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ORIGINAL ARTICLE

Transcriptome-wide m6A methylation profile reveals its potential role underlying drought response in wheat (*Triticum aestivum* L.)

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Abstract

Main conclusion **This study revealed the transcriptome-wide m6A methylation profile under drought stress and found that *TaETC9* might regulate drought tolerance through mediating RNA methylation in wheat.**

Abstract Drought is one of the most destructive environmental constraints limiting crop growth and development. *N*6-methyladenosine (m6A) is a prevalent and important post-transcriptional modification in various eukaryotic RNA molecules, playing the crucial role in regulating drought response in plants. However, the significance of m6A in wheat (*Triticum aestivum* L.), particularly its involvment in drought response, remains underexplored. In this study, we investigated the transcriptome-wide m6A profile under drought stress using parallel m6A immunoprecipitation sequencing (MeRIP-seq). Totally, 4221 m6A peaks in 3733 m6A-modified genes were obtained, of which 373 methylated peaks exhibited differential expression between the control (CK) and drought-stressed treatments. These m6A loci were significantly enriched in proximity to stop codons and within the 3′-untranslated region. Integration of MeRIP-seq and RNA-seq revealed a positive correlation between m6A methylation and mRNA abundance and the genes displaying both differential methylation and expression were obtained. Finally, qRT-PCR analyses were further performed and the results found that the m6A-binding protein (*TaETC9*) showed significant up-regulation, while the m6A demethylase (*TaALKBH10B*) was significantly down-regulated under drought stress, contributing to increased m6A levels. Furthermore, the loss-of-function mutant of *TaECT9* displayed significantly higher drought sensitivity compared to the wild type, highlighting its role in regulating drought tolerance. This study reported the first wheat m6A profile associated with drought stress, laying the groundwork for unraveling the potential role of RNA methylation in drought responses and enhancing stress tolerance in wheat through epigenetic approaches.

Keywords Drought · m6A · MeRIP-seq · RNA methylation · Wheat

Abbreviations

DR	Drought stress
m6A	*N*6-Methyladenosine
IP	Immunoprecipitation
DMG	Differentially methylated genes

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DEG Differentially expressed genes
WYMV Wheat yellow mosaic virus

Introduction

*N*6-methyladenosine (m6A) modification is a methylation modification occurring at the 6th N atom of adenine in RNA molecular, which is one of the most prevalent and crucial RNA modifications in higher eukaryotes (Hu et al. 2022; Li et al. 2023). m6A methylation involves dynamic and reversible writing, reading, and erasing processes (Hu et al. 2019; Zheng et al. 2020). Through the interplay of methyltransferases and demethylases, m6A undergoes dynamic equilibrium within organisms, creating binding sites for m6A-binding proteins and facilitating the proper assembly of RNA secondary structures as well as functioning on RNA transport and processing (Liu et al. 2015).

Previous studies have confirms the significance of m6A in plant growth and development as well as in response to diverse stresses, influencing processes like plant embryogenesis, flower transformation, trichome, leaf and root development, sporogenesis, and fruit maturation (Duan et al. 2017; Scutenaire et al. 2018; Kim et al. 2020; Hu et al. 2021; Song et al. 2021). In Arabidopsis, m6A methylation and mRNA dynamic modification are highly conserved and positively associated with mRNA metabolism, influencing diverse biological processes. For example, the m6A eraser protein (ALKBH10B) regulates flower transformation by modulating the transcription levels of *SPL3*, *SPL9*, and *FT* (Duan et al. 2017). Overexpressionof the human RNA demethylase *FTO* in rice and potato could induce the chromatin opening and transcriptional activation by facilitating extensive demethylation of m6A, and then stimulated root meristem and cell proliferation, leading to increased photosynthetic efficiency and enhanced drought resistance in crops, resulting in a ~ 50% yield increase (Yu et al. 2021). It is also found that m6A modification enhanced the stability of specific salt-tolerant transcripts, thus improving salt tolerance in Arabidopsis and sweet sorghum (Anderson et al. 2018; Zheng et al. 2021). Additionally, some studies have revealed that drought stress could induce the expression of m6A demethylase in sea buckthorn and maize (Miao et al. 2020; Zhang et al. 2021a).

Bread wheat (*Triticum aestivum* L.) is one of the most imporant cereal crops globally with the total cultivation area more than 223 million hectares and the total production of over 778 million tons in 2022/23 seasoning year, providing providing approximately 20% of calories for human consumption annually (FAO 2024; Guo et al. 2022). Due to climate change, drought has emerged as the most destructive environmental factor limiting wheat production, resulting in ~ 5.5% annual yield loss and then heavily threatening global food security (Fahad et al. 2017). Dissection the molecular mechanism and genetic basis underlying drought tolerance holds the promise for breeding of drought-tolerant wheat varieties, which is urgently required to overcome the challenges of population booming and climate change. Previous studies have reported that m6A modification plays a pivotal role in wheat development and stress response. For instance, m6A-seq analysis revealed that m6A methylation modification regulates the expression of different starch synthesis-related genes during grain filling, thereby participating in grain development (Li et al. 2022). *M6A methyltransferase B* (*MTB*) plays a crucial role in wheat and acts as a positive regulator in wheat yellow mosaic virus (WYMV) infection (Zhang et al. 2022). However, the role of m6A playing in response to drought stress remain elusive in wheat at present.

In this study, we performed transcriptome-wide MeRIP-seq analysis to identify the m6A methylation profile under drought stress in wheat and then comparative analysis the m6A pattern as well as gene expression level between drought and normal control conditions. The drought-related differentially methylated genes and differentially expressed genes were also obtained. These results not only provided some drought-related candidate genes for further functional analysis, but also enriched our understanding of the regulatory mechanisms underlying drought tolerance from an epigenetic perspective.

Materials and methods

Plant materials and drought tolerance investigation

The seeds of bread wheat cultivar Chinese Spring was sterilized with the 2% H_2O_2 solution for 30 min, followed by a 2 h distilled water soak. After germination, all seedlings were transplanted into Hoagland's nutrient solution, dividing them into two groups. One group was subjected to drought stress (DR), while the other served as the control (CK) with normal conditions, without adding PEG. When the seedlings were 2 weeks old, the DR group subjected to 19.2% PEG treatment. After seven days of treatment, leaves of two biological replicates were collected from both DR and CK groups, and were stored at − 80 °C. Furthermore, the stop-gain mutant of *Taect9* obtained from wheat cultivar No. Jing411 was purchased from MOLBREEDING Ltd Com (Shijiazhuang, China). It was first verified by specific primer PCR and Sanger sequencing to obtain the pure strains. Subsequently, seeds of both wild-type (WT) and mutant lines were germinated and transferred to a 1/2 Hoagland solution, growing until they reached the three-leaf stage. Subsequently, drought treatments was conducted with the same as the above method. The phenotypic traits of mutant and WT

plants were comprehensively examined after 14 days under stress and normal conditions, with three biological replicates, including above/below-ground biomass, plant height, and root-related traits. The root-related traits were measured using a root scanner (Wanshen Comp., Hangzhou, China). Statistical analysis was carried out using the Student's *t*-test method.

