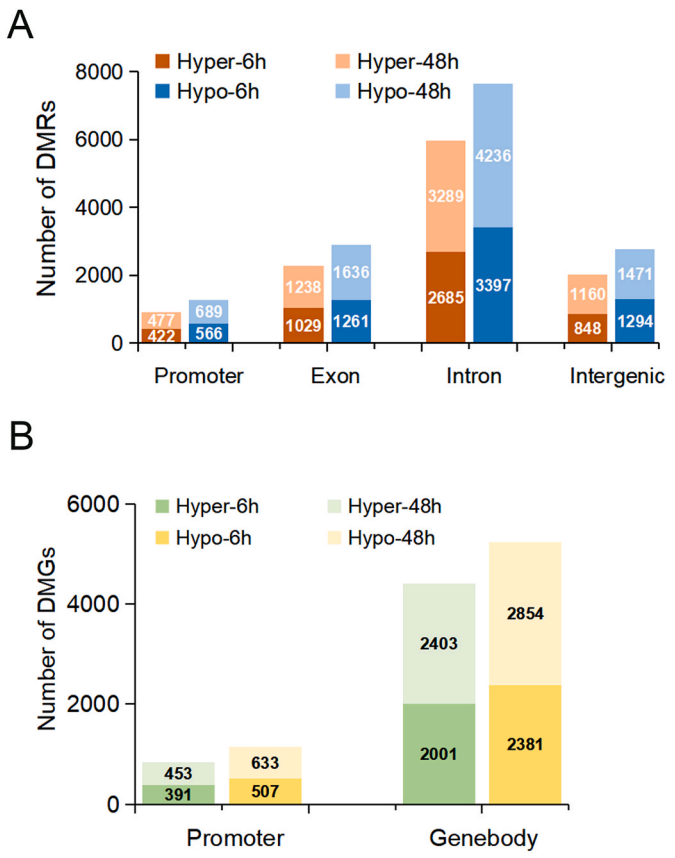

Fig. 3. The distribution of DMRs and DMGs at 6 h and 48 h after *Vibrio* infection. A: The distribution of DMRs in different regions of the gene at 6 h and 48 h after *Vibrio* infection. Deep red and light red represent hyper-methylated regions at 6 h and 48 h, respectively. Deep blue and light blue represent hypo-methylated regions at 6 h and 48 h, respectively. B: The distribution of DMGs in promoter and gene body at 6 h and 48 h, respectively. Deep green and light green represent hyper-methylated genes at 6 h and 48 h. Deep yellow and light yellow represent hypo-methylated genes at 6 h and 48 h, respectively.

LOC109618139), three uncharacterized genes (LOC117682017, LOC105321456, and LOC117686988) showed methylation differences in both promoter and gene body regions (Fig. 5D).

To further determine the role of gene methylation in the immune response of *C. gigas*, we performed GO and KEGG enrichment analysis of these associated genes at two infection stages. The results showed that they were significantly enriched in 437 and 334 GO terms, respectively ($p<0.05$), including 19 GO terms that were common in both treatments, such as inhibition of cysteine-type endopeptidase activity (GO: 1990001), tRNA metabolic process (GO: 0006399), histone H3-R17 methylation (GO: 0034971), and RIG-I signaling pathway (GO: 0039529) (Fig. S2, Table S6, and Table S7). In addition, the KEGG enrichment analysis revealed that 42 and 47 genes were significantly enriched to 33 and 22 pathways at 6 h and 48 h, respectively ($p < 0.05$) (Table S6, Table S7). Totally, 13 pathways were enriched at two stages related to immune pathways including NF-kappa B signaling pathway (ko04064), TNF signaling pathway (ko04668), Transcriptional misregulation in cancer (ko05202), and Apoptosis (ko04210). It is worth noting that the RIG-I-like receptor signaling pathway (ko04622), Inflammatory mediator regulation of TRP channels (ko04750), DNA repair and recombination proteins (ko03400) were only enriched in G6h while Ubiquitin-mediated proteolysis (ko04120), and mTOR signaling pathway (ko04150) were only enriched in G48h (Fig. 6A and B).

