1 **N⁶-methyladenosine RNA modification regulates strawberry fruit ripening**
2 **in an ABA-dependent manner**

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4 **Leilei Zhou^{1,2,#}, Renkun Tang^{1,2,#}, Xiaojing Li^{1,2}, Shiping Tian^{1,2}, Bingbing Li³,**
5 **Guozheng Qin^{1,2*}**

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7 ¹Key Laboratory of Plant Resources, Institute of Botany, Innovation Academy for Seed
8 Design, Chinese Academy of Sciences, No.20 Nanxincun, Xiangshan, Haidian District,
9 Beijing 100093, China.

10 ²University of Chinese Academy of Sciences, Beijing 100049, China.

11 ³College of Horticulture, China Agricultural University, Beijing 100193, China.

12

13 [#]These authors contributed equally

14

15 ^{*}Correspondence: gzqin@ibcas.ac.cn

16

17 **Abstract**

18

19 **Background:** Epigenetic marks, such as DNA methylation, play pivotal roles in regulating
20 ripening of both climacteric and non-climacteric fruits. However, it remains unclear whether
21 mRNA m⁶A methylation, the epitranscriptome, is functionally conserved for ripening control.

22

23 **Results:** Here we show that m⁶A methylation, which has been revealed to regulate ripening
24 of tomato, a typical climacteric fruit, displays a dramatic change at ripening onset of
25 strawberry, a classical non-climacteric fruit. The m⁶A modification in the coding sequence
26 (CDS) regions appears to be ripening-specific and tends to stabilize the mRNAs, whereas
27 m⁶A around the stop codons and within the 3' untranslated regions is generally negatively
28 correlated with the abundance of the mRNAs. We identified thousands of transcripts with
29 m⁶A hypermethylation in the CDS regions, including those of *NCED5*, *ABAR*, and *AREB1* in
30 the abscisic acid (ABA) biosynthesis and signaling pathway. We demonstrated that the
31 methyltransferases MTA and MTB are indispensable for normal ripening of strawberry fruit,
32 and MTA-mediated m⁶A modification promotes mRNA stability of *NCED5* and *AREB1*,
33 while facilitates translation of *ABAR*.

34

35 **Conclusion:** Our findings uncover that m⁶A methylation regulates ripening of
36 non-climacteric strawberry fruit by targeting ABA pathway, which is distinct from that in
37 climacteric tomato fruit.

38

39 **Key words**

40 m⁶A methylation, m⁶A methyltransferase, abscisic acid, mRNA stability, translation

41 efficiency, strawberry, fruit ripening

42

43 Introduction

44 As the most prevalent chemical modification in eukaryotic messenger RNAs (mRNAs),
 45 N⁶-methyladenosine (m⁶A) has been demonstrated to functionally modulate multiple
 46 biological processes through interfering mRNA metabolism [1-4]. In mammals, m⁶A
 47 methylation has been unveiled to play critical roles in regulating various physiological and
 48 pathological processes, such as embryonic and post-embryonic development, cell circadian
 49 rhythms, and cancer stem cell proliferation [5-10]. The m⁶A marks in mammals are
 50 dominantly installed by the methyltransferase complex composed of the stable catalytic core
 51 that is formed by methyltransferase-like 3 (METTL3) and METTL14 [11-13], the Wilms
 52 tumor 1-associating protein (WTAP) [14], and other concomitant functional elements [15-17].
 53 The removal of m⁶A marks is executed by two m⁶A demethylases, fat mass and
 54 obesity-associated protein (FTO) and alkylated DNA repair protein AlkB homolog 5
 55 (ALKBH5) [18, 19]. The m⁶A modification are recognized by the reader proteins, such as
 56 YTH-domain family proteins and specific RNA binding proteins (RBPs), which mediate the
 57 downstream effects of m⁶A methylation [4]. In plants, the m⁶A methylation machineries have
 58 been characterized in the model plant *Arabidopsis thaliana* to modulate development
 59 processes such as shoot stem cell proliferation, trichome branching, and floral transition
 60 [20-23]. Moreover, m⁶A has been demonstrated to play pivotal roles in mediating
 61 sporogenesis in rice [24] and regulating stress responses in maize [25]. By contrast, the m⁶A
 62 methylation machineries as well as the characteristics and functions of m⁶A in regulating
 63 physiological processes of horticultural crops remain largely unknown.

64 Fleshy fruits, which are enriched with nutrients, such as flavor compounds, fiber, vitamins

65 and antioxidants, represent one of the commercially valuable structures of horticultural crops.
 66 As an important component of diets, fleshy fruits are indispensable for human health [26].
 67 The ripening of fleshy fruits, which is characterized by dramatic changes in color, texture,
 68 flavor and aroma compounds [27], is a complex, genetically programmed process that
 69 impacts fruit nutritional quality and shelf life. Fruit ripening is regulated by both
 70 environmental and internal cues, including light, temperature, phytohormones, and
 71 developmental genes [28, 29]. Based on the different ripening mechanisms, fruits are
 72 classified into two groups: climacteric (e.g. tomato, apple, banana, and avocado) and
 73 non-climacteric (e.g. strawberry, grape, and citrus) [30]. Phytohormone ethylene is essential
 74 for the ripening of climacteric fruits [31, 32], and substantial insights have been made toward
 75 ethylene biosynthesis, ethylene perception and signal transduction, and downstream gene
 76 regulation [33]. In comparison, the ripening of non-climacteric fruits is thought to be abscisic
 77 acid (ABA)-dependent [32, 34], although the regulation of ABA pathway is poorly
 78 understood. A comprehensive understanding of the common regulatory mechanisms
 79 underlying ripening in climacteric and non-climacteric species has great potential for
 80 improving fruit quality and maintaining shelf-life.

81 Recently, it was shown that epigenetic marks, including DNA methylation and histone
 82 posttranslational modifications, play critical roles in the regulation of fruit ripening [35]. In a
 83 previous study, we uncovered that mRNA m⁶A methylation, which is considered as an mRNA
 84 “epitranscriptome”, exhibits dynamic changes during fruit ripening of tomato, a typical
 85 climacteric fruit [36]. Mutation of *SLALKBH2*, the m⁶A RNA demethylase gene, delays fruit
 86 ripening [36], indicating that m⁶A modification participates in the ripening control of

climacteric fruit tomato. However, whether m⁶A is evolutionarily conserved among different types of fruits has not been defined. Moreover, the regulatory function of m⁶A in ripening of non-climacteric fruits remains elusive.