RNA extraction, m6A-immunoprecipitation (IP) and library construction

The full-length mRNA from total RNA sample were isolated by the Arraystar Seq-StarTM poly (A) mRNA Isolation kit (Aksomics, Shanghai, China). After purification, the mRNA was chemically fragmented by incubating at 94 °C in 20 µL buffer (10 mM Zn^{2+} and 10 mM Tris-HCI at pH 7.0) for 5–7 min. The mRNA fragment of approximately 100 nt was recovered via agarose gel electrophoresis. Subsequently, the fragmented mRNA (1/10th retained as input control) was combined with 2 µL of anti-m6A rabbit polyclonal antibody (No. 202003, Synaptic Systems) to create a 500 µL IP reaction system, which was incubated at 4 °C for 2 h. It was then incubated with blocked Dynabeads for another 2 h at 4 °C. The material was washed three times with 1×IP buffer (10 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1% NP-40), followed by two washes with 1 × Wash buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1% NP-40). Finally, an elution buffer (200 µL) containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.05% SDS, and 40 U of proteinase K (No. 19131, Qiagen) was incubated at 50 °C for 30 min to remove the m6A antibody-enriched mRNA fragments from Dynabeads. The final mRNA extraction involved phenol–chloroform extraction followed by ethanol precipitation. The KAPA Stranded mRNA-seq Kit (Illumina) was used to generate sequencing libraries for both the m6A-enriched mRNAs and input mRNAs. Finally, these libraries were subsequently sequenced on the HiSeq 4000 platform at Aksomics Inc. (Shanghai, China).

m6A profile analysis

The raw data were processed using FASTP software with default settings to remove reads containing adapter contamination and low-quality bases (Chen et al. 2018). HISAT2 software aligned the reads to the Triticum aestivum reference genome (IWGSCV1.1 version) (Kim et al. 2015). Both the IP and input data were analyzed with the R package exomePeak (Meng et al. 2014). The motif analysis utilized MEME and HOMER software (Heinz et al. 2010; Bailey et al. 2015). The identified peaks were annotated by intersecting with gene architecture using the R package ChIPseeker (Yu et al. 2015). StringTie software calculated fragments per kilobase of transcript per million fragments mapped (FPKM) values (Pertea et al. 2015). We employed the R package edgeR to select differentially expressed genes, requiring a fold change > 1.5 or < 0.5 and a p-value < 0.05 (Robinson et al. 2010). GO and KEGG analyses were conducted using AgriGO and TBtools, respectively (Tian et al. 2017; Chen et al. 2020).

qRT-PCR analysis

Samples from the CK and DR groups, along with control and drought samples, were analyzed using gene-specific primers (Table S1) via quantitative real-time PCR (qRT-PCR). qRT-PCR analysis was performed, and fold changes were determined employing the $2^{-\Delta\Delta Ct}$ method, as described by Livak and Schmittgen (2001).

Results

m6A methylation profile under drought stress in wheat

Transcriptome-wide MeRIP-seq was conducted on wheat leaves exposed to drought conditions. After filtering, we obtained high-quality sequence data from two biological replicates, achieving an alignment rate > 89% (Table S2). Consistent with the results in barley, the m6A peaks were observed across the entire genome and enriched in the telomeres of each chromosome (Fig. 1A) (Su et al. 2022). Peak calls for both normal and drought conditions were predominantly located in transcription end sites (TES) (Fig. S1). In the normal group (CK; Table S3), we identified 2100 m6A peaks, while the drought group (DR; Table S4) had 3447 m6A peaks, with an overlap of 1326 peaks, resulting in a total of 4221 m6A peaks in wheat (Fig. 1B). These m6A peaks were associated with 1875 and 3183 genes in CK and DR, respectively, with 1325 shared gene. Furthermore, we conducted a comprehensive investigation of the distribution enrichment of these m6A peaks in the two groups based on gene structure information (IWGSC v1.1). The results revealed that the majority of m6A peaks were predominantly located near 3′-UTRs and stop codons (Fig. 1C). Additionally, the drought condition induced global hypermethylation of m6A compared to CK (Fig. 1D). Most m6A peaks were anchored to the core motif sequences of 5′-KUACAD-3′ and 5′-GARGARS-3′ (Fig. 1E). We also examined the abundance of m6A-modified sites per gene and found that the majority of genes (97% in both CK and DR) had one or two m6A-modified sites. Notably, the number of genes with more than two m6A-modified sites was lower in the DR group (0.72%) compared to the CK group (1.44%). However, it's worth noting that genes uniquely modified with m6A in the DR group exhibited a higher degree of methylation

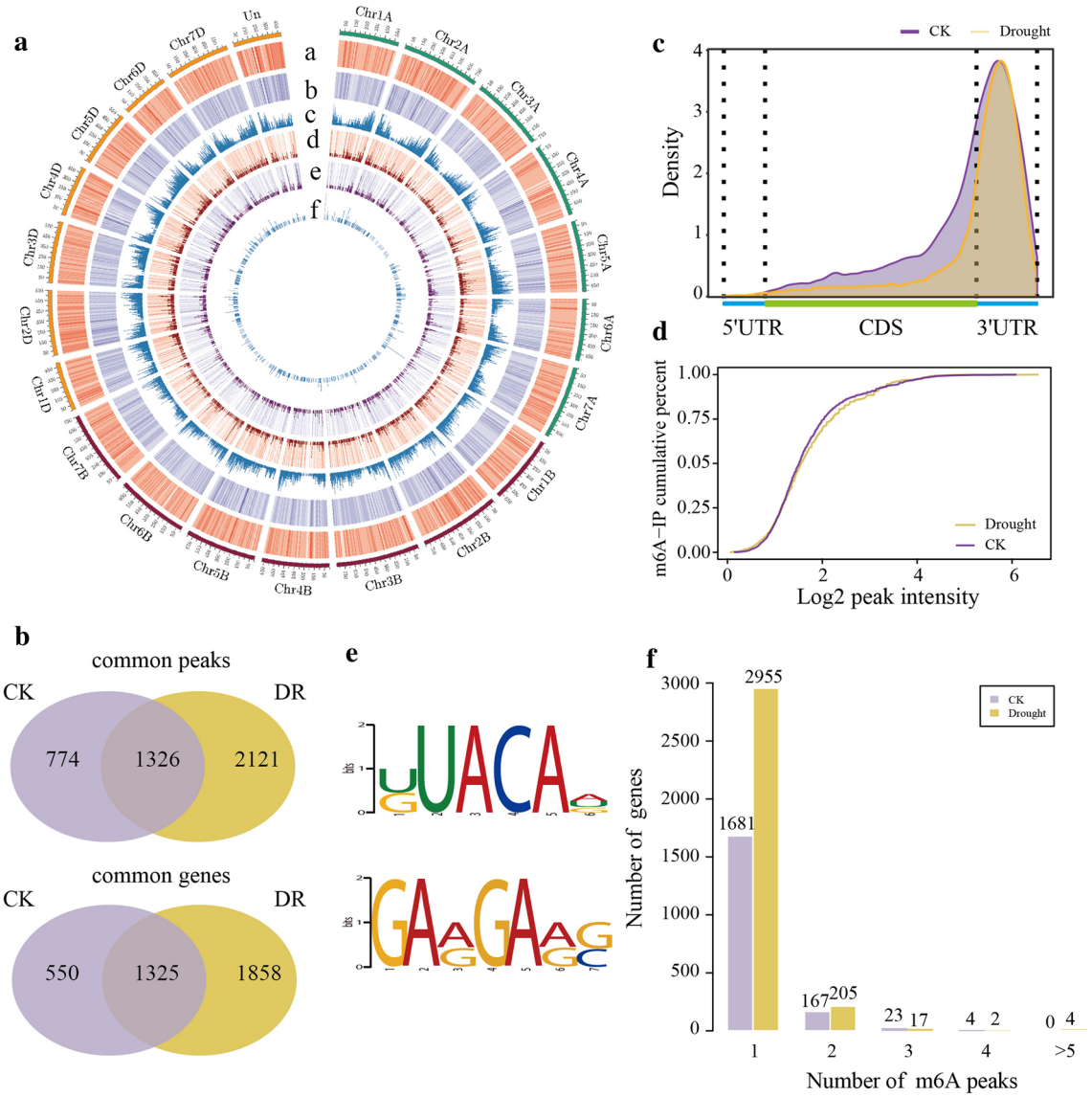


Fig. 1 Overview of m6A profiles in wheat leaf under drought (DR) and control (CK) conditions. **A** Circos plot illustrating the relationship between the distance and density of m6A peaks and gene expression abundance across wheat chromosomes. Circles a, b: the expression level of transcripts under the DR (a) and CK (b) conditions. Circle c: differentially expressed genes between DR and CK. Circles d, e: m6A peak enrichment under the DR (d) and CK (e) conditions. Circle f: differentially m6A peaks between DR and CK. **B** Upper panel: the numbers of the DR-specific and CK-specific m6A as well as their shared m6A peaks. Lower panel: the numbers of the DR-specific and CK-specific m6A as well as their shared m6A genes. **C** The distribution and density of m6A peak in the transcripts (5′-UTR, CDS and 3′-UTR). **D** Cumulative distribution of log2 peak intensity of m6A modification under DR and CK conditions. **E** Top two m6A motifs were enriched in all of the identified m6A peaks. **F** Comparison of the number of genes with different m6A peaks under the DR and CK conditions

compared to those in the CK group (Fig. 1F). These findings indicated that distinct m6A modification patterns under drought and control conditions.