The interaction network was constructed using DEGs that were chosen based on the findings of enrichment analysis and correlation analysis (Fig. 7A and B). The key genes of *TRAF2* (LOC105344264, TNF receptor-associated factor 2), *BIRC3* (LOC105334814, baculoviral IAP repeat-containing protein 3, the homolog of human *BIRC2*), *IRF8* (LOC105317636, Interferon Regulatory Factor 8), and uncharacterized gene LOC117691871 (the homolog of human *BIRC2*) showed significant changes in both infection time points, and they may play a key role in the NF-κB signaling pathway. These genes showed different methylation patterns after infection with *V. alginolyticus*. For instance, the *TRAF2* and *IRF8* were hyper-methylated in the promoter, while they were significantly down-regulated in gene expression. The *BIRC3* was hypo-methylated in the gene body, and it was also significantly down-regulated. The LOC117691871 was hyper-methylated in the gene body, and it was significantly up-regulated (Fig. 7C). The *CASP7* (LOC105339898, uncharacterized protein LOC105339898), and *PARP3* (LOC117683515, protein mono-ADP-ribosyltransferase PARP3-like; LOC10534414, protein mono-ADP-ribosyltransferase PARP3) were only identified at 6 h after infection. In contrast, four BIRC gene family members, the homolog of human *BIRC2* (LOC105334815, LOC117692263, LOC117680358, LOC117691853), ubiquitin carboxyl-terminal hydrolase CYLD-like (the homolog of human *CYLD*) (LOC117692600), *EIF4E* (LOC105327880, eukaryotic translation initiation factor 4E), and *EIF2B5* (LOC105320319, translation initiation factor eIF-2B subunit) were only identified at 48 h after *Vibrio* infection.

4. Discussion

Epigenetic processes constitute an important gene regulation mechanism in organisms that can affect gene activity without changing the DNA sequence [55–57]. DNA methylation is a fundamental epigenetic process that plays crucial roles in the regulation of gene expression and genomic stability [58]. Currently, it has been extensively investigated in mammals, but it has not yet been fully explored in aquatic species, especially mollusks. Oysters, a group of bivalves that inhabit intertidal regions with complex environments, are subjected to numerous environmental stressors, including pathogens [59]. In recent years, *vibriosis* has been widely recognized to be associated with mass summer mortality of oysters [60–62]. In this point of view, studies have been performed to investigate disease resistance against *Vibrio* infection in oysters [63–66]. With the advancement of high-throughput sequencing, whole genome methylation sequencing has been more and more widely used for investigating the role of DNA methylation involved in response to *Vibrio* infection [67–69]. In this study, we used WGBS to elucidate the dynamics of DNA methylation in response to *Vibrio alginolyticus* infection in the *Crassostrea gigas*.

Our WGBS analysis showed that DNA methylation occurred most often at the CpG sites which were mainly located in the gene body. This is consistent with the findings observed in other invertebrates [70], suggesting the conservative feature of methylation in the *C. gigas*. It's well recognized that the DNA methylation level is lower in invertebrates than that in vertebrates [71]. In our work, the genome-wide methylation level of the gill was approximately 1 %, which was consistent with previous reports in the *C. gigas* [72–74]. However, it has been reported that both *Drosophila melanogaster* and *Caenorhabditis elegans* lack DNA methylation in their genomes [75]. In contrast, the methylation levels are about 3 % and 4 % in Yesso scallop (*Patinopecten yessoensis*) [76] and sea cucumber (*Apostichopus japonicas*) [77], respectively. This demonstrates that overall DNA methylation levels differ among species [72]. In this study, we identified 11,502 and 14,196 DMRs from group comparisons of G6h-vs-G0h and G48h-vs-G0h, respectively. The DMRs in both groups were mostly located in the intron and were almost 6 times higher than those in the promoter regions, indicating that DNA methylation usually occurs in the transcription regions in the *C. gigas* which was consistent with previous reports [35,78]. Meanwhile, we also observed that more DMRs were identified at 48 h than at 6 h after infection. At both time points, the number of hypo-methylated regions in each group was greater than the number of hyper-methylated regions, suggesting that the methylation could be altered by demethylation in the

