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Transcriptome-wide methylated RNA immunoprecipitation sequencing profiling reveals m6A modification involved in response to heat stress in *Apostichopus japonicus*

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Abstract

Background Global warming-induced environmental stresses have diverse effects on gene expression and regulation in the life processes of various aquatic organisms. N6 adenylate methylation (m6A) modifications are known to influence mRNA transcription, localization, translation, stability, splicing, and nuclear export, which are pivotal in mediating stress responses. *Apostichopus japonicus* is a significant species in aquaculture and a representative of benthic organisms in ecosystems, thus there is a growing need for research on its heat stress mechanism.

Results In this study, m6A-modified whole transcriptome profiles of the respiratory tree tissues of *A. japonicus* in the control (T18) and high-temperature stress (T32) groups were obtained using MeRIP-seq technology. The results showed that 7,211 common m6A peaks, and 9,459 genes containing common m6A were identified in three replicates T18 and T32 groups. The m6A peaks were found to be highly enriched in the 3' untranslated region, and the common sequence of the m6A peak was also enriched, which was shown as RRACH (R = G or A; H = A, C, or U). A total of 1,200 peaks were identified as significantly differentially enriched in the T32 group compared with the T18 group. Among them, 245 peaks were upregulated and 955 were downregulated, which indicated that high temperature stress significantly altered the methylation pattern of m6A, and there were more demethylation sites in the T32 group. Conjoint analysis of the m6A methylation modification and the transcript expression level (the MeRIP-seq and RNA-seq data) showed co-differentiated 395 genes were identified, which were subsequently divided into four groups with a predominant pattern that more genes with decreased m6A modification and up-regulated expression, including HSP70IV, EIF2AK1, etc. GO enrichment and KEGG analyses of differential m6A peak related genes and

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co-differentiated genes showed the genes were significantly associated with transcription process and pathways such as protein processing in the endoplasmic reticulum, Wnt signaling pathway, and mTOR signaling pathway, etc.

Conclusion The comparisons of m6A modification patterns and conjoint analyses of m6A modification and gene expression profiles suggest that m6A modification was involved in the regulation of heat stress-responsive genes and important functional pathways in *A. japonicus* in response to high-temperature stress. The study will contribute to elucidate the regulatory mechanism of m6A modification in the response of *A. japonicus* to environmental stress, as well as the conservation and utilization of sea cucumber resources in the context of environmental changes.

Keywords *Apostichopus japonicus*, N6 adenylate methylation, Heat stress, RNA methylation, Genes and functional pathways

Introduction

Temperature is a major environmental factor with profound effects on all levels of biological organization, from molecules to ecosystems [1]. Global warming has caused persistently high temperatures and numerous incidents of overheating in recent decades. Animals experience heat stress when the ambient temperature rises above their optimum physiological range, which substantially impacts their daily activities. Studies have increasingly shown that epigenetic modifications can rapidly respond to environmental variability, regulating gene expression, physiological state, and phenotype, in adapting to environmental changes or responding to environmental stress [2–6]. Numerous studies on mammals and other terrestrial animals have shown how environmental conditions, such as high temperatures, affect several aspects of epigenetics [7–11]. High temperatures are major environmental stressors for marine animals, and it is possible that epigenetic modifications significantly affect how they respond to and adapt to the rising seawater temperatures brought on by global warming [12, 13].

Typically, epigenetic mechanisms regulate gene transcription through various pathways, including DNA methylation, small RNAs, ATP-dependent chromatin remodeling, histone variants, histone modifications, histone chaperones, and long non-coding RNAs. Certain epigenetic modifications and modifier proteins control the expression of heat-responsive genes in organisms under high-temperature stress, limiting heat-related damage and enabling adaptation to environmental changes [14]. RNA methylation is a significant area of epigenetic research. It plays a crucial role in regulating gene expression, splicing, RNA editing, RNA stability and degradation, and is considered an important method for gene expression regulation [15, 16]. Over 160 types of chemical modifications have been identified in RNA molecules to date [17, 18], with methylation accounting for over 60% [19]. The most common methylations include m6A, m5C, and m1C RNA methylation [19, 20]. The most abundant post-transcriptional modification of mRNAs in animals is N6-methyladenosine (m6A), first identified in the 1970s [21], with conserved motifs containing DRACH (A is methylated, D=A/G/U, R=A/G, H=A/C/U) [22–24]. The m6A modification status is regulated by 'writers' (methyltransferases), 'erasers' (demethylases), and 'readers' (binding proteins) [25, 26], ultimately affecting various stages of post-transcriptional RNA metabolism, including localization [27], splicing [28], translation [29], degradation [30]and stability [31]. The importance of m6A for controlling gene expression and its function in the life cycle is becoming increasingly clear alongside academic progress in this field. Since 2012, m6A research has employed antibodies that specifically bind to m6A-containing mRNA [32]. Subsequently, next-generation sequencing technologies have facilitated transcriptome-wide sequencing of enriched mRNA fragments, the MeRIP seq technology for sequencing the m6a site has been developed, which can capture unstable or low expression RNA splicing isoforms, enrich sequencing data, and improve the accuracy and efficiency of analysis.

Studies on whether m6A levels and the expression of m6A-related genes differ between mammalian heat stress groups and controls are now being conducted on a ongoing basis, heat shock proteins and genes related to the unfolded protein response (UPR) have been confirmed to be involved in in protein homeostasis and be crucial for cellular defense against thermal stress [33, 34]. However, the role of m6A methylation modification under heat stress conditions has been rarely reported in lower vertebrates, especially marine invertebrates. *Apostichopus japonicus* is a temperate species that is mainly found on the coasts of East Asia. Due to its high nutritional and medicinal value, the sea cucumber *A. japonicus* has become a popular food in China [35], which has led to progress in the Chinese sea cucumber farming industry. The industry faces a major issue as pond aquaculture suffers from regular mass fatalities of sea cucumbers due to persistently high temperatures in the pond water induced by summer heat waves [36]. Sea cucumbers, as benthic echinoderms important to marine ecosystems, are representative organisms for studying how marine ecosystems respond to temperature fluctuations. Consequently, studies on the effects of heat stress on sea cucumbers have gained more attention. In earlier research focused

more on transcriptome expression and gene function analysis, numerous important stress-responsive genes, including heat shock proteins, members of the transferrin superfamily, the complement system, pattern recognition receptors, and those involved in signal transduction, were discovered as regulated and altered [37–40]. However, the exact regulators of these differentially expressed genes and the involvement of m6A modification in this regulatory process remain unknown.

Overall, research on *A. japonicus* has been limited regarding the localization of m6A motifs in their whole transcriptome under stress conditions and their relationship with gene expression regulation. In this study, the high-throughput sequencing technique MeRIP-seq was innovatively applied to obtain the m6A transcriptome profile of respiratory tree tissues of *A. japonicus* under normal (T18) and heat stress (T32) conditions. Transcriptomes from samples were collected and analyzed to understand the role of m6A and provide a foundation for its characterization. This study investigates the changes in m6A modification in *A. japonicus* under heat stress from an epigenetic perspective, potentially offering new insights into the response of marine organisms to environmental heat stress.

Results

General features of m6A methylation in heat-stressed *A. japonicus*

Input and IP libraries were sequenced under normal and heat stress conditions using the Illumina NovaSeq™ 6000. RNA sequencing produces raw data that requires preprocessing, and clean data can be obtained by filtering out defective sequences using fastp. Nearly 80 million reads were obtained in each library. Following the raw data screening and quality control process, each library yielded close to 40 million reads, with all Q30 values over 92.80%. In this study, the proportion of valid reads that were mapped to the reference genome was between 68.42 and 76.46%, with over 45% of valid reads being uniquely mapped to the genome. The quality control and mapping statistics of the MeRIP-seq data for all samples are presented in Table 1, indicating that all samples were of high quality and could be used for subsequent analyses.