In the present study, we performed transcriptome-wide m⁶A methylation (m⁶A methylome) in strawberry, a classical non-climacteric fruit, and revealed that m⁶A represents a prevalent modification in the mRNAs of strawberry fruit. Compared to the dynamic changes in m⁶A modification around the stop codons or within the 3' untranslated regions during the ripening of tomato fruit, a specific enrichment of m⁶A in the coding sequence (CDS) region, which tends to be positively correlated with the abundance of the transcripts, was observed in the ripe strawberry fruit. We demonstrated that, mediated by the methyltransferases MTA and MTB, m⁶A modification enhances mRNA stability or promotes translation efficiency of genes in the ABA biosynthesis and signaling pathway, thereby facilitating the ripening of strawberry fruit. Our study uncovers the regulatory effects of m⁶A methylation on non-climacteric strawberry fruit ripening, and reveals a direct role for m⁶A methylation in the regulation of key elements in the ABA pathway.

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Results

m⁶A methylation is a common feature of mRNAs in strawberry fruit

To investigate whether m⁶A methylation participates in modulating ripening of non-climacteric fruits, we performed m⁶A-seq [37] to characterize m⁶A methylomes on diploid woodland strawberry (*Fragaria vesca*) at three developmental stages, i.e. S6 (the growth stage 6, approximately 15 days post-anthesis (DPA)), RS1 (the ripening stage 1, 21

DPA), and RS3 (the ripening stage 3, 27 DPA) (Fig. 1a) [38]. The transition from S6 to RS1 represents the initiation of ripening, and that from RS1 to RS3 indicates the phase after ripening initiation. The m⁶A methylome libraries were prepared with three independently biological replicates and subjected to massively parallel sequencing according to the standard m⁶A-seq protocols [37]. High Pearson correlation coefficients were observed between biological replicates, indicating reliable repeatability (Additional file 1: Figure S1, S2). A total of 24-37 million raw reads were generated for each library (Additional file 2: Table S1), and this sequencing depth is comparative to that observed in mammal (11-24 million reads) [39], rice (23-47 million reads) [40], and tomato (20-30 million reads) [36]. After adaptor trimming and reads filtration, 24-37 million clean reads were remained at each library, and almost 95% of these reads were uniquely aligned to the strawberry genome v1.1, representing high mapping quality (Additional file 2: Table S1). The peak-calling algorithm was used to identify m⁶A peaks with an estimated false discovery rate (FDR) < 0.05 [37], and only those consistently detected in all three biological replicates, which we called confident m⁶A peaks, were used for subsequent analysis. We identified 9778, 10853 and 10095 confident m⁶A peaks within 8934, 8990 and 8374 gene transcripts, in fruit at S6, RS1, and RS3, respectively (Fig. 1b; Additional file 2: Table S2-S4).

The m⁶A-seq results were validated by independent m⁶A immunoprecipitation followed by qPCR (m⁶A-IP-qPCR) analysis. Three m⁶A peak-containing transcripts, as well as three m⁶A peak-free transcripts were randomly selected and examined (Additional file 1: Figure S3a). As expected, m⁶A enrichment was only observed in transcripts containing m⁶A peaks, but not in those without m⁶A peaks (Additional file 1: Figure S3b), indicating that our m⁶A-seq data

were accurate and robust.

Based on the parallel RNA-seq analyses, we estimated that the transcriptome of diploid woodland strawberry contains 0.6-0.7 m⁶A peaks per actively expressed transcript, which shows FPKM (fragments per kilobase of transcript per million fragments mapped) ≥ 1 ([Additional file 2: Table S5](#)). These levels are comparable with those observed in *Arabidopsis* or tomato [36, 41], demonstrating that m⁶A modification is a common feature of mRNA in strawberry fruit. Most of the m⁶A-containing transcripts (>86%) possess one m⁶A peak. Intriguingly, the percentage of transcripts harboring two or more m⁶A peaks increases dramatically when the strawberry fruit turn to ripen, which changes from 2.95% at S6 to 13.07% at RS1, and 13.63% at RS3 ([Fig. 1c](#)), raising the possibility that new m⁶A peaks generate at the initiation stage of ripening. Gene Ontology (GO) enrichment analysis indicated that m⁶A modification appears in genes in multiple signaling pathways and cellular processes ([Fig. 1d](#)).

m⁶A distribution exhibits a dramatic change at the initiation stage of strawberry fruit ripening

We then evaluated the distribution of m⁶A peaks in the whole transcriptome of strawberry fruit. The transcript was divided into five non-overlapping segments: transcription start site (TSS, 100-nucleotide window centered on the TSS), 5' untranslated region (UTR), coding sequence (CDS), stop codon (100-nucleotide window centered on the stop codon), and 3' UTR. As shown in [Fig. 2a](#), m⁶A modifications in all three samples (fruit at S6, RS1, and RS3 stages) were highly enriched around the stop codon and within the 3' UTR, but the

percentage of peak summits within these regions declined markedly in the ripening process (from S6 to RS1 or RS3; indicated by green arrowheads). Surprisingly, a substantial increase in percentage of peak summits was concurrently observed in the CDS region adjacent to the start codon in fruit at RS1 or RS3 compared to those at S6 (indicated by red arrowheads). This is different from that observed in tomato, which shows no dramatic changes in percentage of peak summits at the initiation stage of ripening [36].

The percentage of peak summits locating in the CDS region increased from 5.18% to 23.26% from S6 to RS1, but displayed no distinct changes from RS1 to RS3 (Fig. 2b). In comparison, the percentage of peak summits falling into the stop codon region and the 3' UTR decreased from 39.95% and 53.81%, to 34.22% and 38.99%, respectively, from S6 to RS1, with no obvious changes observed from RS1 to RS3 (Fig. 2b). After segment normalization by the relative fraction that each segment occupied in the transcriptome, the m⁶A enrichment in fruit at RS1 or RS3 consistently exhibit a preferential localization in CDS region, besides enriched around the stop codon and within the 3' UTR (Fig. 2c). Thus, the transcriptome-wide m⁶A distribution displays a dramatic change at the initiation stage of ripening, but not after ripening initiation.

Several high-confidence sequence motifs were identified within the m⁶A peaks (Additional file 1: Figure S4), by using the hypergeometric optimization of motif enrichment (HOMER; <http://homer.ucsd.edu/homer/>) [42]. The conserved RRACH consensus sequence observed in mammals [43], where R represents adenosine (A) or guanosine (G), underlined A indicates m⁶A, and H represents A, cytidine (C), or uridine (U), appears in the list, whereas the UGUA sequence motif found in *Arabidopsis* [22], tomato [36], and maize [25] was not identified.

