



Unique features of mRNA m⁶A methylomes during expansion of tomato (*Solanum lycopersicum*) fruits

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Abstract

N⁶-methyladenosine (m⁶A) is the most abundant internal modification in eukaryotic messenger RNA. Although the role of m⁶A has been demonstrated in many biological processes, including embryonic development, flowering time control, microspore generation, fruit ripening, and stress responses, its contribution to other aspects of plant development still needs to be explored. Herein, we show the potential link between m⁶A deposition and the expansion of tomato (*Solanum lycopersicum*) fruits through parallel m⁶A-immunoprecipitation-sequencing (m⁶A-seq) and RNA-seq analyses. We found that global m⁶A levels increased during tomato fruit expansion from immature green to mature green stage. m⁶A-seq revealed that thousands of protein-coding genes are m⁶A-modified mainly in the 3'-untranslated regions. m⁶A-seq and RNA-seq analyses showed a positive association between m⁶A methylation and mRNA abundance. In particular, a large number of fruit expansion-related genes involved in hormone responses and endoreduplication were m⁶A modified and expressed more actively than the non-m⁶A-modified genes, suggesting a potential role of m⁶A modification in tomato fruit expansion. Importantly, altering m⁶A levels by direct injection of 3-deazaneplanocin A (DA; m⁶A writer inhibitor) or meclofenamic acid (MA; m⁶A eraser inhibitor) into tomato fruits suppressed fruit expansion; however, injection of exogenous DA or MA accelerated or delayed fruit ripening, respectively. Collectively, these results suggest a dynamic role of m⁶A methylation in the expansion and ripening of tomato fruits.

Introduction

RNA molecules are heavily modified co-transcriptionally and posttranscriptionally in all living organisms. To date, over 150 different chemical modifications of cellular RNAs have been identified (Cantara et al., 2011; Boccaletto et al., 2018). In particular, recent advances in high-throughput sequencing technologies have uncovered N⁶-methyladenosine (m⁶A) as the most abundant, dynamic, and reversible mRNA modification

in eukaryotes (Liu and Pan, 2016; Covelo-Morales et al., 2018). The installation and removal of m⁶A marks are catalyzed by methyltransferases (referred to as “writers”) and demethylases (referred to as “erasers”), respectively (Meyer and Jaffrey, 2017; Hu et al., 2019; Zheng et al., 2020). Dynamic and reversible m⁶A modifications in plants are modulated by writer components, including methyltransferase A (MTA), MTB,

FKBP12-INTERACTING PROTEIN 37, VIRILIZER, and an E3 ubiquitin ligase HAKAI (Zhong et al., 2008; Bodi et al., 2012; Růžička et al., 2017; Zhang et al., 2019), and eraser proteins, such as Alpha-Ketoglutarate-Dependent Dioxygenase homolog 9B (ALKBH9B) and ALKBH10B (Duan et al., 2017; Martínez-Pérez et al., 2017). An increasing body of evidence has demonstrated the importance of m⁶A RNA methylation in controlling mRNA metabolism, including RNA processing, stability, splicing, nuclear-to-cytoplasmic export, and translation control (Wang et al., 2014; Meyer et al., 2015; Wang et al., 2015; Xiao et al., 2016; Coots et al., 2017; Shi et al., 2017; Luo et al., 2020), which are all crucial for various biological processes in plants, such as embryogenesis, floral transition, development of trichomes, leaves and roots, sporogenesis, fruit ripening, and abiotic stress responses (Zhong et al., 2008; Bodi et al., 2012; Duan et al., 2017; Růžička et al., 2017; Arribas-Hernández et al., 2018; Scutenaire et al., 2018; Wei et al., 2018; Zhang et al., 2019; Arribas-Hernández et al., 2020; Kim et al., 2020; Hu et al., 2021; Shao et al., 2021; Song et al., 2021). Although these findings have demonstrated the vital roles of m⁶A methylation in plant development at different development stages, the functional roles of m⁶A modification in the development of crops are largely unknown.

Tomato (*Solanum lycopersicum*) is a widely cultivated horticultural crop and serves as a valuable model plant to study fruit-related traits, including fruit expansion, quality, and ripening. Being an important part of human diet, the quality of tomato fruits, which depends on the developmental process of expansion and ripening, draws consistent attention. Fruit expansion determines the fruit size, whereas ripening impacts fruit nutritional value and shelf life (Zhou et al., 2019). A previous study has demonstrated that epigenetic 5-methylcytosine DNA methylation plays an important role in the regulation of tomato fruit ripening; disruption of DNA demethylase gene *SIDML2* leads to global DNA hypermethylation and remarkable inhibition of fruit ripening (Lang et al., 2017). Moreover, a recent study has shown that disruption of *SIALKBH2*, an m⁶A RNA demethylase in tomato, is associated with increased stability of *SIDML2* transcripts and delayed fruit ripening, highlighting a molecular link between DNA methylation and m⁶A RNA methylation during fruit ripening (Zhou et al., 2019). In addition, a recent study has demonstrated that m⁶A methylation regulates strawberry fruit ripening in an abscisic acid (ABA)-dependent manner (Zhou et al., 2021). Although these studies suggest the crucial role of epitranscriptomic RNA m⁶A methylation in regulating the fruit ripening process, the importance of m⁶A methylation in the expansion of fruits, including tomato, has never been explored.

To understand the role of mRNA m⁶A methylation in the expansion of tomato fruits, we performed transcriptome-wide m⁶A RNA immunoprecipitation-sequencing (m⁶A-seq) and RNA-seq of tomato fruits at different expansion stages and assessed the relation between m⁶A modification and the abundance of RNA transcripts related to cell expansion, hormone response, and endoreduplication. Herein, we

investigated the ways by which m⁶A methylation affected gene expression at different developmental stages and suggested a link between m⁶A modification and tomato fruit expansion.

Results

Global m⁶A level increases during the expansion of tomato fruits

Considering the results from a previous report on the decrease in m⁶A methylation during the ripening of tomato fruits (Zhou et al., 2019), we first aimed to investigate how m⁶A levels are altered during the expansion stages of tomato fruits. We selected three time points, 12, 20, and 28 d after flowering (DAF), which showed clear differences in the fruit expansion (Figure 1A); it is evident that the fresh weight, fruit diameter, and fruit length of the tomatoes increased during the expansion stages (Figure 1B). Next, we analyzed the m⁶A levels in tomato fruits during different expansion stages from immature green to mature green stages (Figure 2A) and found that m⁶A levels in both total RNA and mRNA increased considerably in an expansion stage-dependent manner (Figure 2, B and C). Considering that m⁶A modification in rRNA can also affect developmental programs in animals (Ignatova et al., 2020), we measured the m⁶A levels in rRNA but found no significant changes in rRNA m⁶A levels in tomato fruits during different expansion stages (Supplemental Figure S1), suggesting that rRNA m⁶A methylation is not associated with tomato fruit expansion. These dynamic changes in m⁶A levels during the different developmental stages suggest that m⁶A modification in mRNA contributes positively to the expansion of tomato fruits.