Genome-wide peak scanning was performed using the R package exomePeak to obtain peak locations on the genome, peak length information, etc. The comparison of read segment distributions between input and IP samples led to the identification of m6A peaks, which were actually identified as m6A modification sites. About 90% of the transcripts were made up of genes with 1–3 m6A peaks; the dominant type had only one peak, making up roughly 60% of the transcripts; genes with more than three peaks were a minority, making up less than 10% (Fig. 1A). Furthermore, the T32 group had fewer transcripts containing m6A sites than the T18 group. The distribution of the m6A peaks in different gene functional elements was analyzed, and the results are shown in Fig. 1B. Reads map to and are distributed along the CDS, 5'UTR, and 3' UTR, with m6A peaks generally enriched in the 3'UTR and stop codon regions in *A. japonicus*. The m6A peak subgenomic mapping proportions for the T18 and T32 group transcriptomes were investigated (Fig. 1C and D), showing that the largest number of m6A peaks were clearly located in the 3' untranslated region (3'UTR). Specifically, the 3'UTR accounted for 44.52% in the T18 group and 44.28% in the T32 group. Comparably, the two groups' distribution of percentages showed that the 5'UTR had the lowest proportion (24.21%), followed by exons (31.27%) for the T18 group, the 5'UTR (23.84%), and exons (31.88%) for the T32 group.

The pattern analysis software HOMER was used to find high-confidence patterns in the peak areas. As shown in Fig. 1E and F, the first five m6A consensus motifs were located in the T18 and T32 groups, and these groups identified the classical RRACH (R=purine; A=m6A; H=non-guanine base) motifs.

The relationship between the m6A peaks and the expression level is shown in Fig. 2. It can be inferred

Table 1 Statistics and quality control of raw data generated by sequencing and read alignment statistics

Sample ID	Raw reads	Valid reads	Valid%	Q20%	Q30%	GC%	Mapped reads	Unique mapped reads
T18_R1_IP	41,864,076	41,861,324	99.99	97.55	93.26	41.01	28,640,207(68.42%)	18,899,102(45.15%)
T18_R1_input	53,373,728	53,372,446	100.00	98.10	94.39	42.45	37,321,235(69.93%)	24,849,440(46.56%)
T18_R2_IP	32,314,072	32,305,110	99.98	97.27	92.80	42.97	22,678,528(70.20%)	14,559,990(45.07%)
T18_R2_input	52,117,034	52,115,296	100.00	98.38	95.09	44.25	39,845,912(76.46%)	24,284,845(46.60%)
T18_R3_IP	46,593,366	46,587,660	99.98	97.31	92.85	41.34	31,971,994(68.63%)	21,183,180(45.47%)
T18_R3_input	53,385,892	53,384,418	99.99	98.32	94.97	42.95	38,943,974(72.95%)	25,828,491(48.38%)
T32_R1_IP	42,944,198	42,941,126	99.99	97.48	93.08	41.91	30,227,824(70.39%)	19,609,774(45.67%)
T32_R1_input	54,856,904	54,855,394	100.00	98.20	94.69	42.20	38,147,807(69.54%)	25,543,657(46.57%)
T32_R2_input	55,215,192	55,213,804	100.00	98.24	94.78	42.62	39,947,510(72.35%)	26,834,920(48.60%)
T32_R2_IP	51,522,718	51,519,100	99.99	97.56	93.30	41.79	36,496,331(70.84%)	23,888,900(46.37%)
T32_R3_IP	50,726,554	50,722,882	99.99	97.62	93.39	41.55	35,110,869(69.22%)	23,021,681(45.39%)
T32_R3_input	50,685,594	50,684,146	100.00	98.28	94.86	42.78	36,906,483(72.82%)	24,303,969(47.95%)

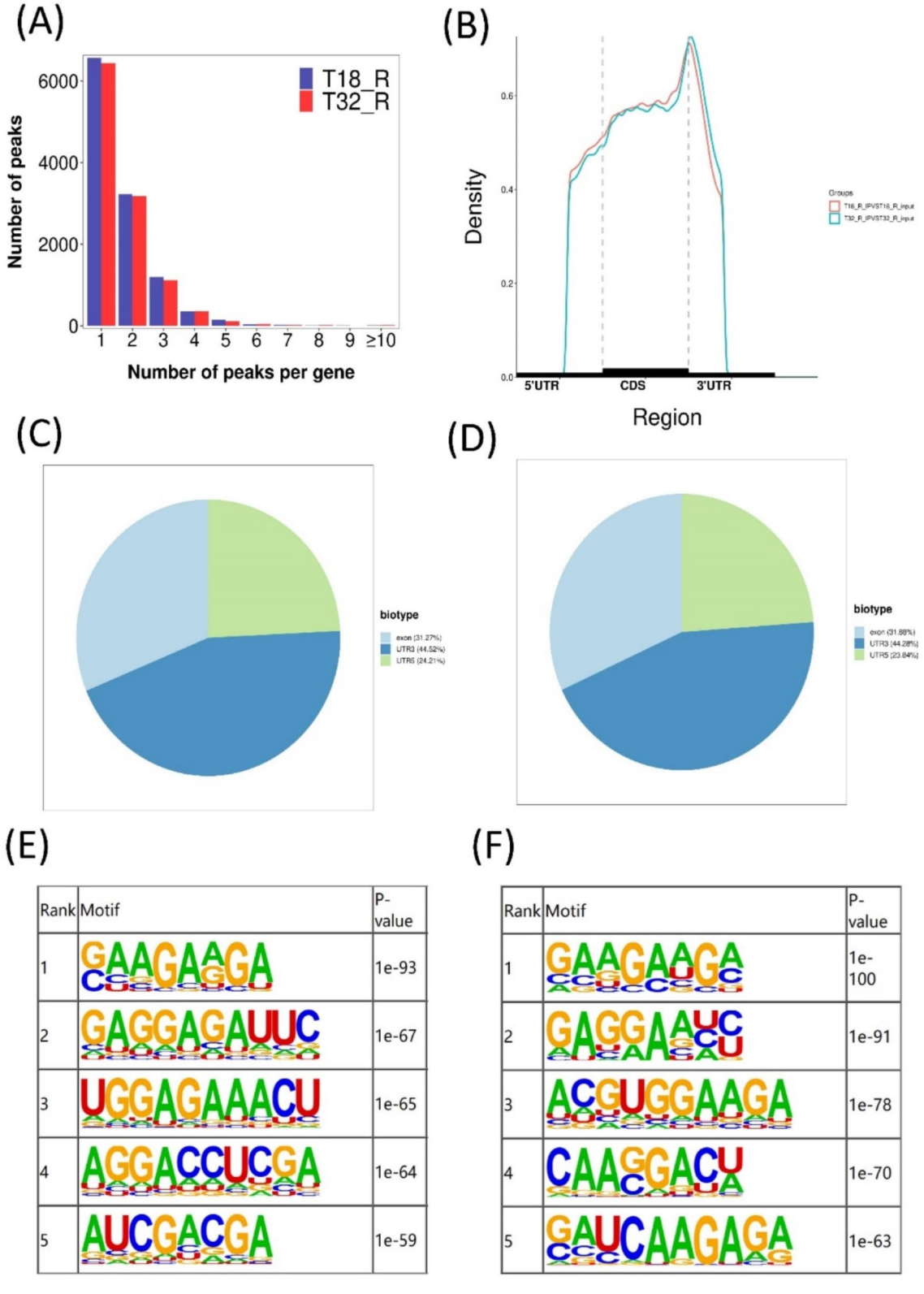

Fig. 1 m6A topological patterns in *A. japonicus*. (**A**) The proportion of genes containing different numbers of m6A peaks. (**B**) Distribution of m6 A methylation groups along transcripts. Each transcript is divided into three sections: 5 UTR, CDS and 3 UTR: red line: normal group (T18); green line: heat stress group (T32). The distribution of m6A peaks within transcripts is divided into 5'UTR, 1st exon, 3'UTR, and other exons. (**C**) T18 group; (**D**) T32 group; The blue part is the T18 group; the red part is the T32 group; the consensus motifs enriched with m6A peaks in the T18 group (**E**) and T32 group (**F**)

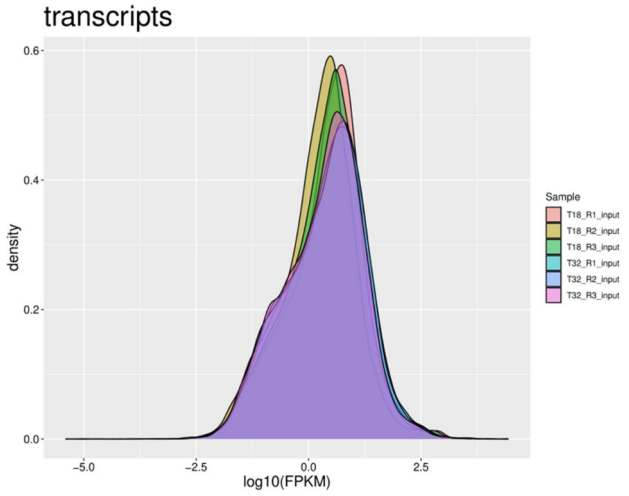

Fig. 2 Estimation of m6A peaks density in *A. japonicus* transcripts

that the density of the m6A peaks increases with the increase in expression level, reaches its peak at around 0.14 expression levels, and then decreases. Meanwhile, the Cricos plot (Fig. 3) was used to more visually display the distribution of gene expression, peak density, and differential gene density across different samples. Every chromosome was found to have peaks and differential peaks, with chromosomes 2, 6, 13, and 15 showing the highest distribution of peaks. Furthermore, at the genomic scale, the number of m6A peaks has higher density in the vicinity of chromosomal telomeres.