To determine the correlation between m6A modification and gene expression, a comparative analysis was performed on six RNA-seq datasets obtained from bread wheat seedlings under leaves both normal and drought conditions (Sequence Read Archive database, accession no. SRP098756). All expressed genes were categorized based on their expression abundance (FPKM) into three groups: low expression (FPKM < 1), medium expression (FPKM > 1 and < 5), and high expression (FPKM > 5) (Zhang et al. 2021b). Subsequently, the proportions of genes in each category are presented in Fig. 2A. In both the CK and DR groups, high expression genes were predominantly m6A-modified, while non-m6A genes were mainly in the low

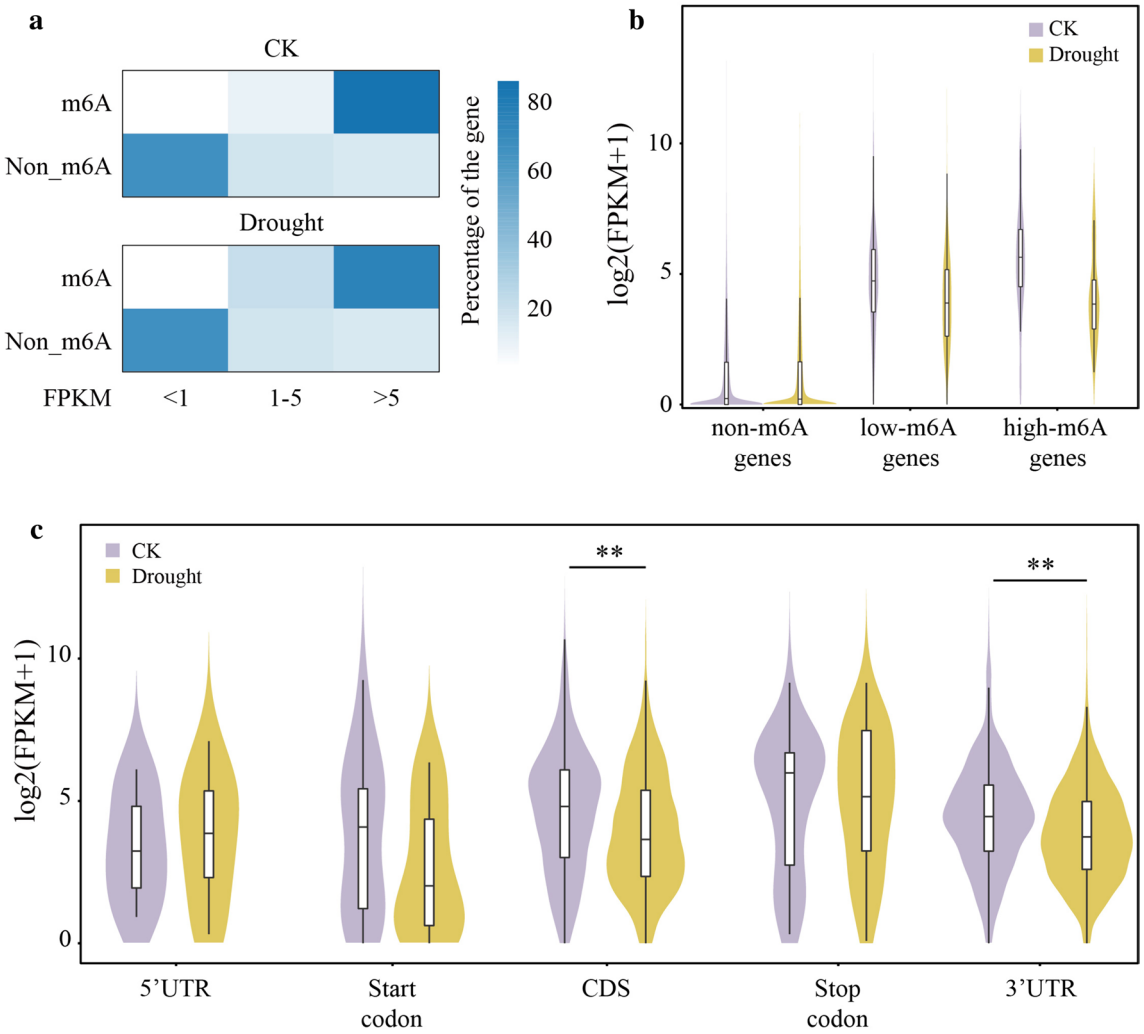


Fig. 2 Impact of m6A methylation on wheat gene expression under the DR and CK conditions. **A** The percentage of m6A methylated and un-methylated genes with differential expression levels under the DR and CK conditions. (FPKM < 1, 1–5, and > 5). Various color densities represent the distinct proportions of the corresponding gene. **B** Comparison of the expressions of the genes with different m6A levels under CK and DR conditions. non-m6A gene (m6A site = 0), low-m6A gene (m6A site < 3), high-m6A gene (m6A site ≥ 3). **C** Under both the CK and DR conditions, the genes are categorized into five groups (3′-UTR, start codon, CDS, stop codon, and 5′-UTR) based on the m6A peak annotation within each gene. Asterisks indicate significant differences (**$P \leq 0.01$; Student's *t*-test) between the DR and CK conditions

expression category (Fig. 2A). Compared to the CK group, the DR group showed a decreasing proportion of highly expressed m6A-methylated genes (Fig. 2A). Clearly, m6A-modified genes exhibited significantly higher expression levels in both CK and DR groups compared to non-m6A-modified genes. Further categorizing genes as non-m6A (m6A site = 0), low-m6A (m6A site < 3), and high-m6A (m6A site ≥ 3), genes with high m6A modification in both conditions had higher expression than those with low m6A modification (Fig. 2B), which was consistent with a previous barley study (Su et al. 2022). The expression of high-m6A and low-m6A genes was lower in the DR group compared to the CK group. Additionally, we investigated the correlation between m6A-modified gene expression and the proximity of m6A binding sites to stop codon regions. Our results showed that genes with m6A sites near stop codon regions exhibited higher expression levels than those located elsewhere (Fig. 2C). Thus, these findings demonstrate the impact of drought stress on wheat m6A methylation distribution and density.

Differentially methylated genes in response to drought stress

To further identify differential methylation peaks between the CK and DR groups, a comparative analysis was

conducted using the m6A-seq dataset from the drought group versus the CK group. We identified a total of 373 differentially methylated sites, comprising 151 hyper-methylated and 222 hypo-methylated m6A sites (fold change > 1.5, p-value < 0.05) (Fig. 3A). These sites are associated with 362 differentially methylated genes (DMGs), including 149 hyper-methylated and 213 hypo-methylated genes. Furthermore, we randomly selected two distinct differentially methylated sites from the hyper-methylated and hypo-methylated datasets, corresponding to the genes TraesCS5B02G409400 (hyper-methylated) and TraesCS1D02G375100 (hypo-methylated). We used IGV software to visualize changes in methylation intensity (Fig. 3B). Gene Ontology (GO) enrichment analysis of all DMGs indicated significant enrichment in processes such as photosynthesis, light reaction, NADP metabolic process, oxidoreduction coenzyme metabolic process, water transport, chloroplast, oxidoreductase activity, RNA binding, and chlorophyll-binding (Fig. 3C). KEGG pathway analysis showed that most DMGs were enriched in metabolism, synthesis pathways, and photosynthesis-related