Important features of m⁶A profile during the expansion of tomato fruits

To understand the association between m⁶A modification and fruit expansion, we conducted m⁶A-seq and RNA-seq for the tomato fruits collected 12, 20, and 28 DAF. We generated a total of 65–74, 64–64, and 80–84 million reads for the tomato fruits at 12, 20, and 28 DAF, respectively, among which 53–59, 51–52, and 64–67 million distinct reads, respectively, were uniquely aligned to the Tomato Genome SL3.0 (Supplemental Table S1). Transcriptome-wide location of m⁶A modification was identified using an algorithm as described previously (Meng et al., 2014). The confident m⁶A peaks obtained from two biological replicates which showed a high Pearson's correlation coefficient were used for subsequent bioinformatics analyses (Supplemental Figure S2). The validity of our m⁶A-seq data was further established by m⁶A-IP-qPCR analysis of six randomly selected m⁶A-containing transcripts (Supplemental Figure S3). Overall, we identified a total of 15,720, 16,547, and 16,381 m⁶A peaks in the tomato fruit at 12, 20, and 28 DAF, respectively (Supplemental Data Set S1), using m⁶A-seq. The majority of protein-coding transcripts (>66%) contained a single m⁶A peak (Supplemental Figure S4A). Consistent with previous

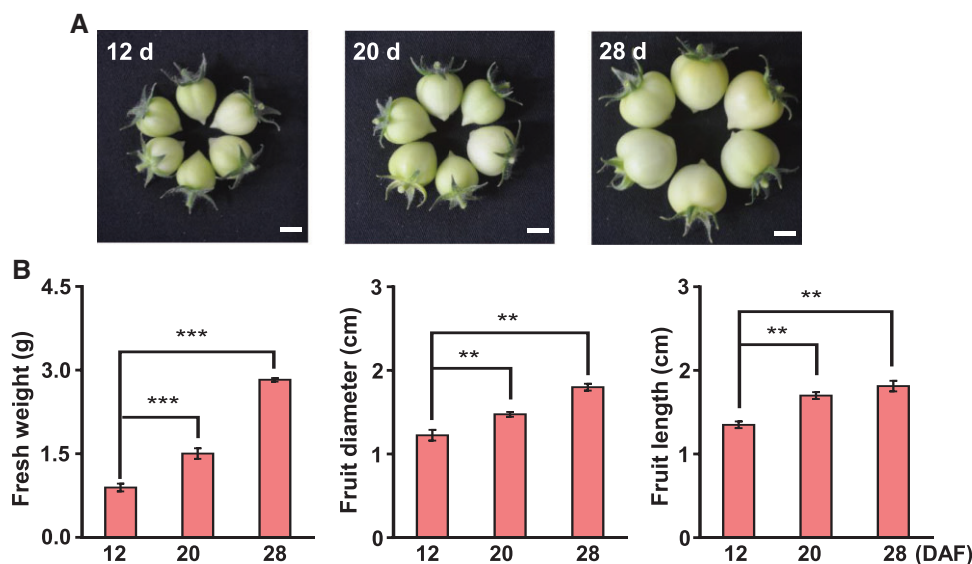


Figure 1 Phenotypes of tomato fruits during the expansion stages. A, Photographs of fresh fruits were taken at 12, 20, and 28 DAF. Scale bar = 1 cm (B). The fresh weight, diameter, and length of the fruits were measured on the indicated days. Data represent means \pm standard deviation of three biological replicates ($n = 6$), and asterisks indicate significant differences (Student's t test, $**P < 0.01$, $***P < 0.001$).

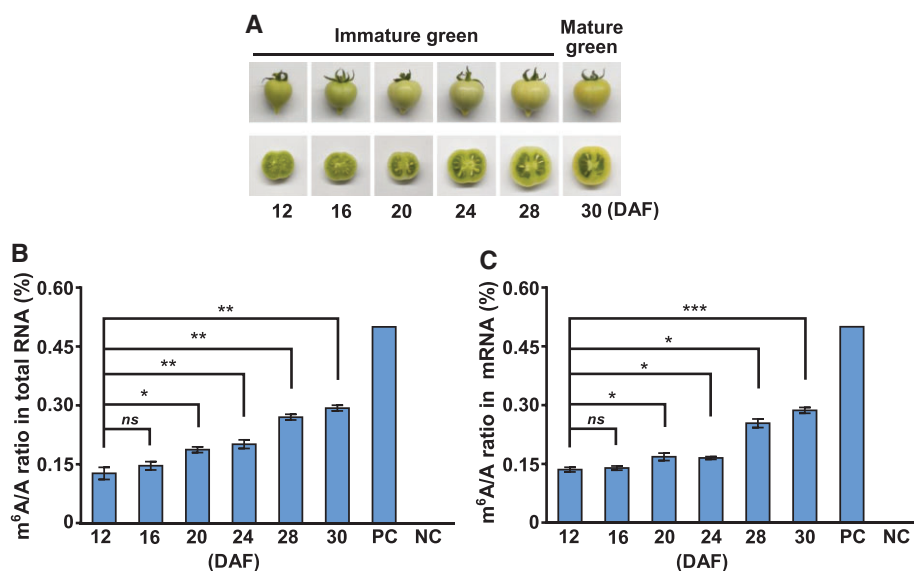


Figure 2 The global m⁶A levels in total RNA and mRNA during tomato fruit expansion stages. A, Photographs of the fruits at different expansion stages of tomato. The levels of m⁶A in (B) total RNA and (C) mRNA at different stages of fruit expansion are shown in (A). Data represent means \pm standard deviation of three biological replicates ($n = 4$), and asterisks indicate significant differences (Student's t test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). ns, not significant. PC, positive control. NC, negative control.

studies conducted in other plants and mammals, the distribution of m⁶A peaks in protein-coding mRNA in all groups was enriched mainly in the 3'-untranslated region (UTR) and relatively less in the 5'-UTR and coding sequences (CDS) (Supplemental Figure S4B). Gene ontology (GO) analysis of the biological function of m⁶A-modified genes revealed that m⁶A-containing genes were involved in various cellular functions, including protein metabolism, DNA and RNA processing, and transcription regulation (Supplemental Figure S4C).