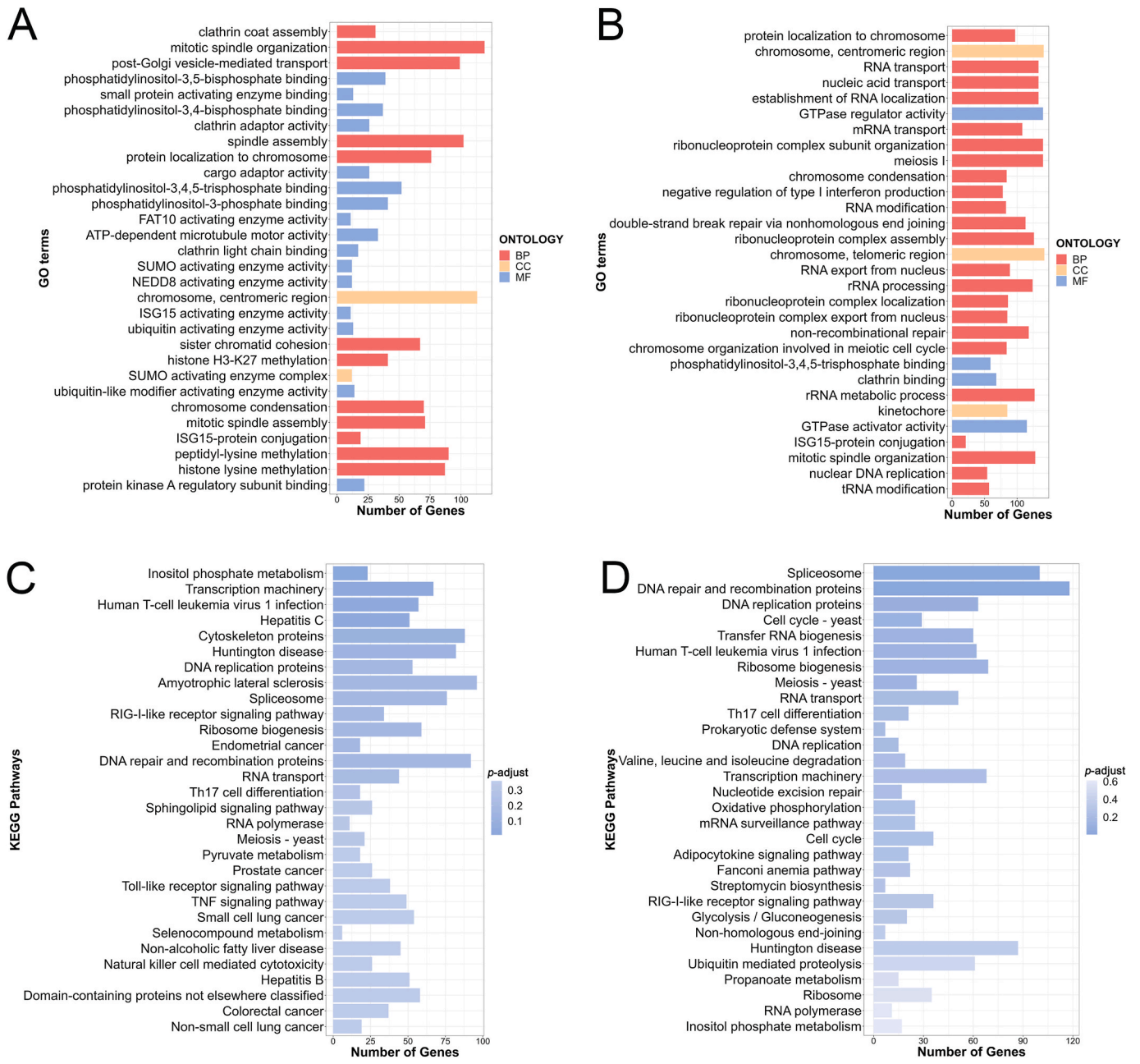

Fig. 4. GO and KEGG enrichment analysis of DMGs identified at 6 h and 48 h after *Vibrio* infection, respectively. A, B: The top 30 GO terms of DMGs in ascending order of *p*-adjust at 6 h (A) and 48 h (B). BP: Biological Process, CC: Cell Component, MF: Molecular Function. C, D: The top 30 KEGG pathways of DMGs in ascending order of *p*-adjust at 6 h (C) and 48 h (D). The darker color indicates the lower *p*-adjust value.

C. gigas upon *V. alginolyticus* infection.

The KEGG analysis of DMGs revealed that the transcription process (ko03021) was the most significant enrichment pathway at 6 h post-infection. Investigations in some other invertebrates also found that DMGs were enriched in transcription [79]. This is probably because DNA methylation in invertebrates usually occurs in the transcription region and is closely related to expression [80]. On the other hand, many terms about RNA transport and processing were enriched significantly at 48 h post-infection. Interestingly, GO enrichment analysis indicated that the phosphatidylinositol-3,4,5-trisphosphate binding was significantly enriched at both time points after *Vibrio* infection. The phosphatidylinositol-3,4,5-trisphosphate (PIP3) is one of the most important second messengers in the PI3K/AKT pathway, the localization and activation of these effector proteins may activate this pathway to control a myriad of cellular functions including cell survival, proliferation, cytoskeletal rearrangement, and gene expression [81]. DNA repair and recombination proteins, together with spliceosomes, were considerably enriched in the oysters at 48 h after infection. Many human diseases were associated with aberrant changes in spliceosome components, which may cause splicing defects or alterations [82,83]. In invertebrates, spliceosomes were stated to have a function in the regulation of the specific immune priming in red swamp crayfish (*Procambarus clarkii*) [84] and silk cocoon (*Bombyx mori*) [85]. DNA repair and recombination have been proven to be related to immune response [86,87]. Therefore, we speculate that inositol phosphate metabolism, spliceosome, and DNA repair play potential roles in response to *V. alginolyticus* infection in the *C. gigas*.