Differentially m6A modification between T18 and T32 groups

Different m6A peak analyses showed that 12,284 and 11,641 peaks were specific to the T18 and T32 groups, respectively. There were 7,211 peaks in common (Fig. 4A). Peak-related genes analyses showed that the T18 and T32 groups have 2,125 and 1,834 distinct genes, respectively, with 9,459 genes sharing commonality (Fig. 4B).

A thorough analysis of every m6A peak in the experimental samples was carried out, as shown in Fig. 4C. A total of 1,200 out of 3,280 peaks were identified as significantly differentially enriched in the T32 group compared with the T18 group. Specifically, 245 m6A peaks were upregulated, while 955 peaks were downregulated in the T32 group ($P<0.05$, fold change>1.5). This suggests that heat stress causes a substantial alteration in the m6A

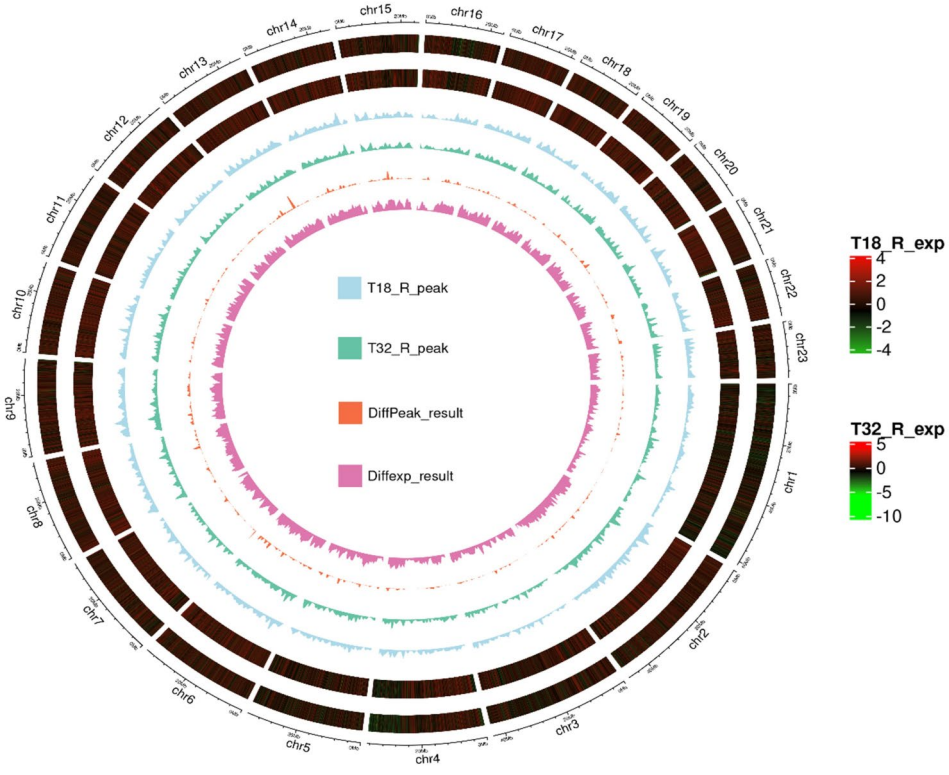

Fig. 3 Circos plot of the genome-wide m6A methylome in *A. japonicus* under heat stress. From outside to inside, the seven rings show (1) Reference genome chromosome position. (2) Gene expression of T18_R sample, taken as log10. (3) Gene expression of T32_R sample, taken as log10. (4) Density of m6A peak of T18_R. (5) Density of m6A peak of T32_R. (6) Distribution of the density of the m6A peak of the difference between T18_R and T32_R. (7) Density distribution of T18_R and T32_R differential genes

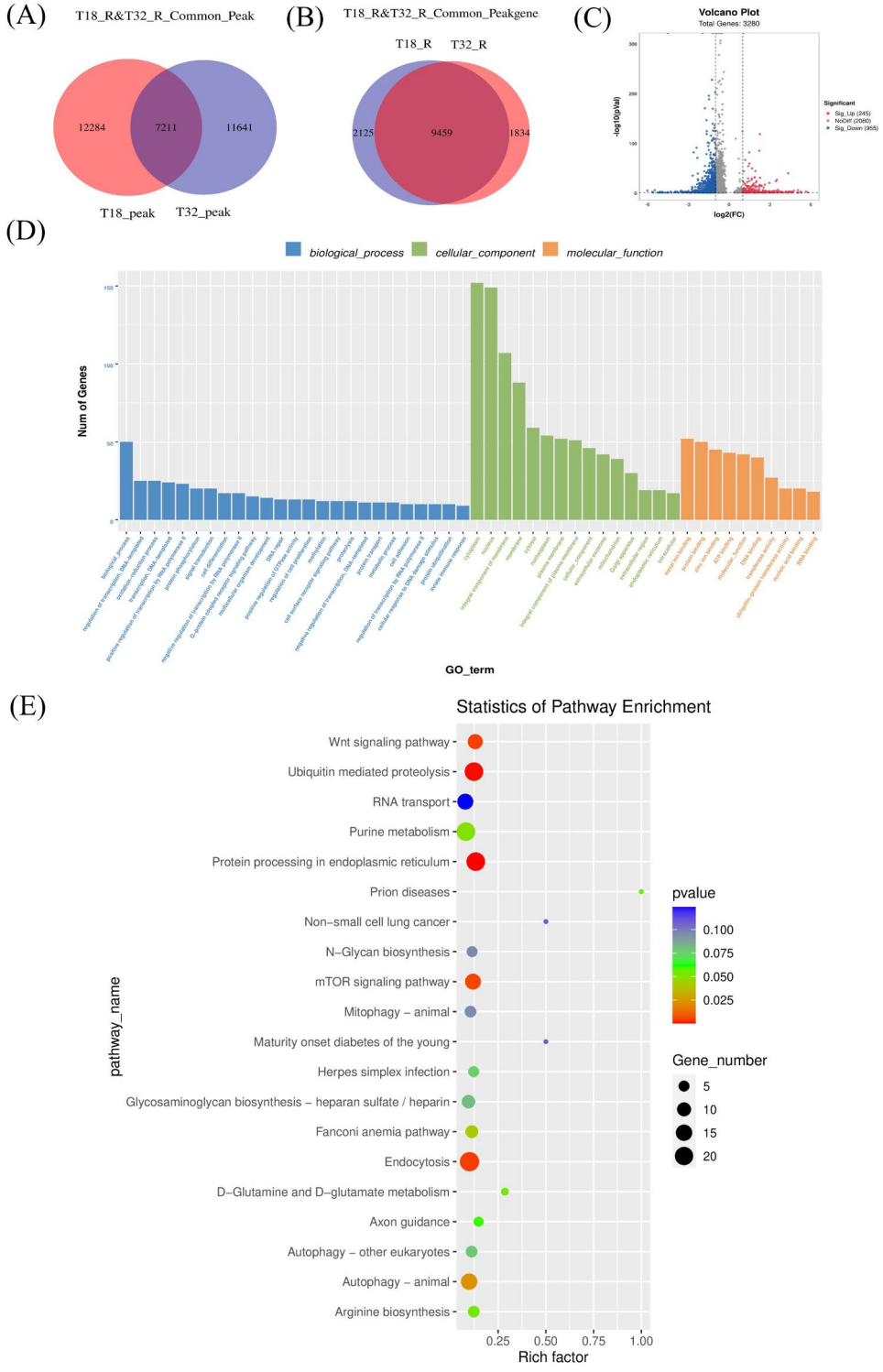

Fig. 4 Differential m6A peaks between two groups. The number of overlapping m6A peaks (**A**) and m6A peak-containing genes (**B**) in the normal (T18) and heat-stressed (T32) groups. (**C**)Volcano plots of the significantly different m6A peaks. (**D**) GO annotation of differential m6A peak-related genes. (**E**) KEGG enrichment analysis of differential m6A peaks related genes

methylation landscape, with more demethylation sites than methylation sites being found.