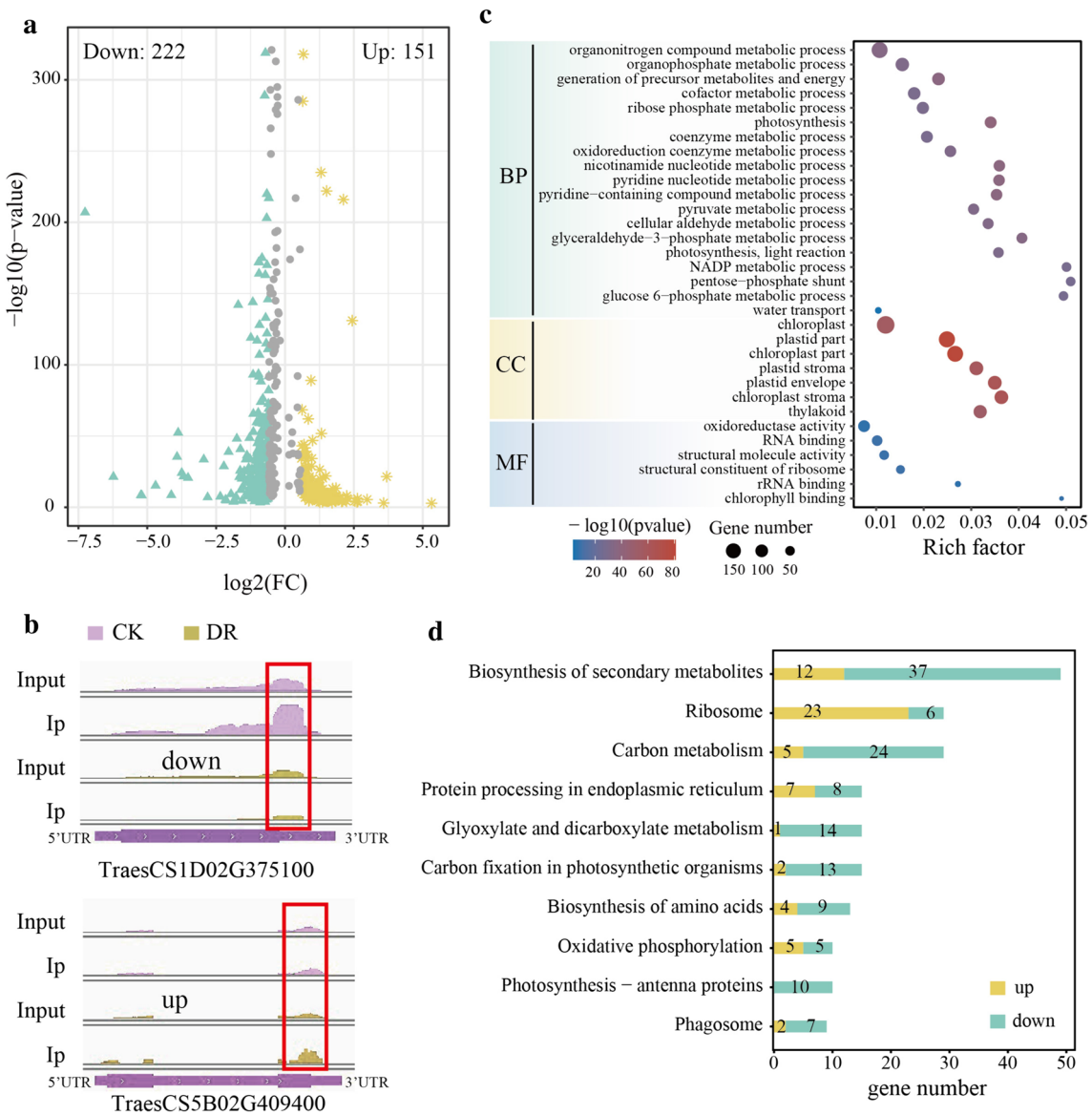


Fig. 3 Global changes in m6A modification under the DR and CK conditions and functional analysis of m6A genes. **A** Identification of 151 hyper-methylated and 222 hypo-methylated m6A peaks in the DR group as compared to CK group (fold change > 1.5, p < 0.05). **B** Highlighting genes with differential m6A peaks that are hypermethylated and hypomethylated. The IP and Input data read from the CK and DR groups are depicted by the blue and red peaks, respectively. The m6A peak is marked by the red box. **C** GO enrichment analysis of differentially methylated genes (DMGs). BP, biological process; CC, cellular component; MF, molecular function. **D** KEGG pathway enrichment analysis of the DMGs

pathways (Fig. 3D). Overall, our findings suggest that m6A modification may mediate diverse biological processes to regulate drought response and tolerance, particularly photosynthesis.

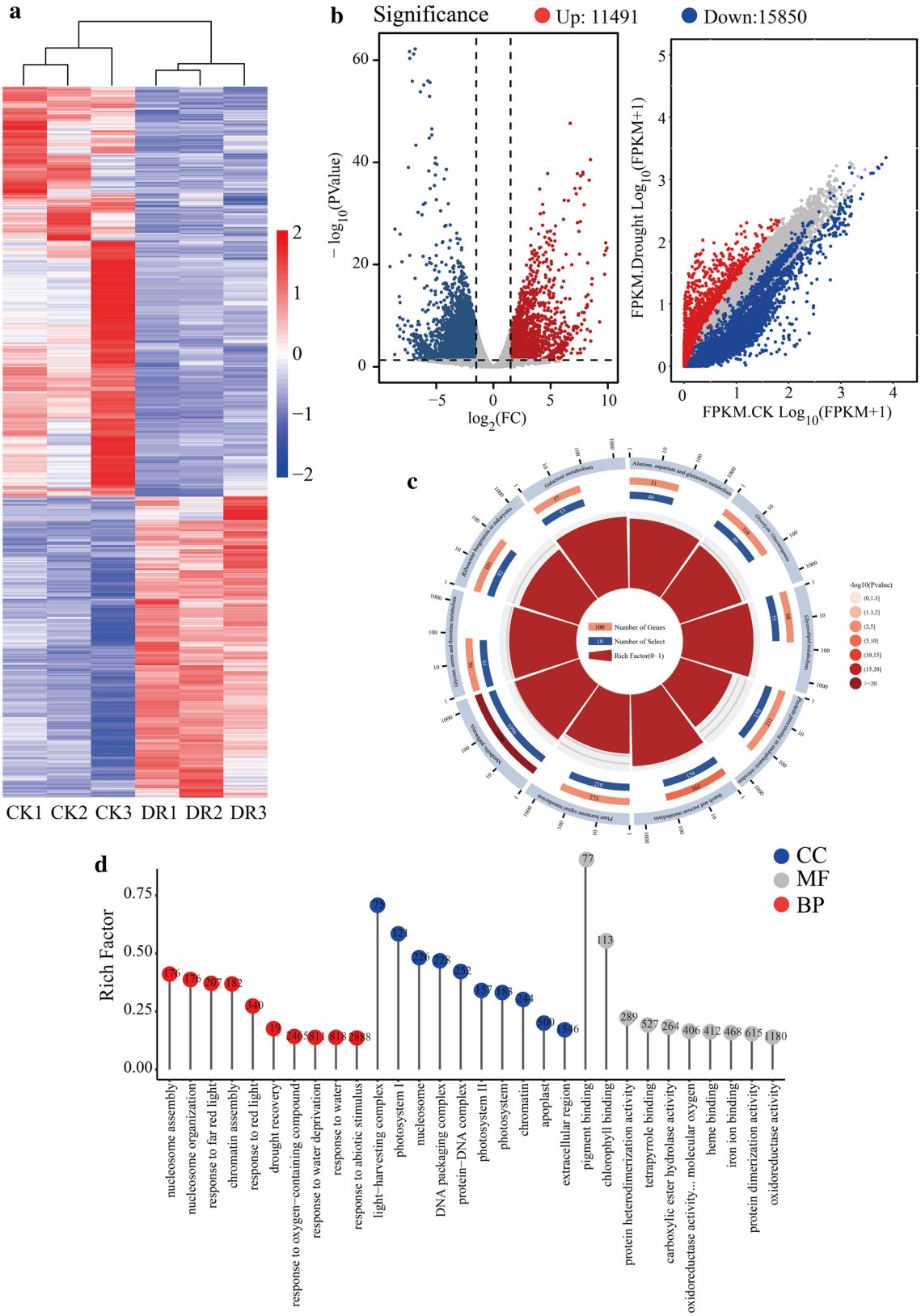
Correlation between differentially methylated genes and differentially expressed genes

To examine the potential link between m6A modification and gene expression, six RNA-seq datasets were utilized from seedling leaves exposed to both drought and control conditions to identify differentially expressed genes (DEGs). Hierarchical clustering analysis of the RNA-seq data revealed distinct global mRNA expression patterns between the CK and DR groups (Fig. 4A). In total, 27,341 DEGs (Fold change > 1.5, P-value < 0.05) were identified through comparative analysis of mRNA expression profiles, with 11,491 up-regulated and 15,850 down-regulated genes (Fig. 4B). KEGG analysis demonstrated significant enrichment of these DEGs in 93 metabolic pathways. Notably, both upregulated and downregulated DEGs were enriched in pathways like glycolysis/gluconeogenesis, starch and sucrose metabolism, galactose metabolism, and plant hormone signal transduction pathways (Fig. 4C). GO analysis revealed significant enrichment of DEGs in processes related to water deprivation response, abiotic stimulus-response, photosystem I, and chlorophyll-binding (Fig. 4D).