Next, to analyze the differences of m⁶A methylome between different stages of fruit expansion, we compared all m⁶A peaks identified in each developmental stage and found that most of the m⁶A peaks (8,596 peaks) were common in all samples, whereas 757, 970, and 920 m⁶A peaks were unique to tomato fruits at 12, 20, and 28 DAF, respectively (Figure 3A). To further evaluate the reliability of our m⁶A-seq data, the m⁶A-containing transcripts identified in the immature green tomato fruits in this study were compared with those identified in the immature green tomato

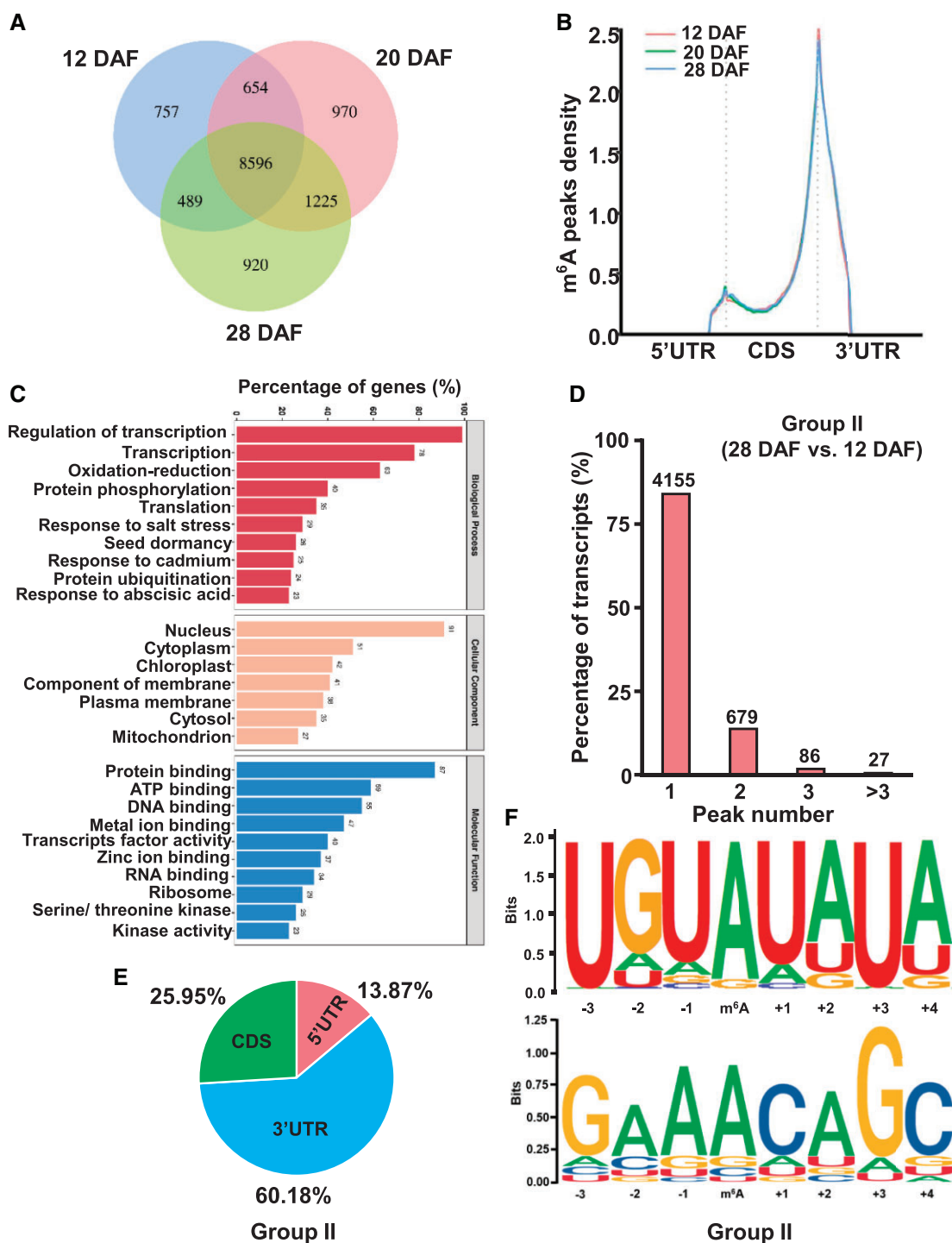


Figure 3 Changes of m^6A methylome during tomato fruit expansion stages. A, Venn diagrams showing the overlap of m^6A peaks in tomato fruits at 12, 20, and 28 DAF. B, GO enrichment analysis of common m^6A -modified genes shown in (A). C, Distribution of m^6A peaks in transcript segments divided into 5'-UTR, coding sequence, and 3'-UTR. D, Proportions of the m^6A -modified transcripts containing different number of m^6A peaks in comparison Group II (28 DAF versus 12 DAF). E, A Pie chart depicting the fraction of m^6A peaks in different gene segments. F, Sequence motifs identified using HOMER in m^6A peaks.

fruits by Zhou et al. (2019). Although the tomato cultivars used in our study and the previous study by Zhou et al. (Micro-Tom versus Ailsa Craig) are different, and the developmental stages investigated in the two studies (28 DAF versus 39 DAF) are not identical, $\sim 83\%$ (7,471/9,047) of the

m^6A -containing transcripts overlapped each other (Supplemental Figure S5), indicating that our m^6A -seq data are reliable. GO analysis of the overlapped m^6A -containing transcripts showed that they are involved in various cellular functions, including transcription regulation, protein

metabolism, DNA and RNA processing, and chloroplast function (Figure 3B). Analysis of the density of m⁶A peaks in all samples revealed similar m⁶A distribution patterns with m⁶A mainly enriched in the 3'-UTR (Figure 3C). To obtain a deeper view of changes in m⁶A landscape during fruit expansion, stages of fruit expansion were divided into the following three comparison groups: Group I, 20 DAF versus 12 DAF; Group II, 28 DAF versus 12 DAF; Group III, 28 DAF versus 20 DAF. We found that differential m⁶A peaks in all comparison groups are mainly modified at one site in the 3'-UTR (Figure 3, D and E; Supplemental Figure S6, A and B). Motif analysis of m⁶A peaks in all comparison groups resulted in the identification of URUAY (R = A/G, Y = U/A) sequence motif, which is a plant-specific motif previously identified in tomato, maize, rice, and Arabidopsis (Wei et al., 2018; Zhou et al., 2019; Miao et al., 2020; Luo et al., 2020; Hu et al., 2021). Interestingly, the RRACH motif found in animals (Dominissini et al., 2012, 2013; Linder et al., 2015), as well as plants, were identified in comparison Groups II and III, but not in Group I (Figure 3F; Supplemental Figure S6C).

Association between m⁶A methylation and the expression of fruit expansion-related genes

m⁶A modification has been shown to affect either negatively or positively the level of transcripts depending on developmental stages or environmental conditions (Shen et al., 2016; Hu et al., 2021). In tomato, m⁶A methylation is negatively associated with mRNA abundance during ripening (Zhou et al., 2019). To evaluate the relation between m⁶A modification and transcript levels during the expansion stages of tomato fruits, we performed RNA-seq analysis of the same samples used for m⁶A-seq. Comparison of the differentially expressed genes (DEGs; fold change > 2; $P < 0.05$; Supplemental Data Set S2) with altered m⁶A levels (fold change > 2; $P < 0.05$) in different comparison groups revealed that ~70% mRNAs in Group I, 78% mRNAs in Group II, and 81% mRNAs in Group III with increased or decreased m⁶A levels tended to enhance or reduce transcript levels, respectively, whereas only ~30% in Group I, 22% in Group II, and 19% in Group III with increased or decreased m⁶A levels tended to negatively affect transcript levels (Figure 4, A and B; Supplemental Figure S7A). These results suggest an overall positive association between m⁶A modification and transcript levels during the expansion of tomato fruits. This highlights the multifaceted effects of m⁶A modification on gene expression during the different developmental stages in tomato fruits. To understand the function of m⁶A-modified DEGs, we performed GO analysis and found that a majority of the m⁶A-modified DEGs were involved in cellular functions related to photosynthesis, chlorophyll-binding, and cell wall organization (Figure 4, C and D; Supplemental Figure S7B).