To further assess the role of m6A modification in *A. japonicus* in response to heat stress, the biological functions of differential m6A peak-related genes were analyzed. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) analysis were performed on all genes connected to the differential m6A peak. Figure 4D displays the significantly enriched GO elements sorted by P-value. As seen in Fig. 4D, the distribution of the number of differential genes on GO terms enriched in biological process (TOP25), cellular component (TOP15), and molecular function (TOP10) is depicted in the bar chart of GO enrichment analysis results. A total of 2503 m6A peak-associated genes were enriched, including 1568 genes in the biological process (BP) category, 594 genes in the molecular function (MF) category, and 341 genes in the cell component (CC) category. In terms of biological processes, m6A-containing genes were mainly involved in biological processes, regulation of transcription, DNA-templated, oxidation-reduction process, transcription, DNA-templated. Cellular components were mainly related to the cytoplasm, nucleus, and integral component of the membrane. Zinc ion binding, protein binding, metal ion binding, and ATP binding showed significant enrichment in terms of molecular function. The KEGG analysis results showed that m6A-containing genes were significantly associated with Protein processing in the endoplasmic reticulum, Ubiquitin-mediated proteolysis, Wnt signaling pathway, mTOR signaling pathway, and Endocytosis (Fig. 4E).

Conjoint analysis of MeRIP-seq and RNA-seq data

To explore the potential role of m6A modification in gene expression, the transcriptome profiles of the normal group (T18) and the heat stress group (T32) were examined. There were 19,561 genes in total, of which 17,175 were co-owned, however, the T32 group expressed more genes (Fig. 5A), with 1,452. Volcano maps were used to illustrate the overall distribution of differentially expressed genes. In the heat-stressed (T32) group, compared to the normal (T18) group, 2459 genes were up-regulated and 1937 were down-regulated (Fig. 5B). The RNA-seq and MeRIP-seq data were combined to elucidate the relationship between m6A modification and gene expression. Co-differential gene analysis was performed by a combination of differentially expressed genes and differential m6A peaks. Four-image mapping of differentially m6A methylated and differentially expressed genes was created to provide a visual depiction of the relationship between m6A methylation changes and gene expression (Fig. 5C). The result showed that 395 genes were identified as significant co-differentiated genes with the following screening criteria: m6A peaks and fold change in expression levels ≥ 1.5 (Fig. 5C) (Table S1). It was found that 202 genes were significantly different in m6A down-regulation and their expression was significantly up-regulated, such as heat shock protein 70 (HSP70IV), eukaryotic translation initiation factor 2 alpha kinase 1 (EIF2AK1), MAPK-associated protein 1 (MAPKAP1); 66 different genes were significantly different in m6A up-regulation and their expression was significantly up-regulated, such as Heat shock gene 67Bc (Hsp67Bc), gamma-glutamylcyclotransferase (GGCT), GTPase-activating protein BEM2 (sup-9), tudor domain containing 1 (TDRD1). Similarly, 115 genes were significantly different in m6A down-regulation and their expression was significantly down-regulated, such as frizzled class receptor 5 (FZD5), 5-hydroxymethylcytosine binding, ES cell-specific (HMCES), tripartite motif containing 2 (TRIM2), nucleolar protein 4 like (NOL4L), while 12 genes were significantly different in m6A up-regulation and their expression was significantly down-regulated, such as 2-hydroxyacyl-CoA lyase 1 (HACL1), protein kinase, DNA-activated, catalytic subunit (PRKDC).

All differential genes were subjected to GO and KEGG analyses. The top 25 BP, top 15 CC, and top 10 GO terms of MF in these three categories are shown in Fig. 6A. Terms like oxidation-reduction process, positive regulation of transcription by RNA polymerase II, and regulation of transcription, show more important in the biological process category. The cellular components are abundant in cytoplasm, nucleus, and integral component of membrane. Protein binding, molecular function, and ATP binding all have a strong relationship with molecular function. Using KEGG enrichment analysis, we discovered that the majority of the differential genes were enriched in the protein processing in endoplasmic reticulum (Fig. 6B), as well as other pathways like the mTOR signaling system, the Wnt signaling pathway, and the animal autophagy pathway. Based on multi omics analysis, it is proposed that some genes play a certain role in m6A methylation regulation under heat stress conditions.

An Integrated Genomics Viewer (IGV) was used to examine the m6A peak profiles of the EIF2AK1 and HSP70IV genes (Fig. 7), and the results also showed significant differences in the distribution of peaks.

Discussion

Overview of m6A characteristics of respiratory tree in *A. japonicus* under heat stress

Improving our understanding of the molecular mechanisms underlying heat stress responses is crucial for addressing heat stress issues in marine organisms. *A. japonicus* is a typical representative of marine benthic animals and an important component of marine

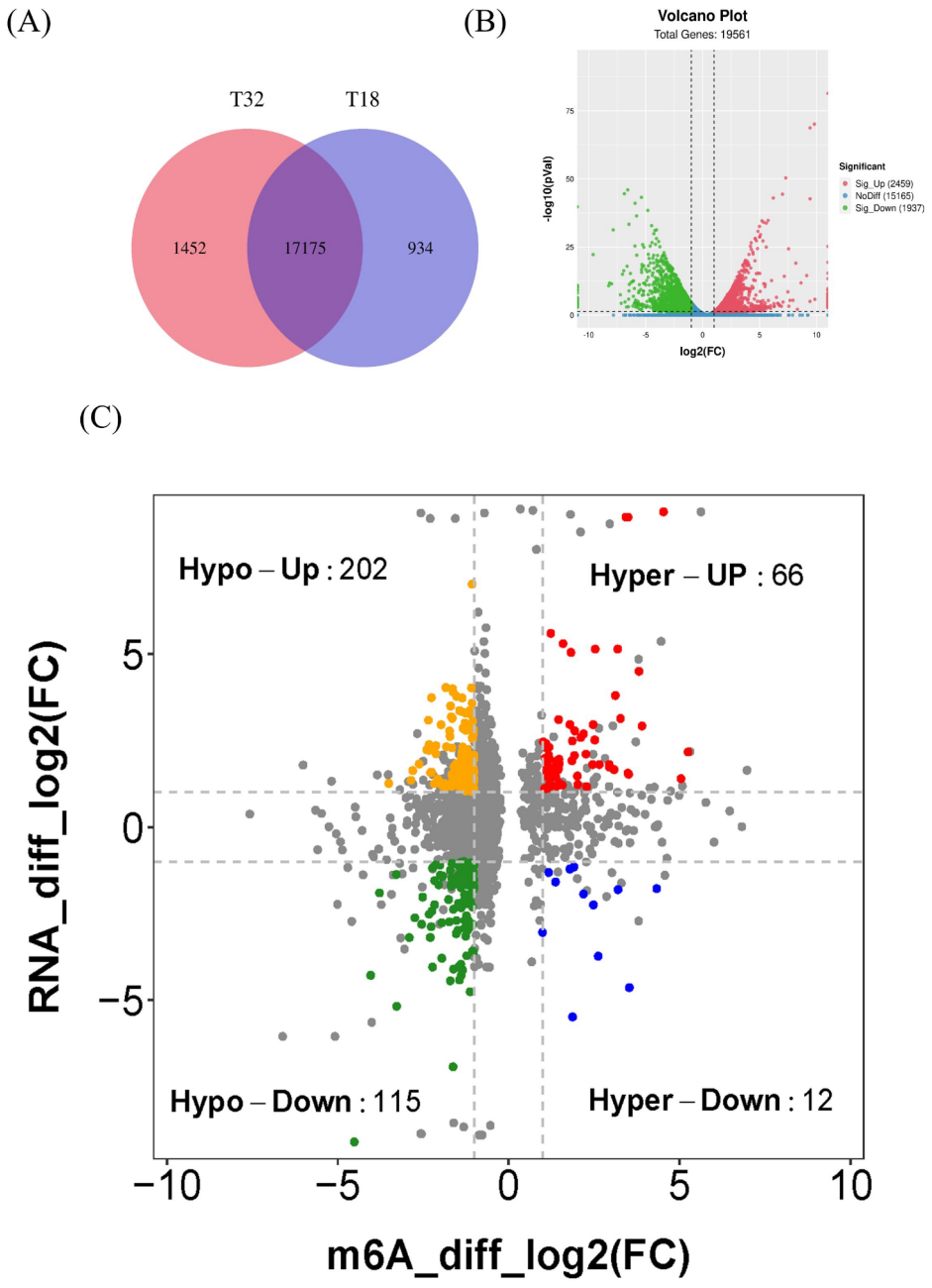

Fig. 5 Conjoint analysis of MeRIP-seq and RNA-seq data. (**A**) Venn diagram showing the numbers of genes expressed in the normal (T18) and heat-stressed (T32) groups. (**B**) Volcano plots showing differentially expressed genes. (**C**) Four-quadrant diagram showing the distribution of genes with significant changes in both m6A modification and mRNA expression