By integrating m6A-seq and RNA-seq data and comparing the DR group with the CK group, 143 DEGs were identified with DMGs (Fig. 5A; Table S5). Among these, 11 genes displayed both m6A hypermethylation and upregulated expression (Hyper-up), while 27 genes exhibited m6A hypermethylation and downregulated expression (Hyper-down). Furthermore, 17 genes featured m6A hypomethylation and upregulated expression (Hypo-up), and 88 genes demonstrated m6A hypomethylation coupled with downregulated expression (Hypo-down) (Fig. 5A). The KEGG analysis of drought-responsive genes in the four-quadrant plot enriched 21 pathways, including metabolic pathways, carbon fixation in photosynthetic organisms, photosynthesis, and pyruvate metabolism (Fig. 5B). GO enrichment analysis indicated significant enrichment of these genes in the photosynthesis process, glyceraldehyde-3-phosphate metabolic process, oxidoreduction coenzyme metabolic process, pigment binding, and chlorophyll-binding (Fig. 5C). Based on the m6A RNA methylation and mRNA expression levels of these differentially expressed m6A-modified genes (Fig. 6A), an interaction network analysis was performed to gain insights into their functions (Fig. 6B). According to the KEGG enrichment pathway, the protein interaction network was categorized into metabolism, photosynthesis, and ribosome. In the ribosomal category, all genes belonged to the ribosomal protein (RP) gene family, including 3 small ribosomal proteins (RPS) (TraesCS3B02G303000, TraesCS5B02G409400, TraesCS2B02G623400) and 9 large ribosomal protein (RPL) subunits (TraesCS3D02G271800, TraesCS6D02G094200, TraesCS4B02G213600, TraesCS7A02G145700, TraesCS1D02G227300, TraesCS3B02G268900, TraesCS5B02G154300, TraesCS3B02G271800, TraesCS5A02G279400). Previous studies have shown that the RPL gene family encodes key proteins that alleviate the effects of drought stress in plants (Moin et al. 2017). In the photosynthesis category, most genes had high connections with others, and the top 3 node degrees belonged to central genes, serving as hub genes in the protein interaction network. This indicated that TraesCS6D02G247400 (Phosphoribulokinase), and TraesCS1A02G313300 (Phosphoglycerate kinase) might play crucial roles in wheat's response to drought stress. Additionally, there were eight genes (TraesCS5A02G229300, TraesCS4A02G226900, TraesCS1D02G375100, TraesCS6A02G159800, TraesCS2A02G204800, TraesCS7A02G227100, TraesCS5A02G454200, and TraesCS2D02G209900) in the photosynthetic category belonging to the LHC gene family. In the metabolic category, TraesCS7B02G065200 (*Gpx4*) emerged as a potentially significant gene in wheat's response to drought stress, given its strong correlation with other genes in the protein interaction network.

TaECT9 and TaALKBH10B may be related to increase m6A methylation levels in response to drought stress

The m6A regulatory machinery comprises three core components: writer, eraser and reader. In a previous study on wheat, 20 writers, 29 erasers, and 41 readers were documented (Yue et al. 2019). To investigate the genes influencing m6A levels in response to drought, we conducted a comprehensive analysis of these m6A-regulated genes using RNA-seq data. The analysis revealed differential expression of 27 m6A-regulated genes between DR and CK condition. Specifically, 7 writers and 15 readers were up-regulated, while 3 eraser exhibited up-regulation, and 2 eraser showed down-regulation (Fig. 7A). Subsequently, we confirmed the expression levels of these 11 m6A-regulated genes using qRT-PCR. The results indicated that TraesCS1D02G109300, responsible for encoding the reader protein ECT9 (*TaECT*9), exhibited a substantial up-regulation in response to drought stress. Conversely, TraesCS7A02G215100, encoding the eraser protein ALKBH10B (*TaALKBH10B*), demonstrated a significant down-regulation. The remaining m6A-regulated genes did not exhibit differential expression under DR conditions (Fig. 7B). Hence, the results suggest that the increased m6A methylation abundance under DR conditions is attributed to the increased expression of the m6A-binding protein *TaECT9* and reduced expression of the m6A demethylase


a
CK1 CK2 CK3 DR1 DR2 DR3
b
Significance
Up: 11491
Down:15850
− log10(PValue)
log2(FC)
FPKM.Drought Log10(FPKM+1)
FPKM.CK Log10(FPKM+1)
c
Number of Genes
Number of Select
Rich Factor(0−1)
-log10(Pvalue)
d
CC
MF
BP
Rich Factor
nucleosome assembly
nucleosome organization
response to far red light
chromatin assembly
response to red light
drought recovery
response to oxygen−containing compound
response to water deprivation
response to water
response to abiotic stimulus
light−harvesting complex
photosystem I
nucleosome
DNA packaging complex
protein−DNA complex
photosystem II
photosystem
chromatin
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protein heterodimerization activity
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oxidoreductase activity... molecular oxygen
heme binding
iron ion binding
protein dimerization activity
oxidoreductase activity

◂**Fig. 4** Summary of the RNA-seq analysis depicting the differentially expressed genes (DEGs) in the DR group as compared to the CK group, along with the functional analysis of these genes. **A** The expression level of DEGs between DR and CK conditions based on RNA-seq data. Red, white, and blue colors represent the up-, non-significant, and down-regulation of mRNA levels, respectively. **B** Left: volcano analysis of the DEGs. Right: scatter plot of the DEGs. **C** KEGG pathway enrichment analysis of the DEGs. **D** GO enrichment analysis of DEGs. *BP* biological process, *CC* cellular component, *MF* molecular function

gene *TaALKBH10B*. *TaECT9* and *TaALKBH10B* emerge as potential key candidate genes governing drought response and tolerance by modulating RNA m6A methylation in wheat.

Phenotypic traits and drought tolerance identification of mutant *Taect9*

To gain insights into m6A's impact on drought stress, this study utilized Sanger sequencing to obtain stable homozygous mutant lines of the gain-of-stop (loss-of-function) mutant *Taect9* (with Trp^{344} mutated from TGG to TAG), and comprehensive analyses of its phenotypic traits and drought tolerance were then conducted (Fig. 8A). Results revealed that, compared to normal conditions, both the WT and mutants exhibited reduced growth vigor under drought treatment. There were significant reductions in total biomass, both aboveground and belowground biomass, and a reducing root-to-shoot ratio (Fig. 8B and C). Drought treatment also yielded significant differences in root-related traits between *Taect9* and WT (Fig. 8B and C). Furthermore, when considering the drought tolerance index (DTI) based on total biomass, WT and *Taect9* scored 0.63 and 0.39 respectively, thus demonstrating that the loss-of-function of ECT9 significantly impeded wheat's drought tolerance of wheat (Fig. 8C). Notably, *Taect9* displayed significantly shorter root length, reduced root surface area, and smaller root volume compared to WT (Fig. 8C), suggesting that ECT9 may regulate root growth to enhance drought tolerance in wheat.