To further reveal the role of m⁶A modification in the regulation of tomato fruit expansion, we analyzed the m⁶A patterns in the genes related to fruit expansion (Supplemental

Table S2). Many genes that induce or repress fruit expansion contain m⁶A marks at one or all stages of fruit expansion (Supplemental Table S2). Moreover, RNA-seq analysis revealed that genes with m⁶A methylation were expressed more actively than genes without m⁶A modification (Figure 5; Supplemental Figure S8). Notably, a large number of genes involved in auxin and gibberellic acid signaling and endoreduplication, as well as several genes related to cell expansion and pericarp thickness contain m⁶A modification.

Altered m⁶A modification by direct injection of writer or eraser inhibitor represses the expansion of tomato fruits

To further evaluate the effect of m⁶A modification on fruit development, we employed a method of direct injection of m⁶A writer or eraser inhibitor into tomato fruits (Supplemental Figure S9). The inhibitors used in this study were 3-deazaneplanocin A (DA) and Meclofenamic acid (MA). DA acts as an S-adenosylhomocysteine synthesis inhibitor and is known to inhibit m⁶A methylation in animal cells (Fustin et al., 2013, 2018), whereas MA is a well-characterized inhibitor of the fat mass and obesity-associated protein, an m⁶A demethylase in animals (Huang et al., 2015). We injected DA or MA solution into the tomato fruits at 12 DAF and measured the size of tomato fruits 7 d later. We found that the injected fruits were smaller than those with mock treatment; the fresh weight, diameter, and length of the inhibitor-injected fruits decreased (Figure 6, A and B), indicating that DA or MA treatment inhibits tomato fruit expansion. Contrary to the inhibitory effects of DA and MA on tomato fruit expansion, DA or MA treatment accelerated or delayed fruit ripening, respectively (Figure 6A). To verify whether these changes in fruit development were due to altered m⁶A modification by the inhibitors, we analyzed m⁶A levels in tomato fruits subjected to DA or MA treatment. We found that DA and MA treatment decreased and increased m⁶A levels in RNA, respectively, compared with mock treatment (Figure 7A), suggesting that changes in the expansion and ripening of tomato fruits are because of altered m⁶A levels modulated by inhibitor treatment. These results are in line with a previous report that suggested that m⁶A levels gradually decreased during the ripening stage of tomato fruits and that increased m⁶A levels because of m⁶A demethylase inhibition were associated with delayed fruit ripening (Zhou et al., 2019). Considering that DA and MA are not specific inhibitors to the methylation and demethylation in RNA, we evaluated whether DA and MA affect methylation in DNA. The results showed that the levels of 6mA and 5mC in DNA were not significantly altered by DA and MA treatment (Supplemental Figure S10), suggesting that DA and MA do not influence DNA methylation during the expansion and ripening of tomato fruits. To further elucidate how altered m⁶A methylation affects fruit expansion, the expression levels of genes involved in fruit expansion were analyzed. Notably, transcript levels of several m⁶A-modified genes were altered after DA or MA treatment,

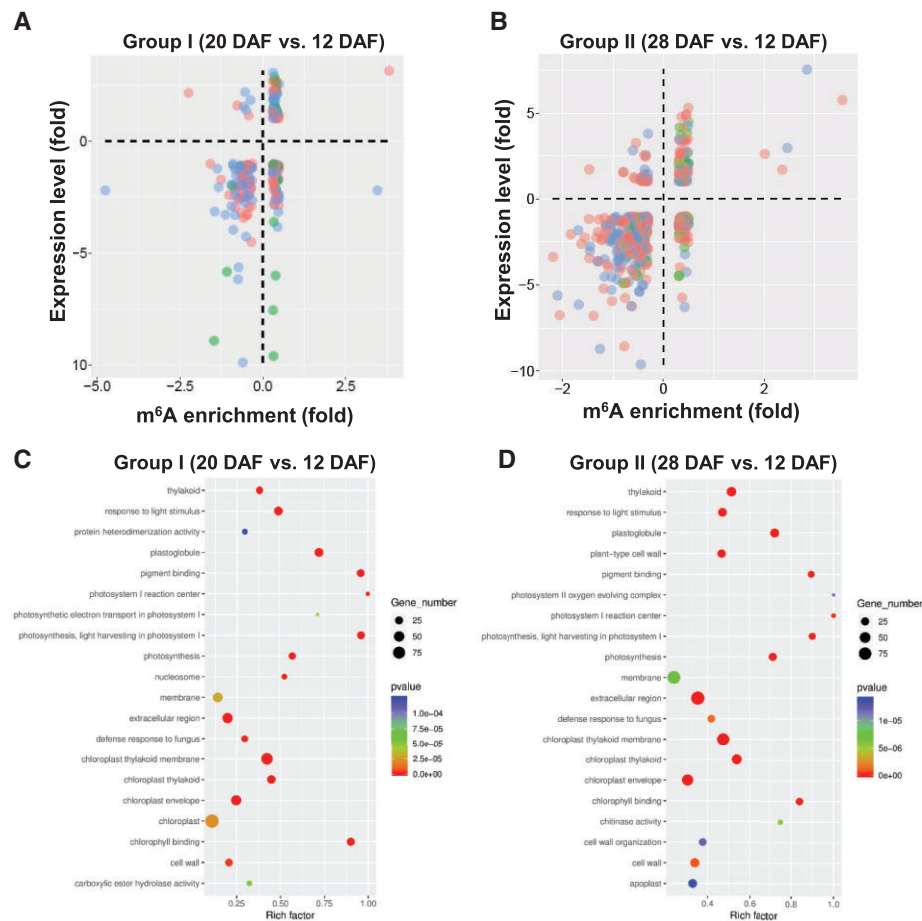


Figure 4 Association between m^6A methylation and gene expression. A and B, Association between m^6A modification and the expression of m^6A -containing genes in Groups I and II. C and D, GO analysis of the m^6A -containing DEGs identified in comparison Groups I and II.

whereas the levels of non- m^6A -modified genes were not affected by inhibitor treatment (Figure 7B; Supplemental Figure S11). Collectively, our results point to the importance of m^6A methylation in regulating the expansion of tomato fruits as well as the ripening of tomato fruits.