The NF-κB family of transcription factors is activated by canonical and non-canonical signaling pathways, both of which have different activation mechanisms but similar functions in mediating the immune

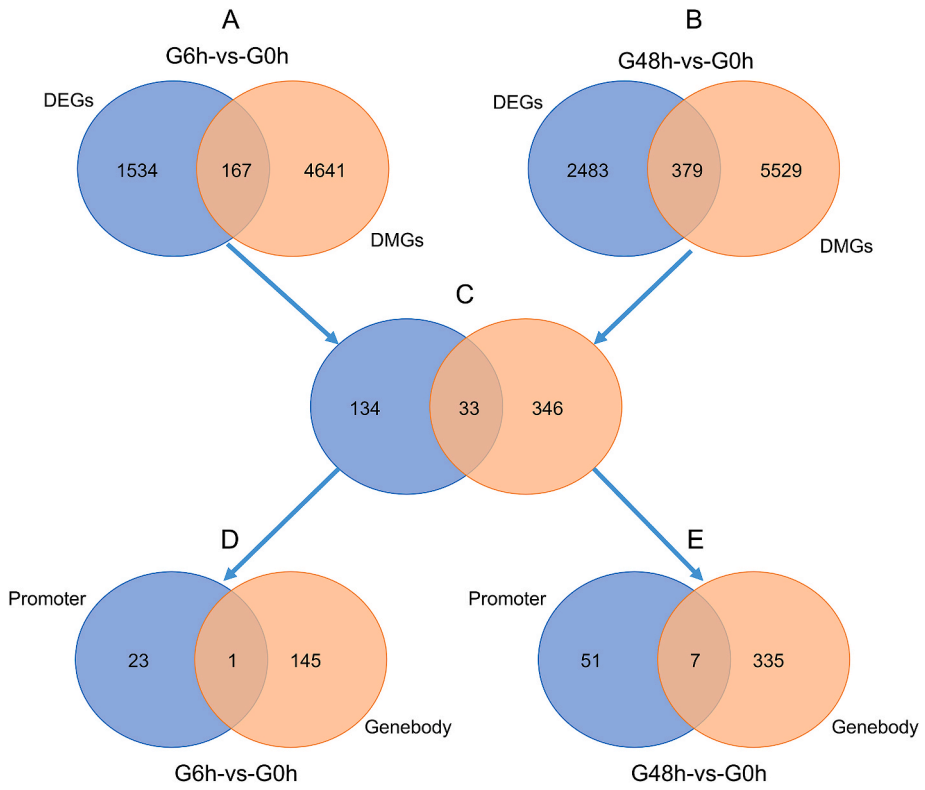

Fig. 5. Venn diagrams of differentially methylated genes and differentially expressed genes. A, B: The number of DMGs, DEGs, and DMG-related DEGs identified at 6 h (G6h-vs-G0h) after infection (A) and at 48 h (G48h-vs-G0h) (B). C: The overlapping genes of DMG-related DEGs between two time points. D, E: The location of DMG-related DEGs in G6h-vs-G0h (D) and G48h-vs-G0h (E).