ecosystems. It is critical to investigate how sea cucumbers, *A. japonicus*, respond to high temperatures given the current state of global warming and the frequency of extreme weather events. It can offer technological and scientific support for the preservation and management of sea cucumber resources, the stability and well-being of marine environments, and the long-term growth of the sea cucumber aquaculture industry. A growing body of research shows that epigenetic events are intimately linked to variations in the external environment, and that epigenetics is essential to many critical life processes, and the epigenetic characteristic study of aquatic organism response to environmental stress has also gained increasing attention in recent years. Aquatic species' physiological and metabolic processes, as well as gene expression, can change in response to environmental changes, potentially involving the control of epigenetic mechanisms [41–43]. RNA modification is a form

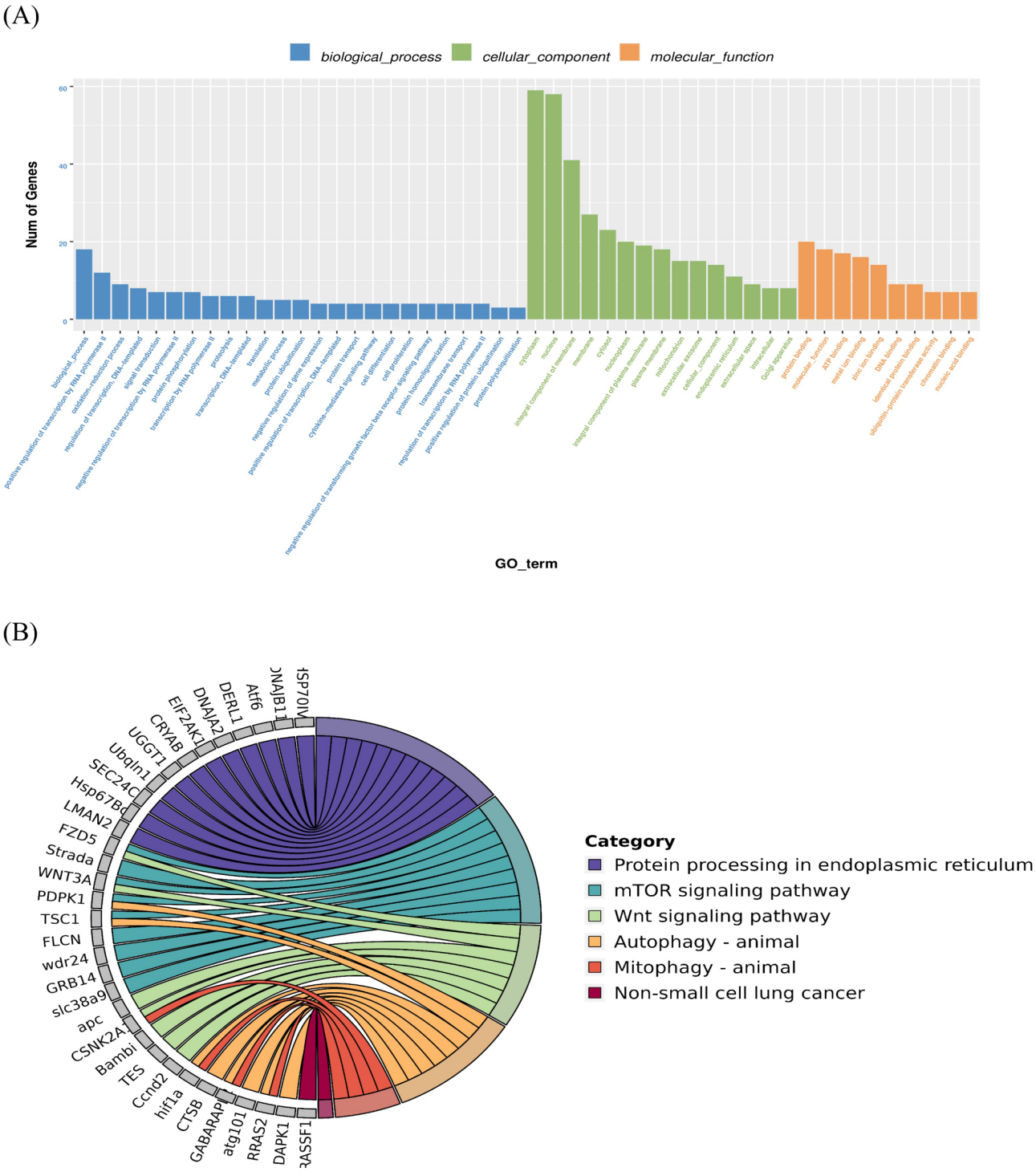

Fig. 6 Conjoint analysis of MeRIP-seq and RNA-seq data. (**A**) GO annotation of genes with significant changes in m6A modification and mRNA expression. (**B**) Circle plot of the KEGG enrichment analysis of the m6A differentially modified genes

of post-transcriptional epigenetic regulation, and m6A RNA methylation, as the most prevalent, abundant, and conserved internal RNA modification, is essential for controlling the expression of genes related to cell stress response [44]. The study of how RNA modification changes in *A. japonicus* response to high temperatures in the environment from the standpoint of m6A RNA methylation is a novel investigation into the epigenetic regulation mechanism of adversity response and environmental adaptation in marine organism, which could