These data suggest the complexity of m⁶A modification among various species.

m⁶A methylation overall affects mRNA abundance during the ripening of strawberry fruit

To gain insight into the potential roles of m⁶A in the regulation of strawberry fruit ripening, we next sought for transcripts showing differential m⁶A peaks, which exhibit a fold change $\geq$ 1.5 and P value < 0.05 in m⁶A enrichment between the samples, by comparing the m⁶A methylomes. A total of 1608 hypermethylated m⁶A peaks and 865 hypomethylated m⁶A peaks, corresponding to 1398 and 790 transcripts, respectively, were identified in fruit at RS1 compared to those at S6 ([Fig. 3a](#); [Additional file 2: Table S6, S7](#)). By contrast, only 113 hypermethylated m⁶A peaks and 102 hypomethylated m⁶A peaks, which were distributed in 107 and 90 transcripts, respectively, were identified in fruit at RS3 compared to those at RS1 ([Fig. 3b](#); [Additional file 2: Table S8, S9](#)). These results confirmed that substantial changes in overall m⁶A methylome occurred at the initiation stage of ripening (from S6 to RS1), but not after ripening initiation (from RS1 to RS3).

The 1608 hypermethylated m⁶A peaks were highly enriched (83.04%) in the CDS region, whereas the 865 hypomethylated m⁶A peaks were mainly distributed around the stop codon (40.66%) or within the 3' UTR (58.59%) ([Fig. 3c](#)). This is in accordance with the results of m⁶A distribution ([Fig. 2](#)), showing that the percentage of peak summits locating in the CDS region increased sharply, while that around the stop codon or within the 3' UTR declined, during from S6 to RS1. Interestingly, of the 1608 hypermethylated m⁶A peaks, 1424 (88.56%) fell into the newly generated peaks, which represents ripening-specific peaks ([Additional file](#)

2: Table S10). For the differential m⁶A peaks identified after ripening initiation, both the hypermethylated and hypomethylated m⁶A peaks were highly enriched (over 90%) around the stop codon or within the 3' UTR (Fig. 3d).

Accumulating evidences have confirmed that m⁶A deposition affects mRNA abundance [1, 22, 41, 44]. To assess the potential correlation between m⁶A modification and mRNA abundance in strawberry fruit, we compared the list of transcripts harboring altered m⁶A methylation with the differentially expressed genes (fold change ≥ 1.5 and *P* value < 0.05) obtained from our parallel RNA-seq analyses (Additional file 2: Table S11, S12). Among the 1398 transcripts with hypermethylated m⁶A peaks in fruit at RS1 compared to those at S6, 440 and 147 transcripts displayed higher and lower expression levels, respectively (Fig. 3e; Additional file 2: Table S13). The distribution features of m⁶A modifications in these transcripts (Fig. 3c) suggest that m⁶A depositions in CDS region overall exhibit a positive effect on mRNA abundance. Accordingly, among the 790 transcripts showing hypomethylated m⁶A peaks in fruit at RS1 compared to those at S6, 267 transcripts showed higher expression levels, whereas only 51 transcripts exhibited lower expression levels (Fig. 3f; Additional file 2: Table S14). The negative role of m⁶A modifications on mRNA abundance was also observed in transcripts with hypermethylated (Fig. 3g) or hypomethylated (Fig. 3h) m⁶A peaks that were identified after ripening initiation (Additional file 2: Table S15, S16). Considering the distribution characteristics of m⁶A within these transcripts (Fig. 3c, d), it was proposed that, consistent with the observation in tomato fruit [36], m⁶A depositions around the stop codon or within the 3' UTR are generally negatively correlated with the abundance of the transcripts in strawberry fruit.

To further evaluate the potential correlation between m⁶A deposition and mRNA abundance, the changes in transcript levels were separately explored, based on the m⁶A distributions, for transcripts showing differential m⁶A modification at the initiation stage of ripening (from S6 to RS1). Gene expression profiles showed that most of the differential m⁶A-modified transcripts (approximately 75%) of the 5' UTR, CDS, stop codon, and 3' UTR categories exhibited relatively higher transcript levels (Fig. 3i), suggesting that m⁶A methylation overall affects mRNA abundance upon initiation of strawberry fruit ripening. Notably, 73 transcripts concurrently harboring hypermethylated m⁶A peaks in the CDS region and hypomethylated m⁶A peaks around the stop codon or within the 3' UTR were identified at this process. Of these, 34 transcripts were expressed at higher levels in fruit at RS1 compared to those at S6, while only 2 were expressed at lower levels (Fig. 3j), implying that the effects of m⁶A methylation on the abundance of the transcripts may be overlaid.

Notably, hundreds of ripening-induced and ripening-repressed genes, which display significantly higher or lower expression in RS1 compared to S6 (Additional file 2: Table S11), exhibit differential m⁶A modification (Additional file 2: Table S13, S14), implicating the involvement of m⁶A methylation in the regulation of strawberry fruit ripening.

Genes in ABA biosynthesis and signaling pathway exhibit differential m⁶a methylation upon ripening initiation

The plant hormone ABA has been elucidated to be essential for strawberry fruit ripening [32].

Two core ABA signal transduction pathways, the 'ABA-PYR/PYL-PP2C-SnRK2-AREB/ABF' pathway [45, 46] and the

241 ‘ABA-ABAR-WRKY40-ABI5’ pathway [47], have been proposed in *Arabidopsis*,
 242 respectively (Fig. 4a). In the m⁶A-seq analysis, we found that transcripts of key genes in ABA
 243 biosynthesis and signaling pathway, including *9-cis-epoxycarotenoid dioxygenase 5* (*NCED5*)
 244 [38], *putative ABA receptor* (*ABAR*) [34], and *ABA-responsive element-binding protein 1*
 245 (*AREB1*) [46], exhibit hypermethylation in the CDS region at the initiation stage of
 246 strawberry fruit ripening (from S6 to RS1) (Fig. 4b, c). *NCED5* encodes the rate-limiting
 247 enzyme for ABA biosynthesis, while *ABAR* and *AREB1* encode an ABA receptor and an
 248 ABA-responsive element, respectively. All three genes have been proposed to regulate
 249 strawberry fruit ripening [34, 38, 48]. The differential m⁶A modifications were confirmed by
 250 m⁶A-IP-qPCR analysis (Fig. 4d). The transcript levels of *NCED5* and *AREB1*, but not *ABAR*,
 251 increased significantly in fruit at RS1 compared to those at S6 as revealed by both RNA-seq
 252 (Fig. 4e) and quantitative RT-PCR analyses (Fig. 4f), indicating a positive correlation
 253 between m⁶A depositions and mRNA abundances. It is noteworthy that similar results of m⁶A
 254 enrichment as well as transcript levels of these three genes were found upon ripening
 255 initiation of the octoploid cultivated strawberry (*Fragaria* × *ananassa*) (Additional file 1:
 256 Figure S5).