Discussion

Despite the increasing understanding of the crucial roles of RNA m^6A methylation in various biological processes, the importance of m^6A methylation in the expansion of fruits, including tomato, has never been explored. In this study, we showed a close association between m^6A modification and the expansion of tomato fruits. Notably, overall m^6A levels increase during tomato fruit expansion (Figure 2), which is in contrast to that seen in tomato fruit ripening (Zhou et al., 2019). As m^6A methylation is a dynamic and reversible process with regulatory functions (Yue et al., 2015; Liu and Pan, 2016; Meyer and Jaffrey, 2017; Hu et al., 2019; Zheng et al., 2020), differential m^6A methylation patterns during the different stages of fruit development suggest that the expression and/or activity of m^6A writers and erasers are modulated depending on the developmental stages of fruits. Through parallel m^6A -seq and RNA-seq analyses of tomato

fruits at different expansion stages, we demonstrated that a positive association exists between m^6A modification and the expression levels of the genes related to cell expansion, hormone signaling, and endoreduplication, which play a crucial role in the expansion of tomato fruits.

The m^6A methylome identified in tomato fruits at different expansion stages contains far more m^6A -modified genes than those identified in tomato fruits during ripening (Zhou et al., 2019), but it has a similar number of genes compared with those in other plants species, including Arabidopsis and maize (Shen et al., 2016; Luo et al., 2020; Hu et al., 2021). In this study, m^6A -seq revealed the presence of m^6A marks predominantly in the 3'-UTR of mRNAs in tomato fruits during all expansion stages (Figure 3), which is in line with the findings of other studies that have demonstrated the biased deposition of m^6A marks near the stop codon and in the 3'-UTR in plants under normal and stress conditions (Shen et al., 2016; Zhang et al., 2019; Zhou et al., 2019; Luo et al., 2020; Hu et al., 2021). These results suggest that although the abundance of m^6A could vary across species or depending on environmental stimuli, the deposition of m^6A marks primarily in the 3'-UTR of mRNAs is an evolutionarily conserved feature. Notably, as the major m^6A motif,

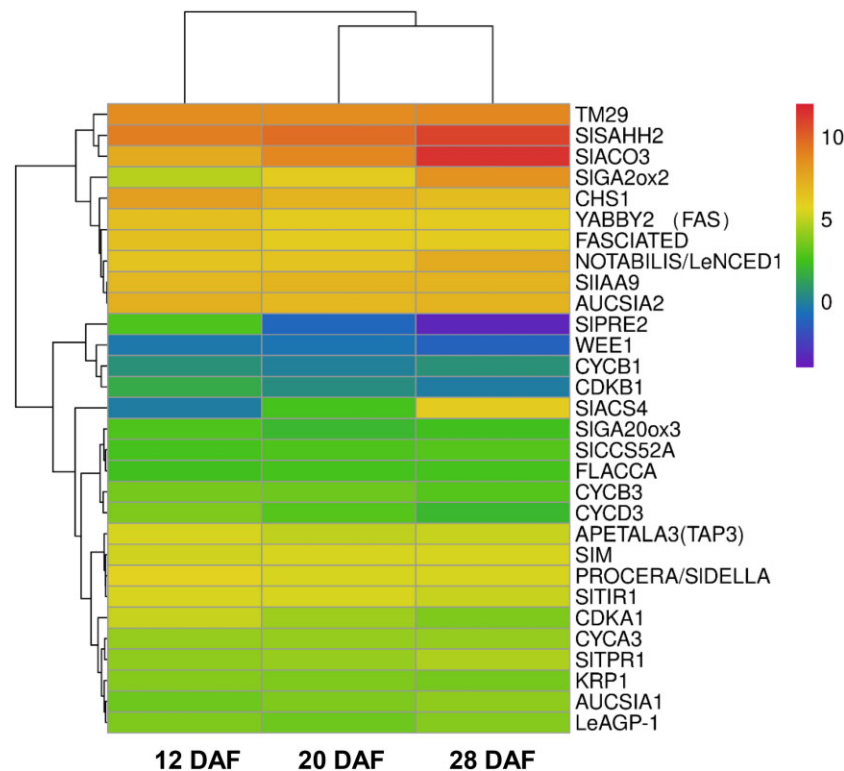


Figure 5 Expression levels of m⁶A-modified genes associated with tomato fruit expansion. Total RNA was extracted from tomato fruits at 12, 20, and 28 DAF and was subjected to RNA-seq analysis. The Heat Map represents average read depth obtained from two biological replicates.

we identified conserved URUAY (R = A/G, Y = U/A) sequence (Figure 3; Supplementary Figure S6), which is a previously identified plant-specific motif (Wei et al., 2018; Zhou et al., 2019; Luo et al., 2020; Miao et al., 2020; Hu et al., 2021). In addition, RRACH-like motifs, which were identified in animals (Dominissini et al., 2012, 2013; Meyer et al., 2012; Linder et al., 2015) and two plant species (Arabidopsis and maize; Duan et al., 2017; Miao et al., 2020) were also identified (Figure 3, Supplemental Figure S6). To determine the precise m⁶A motifs in plants, it is highly desirable to employ single-nucleotide resolution mapping of m⁶A, which has been successfully used in human and mouse mRNAs (Linder et al., 2015). Moreover, as all the transcripts that contain these conserved motifs are not methylated, it is important to determine how certain transcripts are selected for methylation by m⁶A writers. Several cellular factors, including transcription factors, histone marks, and microRNAs, have been shown to affect de novo m⁶A deposition at specific loci in animal transcripts (Chen et al., 2015; Shi et al., 2019; Huang et al., 2020); similarly, it will be interesting to determine the underlying mechanisms by which specific m⁶A motifs are selected for methylation in diverse plant species depending on the developmental and environmental cues.

The impact of mRNA m⁶A modification on gene expression is multifaceted. In mammals, m⁶A modification found mainly in the 3'-UTR of mRNA is negatively associated with gene expression (Yue et al., 2018). Several studies in Arabidopsis have demonstrated a negative link between m⁶A modification and gene expression under normal

conditions but a positive association under salt stress conditions (Anderson et al., 2018; Hu et al., 2021). Contrary to a previous study that showed an overall negative link between m⁶A modification and gene expression during the ripening of tomato fruits (Zhou et al., 2019), our results demonstrated an overall positive association between m⁶A modification and mRNA abundance during the expansion of tomato fruits (Figure 4). This opposite association between m⁶A methylation and gene expression during the different developmental stages of tomato fruits raises an intriguing question of how m⁶A modification differentially affects mRNA abundance depending on the developmental process of tomato fruits. Interestingly, a recent study has demonstrated an overall positive link between m⁶A modification and gene expression during the ripening of strawberry fruits (Zhou et al., 2021). Several studies have demonstrated that specific RNA-binding proteins (referred to as “readers”) recognize and bind m⁶A marks and play a vital role in determining mRNA fate (Hu et al., 2019; Huang et al., 2020; Shao et al., 2021). In particular, several YT521-B homology (YTH) domain-containing proteins as m⁶A readers have been identified to regulate either negatively or positively mRNA stability in animals and plants (Du et al., 2016; Shi et al., 2017; Huang et al., 2018; Wei et al., 2018; Baquero-Perez et al., 2019; Song et al., 2021). Considering the fact that many RNA-binding proteins, including YTH proteins, K-homology proteins, CCHC zinc-finger proteins, and RNA-recognition motif proteins, have been identified as potential m⁶A readers in mammalian cells (Edupuganti et al., 2017), it will be