Discussion

m6A methylation is a widespread post-transcriptional RNA modification in eukaryotes, present in various RNA types, including mRNAs, tRNAs, miRNAs, and long-stranded noncoding RNAs, and is crucial for plant adaptation to biotic and abiotic stresses (Cantara et al. 2011; Wei et al. 2017; Arribas-Hernandez and Brodersen 2020). However, the characteristics and regulatory mechanisms of m6A in wheat response to drought stress remain unclear. In this study, we elucidated the m6A modification patterns under control and drought stress conditions in wheat. A total of 4221 m6A peaks and 3733 m6A-modified genes were detected, of which 373 methylated peaks exhibited differential expression between the CK and DR groups. In detail, 2100 and 3447 m6A sites were found in CK and DR groups respectively, of which 1326 were shared by both groups, with 774 CK-specific and 2121 DR-specific sites. This corresponds to 1325 shared m6A-modified genes, including 550 CK-specific and 1858 DR-specific genes. These findings indicated the involvement of m6A modification in response to drought stress in wheat.

We found that the distribution pattern of m6A in wheat is similar to that of other plants, such as Arabidopsis, sorghum, rice and sea buckthorn, with a predominant enrichment in the 3′-UTR region and stop codon region (Luo et al. 2014; Du et al. 2020; Miao et al. 2020; Zhang et al. 2021a). These results indicated that the distribution pattern of m6A methylation was conserved among plants. In mammals, m6A modification was predominantly observed in the 3′-UTR of mRNA and is typically associated with reduced gene expression levels (Luo et al. 2014; Wang et al. 2015). However, contrasting patterns have been identified in plants, such as Arabidopsis, where m6A modification exhibited a negative correlation with gene expression under normal conditions but switched to a positive correlation under specific stress conditions, such as salt stress (Anderson et al. 2018; Hu et al. 2021). Moreover, m6A modification generally showed a positive correlation with mRNA abundance during fruit expansion and other specific processes in tomato (Zhou et al. 2019). In our this study, m6A modification was positively correlated with mRNA abundance in wheat under drought stress, suggesting a role for m6A modification in regulating gene expression under drought stress. Meanwhile, the m6A peak in the stop codon region was accompanied by higher gene expression levels, followed by the 3′-UTR region.

The RRACH motif is the most common motif in m6A methylation peaks in mammals, and this phenomenon was also found in Arabidopsis and maize (Luo et al. 2014; Du et al. 2020; Miao et al. 2020). The GAGGGA G motif in wheat grain development and the URUAH (R = A/G, H = A/C/U) motif in maize seedlings were found to be enriched around m6A sites (Du et al. 2020; Li et al. 2022). Tissue-specific m6a peaks enriched in rice included UGWAMH, UGUAMM, and RAG RAG (R = A/G, H = A/C/U, W = A/U, M = A/C) (Zhang et al. 2019). In this study, the KUACAD motif was significantly enriched in wheat seedling m6a-seq data, suggesting that this motif may also be enriched around m6A sites in plants. These results indicate that the diversity of m6A motifs across different species increases the complexity of m6A methylation and imply potential modulation of the methylation process by diverse RNA-binding proteins.

By integrating m6A-seq and RNA-seq dataset, we identified 143 genes with disparities in both m6A levels and

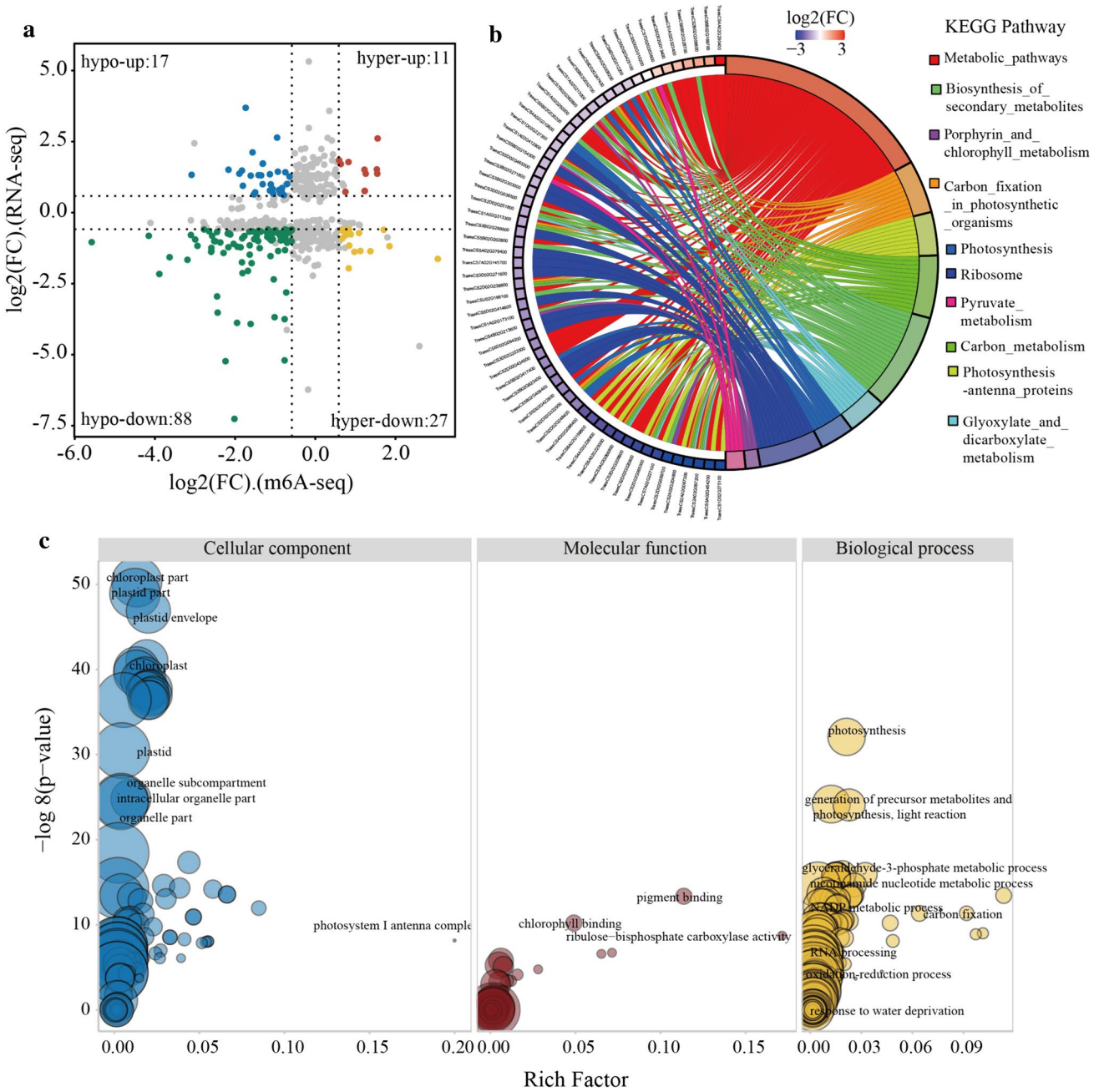

Fig. 5 Conjoint analysis of DMGs and DEGs. **A** Identification of the genes with a significant change in both m6A and mRNA levels under the DR condition as compared to the CK condition. **B** KEGG pathway enrichment analysis of genes with significant changes in both m6A and mRNA levels. **C** GO enrichment analysis of the genes with a significant change in both m6A and mRNA levels

mRNA abundant, half of which exhibited a negative correlation with m6A modification. GO enrichment analysis showed these genes involved multiple biological processes, including metabolism, photosynthesis, and ribosomal pathways. Previous studies have demonstrated that maintaining photosynthetic capacity was a fundamental trait of drought-resistant crops (Ma et al. 2016). In this study, TraesCS6D02G247400, encoding phosphoribulokinase, had differentially methylated peaks (DMPs) in response to drought. Phosphoribosinase plays a pivotal role in CO_2 assimilation in the calvin cycle by phosphorylating of ribulose 5-phosphate to ribulose 1,5-diphosphate (Shi et al. 2014). It has been reported that the level of phosphoribokinase protein remains consistent in the roots of water-tolerant grass canines, while decreasing in water-stress-sensitive grass species (Zhao et al. 2011). Similarly, phosphoribokinase levels decreased in alfalfa