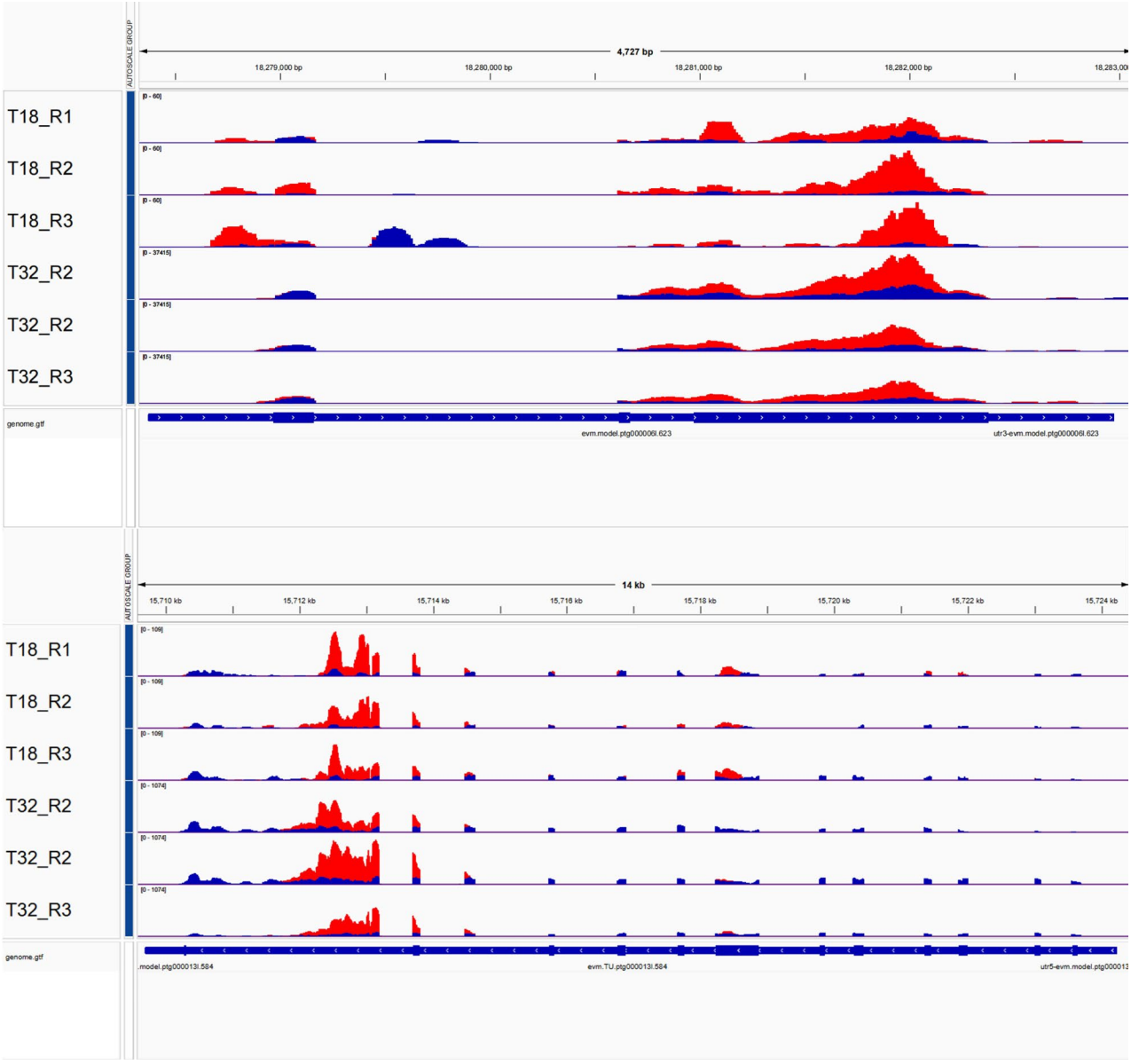

Fig. 7 IGV software revealed the expression profile of m6A methylation-modified differentially expressed genes

be helpful for developing strategies for aquaculture and conservation efforts in the face of climate change.

In this study, MeRIP-seq technology was used to generate the m6A map related to heat stress in the respiratory tree tissue of *A. japonicus*. The distribution of m6A peaks revealed that the m6A modification in the respiratory tree tissue of sea cucumber was mainly located in the 3 'downstream region of the gene, a pattern observed in various organisms, including soybean [45], rice, and barley [46, 47]. Studies on the response of bovine mammary epithelial cells to heat stress [34] have shown that the m6A peak near the stop codon was significantly enriched, and two obvious peaks were also observed near the start codon and the 3 'UTR region. Less than 10% of transcripts had more than three peaks; the remaining approximately 90% had one to three peaks. The proportion of transcripts with m6A peaks in the heat stress group was lower than in the normal temperature control group. The results of m6A methylation in mouse liver under high-fat feeding conditions [48] and the transcripts containing 1–3 peaks in the m6A study of pig liver at different development stages [49] are also dominant, accounting for more than 80%. The genomically displayed m6A RNA methylation map of *A. japonicus* respiratory tree tissue is a novel application in the field of marine invertebrate epigenetics research. The findings demonstrated that distinct chromosomes and chromosome regions have distinct distribution densities of m6A

peaks. A similar method was used in the study of rhizobia promoting soybean root growth under cadmium stress [45] to show that m6A peaks exhibit high density near chromosome telomeres. It is inferred that distinct regulation modalities for gene expression patterns may be represented by m6A sites located in different transcript regions. Differences in m6A locus distribution abundance across various chromosomes and genomic regions may relate to variations in gene function execution, environmental conditions, and among species.

In this study, HOMER, a pattern analysis software was used to determine the existence of the classical RRACH motif (where R=G or A; H=A, C, U) in the m6A modification of *A. japonicus*. The process by which different proteins attach to particular groups at methylation sites initiates RNA methylation and demethylation changes. RNA methylation-related enzymes identify such groups, which are fundamentally biologically significant patterns of nucleic acid sequences that can influence gene expression through binding [50, 51]. Identifying these patterns has been widely acknowledged as being important for understanding the mechanisms regulating gene expression, a crucial area of scientific research. In previous studies, such as the heat stress response in sheep [52], the sequence GGACA was identified in the analysis of m6A mRNA methylation in liver tissue. Similarly, the GGACU sequence was observed in the study of the heat stress response in bovine mammary epithelial cells [49]. The GGACC sequence and the classical RRACH motif (R=G or A; H=A, C, U) mentioned in this study are part of the same shared sequence pattern.

Function of genes related to differential m6A peak and differentially expressed genes

Genes implicated in the biological process were mostly linked to transcriptional control, DNA templating, and other transcriptional processes in the GO enrichment analysis of differential m6A peak-related genes. KEGG enrichment analysis revealed that differential m6A peak-related genes were enriched in important functional pathways such as protein processing in the endoplasmic reticulum, ubiquitin-mediated proteolysis, the Wnt signaling pathway, the mTOR signaling pathway, and endocytosis. A growing body of research shows that m6A modifications occur in response to stress, connecting a complex network involving translational, post-transcriptional, and transcriptional processes to external stress [53, 54]. Yu, J., et al. [55] showed that the differential expression of HSPs modulated by m6A may depend on the m6A site and the abundance of the target gene. Zhou et al. [56] found changes in m6A in the mouse embryonic fibroblast (MEF) cell line before and after heat shock stress and proposed that these differential genes may participate in various metabolic pathways in vivo. The reported transcriptome, proteome, and metabolome studies of *A. japonicus* in response to high-temperature stress also demonstrated that temperature and heat stress triggered the expression of relevant regulatory genes, which in turn mediated the metabolism and signaling pathways of oxidative stress, protein conformation processing and folding, autophagy, and apoptosis [57–59]. Further investigation into the relationship between m6A modification and transcriptional expression is necessary to determine whether alterations in m6A modification in response to temperature fluctuations in sea cucumbers are connected to variations in gene expression and specific the process and ways of this regulatory linkage.

Differential gene analysis in conjunction with differentially expressed transcriptome genes and genes related to the m6A peak were conducted to figure out the association between m6A alteration and gene expression in *A. japonicus* during response to high-temperature stress. Reduced m6A modification accounted for a rather high proportion (317 genes) of the 395 co-differentially expressed genes, according to the four-quadrant diagram of differentially expressed and differentially methylated m6A peak-related genes. This included 202 genes with up-regulated expression and 115 genes with down-regulated expression. Only 78 genes exhibiting elevated m6A alteration exhibited significant differences, comprising 66 significantly up-regulated genes and 12 considerably down-regulated genes. In general, there were more genes with decreased m6A modification and up-regulated expression than those with increased m6A modification and down-regulated expression. Current research on the m6A regulation mechanism focuses on controlling mRNA stability, splicing, translation efficiency, and processing of microRNA precursors [60–62]. m6A mediates various effects on mRNA metabolism by influencing interactions with RNA-binding proteins [63, 64]. Additionally, m6A affects mRNA processing and function, including splicing of mRNA precursors, 3' end processing, nuclear export, translation, and mRNA degradation [65]. These studies demonstrate that there is a complex link between m6A modification and gene expression. In addition, gene expression is a comprehensive regulation result of multiple levels, including epigenetic regulation, and m6A modification is only one of many epigenetic modifications. Based on these, we conclude that the decrease and increase of m6A modification do not directly lead to the up or down regulation of gene expression; rather, the precise effect is determined by the combined influence of numerous variables. The co-differentiation analysis of the transcriptome and m6A peaks in this study also showed that m6A modification and the expression of corresponding genes exhibited a variety of regulation forms under high-temperature stress in sea cucumber respiratory tree tissue, with a predominant

pattern where m6A modification decreased and gene expression increased. It was hypothesized that multiple regulatory modes including RNA modification controlled a large number of functional genes in sea cucumbers that responded to heat stress to adapt to environmental stress. Genes with reduced m6A modification and up-regulated expression, such as HSP70IV, EIF2AK1, MAPKAP1, etc., may play a more active role.

Important heat stress pathways regulated by m6A methylation

The protein processing in the endoplasmic reticulum, the Wnt signaling pathway, and the mTOR signaling pathway were enriched in the KEGG analysis of genes related to differential m6A peaks and in the KEGG analysis of genes related to co-differentially expressed m6A peaks and the transcriptome. These pathways are considered to adjust life processes to cope with unfavorable conditions by managing signal transduction, regulating gene expression, and improving metabolic flexibility when *A. japonicus* meets a heat-stress environment.