257 To better understand how m⁶A methylation affects the abundance of the transcripts, we
 258 examined the mRNA stability of *NCED5*, *AREB1*, and *ABAR* by monitoring the degradation
 259 rate of mRNAs in the presence of transcription inhibitor actinomycin D. The intact CDS
 260 sequences of the three genes and their mutated forms in which the potential m⁶A sites
 261 identified in m⁶A-seq were mutated from A to cytidine (C) or guanosine (G) were separately
 262 inserted into the vector for transient expression in the *Nicotiana benthamiana* leaves (Fig. 4g).

As shown in Fig. 4h, the mRNAs of *NCED5*, *AREB1*, and *ABAR* degraded obviously after actinomycin D treatment. The degradation rate was substantially increased for mRNAs of *NCED5* and *AREB1*, but not *ABAR*, in the mutated form (Fig. 4h), concomitant with significantly diminished m⁶A depositions (Fig. 4i), indicating that site-specific m⁶A modification stabilizes mRNAs of *NCED5* and *AREB1*.

The m⁶A modification in the CDS region has been demonstrated to affect translation efficiency beyond mRNA stability [3]. We next investigated whether m⁶A methylation modulates translation efficiency of *NCED5*, *AREB1*, and *ABAR*, which was determined by calculating the abundance ratio of mRNA in the polysomal RNA versus the total RNA [49] (Fig. 4j). The translation efficiency of *ABAR* rather than *NCED5* and *AREB1* was significantly decreased when the potential m⁶A modification site was mutated (Fig. 4k), demonstrating that m⁶A methylation facilitates translation of *ABAR*. Collectively, these data suggest that critical genes in ABA pathway undergo m⁶A-mediated post-transcriptional regulation, which promotes mRNA stability or facilitates translation.

Characterizations of m⁶A methyltransferases in strawberry fruit

Having observed the changes in m⁶A methylation in a large number of transcripts (Fig. 3), including those of ABA biosynthesis and signaling genes (Fig. 4), during the ripening of strawberry fruit, we next explored the mechanistic basis. We speculate that the ripening-specific hypermethylation in the CDS region is regulated by mRNA m⁶A methyltransferases. In mammals, METTL3 and METTL14 form a stable heterodimer wherein METTL3 serves as the m⁶A catalytic subunit and METTL14 facilitates RNA binding [12, 13]

(Fig. 5a). The m⁶A methyltransferase complexes are conserved between mammals and plants [50], and the *Arabidopsis* mRNA adenosine methyltransferase (MTA) and MTB appears to be the homologs of METTL3 and METTL14, respectively [51, 52]. We identified the single homologs of *Arabidopsis* MTA and MTB in the genome of the diploid woodland strawberry. Phylogenetic analysis indicated that MTA and MTB exhibit high similarity among plant species and are evolutionarily conserved with mammal METTL3 and METTL14 (Fig. 5b). Both MTA and MTB in strawberry contain the highly conserved MT-A70 domain (Additional file 1: Figure S6), which displays extremely similar protein sequences to those observed in mammals and *Arabidopsis* (Additional file 1: Figure S7).

Transcriptome analysis showed that *MTA* and *MTB* increased significantly at the initiation stage of ripening (from S6 to RS1) (Fig. 5c). The homolog genes of *MTA* and *MTB* in the octoploid cultivated strawberry also exhibited increased expression from white (Wt) stage to initial red (IR) stage (Fig. 5d, e), suggesting that the two methyltransferases may play important roles in modulating strawberry fruit ripening.

To explore the possibility that MTA and MTB in strawberry function in the form of heterodimer as METTL3 and METTL14 in mammals [12, 13], we subsequently analyzed the interactions between MTA and MTB using the yeast two-hybrid (Y2H) system. As shown in Fig. 5f, the yeast cells co-expressing AD-MTA and BD-MTB, but not the negative controls, displayed normal growth on the selective SD/-Leu-Trp-His (-LWH) and SD/-Leu-Trp-His-Ade (-LWHA) solid medium and turn to blue with the addition of X- α -gal, indicating that MTA interacts with MTB. The interactions between MTA and MTB were further verified by the split luciferase complementation imaging (LCI) assay, in which the

luciferase activity was detected when MTA-nLUC and cLUC-MTB were co-expressed in *N. benthamiana* leaves (Fig. 5g). It should be noted that, compared with the MT-A70 domain of MTB, the full-length MTB protein exhibit relatively weaker combining capacity with MTA (Fig. 5g). Subcellular localization analysis by transiently expressing MTA-mCherry and MTB-eGFP fusion proteins in *N. benthamiana* leaves showed that MTA is present in both the nucleus and cytoplasm, while the MTB protein is specifically localized in the nucleus (Fig. 5h). Interestingly, when MTA-mCherry was co-expressed with MTB-eGFP, the two proteins tend to colocalization in the nucleus (Fig. 5i).

m⁶A methyltransferases positively regulate strawberry fruit ripening

We subsequently examined the function of MTA and MTB in strawberry fruit ripening. The RNA interference (RNAi) and overexpression constructs of *MTA* or *MTB* under the control of a 35S cauliflower mosaic virus promoter were agroinfiltrated into the octoploid strawberry fruit according to the reported procedures [53]. By comparing the fruits of RNAi with the control, we found that suppression of either *MTA* or *MTB* delayed fruit ripening (Fig. 6a). A visible color change was observed at the seventh days after agroinfiltration in the control, whereas the *MTA* or *MTB* RNAi fruits were almost green at this stage (Fig. 6a). Conversely, overexpression of *MTA* or *MTB* accelerated fruit ripening (Fig. 6a). Gene expression analysis indicated that *MTA* and *MTB* were successfully silenced in the RNAi fruits while enhanced in the overexpressed fruits (Fig. 6b). The ripening genes *chalcone synthase* (*CHS*) and *polygalacturonase 1* (*PGI*) displayed a noticeable decrease in the *MTA* or *MTB* RNAi fruits, but were dramatically enhanced in the overexpressed fruits (Fig. 6b). These results suggest

that *MTA* and *MTB* are necessary for normal fruit ripening of strawberry.