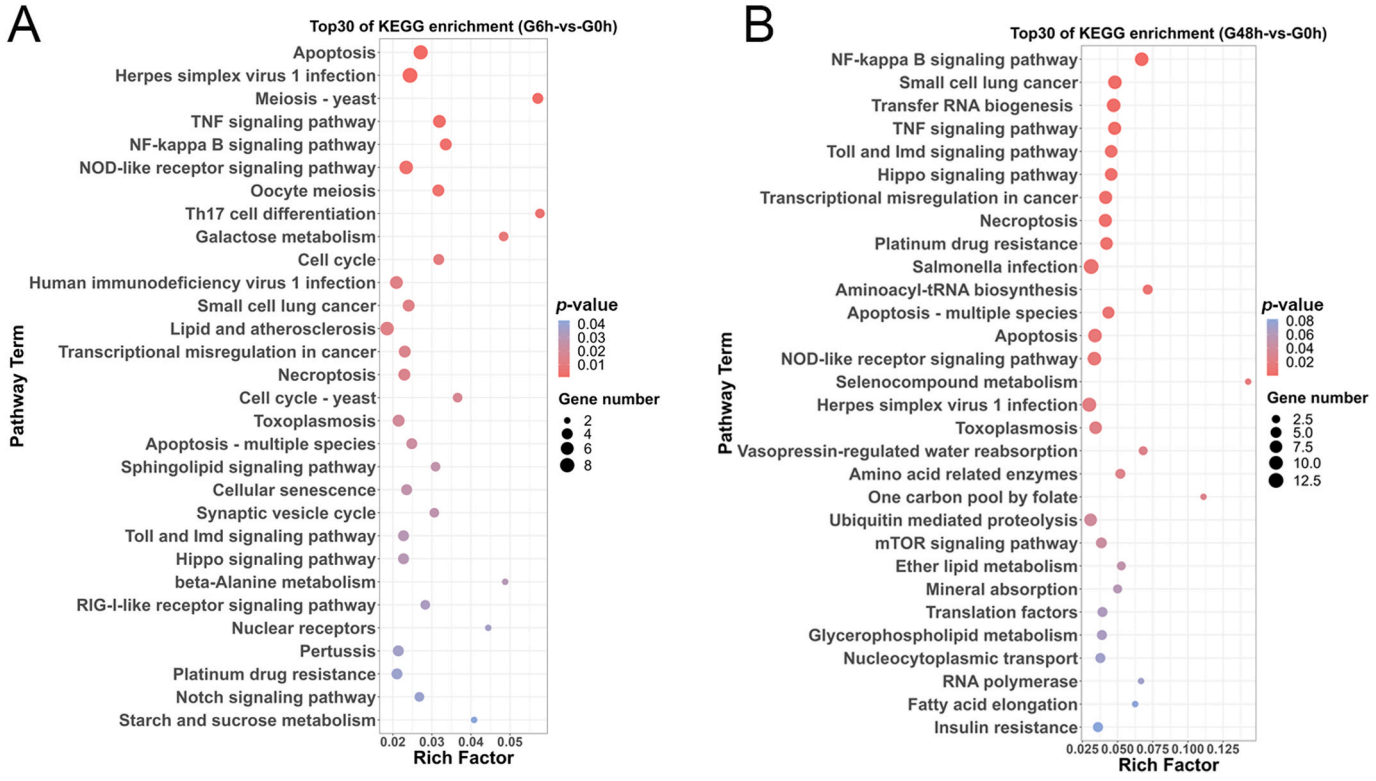

Fig. 6. Enrichment analysis of KEGG pathways on associated genes in two time points. The rich factor is the ratio of the number of associated genes in this pathway term to the number of all genes in this pathway term. A greater rich factor means greater intensiveness. The bigger circle indicates the larger the number of genes. KEGG enrichment analysis was conducted by the hypergeometric test with *p*-value $\leq$0.05 as a threshold. The smaller *p-value* indicates the higher enrichment significance.