Protein processing in the endoplasmic reticulum is an important process for cells to synthesize and modify proteins. Unfolded proteins are present in the endoplasmic reticulum, and a moderate unfolded protein response can effectively protect cells under normal circumstances. Nevertheless, prolonged endoplasmic reticulum stress will trigger cell apoptosis if it results in a significant buildup of unfolded proteins that exceeds the ability of the cell to respond [66]. Recent research has shown that high-temperature stress can induce endoplasmic reticulum stress, initiating the unfolded protein response, promoting the clearance of misfolded proteins, and protecting cells from heat stress-induced damage [67–69]. Based on these findings, it is presumed that heat stress of 32° C causes misfolded proteins to accumulate in the respiratory tree tissue of *A. japonicus* by interfering with proper protein folding during synthesis. Heat shock protein and endoplasmic reticulum stress-related genes can rapidly respond to heat stress to eliminate misfolded proteins, and in this process, changes in the m6A methylation of these gene transcripts may help control gene expression and translation. In previous studies on the function of METTL3, the most famous m6A methyltransferase in sea cucumber, it was confirmed that m6A modification was involved in the endoplasmic reticulum-associated degradation pathway, promoting the accumulation of ubiquitinated proteins and endoplasmic reticulum stress, and further activating apoptosis in coelomic cells through related pathways [70].

The Wnt signaling pathway is a sophisticated signal transduction system that functions to control several crucial biological processes in living things, including differentiation, cell division, and proliferation [71]. It is currently believed that Wnt signals are delivered through at least three different intracellular pathways, of which the one that activates gene transcription through β-catenin is known as the classical Wnt signaling pathway. Wnt is a glycoprotein that is essential to this signaling pathway and can be generated or released by resident effector cells. A variety of proteins within the cell are involved in Wnt-mediated signaling, including Dishevelled (Dvl), serine/threonine protein kinase Gsk3 (GSK-3), axin protein (Axin), APC regulator of WNT signaling pathway (APC), and β-catenin. Studies have shown that the Wnt/β-catenin signaling pathway is involved in oxidative stress regulation [72], and activated Wnt/β-catenin signaling increases the expression and activity of SOD and GSH-Px, which effectively maintains the balance between oxidative and antioxidant systems, and reduces cellular damage under oxidative stress conditions [73]. ROS are key intracellular signaling mediators that regulate Wnt/β-catenin signaling in a redox-dependent manner [74]. High temperatures can create heat stress in biological organisms, which can lead to abnormalities in the organism's antioxidant function due to the creation of excessive free radicals [75, 76]. It was also indicated that high temperatures induce oxidative stress in studies about heat stress on sea cucumber *A. japonicus* [58, 59]. Combining the results of this study with previous research, it is inferred that the ROS level in *A. japonicus* would increase dramatically under high-temperature environmental stress, which might cause serious damage to cellular structures. Excessive ROS may activate the Wnt/β-catenin signaling pathway to increase antioxidant-related gene expression and reduce the cellular damage caused by high temperature. In this process, signaling molecules in the Wnt/β-catenin signaling pathway are regulated by m6A modification, which is involved in the pathway's activity. In this process, signaling molecules in the Wnt/β-catenin signaling pathway are regulated by m6A modification, which is involved in the pathway's activity. The regulation of the Wnt/β-catenin pathway in hepatoblastoma (HB) and ovarian epithelial cancer cells (EOC) has also been confirmed to involve the m6A modification of catenin beta-1 (CTNNB1), cyclin D1 (CCND1), NKD1, an inhibitor of WNT signaling pathway 1, frizzled class receptor 10 (FZD10), and other important genes [77].

The mTOR signaling pathway is downstream of the Wnt signaling pathway and is currently the focus of many studies that are examining the effects of heat stress on the life regulation processes of organisms through this pathway [78]. According to the correlation analysis in this study, differential m6A peak-related genes and differentially expressed genes were enriched in the mTOR signaling pathway. Sensing changes in the internal and external surroundings of cells, the mTOR signaling pathway is a

crucial intracellular signal transduction route that acts as the fundamental link in growth regulation, controlling cell survival and activity [79]. It consists of the mTORC1 and mTORC2 complexes. mTORC1 is mainly involved in protein synthesis, cell growth, and autophagy, and its core pathways and genes are inextricably linked with heat stress [80]. Studies have suggested that mTORC1 can promote cell growth and proliferation by partially increasing WTAP levels, leading to increased m6A modification and the degradation of specific transcripts [81]. mTORC1 can also increase the level of S-adenosylmethionine and the abundance of the methyltransferase complex (WTAP), promoting m6A modification [82]. The findings of our investigation indicate that m6A modification exists in genes related to the mTOR signaling pathway and regulates the pathway's activity. It can be inferred that m6A modification plays an important role in the response of the sea cucumber *A. japonicus* to environmental temperature changes, and the biological functions involved in the mTOR signaling pathway interact with m6A modification activities, jointly participating in the heat stress response of the sea cucumber *A. japonicus*.

The protein processing pathway, Wnt signaling pathway, and mTOR signaling pathway within the endoplasmic reticulum are all closely related. Protein processing in the endoplasmic reticulum regulates proteins related to the Wnt signaling pathway, and the Wnt pathway is also involved in regulating mTORC1 activity. These three pathways are interconnected with autophagy, which is rapidly activated under various stress conditions such as high temperature, hypoxia, oxidative stress, and protein aggregation [83]. The autophagy pathway is also enriched in the KEGG analysis of genes related to the co-differentiation between m6A peaks and the transcriptome in *A. japonicus* in this study. The aforementioned data suggests that the endoplasmic reticulum's protein processing, Wnt signaling, mTOR signaling, and autophagy pathways in *A. japonicus* work in concert to control cellular metabolism and repair. The proposed molecular mechanism of interaction between pathways and their synergistic response to heat stress is shown in Fig. 8. These pathways preserve intracellular stability and aid the organism in adapting to challenging circumstances. During this process, m6A modification is deeply involved.