We next explored how the ripening of strawberry was regulated by *MTA*, the core component of the methyltransferase complexes with m⁶A catalytic activity. The *MTA*-overexpressed fruits displayed higher m⁶A levels than the control as revealed by LC-MS/MS (Fig. 6c). RNA immunoprecipitation (RIP) analysis indicated that the *MTA* directly binds to the transcripts of the ABA biosynthesis or signaling genes *NCED5*, *ABAR*, and *AREB1* (Fig. 6d). Accordingly, the m⁶A enrichments in the transcripts of *NCED5*, *ABAR*, and *AREB1* (Fig. 6e) and the corresponding mRNA levels (Fig. 6f) were significantly increased in the overexpressed fruits compared to the control. The mRNA stability assay demonstrated that mRNA of *NCED5* and *AREB1*, but not that of *ABAR*, degraded more slowly in the *MTA*-overexpressed fruits compared to the control (Fig. 6g). By contrast, *MTA* overexpression markedly enhanced the translation efficiency of the *ABAR* mRNA, but showed no significant effects on that of *NCED5* and *AREB1* (Fig. 6h). Together, these results suggest that *MTA* may regulate strawberry fruit ripening by targeting genes in the ABA pathway, leading to the increase in mRNA stability or translation efficiency of these genes.

***MTA*-mediated m⁶A methylation modulates genes encoding translation initiation factors and elongation factors**

Due to the critical role of ABA in the regulation of fruit ripening of strawberry, we evaluated whether *MTA* overexpression affects translation efficiency of other gene in the ABA signaling pathway. We found that genes, such as *WRKY DNA-binding protein 40* (*WRKY40*), exhibited significantly enhanced translation efficiency when the *MTA* was overexpressed (Additional

file 1: Figure S8). This could not be reasonably explained by m⁶A deposition because the transcripts of these genes are not m⁶A-modified according to our m⁶A-seq datasets. We speculate that MTA may regulate translation efficiency of numerous transcripts beyond direct m⁶A installation. As expected, *MTA* overexpression also elevated the translation efficiency of a number of transcripts of ripening-related genes without m⁶A modification, such as *PGI* relevant to firmness and *dihydroflavonol 4-reductase (DFR)* associated with anthocyanin biosynthesis (Additional file 1: Figure S8).

To investigate the possible mechanisms, we mined our m⁶A-seq and RNA-seq data and found that the transcripts of genes encoding translation initiation factors (*EIF2*, *EIF2B*, *EIF3A*, and *EIF3C*) and elongation factors (*EF1A*), which play pivotal roles in facilitating protein synthesis by promoting the initiation and elongation of mRNA translation, respectively, exhibited m⁶A hypermethylation in the CDS region upon ripening initiation of strawberry fruit (Fig. 7a, b), concomitant with an increase in the transcript levels of these genes (Fig. 7c). Similar results were observed in the ripening process of the octoploid strawberry fruit (Fig. 7d, e). RIP analysis showed direct interactions between MTA and the transcripts of *EIF2*, *EIF2B*, *EIF3A*, and *EIF3C*, and *EF1A* (Fig. 7f). More importantly, the m⁶A enrichment in these gene transcripts (Fig. 7g) and the mRNA abundance (Fig. 7h) increased noticeably when *MTA* was overexpressed. The mRNA degradation rates were obviously reduced in the *MTA*-overexpressed fruits compared to the control (Fig. 7i), implying that *MTA*-mediated m⁶A modification stabilizes mRNAs of these genes. We propose that, in addition to direct modulation of translation efficiency via m⁶A installation in the target transcripts, MTA may indirectly modulate translation efficiency via m⁶A-mediated

regulation of the translation initiation factors or elongation factors.

Discussion

m⁶A methylation participates in the regulation of strawberry fruit ripening

Compared to the understanding of the molecular basis underlying fruit ripening in climacteric fruits such as tomato, our knowledge regarding the regulation of ripening in non-climacteric fruits, e.g. strawberry, is still limited. Recently, it was elucidated that the ripening of strawberry involves the remodeling of DNA methylation [54]. However, it is unclear whether m⁶A methylation, which has been revealed to modulate the ripening of tomato fruit [36], is involved in the regulation of strawberry fruit ripening. In the present study, we found that m⁶A methylation represents a widespread mRNA modification in strawberry fruit and exhibits a dramatic change at the initiation stage of ripening. Overexpression of *MTA* or *MAB*, the m⁶A methyltransferase genes, promotes fruit ripening, whereas repression of either gene delays ripening (Fig. 6a, b), demonstrating that m⁶A methylation participates in the regulation of strawberry fruit ripening.

The transcript levels of *MTA* and *MTB* increased significantly upon ripening initiation of strawberry fruit (Fig. 5c-e), which may account for the m⁶A hypermethylation in the CDS region. It should be noted that the m⁶A methyltransferase genes in tomato express stably and no obviously global m⁶A hypermethylation was observed during fruit ripening [36]. By contrast, it is the m⁶A demethylase *SIALKBH2* that positively regulates tomato fruit ripening through mediating the mRNA stability of *SIDML2*, a key DNA demethylase gene determining the DNA methylation patterns during ripening [36]. Due to the pivotal role of DNA

methylation in the regulation of fruit ripening in both climacteric and non-climacteric fruits, it is reasonable to assume that m⁶A modification may modulate strawberry fruit ripening by modulating the DNA methylation machinery.

Strawberry undergoes an overall loss of DNA methylation during ripening, and the reprogramming is governed by components in the RNA-directed DNA methylation (RdDM) pathway rather than those in the demethylation pathway [54]. However, our m⁶A-seq data indicated that there was no differential m⁶A modification in the transcripts of DNA methyltransferase genes in the RdDM pathway during strawberry fruit ripening (Additional file 1: Figure S9), suggesting that distinct mechanisms underlie the m⁶A-mediated ripening regulation in strawberry fruit.

m⁶A methylation regulates strawberry fruit ripening by targeting ABA pathway

The phytohormone ABA plays crucial roles in plant growth, development, and stress responses [55]. The ripening of non-climacteric fruits, such as strawberry, has been revealed to be ABA-dependent [34]. Identification and functional definition of the core elements in ABA biosynthesis and signal transduction pathway have expanded our understanding of the mechanistic roles of ABA-mediated regulation of non-climacteric fruit ripening.