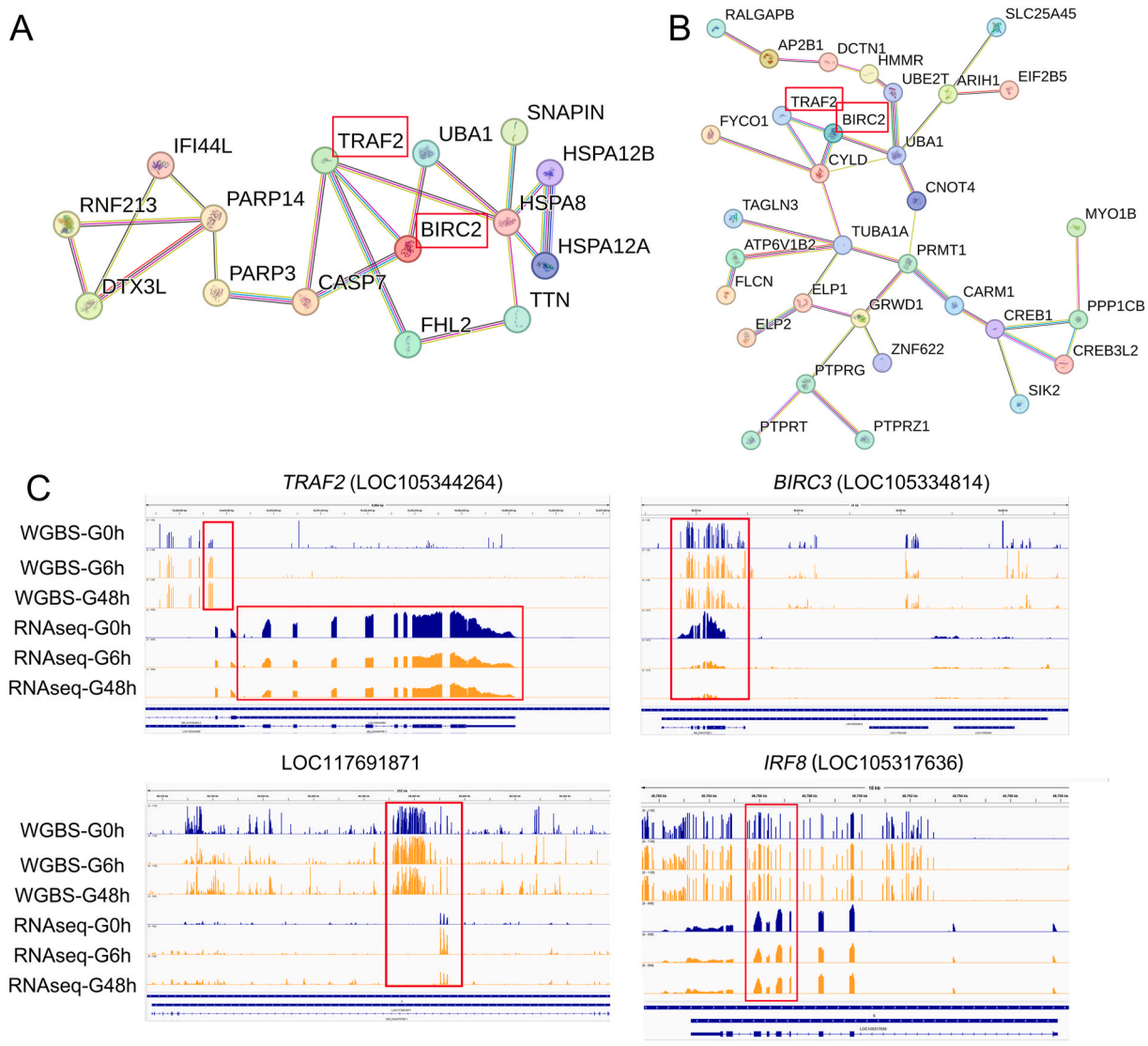

Fig. 7. The key genes between the DNA methylation and gene expression. A, B: Interaction network of DMG-related DEGs at two time points. These genes were analyzed using the STRING database. Line colors indicated the type of interaction. A blue line means known interaction from the curated database, a purple line means known interaction from experimentally determined, a green line means predicted interaction of gene neighborhood, a red line means predicted interaction of gene fusions, a deep blue line means predicted interaction of gene co-occurrence, a yellow line means textmining, a black line indicates co-expression and a light blue line means protein homology. C: Visualizations of methylation (WGBS) and expression (RNA-seq) between G0h, G6h, and G48h for *TRAF2* (LOC105344264), *BIRC3* (LOC105334814), LOC117691871, and *IRF8* (LOC105317636).

response such as the inflammatory response [88]. The classical NF-κB pathway can be activated by various stimuli such as TNF-α, LPS, and IL-1β and mediated by cell surface receptors such as IL-1R, TLR, TNFR, and antigen receptors, and by various adaptor proteins and signaling kinase IKK [89]. However, in invertebrates, more studies have focused on the TLR-mediated NF-κB pathway, while the TNFR-mediated pathway has rarely been mentioned [90–92]. The association of DMGs with DEGs after *Vibrio* infection revealed their significant connection in the nuclear factor kappa-B (NF-κB) signaling pathway. In the *C. gigas*, this pathway also plays important roles in several developmental, cellular, and immune response processes [93] and it is mainly mediated by TLR [94].

In our study, we found that the expression and methylation levels of *TRAF2* (LOC105344264), *BIRC3* (LOC105334814, and LOC117691871, the homolog of human *BIRC2*), and *IRF8* (LOC105317636) were both significantly enriched in this pathway at two time points after infection. The distinct methylation patterns following *V. alginolyticus* infection suggested specific differences in the methylation patterns of different genes which could both regulate gene expression. Studies in humans have also demonstrated that *TRAF2* and *BIRC3* may play a key role in cell inflammation and cell survival by activating the TNFR-mediated NF-κB pathway [95]. *TRAF2* was highly expressed in gastric cancer (GC) patients by DNA hypo-methylation [96] and could be methylated by *SMYD2* for the maintenance of NF-κB activation during inflammatory diseases [97]. However, the specific roles of *TRAF2* and *BIRC3* in this pathway have not been studied in invertebrates, especially marine invertebrates. In the *C. gigas*, a total of 15 TRAF family members have been identified [98], and *TRAF2* and *TRAF6* have been demonstrated to be crucial components of immunological defense mechanisms in response to pathogens [99,100]. Baculoviral inhibitors of apoptosis repeat-containing proteins (*BIRCs* or *cIAPs*) are negative regulators of apoptosis which have also been linked to the complex and subtle control of apoptosis and other immune responses in the *C. gigas* [101,102]. Interferon regulatory factor 8 (*IRF8*) is also a key regulator of innate immune receptor signaling, which regulates cell growth and differentiation to resist pathogen invasion. In vertebrates, the DNA methylation