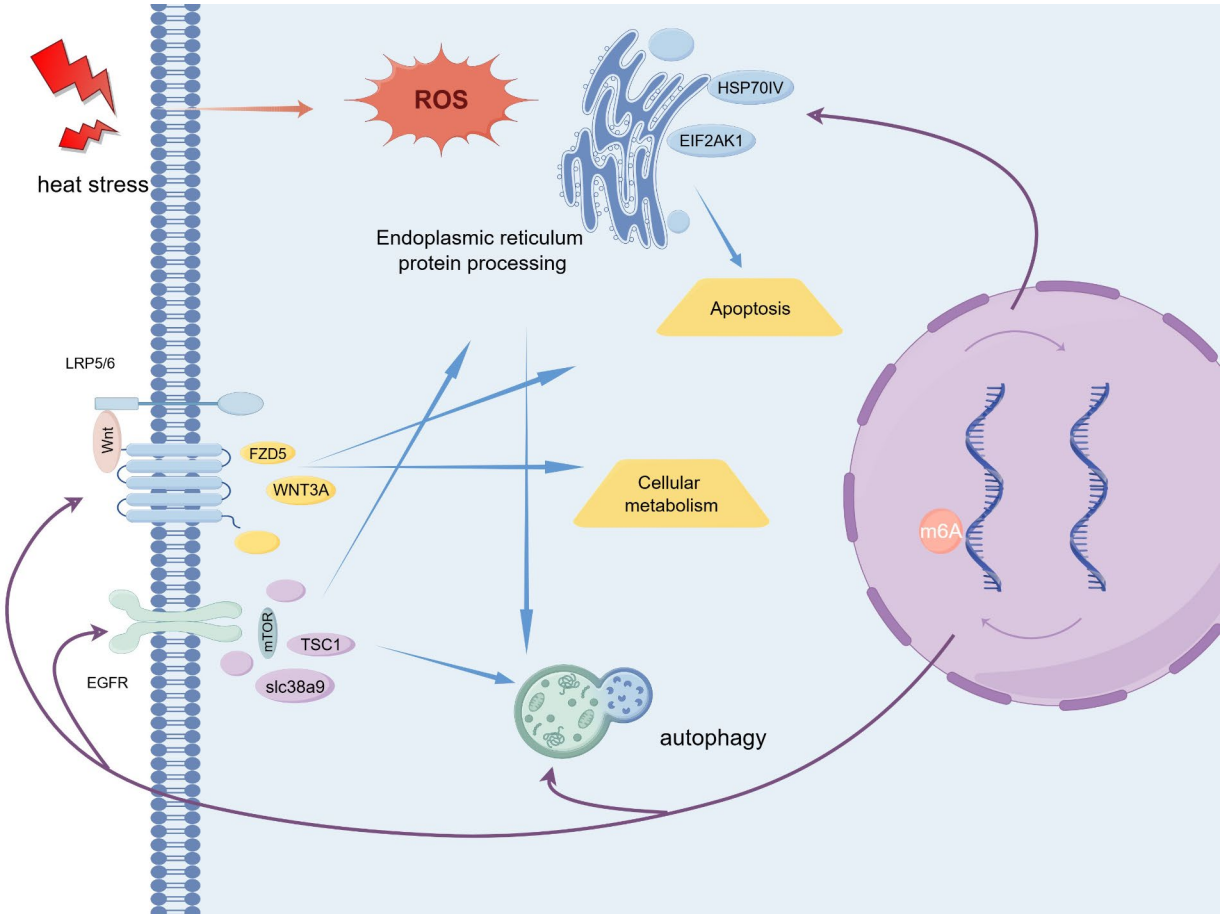

Fig. 8 Effect of heat stress on cells through the m6A modification. The proposed molecular mechanism by which heat stress affects m6A is shown. The cell signaling pathways enriched in the KEGG analysis are indicated.

Conclusion

Under the background of global warming, it is urgent to study the heat stress of *Apostichopus japonicus*. This study investigated the characterization of whole m6A transcriptome profile of *A. japonicus* response to high-temperature, made conjoint analysis of m6A modification and gene expression and revealed that heat stress-responsive genes, like HSP70IVE and IF2AK1 etc., and important pathways, like protein processing endoplasmic reticulum pathway etc., were involved in the response to environmental heat stress. By elucidating the epigenetic regulatory mechanisms at play, our work sheds light on the complex interplay between gene expression and environmental stress. This comprehensive analysis not only enhances our understanding of how marine organisms adapt to thermal challenges but also holds promise for informing conservation and aquaculture strategies in the face of climate change. Since the precise regulation approach by which m6A modifies transcripts to alter gene function is not yet clear and research in this field is very restricted, our next research will focus on the relationship between specific m6A modification sites of key functional genes and gene expression levels under stress conditions.

Materials and methods

Temperature treatment experiment and sampling

The *A. japonicus* samples were sourced from Shandong Anyuan Aquatic Products Co., Ltd. The samples were bred in the same batch and during the same growth period. A specification of 106 g±15.3 g of *A. japonicus* were selected, and were temporarily raised in the laboratory at 18 ℃ for more than 7 days. After acclimatization, 30 samples of *A. japonicus* were divided into the T18 and the T32, which were placed into three cultured glassware. The volume of seawater in each tank was 50 L, temperature control heating rods were used to heat the seawater at a rate of 1℃ per 12 h. When the water temperature reaches 32 ℃, the *A. japonicus* is temporarily raied for 24 h, and samples of the respiratory tree are immediately taken from control and heat-treated groups. 3 samples from each treatment were collected from all the aforementioned *A. japonicus* for three biological replicates. The sampling process is rapidly carried out under low-temperature freezing conditions, and the tissue samples were immediately frozen in liquid nitrogen, and then stored in a refrigerator at -80 ℃.

Library construction and RNA sequencing

Total RNA was isolated and purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's procedure. The RNA amount and purity of each sample were quantified using NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA), and the RNA integrity was assessed by Bioanalyzer 2100 (Agilent, CA, USA). Poly (A) RNA was purified from 50 μg total RNA using Dynabeads Oligo (dT)25-61005 (Thermo Fisher, CA, USA) by two rounds of purification. Then the poly(A) RNA was fragmented into small pieces using a Magnesium RNA Fragmentation Module (NEB, cat. e6150, USA) under 86℃ 7 min. The cleaved RNA fragments were incubated for 2 h at 4℃ with m6A-specific antibody (No. 202003, Synaptic Systems, Germany) in IP buffer (50 mM Tris-HCl, 750 mM NaCl and 0.5% Igepal CA-630). The IP RNA was reverse-transcribed to cDNA by SuperScript™ II Reverse Transcriptase (Invitrogen, cat. 1896649, USA), which was next used to synthesize U-labeled second-stranded DNAs with E. coli DNA polymerase I (NEB, cat.m0209, USA), RNase H (NEB, cat. m0297, USA) and dUTP [84] Solution (Thermo Fisher, cat.R0133, USA). An A-base was added to the blunt ends of each strand, preparing for ligation to the indexed adapters. Dual-index adapters were ligated to the fragments, and size selection was performed with AMPureXP beads. After the heat-labile UDG enzyme (NEB, cat.m0280, USA) treatment of the U-labeled second-stranded DNAs, the ligated products were amplified with PCR by the following conditions: initial denaturation at 95℃ for 3 min; 8 cycles of denaturation at 98℃ for 15 s, annealing at 60℃ for 15 s, and extension at 72℃ for 30 s; and then final extension at 72℃ for 5 min. At last, we performed paired-end sequencing (PE150) on an Illumina Novaseq™ 6000 platform (LC-Bio Technology CO., Ltd., Hangzhou, China) following the vendor's recommended protocol.

Biological information analysis

fastp software (https://github.com/OpenGene/fastp) [85] was used to remove the reads that contained adaptor contamination, low quality bases and undetermined bases with default parameter. The processing steps include: removing reads with adapters, removing reads with N (N means that the proportion of unidentified bases is greater than 5%), removing low-quality reads (the number of bases with a quality value of Q≤10 accounts for more than 20% of the whole read), counting the amount of raw sequencing, effective sequencing, Q20, Q30, GC content, and performing comprehensive evaluation. Meanwhile, sequence quality of IP and Input samples were verified using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) [86] and RseQC (http://rseqc.sourceforge.net/) [87]. Then we used HISAT2 (http://daehwankimlab.github.io/hisat2) [88] to map reads to the reference genome *A. japonicus* (Version: NCBI-ASM275485v1). Peak calling and diff peak analysis were performed by R package exomePeak (https://www.bioconductor.org/packages/3.3/bioc/html/exomePeak.html) [89, 90], and peaks were annotated by intersection

with gene architecture using R package ANNOVAR (http://www.openbioinformatics.org/annovar/) [91].

Expression and function analysis of multilayer genes

HOMER (http://homer.ucsd.edu/homer/motif) was used for de novo and known motif finding followed by localization of the motif with respect to peak summit. GO and KEGG enrichment analyses were performed for peak related genes using the GOseq R package, with a focus on GO terms with a corrected P-value < 0.05. In this context, DEGs were mapped to GO Terms within the Gene Ontology database (http://www.geneontology.org/), with gene numbers calculated for every term. The significantly enriched GO Terms in DEGs, compared to the genome background, were identified through a hypergeometric test. KEGG(http://www.kegg.jp/), the Kyoto Encyclope dia of Genes and Genomes, provided valuable pathway information for our analysis. And StringTie (https://ccb.jhu.edu/software/stringtie) was used to perform express ion level for all transcripts and genes from input libraries by calculating FPKM (total exon fragments /mapped reads (millions) × exon length (kB)) [92]. The differentially expressed transcripts and genes were selected with log2 (fold change) ≥ 1 or log2 (fold change) ≤ -1 and p value < 0.05 by R package edgeR (https://bioconductor.org/packages/edgeR) [93].

Abbreviations

m6A	N6-methyladenosine
CDS	Coding sequence
3'UTR	3'Untranslated region
MeRIP-seq	Methylated RNA immunoprecipitation sequencing
GO	Gene Ontology
KEGG	Kyoto Encyclope dia of Genes and Genomes
DEGs	Diferentially expressed genes

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10972-1.

Supplementary Material 1

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Not applicable.

Author contributions

YNS and YMS conceived and designed the study. SYL, XHH and XHX performed experiments. YWF, JMY, RBX and GHS performed bioinformatics analyses. YNS wrote and GHS revised the manuscript. The author(s) read and approved the final manuscript.

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Data availability

The datasets generated and analyzed during the current study are available in the NCBI Sequence Read Archive under the accession number PRJNA1149111. https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1149111.

Declarations

Ethics approval and consent to participate

The animal study was approved by the Institutional Animal Care and Use Committee of the Ludong University (protocol number LDU-IRB20210308NXY). The study was conducted in accordance with the local legislation and institutional requirements.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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