The ABA biosynthesis rate-limiting enzyme NCED was reported to play an essential role in the ripening of strawberry fruit [34, 38]. Moreover, several critical constituents of ABA signaling, including the ABA receptor FaPYR1 and FaABAR, the type 2C protein phosphatase FaABI1, and the SNF1-related kinase FaSnRK2.6, have been revealed to be indispensable for normal fruit ripening of strawberry [56-58]. Nevertheless, the regulatory

mechanisms underlying ABA biosynthesis and signaling pathway remain largely unknown. In *Arabidopsis*, ABA receptor PYLs could be regulated by post-translational modification, such as phosphorylation [59], tyrosine nitration [60], and ubiquitination [61], which synergistically modulate the abundance and activity of the receptors [55]. In contrast, the downstream signal molecules PP2Cs and SnRK2s are mainly controlled by protein phosphorylation and dephosphorylation for maintaining the appropriate ABA signal transduction [62-65]. The transcription of genes in ABA biosynthesis and signaling pathway displays dynamic changes in various development processes or in response to environmental stresses [55, 66-68], implying that they undergo precise regulation at transcriptional level. However, little is known about the regulation of genes in ABA pathway at the post-transcriptional level.

In this study, we found that *NCED5*, *ABAR*, and *AREB1*, the genes in ABA biosynthesis and signaling pathway undergo m⁶A-mediated post-transcriptional regulation (Fig. 4). The m⁶A modifications promote the mRNA stability of *NCED5* and *AREB1*, while enhance the translation efficiency of *ABAR*. These findings identify a novel layer of gene regulation in ABA biosynthesis and signaling pathway and establish a link between m⁶A-mediated ABA pathway and strawberry fruit ripening. Given the essential roles of ABA in plant development and stress resistance, it is interesting and necessary to explore the regulation of m⁶A methylation on these physiological processes.

m⁶A modification exhibits diverse effects on mRNAs in strawberry

As the most prominent modification in mRNA, m⁶A was initially thought to accelerate

degradation of mRNAs through promoting their transfer from the translatable pool to the decay sites [1]. Later findings have indicated that m⁶A modification also harbors the capacity to stabilize the mRNAs or promotes the translation in various biological processes [3, 22, 69, 70], although the underlying mechanisms are poorly understood, suggesting that m⁶A methylation possesses different regulatory roles on mRNAs [4]. This functional diversity was proposed to be heavily dominated by m⁶A distribution and the local sequence contexts within transcripts. One likely explanation is that the specific sequence contexts around m⁶A marks determine the recruitment of diverse m⁶A readers or other RNA binding proteins (RBPs) that carry distinguishing and even opposite molecular functions [4].

In this study, we observed a dramatic change in m⁶A modification at the initiation of strawberry fruit ripening (Fig. 2). The m⁶A depositions in the CDS region tend to stabilize the mRNAs, whereas those in the 3' UTR or around the stop codon exhibit the opposite effects (Fig. 3). These data suggest that, depending on their distribution, the m⁶A modification plays distinct roles on mRNAs in strawberry. Notably, the differential m⁶A modification in the ripening process of tomato fruit mainly appeared around the stop codon or within the 3' UTR, and these m⁶A depositions were generally negatively correlated with the abundance of the mRNAs. Combining the observation in strawberry and tomato, we propose that m⁶A modification around the stop codon or within the 3' UTR tends to negatively correlated with mRNA abundance in the ripening process of both climacteric and non-climacteric fruits, whereas m⁶A deposition in the CDS region generally stabilize the mRNAs upon ripening initiation of non-climacteric fruits. Interestingly, besides modulating mRNA stability, m⁶A modification also affected translation efficiency of some transcripts. The changes in

translation efficiency might be directly regulated by m⁶A deposition on the transcripts, or indirectly by m⁶A-mediated regulation of translation initiation factors and elongation factors.

In conclusion, our work reveals a regulatory role of m⁶A methylation on the ripening of the non-climacteric strawberry fruit. The molecular basis, which involves the m⁶A-mediated regulation of genes in the ABA biosynthesis and signaling pathway, is distinct from what was described in climacteric tomato fruit ([Additional file 1: Figure S10](#)). These findings provide new insights into understanding the regulatory networks controlling fruit ripening.

Methods

Plant materials

The diploid woodland strawberry (*Fragaria vesca* cv. ‘Hawaii-4’) and the octoploid cultivated strawberry (*Fragaria × ananassa* cv. ‘Benihoppe’) were planted in a greenhouse with standard culture conditions [58]. To accurately determine fruit ages through development, flowers were tagged at the anthesis. Fruits of ‘Hawaii-4’ at the growth stage 6 (S6), the ripening stage 1 (RS1), and the ripening stage 3 (RS3) [38], which were on average 15, 21, and 27 days post-anthesis (DPA), respectively, were harvested, and then frozen in liquid nitrogen. The fruits with the removal of attached achenes were subsequently stored at –80 °C until use. The ‘Benihoppe’ fruits were harvested at the small green (SG), large green (LG), white (Wt), initial red (IR), and full red (FR) stages, respectively, based on the size, weight, shape, and color [71], and then maintained at fresh status for further studies or directly frozen and stored as the ‘Hawaii-4’.

483 **m⁶A-seq and data analysis**

484 The m⁶A-seq was performed according to the method described by Dominissini et al (2013)
 485 [37]. Briefly, total RNAs were extracted from the woodland strawberry fruit at S6, RS1, and
 486 RS3 stages by the plant RNA extraction kit (Magen, R4165-02), and then 300 µg of intact
 487 total RNAs were used for mRNA isolation by the Dynabeads mRNA purification kit (Life
 488 Technologies, 61006). The purified mRNAs were randomly fragmented into ~100
 489 nucleotide-long fragments by incubation at 94 °C for 5 min in the RNA fragmentation buffer
 490 (10 mM Tris-HCl, pH 7.0, and 10 mM ZnCl₂). The reaction was terminated with 50 mM
 491 EDTA, and then the fragmented mRNAs were purified by standard phenol-chloroform
 492 extraction and ethanol precipitation. For immunoprecipitation (IP), 5 µg of fragmented
 493 mRNAs was mixed with 10 µg of anti-m⁶A polyclonal antibody (Synaptic Systems, 202003)
 494 and incubated at 4 °C for 2 h in 450 µL of IP buffer consisting of 10 mM Tris-HCl, pH 7.4,
 495 150 mM NaCl, 0.1% NP-40 (v/v), and 300 U mL⁻¹ RNase inhibitor (Promega, N2112S).
 496 After the addition of 50 µL Dynabeads protein-A (Life Technologies, 10002A), the mixture
 497 was incubated at 4 °C for another 2 h. The beads were subsequently washed twice with
 498 high-salt buffer containing 50 mM Tris-HCl, pH 7.4, 1 M NaCl, 1 mM EDTA, 1% NP-40
 499 (v/v), and 0.1% SDS (w/v) and twice with IP buffer. The m⁶A-containing fragments were
 500 eluted from the beads by incubation with 6.7 mM N⁶-methyladenosine (TargetMol, T6599) in
 501 IP buffer at 4 °C for 2 h, followed by standard phenol-chloroform extraction and ethanol
 502 precipitation. Then, 50 ng of m⁶A-containing mRNAs or pre-immunoprecipitated mRNAs
 503 (the input) were used for library construction by the NEBNext ultra RNA library preparation
 504 kit (NEB, E7530). High-throughput sequencing was conducted on the illumina HiSeq X