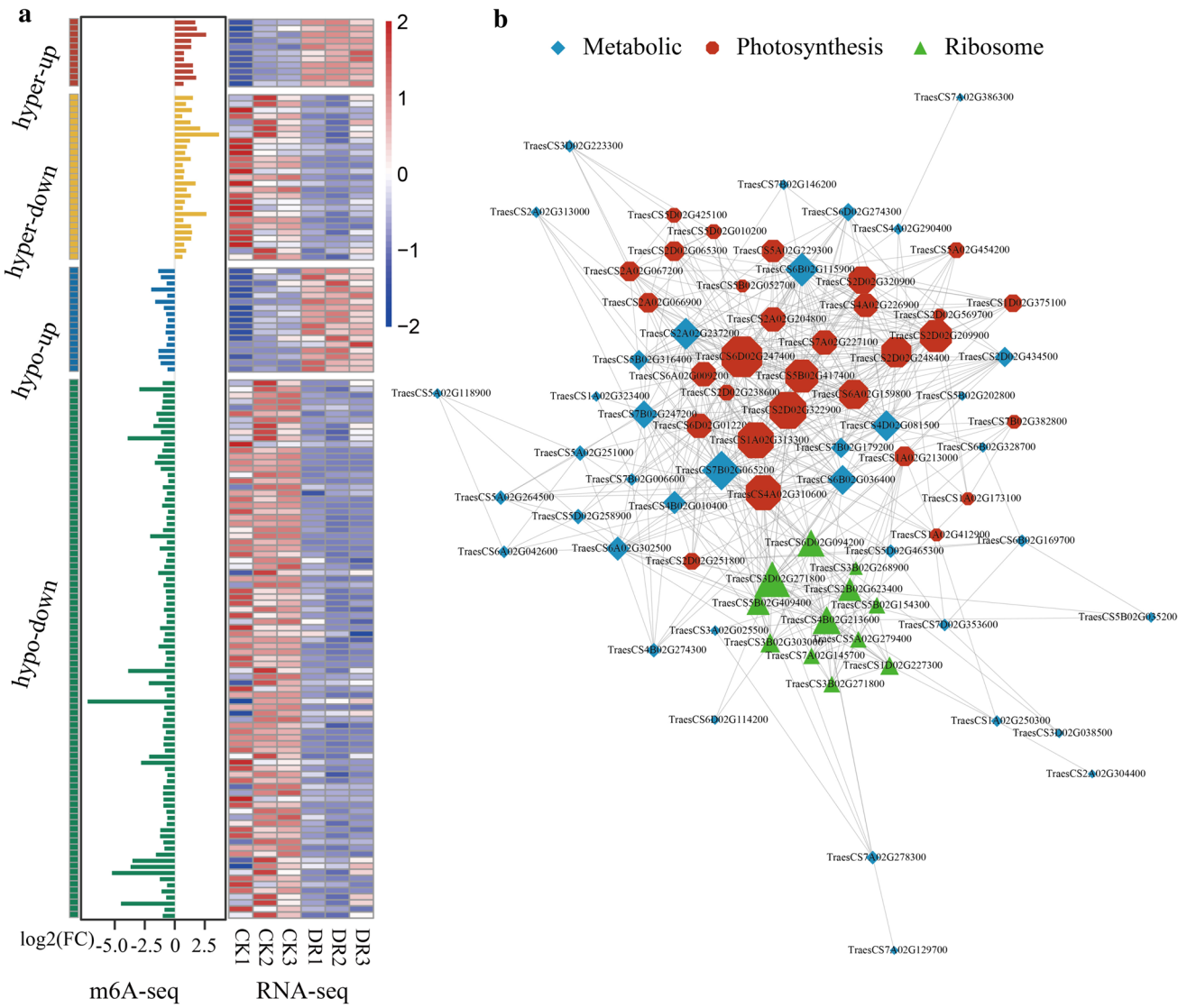

Fig. 6 Identification and regulatory network of the genes with differential m6A and mRNA levels. **A** The m6A and expression level of the hyper-up, hyper-down, hypo-up, and hypo-down regulated genes under the DR and CK conditions. **B** Regulatory interaction network of the m6A-modified genes

and cotton leaves under drought stress (Aranjuelo et al. 2011; Deeba et al. 2012). These results suggested that the m6A modification on TraesCS6D02G247400 might play an potential role in regulating drought response by mediating photosynthesis. Moreover, the LHC gene family significantly contributes to plant stress response and stress resistance regulation. In Arabidopsis, *Lhcb1-6* responds to stomatal movement and participates in ABA signaling by partially altering ROS homeostasis, primarily associated with drought stress responses (Xu et al. 2012). Eight LHC genes were also identified as DEGs with DMPs in this study, including *LHCB1.1* (TraesCS1D02G375100, TraesCS5A02G454200), *LHCB3* (TraesCS2A02G204800), *LHCB4.2* (TraesCS2D02G209900), *LHCB5* (TraesCS4A02G226900), *LHCA1* (TraesCS7A02G227100), *LHCA3* (TraesCS6A02G159800), and *LHCA4* (TraesCS5A02G229300). PrxQ protein interacts with CDSP32 to function in photosynthesis and regulate drought tolerance in Arabidopsis (Lamkemeyer et al. 2006). In this study, TraesCS7B02G065200, the ortholog of *AtPrxQ,* also displayed differential methylation and expression in DR compared to CK.

Finally, combined with RNA-seq analysis and qRT-PCR validation, *TaECT9* (an m6A-binding protein) was confirmed that significantly up-regulated in wheat in response to drought stress. Further investigation of phenotypic traits and drought tolerance of its loss-function mutant demonstrated that *TaECT9* mediates root-related traits to regulate drought tolerance. Therefore, the identified DEGs with DMPs and