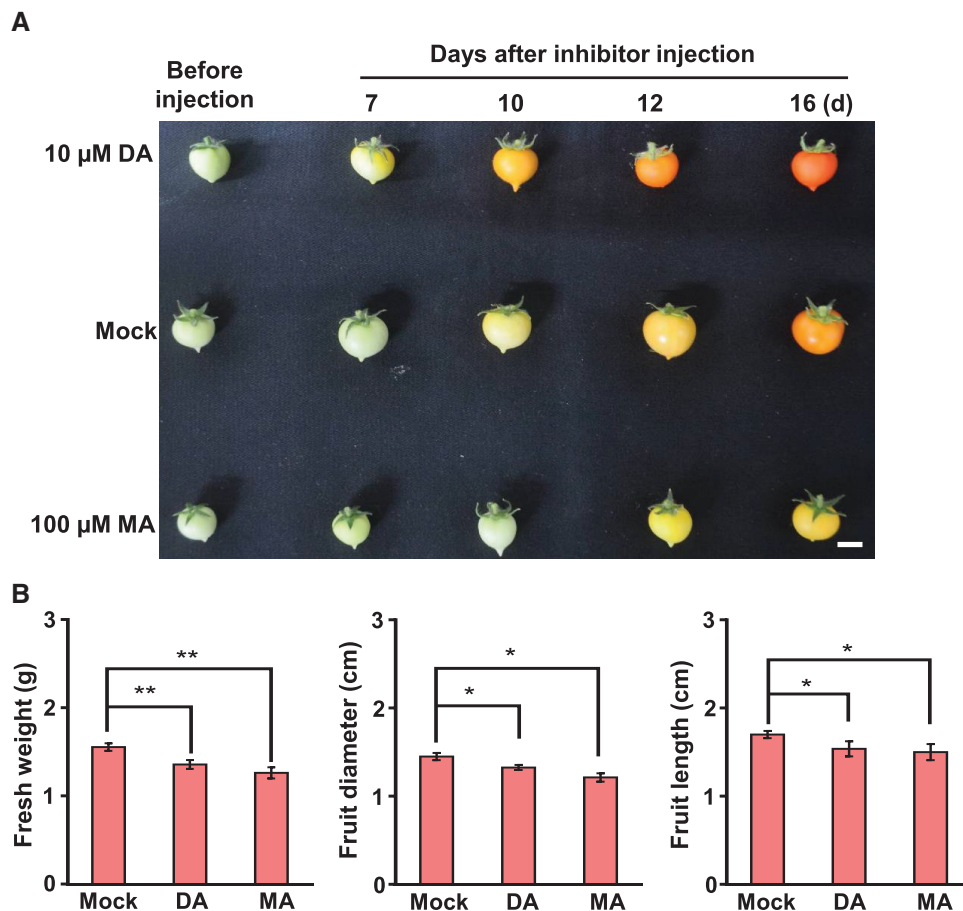


Figure 6 Effects of direct injection of m^6A writer or eraser inhibitor on tomato fruit development. A, Phenotypes of the tomato fruits before and after injection of DA or MA. Scale bar = 1 cm. B, The fresh weight, diameter, and length of the tomato fruits were measured 7 d after injection. Data represent means \pm standard deviation of three biological replicates ($n = 6$), and asterisks indicate significant differences (Student t test, $*P < 0.05$, $**P < 0.01$).

worthy to identify additional m^6A readers that are expressed during the expansion or ripening stages of tomato fruits and determine their roles in regulating the stability and abundance of RNA transcripts during tomato fruit development.

The growth of fruits depends on two cellular processes—cell division and cell expansion. Cell division occurs during the early stages after fruit setting, whereas fruit expansion occurring during the later stages is mainly due to cell expansion, which is influenced by phytohormones, such as auxin and gibberellic acid and endoreduplication. We found that in tomato fruit, a large portion of genes involved in hormone signaling pathways and endoreduplication are m^6A -modified (Supplemental Table S2). Analysis of RNA-seq data further revealed that m^6A -modified genes are expressed at a much higher level than non- m^6A -modified genes (Figure 5; Supplemental Figure S8). These results demonstrated the important role of m^6A modification in fruit expansion through the regulation of hormones and/or endoreduplication. Previously reported m^6A -seq data have also shown m^6A marks on a large number of genes involved in hormone signaling in Arabidopsis and maize (Shen et al., 2016; Luo et al., 2020; Hu et al., 2021). Moreover, recent reports have demonstrated that a decrease in m^6A levels due to

loss-of-function of an m^6A writer component influences the expression of many genes involved in hormone signaling pathways (Shen et al., 2016; Hu et al., 2021). Notably, the phenotypes of Arabidopsis and rice m^6A writer mutants mimic those of hormone-deficit mutant phenotypes, such as dwarfism, small size, and multiple branches (Růžička et al., 2017; Zhang et al., 2019). These observations suggest a close link between m^6A modification and the regulation of hormone signaling, which contributes to fruit development, as well as the normal growth of plants.

Decreased m^6A modification via knockout or knockdown of m^6A writer genes causes severe defects in plant growth and development (Zhong et al., 2008; Shen et al., 2016; Růžička et al., 2017; Zhang et al., 2019); therefore, it is impractical to evaluate the effect of m^6A modification on fruit development by using a transgenic approach. Instead, direct injection of m^6A writer inhibitor, DA (Fustin et al., 2013, 2018), or eraser inhibitor, MA (Huang et al., 2015), into the tomato fruits employed in this study is a valuable alternative to evaluate the effect of m^6A modification on fruit development. Interestingly, both exogenous m^6A writer and eraser inhibitors suppressed the expansion of tomato fruits, whereas writer inhibitor treatment and eraser inhibitor