sequencer with a paired-end read length of 150 bp following the standard protocols. The sequencing was performed with three independent biological replicates, and each RNA sample was prepared from the mix of at least 60 strawberry fruits to avoid individual difference among fruits.

For data analysis, the quality of raw sequencing reads was initially assessed by FastQC tool (version 0.11.7; <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Adaptors and low-quality bases with a score < 20 were trimmed using Cutadapt (version 1.16) [72], and then short reads with a length < 18 nucleotides or reads containing ambiguous nucleotides were filtered out by Trimmomatic (version 0.30) [73]. The remaining reads were aligned to the strawberry reference genome v1.1 (ftp://ftp.bioinfo.wsu.edu/species/Fragaria_vesca/Fvesca-genome.v1.1) by Burrows Wheeler Aligner (BWA; version 0.30) [74]. Mapping quality (MAPQ) of all aligned reads was concurrently evaluated, and only uniquely mapped reads with a MAPQ ≥ 13 were retained for subsequent analysis [37].

The identification of m⁶A peaks was carried out by MACS software (version 2.0.10) [75], using the corresponding input as a control. High-confidence peaks were obtained by a stringent cutoff threshold for MACS-assigned false discovery rate (FDR) < 0.05 . PeakAnalyzer (version 2.0) [76] was applied to annotate the identified peaks to the strawberry genome annotation file (ftp.bioinfo.wsu.edu/species/Fragaria_vesca/Fvesca-genome.v2.0.a2/genes/). To identify the differentially methylated peaks between samples, the m⁶A site differential algorithm [77] was applied with a criterion of fold change in m⁶A enrichment ≥ 1.5 and P value < 0.05 . HOMER

(version 4.7; <http://homer.ucsd.edu/homer/>) [78] was employed to identify the m⁶A motifs with a restricted length of six nucleotides. The differential m⁶A peaks identified between S6 and RS1 stages were used as the target sequences, and the exon sequences without m⁶A peaks were used as the background sequences. Integrated Genome Browser (IGB, version 9.0.2) [79] was used for visualization of m⁶A peaks. Gene Ontology (GO) enrichment was analyzed on the agriGO database (version 2.0; <http://systemsbiology.cau.edu.cn/agriGOv2/>) and only statistically significant terms with a Yekutieli-corrected *P* value < 0.05 were remained [80].

RNA-seq and data analysis

The sequencing reads from the input samples in m⁶A-seq were used for RNA-seq analysis as previously reported [21]. In brief, the uniquely mapped reads with a MAPQ ≥ 13 were assembled by Cufflinks [81]. Gene expression was presented as fragments per kilobase of exon per million mapped fragments (FPKM) by using Cuffdiff [81], which concurrently provides statistical routines for capturing differentially expressed genes. The Benjamini and Hochberg's approach [82] was used to adjust the resulting *P* values for controlling the false discovery rate (FDR). Differential gene expression was defined on basis of a cutoff criterion of FPKM fold change ≥ 1.5 and *P* value < 0.05.

Quantitative RT-PCR analysis

Total RNAs were extracted from the strawberry fruits or *N. benthamiana* leaves using the plant RNA extraction kit (Magen, R4165-02). The extracted RNAs were reverse transcribed into cDNAs by the HiScript[®] III RT SuperMix for qPCR kit (Vazyme, R323-01). The

synthesized cDNAs were then employed as templates for PCR amplification using the ChemQ Universal SYBR qPCR Master Mix (Vazyme, Q711-02-AA) and a StepOne Plus Real-Time PCR System (Applied Biosystems) in the following program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative quantification of gene transcription levels were performed by the cycle threshold (C_T) $2^{(-\Delta CT)}$ method [83]. Strawberry *ACTIN* (gene22626) or *N. benthamiana ACTIN* (*Niben101Scf03410g03002*) was applied to normalize the expression values. The primers for PCR amplification are listed in [Additional file 2: Table S17](#). The experiment was conducted with three biological replicates, and each contained three technical repeats.

m⁶A-IP-qPCR

m⁶A-IP-qPCR was carried out as previously described with minor modifications [84]. Briefly, 5 µg of intact mRNAs were fragmented into ~300 nucleotide-long fragments by an incubation at 94 °C for 30 s in the RNA fragmentation buffer (10 mM Tris-HCl, pH 7.0, and 10 mM ZnCl₂), followed by the addition of 50 mM EDTA to terminate the reaction. The fragmented mRNAs were purified by standard ethanol precipitation and resuspended in 250 µL of DEPC-treated water. Then, 5 µL of fragmented mRNAs was taken as the input control and 100 µL were incubated with 5 µg of anti-m⁶A polyclonal antibody (Synaptic Systems, 202003) at 4 °C for 2 h in 450 µL of IP buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40 (v/v), and 300 U mL⁻¹ RNase inhibitor (Promega, N2112S). The mixture was subsequently incubated with 20 µL of Dynabeads protein-A (Life Technologies, 10002A) at 4 °C for another 2 h. After washing twice with high-salt buffer containing 50 mM Tris-HCl,

571 pH 7.4, 1 M NaCl, 1 mM EDTA, 1% NP-40 (v/v), and 0.1% SDS (w/v) and twice with IP
572 buffer, the m⁶A-containing fragments were eluted by an incubation with 6.7 mM
573 N⁶-methyladenosine (m⁶A; TargetMol, T6599) in 200 µL of IP buffer at 4 °C for 2 h,
574 followed by ethanol precipitation. The immunoprecipitated mRNA fragments were finally
575 resuspended in 5 µL DEPC-treated water. Then, both the m⁶A-containing mRNAs and the
576 input mRNAs were submitted to quantitative RT-PCR by using the primers listed in
577 [Additional file 2: Table S17](#). Relative m⁶A enrichment in specific region of a transcript was
578 calculated using the cycle threshold (C_T) 2^(-ΔCT) method [83]. The value for the
579 immunoprecipitated sample was normalized against that for the input. The experiment was
580 performed with three biological replicates, and each contained three technical repeats.