induced the silencing of *IRF8* expression is associated with the malignant phenotype of Multiple myeloma (MM) [103]. The overexpression of *IRF8*, which was negatively correlated with the promoter methylation status suppressed apoptotic gene expression, thereby inhibited apoptosis in response to Porcine epidemic diarrhea virus (PEDV) stimulation [104]. In invertebrates, *CfIRF8-like* has been reported to interact with TBK1/IKKε family proteins and regulate the host antiviral innate immunity in *Chlamys farreri* [105]. In the *C. gigas*, two *IRF1-like* genes and one *IRF8-like* gene were identified, in which *CgIRF1a* could significantly activate the expression of the ISRE reporter gene, while *CgIRF1b* and *CgIRF8-like* genes showed no regulatory activity of ISRE and NF-κB [106].

The ubiquitin carboxyl-terminal hydrolase cylindromatosis (*CYLD*) is a tumor suppressor that is mutated in familial cylindromatosis, which can negatively regulate NF-κB signaling by deubiquitination [107]. The deubiquitination and inactivation of *TRAF2* is the primary mechanism via which *CYLD* inhibits NF-κB activation [108]. A study in Gastric cancer (GC) patients has shown a significant negative correlation between DNA promoter methylation and *CYLD* expression [109]. Caspase recruitment domain family member (*CARD*) plays a critical role in molecular interaction and regulation of various signaling pathways, such as the activation of caspase and NF-κB signaling pathway in the process of apoptosis or inflammation. In the *C. gigas*, the *CgCARDCP-1* was reported to act as a recognition molecule of LPS and a regulator of NF-κB activation in the oyster immune responses [110].

Several pathway-related genes including *TLR4* (Toll-like receptor 4, LOC105324719), *XIAP* (X-Linked Inhibitor Of Apoptosis, LOC105328062), *gadd45β* (growth arrest and DNA damage-inducible 45β, LOC105327450 LOC105327454), *Bcl-2* (B-cell lymphoma-2, LOC105343381), *PTGS2* (Prostaglandin G/H synthase 2, LOC105344258), and *TNFAIP3* (TNF Alpha Induced Protein 3, LOC105347244) were also identified to exhibit different expression patterns in both infection groups through previous transcriptome analysis even though the methylation analysis indicated that their methylation levels did not change significantly after infection. This may indicate that the immune response is regulated by a network of multiple complex mechanisms. It might be caused by the methylation-induced suppression of gene transcription in key genes like *TRAF2* and *BIRC3*, which might activate the downstream NF-κB pathway leading to numerous changes in gene expression that drive inflammation, cell proliferation, and cell survival in response to the challenge posed by *V. alginolyticus*.

Taken together, we conclude that the NF-κB pathway plays a crucial role in counteracting *V. alginolyticus* infection in the *C. gigas*. The epigenetic alteration altered gene expression and triggered immune-related signaling pathways. A previous study has shown that TLRs or RLRs transduced immune signals by interacting with the corresponding adaptor molecule and finally phosphorylated IκB molecules to realize regulation of *NF-κB* in oysters [111]. On this basis, we found that the classical NF-κB signaling pathway mediated by *TNFR* may also play a role in the innate immunity of the *C. gigas*. Furthermore the expression of key genes such as *TRAF2*, *BIRC2/3*, and *IRF8* were regulated by methylation. Integrative analysis of DNA methylation and transcriptional expression allowed us to propose a predictive model of molecular mechanism underlying immune response to *V. alginolyticus* infection in the *C. gigas* (Fig. 8). When oysters are infected by *Vibrio*, *TNF-α* binds to *TNFR* through its specific receptor, bringing complexes of *RIPK1*, *TRAF2*, *BIRC2/3*, and *TRADD* will be activated. After being ubiquitinated by the ubiquitinating enzymes *BIRC2/3* and *TRAF2*, the adaptor protein *RIPK1* binds on *MAVs* or activates *IκBs* via *TBK1* to control *NF-κB* and *ISRE*. Moreover, the *NF-κB* affects the expression levels of genes linked to the downstream inflammation in response to *vibrio* infection, including *BIRC2/3*, *TRAF2*, *XIAP*, *Bcl-2*, *gadd45β*, *TNFAIP3*, and *PTGS2*. This work provides insights into the role of methylation in the innate immune response to *Vibrio* infection in the *C. gigas*. The epigenetic regulation of key molecules like *TRAF2*, *BIRC3*, and *IRF8* plays a key role in the antiviral and antibacterial innate immune signaling in oysters.