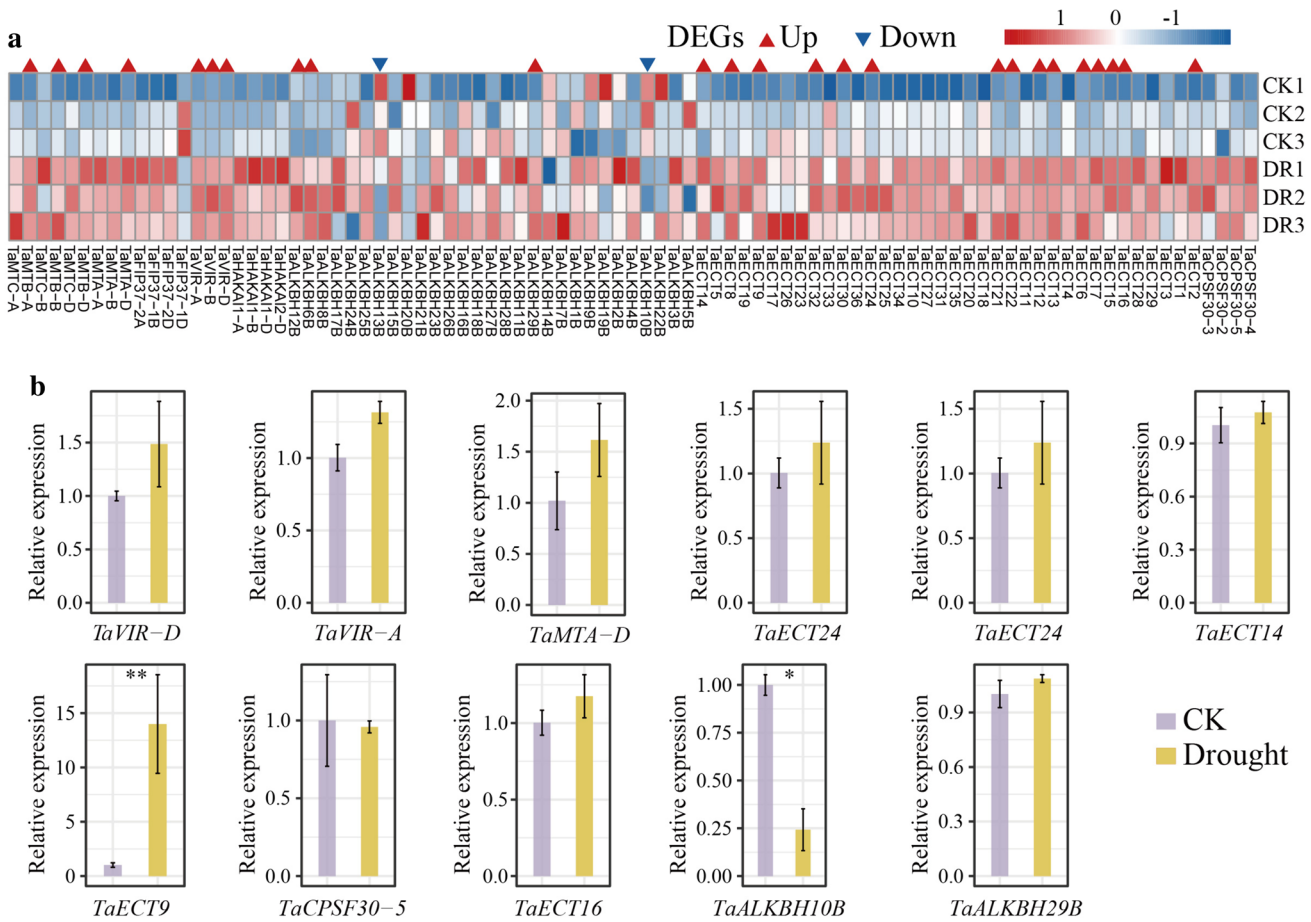

Fig. 7 Validation of the expression levels through qRT-PCR analysis. **A** The expression patterns of wheat m6A machinery-related genes under the CK and DR conditions based on RNA-seq data. **B** Relative mRNA levels of wheat m6A machine-related genes under CK and DR conditions through qRT-PCR analysis. Statistical analyses (mean values ± SD, $n = 3$) were performed using the Student's *t*-test. Asterisks indicate the significant difference when compared to the control. $^{*}P < 0.05$; $^{**}P < 0.01$

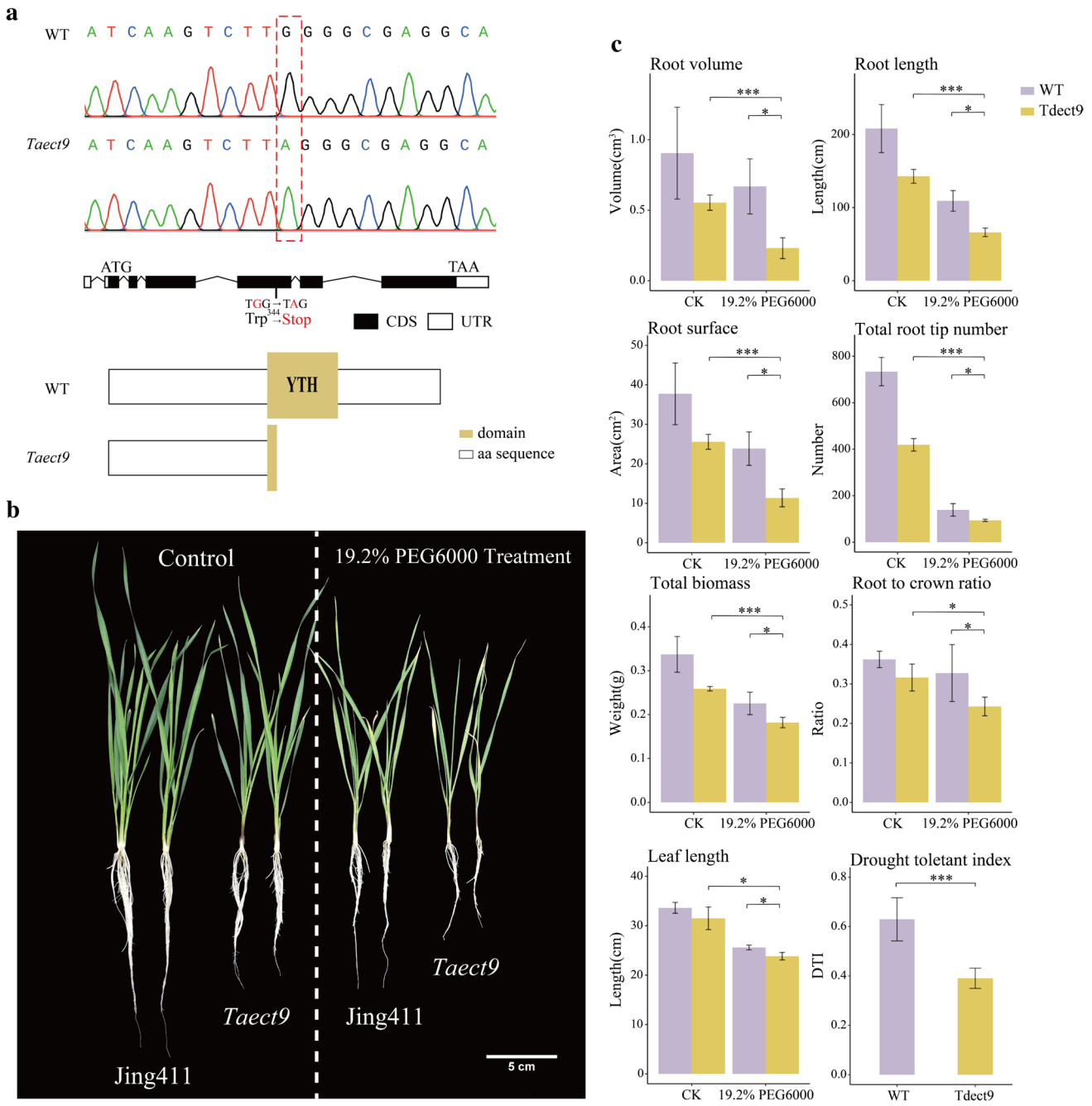

Fig. 8 Drought tolerance investigation of the stop-gain mutant of *TaECT9*. **A** Verification of *Taect9*. DNA sequence alignment at mutation site and mutation site peak diagrams. **B** The WT and *Taect9* seedlings after 14 d of growth under 19.2% PEG6000 treatment. **C** Comparison of the phenotypic performance of WT and *Taect9* under drought treatment, including total biomass, root-to-crown ratio, leaf length, root length, root surface area, total root tip number, root volume, and drought tolerance index (DTI) comparison based on total biomass between WT and *Taect9*. The phenotypic data is collected by three independent biological replicates. Significance analysis (mean values ± SD, $n = 3$) was performed using Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$

m6A-related genes here lay the foundation for revealing the mechanism of m6A-mediated drought response.

Conclusion

This study explored the transcriptome-wide m6A methylation profile of wheat under drought stress, identifying 4221 m6A peaks and 3733 m6A-modified genes. This represents the first m6A methylome association with wheat drought response. When combining m6A-seq and RNA-seq data, the m6A methylation was found to be significantly correlated with mRNA expression abundance. Moreover, 143 genes exhibited differential m6A and mRNA expression levels in DR compared to CK. These genes were notably enriched in photosynthesis, metabolism, and ribosomal pathways, suggesting the potential role of m6A modification in regulating drought response by mediating photosynthesis and RNA metabolism-related processes. Additionally, up-regulated *TaETC9* and down-regulated *TaALKBH10B* likely contribute to increased m6A levels under drought stress. The loss-function mutant of *TaECT9* demonstrated its involvement in regulating drought tolerance. This study identifies candidates for further functional investigations, shedding light on the potential role of RNA methylation in stress response and tolerance.

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Author contributions NX and LX designed and supervised the project. PY and LW collected, generated, and analyzed the data. JY and PW contributed to expression analysis. HJ and LY contributed to plant material collection. PY and JY prepared the draft manuscript. NX and LS reviewed and revised the manuscript.

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Data availability MeRIP-seq data used in this study has been deposited into the Genome Sequence Archive (GSA) database with the accession number of PRJCA009400. RNA-seq data used in this study was downloaded from the Sequence Read Archive database with accession no. SRP098756 (https://www.ncbi.nlm.nih.gov/sra) and other data was provided in Supplemental materials.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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