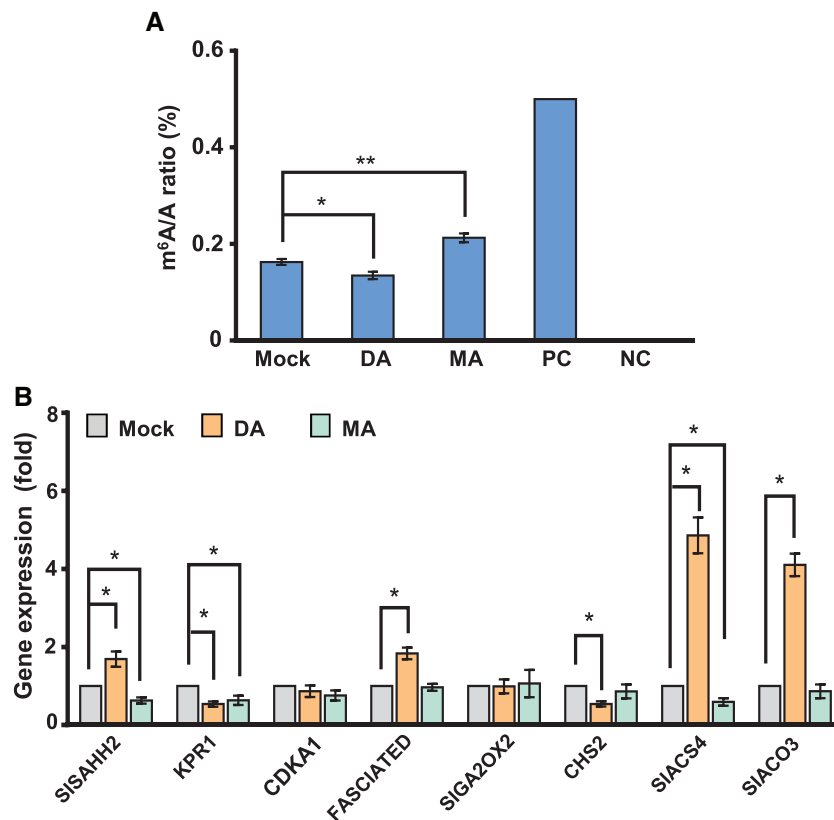


Figure 7 Effects of m⁶A writer or eraser inhibitor on global m⁶A modification and the expression of m⁶A-modified tomato fruit expansion-related genes. A, The levels of m⁶A in total RNA in tomato fruits 7 d after DA or MA injection. B, Transcript levels of tomato fruit expansion-related genes at 7 d after inhibitor injection were determined via RT-qPCR. Data represent means \pm standard deviation of three biological replicates ($n = 6$), and asterisks indicate significant differences (Student's *t* test, * $P < 0.05$, ** $P < 0.01$).

treatment accelerated and delayed fruit ripening, respectively (Figure 6). The delayed ripening of tomato fruits by m⁶A eraser inhibitor treatment and a concomitant increase in m⁶A levels observed in this study is in line with the previous report that has shown an increase in m⁶A levels by knockout of m⁶A demethylase causes delayed fruit ripening (Zhou et al., 2019). These observations indicate that the direct inhibitor injection approach can be used to mimic transgenic approaches. Although the mechanisms by which both m⁶A writer and eraser inhibitors suppress tomato fruit expansion are not known, we postulate that the maintenance of proper m⁶A homeostasis at different developmental stages is critical for fruit development. Notably, the expression of several m⁶A-modified genes involved in fruit expansion, such as KRP1, FASCIATED, SIACS4, and SIACO3, was modulated by either the m⁶A writer or eraser inhibitor treatment, whereas that of non-m⁶A-modified genes was not altered (Figure 7; Supplemental Figure S11), suggesting that m⁶A modification indeed affects the expression of genes involved in fruit growth. Kip-related Protein 1 (KRP1), as a cyclin-dependent kinase inhibitor, has been shown to participate in the control of endoreduplication (Bisbis et al., 2006), which is a critical process to determine cell size in developing tomato fruits. The number of locules is another

important factors contributing to fruit size. FASCIATED is one of the vital loci that negatively affect carpel number and ultimate locule number in tomato fruits (Barrero and Tanksley, 2004). SIACS4 and SIACO3 are two key genes involved in ethylene biosynthesis, which affects fruit development by modulating the levels of auxin-signaling repressor gene, such as SIAA9 (Wang et al., 2009). The decreased KRP1 levels and the increased FASCIATED, SIACS4, and SIACO3 levels observed in the tomato fruits after injection of m⁶A writer or eraser inhibitor (Figure 7) suggest an association between m⁶A levels and the levels of transcripts involved in fruit development. Moreover, GO analysis for all m⁶A-containing transcripts in developing tomato fruits showed an enrichment of genes related to chloroplast function, such as chloroplast biogenesis, photosynthesis, and pigment binding (Figures 3 and 4; Supplemental Figures S4 and S7), which is similar to a previous report demonstrating that a large number of transcripts with m⁶A marks are associated with chloroplast function in Arabidopsis (Luo et al., 2014). Considering that chloroplast biogenesis and function and photosynthesis are critical for the accumulation of starch and nutritional components during early fruit development (Nadakuđuti et al., 2014), our findings support the association between m⁶A modification and chloroplast

function during tomato fruit development. Understanding the molecular mechanisms connecting m⁶A modification to fruit expansion and ripening warrants further research.

In summary, this study uncovered important features of m⁶A methylome associated with the expansion of tomato fruits. Through parallel m⁶A-seq and RNA-seq analyses at three different stages of expansion, we found a positive association between m⁶A modification and mRNA abundance during fruit expansion, which is contradictory to what was observed during tomato fruit ripening. Importantly, we found that many cell expansion-related genes involved in hormone signaling and endoreduplication exhibit increased m⁶A methylation and that m⁶A-modified genes are generally expressed more actively than non-m⁶A-modified genes. Considering that a recent study has proposed a link between DNA methylation and m⁶A RNA methylation during fruit ripening (Zhou et al., 2019), it would be interesting to further determine the importance of DNA methylation as well as RNA methylation in the expansion and ripening of tomato fruits. Given that direct injection of m⁶A writer or eraser inhibitor into tomato fruits can alter global m⁶A levels and gene expression levels, thus affecting the expansion and ripening of tomato fruits, it would be a practical approach to evaluate the effect of m⁶A modification in fruit development. This study not only improves our understanding but also provides valuable background information required for further mechanistic studies on the role of m⁶A methylation in fruit development and ripening, which can be utilized to engineer epitranscriptomic RNA modifications for crop improvement.

Materials and methods

Plant materials and growth conditions

Micro-Tom tomato (*S. lycopersicum*) was used in this study. Micro-Tom seeds were surface sterilized with 70% (v/v) ethanol for 2 min, followed by 1% (w/v) sodium hypochlorite application for 10 min, and then rinsed with distilled water. For seed germination, seeds in distilled water were incubated at 28°C in the dark with gentle agitation for 2–3 d. The germinated seeds were transferred to soil consisting of vermiculite, peat moss, and perlite (in a 3:1:1 ratio) and grown in a growth room at 23 ± 2°C under long-day conditions (16-h-light/8-h-dark cycle). The plants were watered every 3 d. For measuring m⁶A levels, fruit samples were collected at 12, 16, 20, 24, 28, and 30 DAF. For phenotype analysis and m⁶A-seq and RNA-seq, the fruit samples were collected at 12, 20, and 28 DAF.