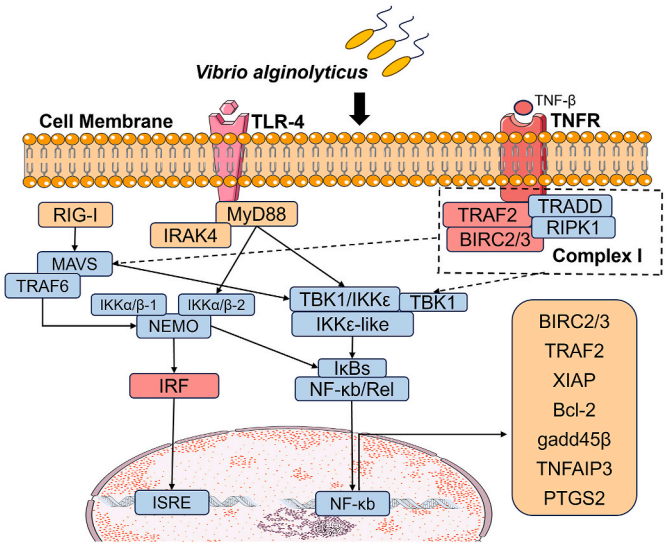

Fig. 8. The epigenetic regulation of NF-κB pathway in response to *V. alginolyticus* infection in the *C. gigas*. When oysters are infected by *Vibrio alginolyticus*, *TNF-α* binds to *TNFR* through its specific receptor, bringing complexes of *RIPK1*, *TRAF2*, *BIRC2/3*, and *TRADD*. After being ubiquitinated by the ubiquitinating enzymes *BIRC2/3* and *TRAF2*, the adaptor protein *RIPK1* binds on *MAVs* to control *NF-κB* and *ISRE* or activates *IκBs* via *TBK1*. Moreover, *NF-κB* affects the expression levels of genes linked to the downstream inflammatory response, including *BIRC2/3*, *TRAF2*, *XIAP*, *Bcl-2*, *gadd45β*, *TNFAIP3*, and *PTGS2*. The genes in the red, orange, and blue boxes represent DMG-related DEGs, DEGs, and other genes, respectively. Abbreviations are: *RIG-I*, Retinoic acid-inducible gene I; *MAVs*, Mitochondrial antiviral signaling protein; *TRAF6*, TNF-receptor associated factor 6; *TRAF2*, TNF-receptor associated factor 2; *MyD88*, Myeloid differential protein-88; *BIRC2/3*, baculoviral IAP repeat-containing protein 2/3; *RIPK1*, Receptor-interacting protein kinase 1; *TRADD*, TNFRSF1A Associated Via Death Domain; *TLR-4*, Toll-like receptor 4; *TBK1*, TANK-binding kinase 1; *IKKα/β*, IκB kinase α/β; *NEMO*, NF-κB essential modulator; *IRF*, Interferon regulatory factor; *ISRE*, Interferon stimulation response element.

5. Conclusion

In this work, whole genome bisulfite sequencing for DNA methylation analysis was carried out in the Pacific oysters at the short-term (6 h) and long-term (48 h) stages after *Vibrio alginolyticus* infection. Our results revealed that epigenetic modification was involved in regulating gene expression to modulate immune signaling pathways in response to *V. alginolyticus* infection. This work provided crucial information regarding the regulation of critical genes after *V. alginolyticus* infection in the *C. gigas* which consideres valuable evidence toward understanding the intricate mechanisms driving survival in marine invertebrates.

Funding

This work was supported by National Natural Science Foundation of China (32341060), the Key Research and Development Program of Shandong Province (2021ZLGX03), the National Key Research and Development Program of China (2022YFD2400300), and the Agriculture Research System of China Project (CARS-49).

CRediT authorship contribution statement

Jian-an Li: Writing – original draft, Formal analysis. **Yameng He:** experimented, Formal analysis. **Ben Yang:** experimented. **Ahmed Mokrani:** revised the manuscript. **Yin Li:** experimented. **Chao Tan:** experimented. **Qi Li:** Supervision, All authors have read and approved

the final manuscript. **Shikai Liu:** Funding acquisition, revised the manuscript.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109705.

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