Measurement of m⁶A/A ratio

Total RNA was extracted from fruit samples using a Plant RNeasy extraction kit (Qiagen, Valencia, CA, USA), and mRNA was isolated from the total RNA using a poly(A) Spin mRNA Isolation Kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. The m⁶A level was determined using an EpiQuik m⁶A RNA Methylation Quantification Kit (EPiGENTEK, Farmingdale,

NY, USA) as described previously (Hu et al., 2021). Briefly, total RNA or mRNA were bound to strip wells containing an m⁶A-specific antibody. After washing the wells, capture and detection antibody solutions were added, and the signals were quantified colorimetrically by reading the absorbance at 450 nm. The m⁶A levels in the samples were calculated using a standard curve. The 6mA and 5mC levels in DNA were determined using a MethylFlash m⁶A DNA Methylation ELISA Kit and MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (EPiGENTEK, Farmingdale, NY, USA), respectively, according to the manufacturer's instructions.

m⁶A-MeRIP-seq and RNA-seq

All experiments regarding RNA preparation and RNA-seq were performed as described previously (Hu et al., 2021). Briefly, the poly(A) mRNAs were fragmented into ~100-nucleotide (nt)-long oligonucleotides using divalent cations, and the cleaved RNA fragments were subjected to m⁶A-immunoprecipitation by incubating with an m⁶A-specific antibody (Synaptic Systems, Goettingen, Germany) in IP buffer (50 mM Tris-HCl, 750 mM NaCl, 0.5% (w/v) Igepal CA-630, and 0.5 µg µL⁻¹ BSA) at 4°C for 24 h. The eluted m⁶A-containing immunoprecipitated fragments (IP fractions) and nonimmunoprecipitated fragments (input control) were used to construct a cDNA library according to a strand-specific library preparation protocol. The average insert size for the paired-end libraries was ~100 ± 50 bp. RNA sequencing was performed at LC-BIO Bio-tech Ltd (Hangzhou, China) using the Illumina Novaseq 6000 platform.

Analysis of m⁶A-seq and RNA-seq data

m⁶A-seq and RNA-seq data were analyzed as described previously (Hu et al., 2021). Briefly, Cutadapt (Martin, 2011) and Perl script in house were used to remove the reads that contained adaptor contamination, low-quality bases, and undetermined bases, after which sequence quality was verified using fastp. HISAT2 (Langmead and Salzberg, 2012) was used to map the reads to the genome of *S. lycopersicum* (version 3.2) with default parameters. Mapped reads of IP and input libraries were used for peak calling in the R package exomePeak (Meng et al., 2014), which identifies m⁶A peaks with bed or bam format that can be adapted for visualization on the IGV software (<http://www.igv.org/>). The called peaks were annotated by intersection with gene architecture using the R package ChIPseeker (Yu et al., 2015). StringTie (Pertea et al., 2015) was used to analyze expression levels of all mRNAs from input libraries by calculating Fragments Per Kilobase of transcript per Million (= total_exon_fragments/mapped_reads (millions) × exon_length (kb)). The differentially expressed mRNAs were selected with log₂ (fold change) > 1 or log₂ (fold change) less than -1 and *P* < 0.05 using R package edgeR (Robinson et al., 2010). The HOMER software-4.10/bin/findMotifs Genome.pl script (<http://homer.ucsd.edu/homer/motif/>) was used to detect conserved sequence motifs followed by localization of the motifs with respect to peak summits using Perl scripts. GO

analysis of m⁶A-modified genes was performed using the OmicStudio tools (<https://www.omicstudio.cn/tool>).

RT-qPCR and m⁶A-IP-qPCR

m⁶A-IP-qPCR was performed as previously described (Dominissini et al., 2013) with minor modifications (Hu et al., 2021). Briefly, the same mRNA used for m⁶A-MeRIP-seq has fragmented into ~300-nt-long fragments in an RNA fragmentation buffer (10 mM Tris-HCl, pH 7.0, 10 mM ZnCl₂). The fragmented mRNA was subjected to m⁶A-IP using an m⁶A-specific antibody as described above. The input RNAs and immunoprecipitated RNAs were reverse-transcribed using reverse transcriptase with random hexamer primers. The levels of each transcript were determined by quantitative real-time PCR conducted in a Rotor-Gene Q thermal cycler (Qiagen) using an SYBR Green PCR kit (Qiagen) and gene-specific primers listed in Supplemental Table S3 as previously described (Park et al., 2020). The PCR cycle threshold value for the immunoprecipitated sample was initially normalized against that of *ACTIN*, which has no internal m⁶A modification and serves as an internal control and then normalized against that of the input RNAs.

Direct injection of inhibitor solution into tomato fruits

The m⁶A writer or eraser inhibitor solution was directly injected into tomato fruits as previously described (Orzaez et al., 2006) with minor modifications. Briefly, tomato fruits at 12 DAF of the immature green stage were infiltrated using a 1-mL syringe with a 0.5-mm diameter needle. The needle was injected up to 3–4 mm into the fruit through the peduncle. Approximately 50 µL of inhibitor solution was injected each try, and the progress of the injection was monitored by observing a slight change in color in the infiltrated area and appearance of water drops through a hydathode. Once the entire fruit surface was infiltrated, several drops of infiltration solution began to run off the hydathode at the tip of the sepals. A total of 100 µL of 10 µM DA or 100 µM MA were introduced into the fruits via two successive injections at a 2-d interval.

Accession numbers

The MeRIP-seq and RNA-seq data used in this study have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database under accession number GSE178904.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. The m⁶A levels in rRNA during tomato fruit expansion stages.

Supplemental Figure S2. Pearson correlation analysis of input and immunoprecipitation reads between sequence profiles from two biological replicates of tomato fruits at 12, 20, and 28 DAF.

Supplemental Figure S3. Validation of m⁶A enrichment for six randomly selected m⁶A-modified genes.

Supplemental Figure S4. The conserved feature of m⁶A methylome in tomato fruits at different expansion stages.

Supplemental Figure S5. Venn diagram showing the overlap of m⁶A peaks in tomato fruits at immature green stage.

Supplemental Figure S6. Comparison of m⁶A methylome in tomato fruits at different expansion stages.

Supplemental Figure S7. Association between m⁶A methylation and gene expression in comparison Group III.

Supplemental Figure S8. Expression levels of non-m⁶A-modified genes associated with tomato fruit expansion.

Supplemental Figure S9. Exogenous application of m⁶A writer or eraser inhibitor into tomato fruits.

Supplemental Figure S10. Effects of m⁶A writer or eraser inhibitor on methylation in DNA.

Supplemental Figure S11. Effects of m⁶A writer or eraser inhibitor on the expression of non-m⁶A-modified tomato fruit expansion-related genes.

Supplemental Table S1. A summary of m⁶A-seq results of tomato fruits at 12, 20, and 28 DAF.

Supplemental Table S2. List of genes related to tomato fruit expansion.

Supplemental Table S3. List of primer pairs used in this study.

Supplemental Data Set S1. Genomic loci, fold enrichment, and associated genes for each m⁶A peak in tomato fruits at 12, 20, and 28 DAF.

Supplemental Data Set S2. Differential m⁶A peaks and gene expression in comparison Groups I, II, and III.

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