581

582 **Identification of strawberry m⁶A methyltransferases and phylogenetic analysis**

583 To identify m⁶A methyltransferases in strawberry, the amino acid sequence of conserved
584 MT-A70 domain (PF05063) was downloaded from the Pfam (<http://pfam.xfam.org/>) and then
585 utilized to search the potential homologs against the strawberry protein dataset
586 (ftp.bioinfo.wsu.edu/species/Fragaria_vesca/Fvesca-genome.v2.0.a2/genes/) using HMMER
587 3.1 with default parameters [85]. The resulting protein sequences were analyzed on the CDD
588 database (<https://www.ncbi.nlm.nih.gov/cdd/>) [86], and only that containing a MT-A70
589 domain were remained as the m⁶A methyltransferase candidates. The known m⁶A
590 methyltransferases METTL3 and METTL14 in mammals and MTA and MTB in *Arabidopsis*
591 were then employed to perform BLASTP-algorithm in NCBI (<https://www.ncbi.nlm.nih.gov/>)
592 to further confirm the identified homologs. For phylogenetic analysis, the sequences of the

identified strawberry m⁶A methyltransferase were aligned with the sequences of m⁶A methyltransferase in mouse (*Mus musculus*), rice (*Oryza sativa*), maize (*Zea mays*), tomato (*Solanum lycopersicum*), tobacco (*Nicotiana benthamiana*), and *Arabidopsis*, using ClustalX 2.1 with standard parameters [87]. The alignment result was imported into MEGA software (version 5.2) to create the phylogenetic tree by using Neighbor-Joining method with 1000 bootstrap replicates [88].

Y2H analysis

Y2H analysis was performed as the previously described [89]. In brief, the coding sequence of *MTA* and *MTB* was amplified from the cDNAs of diploid woodland strawberry and then ligated into the pGADT7 (AD) and pGBKT7 (BD) vectors, respectively. The resulting plasmids were co-transformed into *Saccharomyces cerevisiae* strain AH109 according to the protocols in the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). The yeast cells were cultured on SD/-Leu-Trp (-LW) medium, and then transferred onto the SD/-Leu-Trp-His (-LWH) or SD/-Leu-Trp-His-Ade medium (-LWHA) with or without X- α -gal. Transformants carrying empty pGADT7 (AD) or pGBKT7 (BD) vectors were used as negative controls, and that concurrently carrying pGBKT7-53 and pGADT7-T vectors was used as the positive control. The primers used for vector constructions are listed in [Additional file 2: Table S17](#).

LCI assay

LCI assay was performed as previously reported [90]. Briefly, the coding sequence of *MTA* was cloned into the pCambia1300-nLUC plasmid, and the coding sequence of *MTB* or its

615 MT-A70 domain sequence (*MTB^D*) was cloned into the pCambia1300-cLUC plasmid. The
 616 resulting constructs were separately transformed into *Agrobacterium tumefaciens* strain
 617 GV3101. The agrobacteria were cultured at 28 °C for 18 h in Luria-Bertani (LB) liquid
 618 medium containing 50 µg mL⁻¹ kanamycin, 50 µg mL⁻¹ gentamycin, and 50 µg mL⁻¹
 619 rifampicin. After being pelleted by centrifugation at 5,000 g for 5 min, the agrobacteria were
 620 resuspended in the infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂, and 100 µM
 621 acetosyringone) to a final OD₆₀₀ of 0.5. Then, the suspension was infiltrated into *N.*
 622 *benthamiana* leaves for co-expression of fusion protein nLUC-MTA with MTB-cLUC or
 623 MTB^D-cLUC. After culture for 36 h, the leaves were incubated with 1 mM luciferin dissolved
 624 in ddH₂O supplemented with 0.01% Triton X-100 at room temperature for 5min, and then
 625 observed under a chemiluminescence imaging system (Tanon). Empty vectors expressing
 626 cLUC or nLUC were co-transformed as the negative controls. The primers used for vector
 627 constructions are listed in [Additional file 2: Table S17](#).

628

629 **Subcellular localization**

630 For subcellular localization analysis, the coding sequence of *MTA* and *MTB* was amplified
 631 from the cDNAs of diploid woodland strawberry and then inserted into the
 632 pCambia2300-mCherry and pCambia2300-eGFP plasmids to generate 35S::*MTA-mCherry*
 633 and 35S::*MTB-eGFP* vectors, respectively. The resulting constructs were separately
 634 transformed into *A. tumefaciens* strain GV3101. The agrobacteria were subsequently
 635 infiltrated into *N. benthamiana* leaves for the individual expression of mCherry-tagged MTA
 636 (MTA-mCherry) and eGFP-tagged MTB (MTB-eGFP) or the co-expression of the two fusion

proteins. After culture for 36 h, the mesophyll protoplasts were isolated from *N. benthamiana* leaves as previously reported [91] and observed under a Leica confocal microscope (Leica DMI600CS). Protoplasts expressing eGFP or mCherry were used as negative controls. The primers used for vector constructions are listed in [Additional file 2: Table S17](#).

Agroinfiltration-mediated transient transformation in strawberry fruit

Transient transformation of strawberry fruit mediated by agroinfiltration was performed as previously described [53]. To construct the RNA interference (RNAi) vectors, a ~300 bp fragment targeting the coding sequence region of *MTA* or *MTB* was cloned and inserted into the pCR8 plasmid, and then restructured into the pK7GWIWGD (II) plasmid by using the Gateway LR ClonaseTM Enzyme Mix (Invitrogen, 11791-020). To construct the overexpression (OE) vectors, the coding sequence of *MTA* and *MTB* was amplified and ligated into the pCambia2300-eGFP plasmid to generate 35S::*MTA-eGFP* and 35S::*MTB-eGFP* vector, respectively. The resulting constructs were separately transformed into the *A. tumefaciens* strain GV3101. The agrobacteria were cultured at 28 °C overnight in LB liquid medium supplemented with 50 µg mL⁻¹ kanamycin, 50 µg mL⁻¹ gentamycin, and 50 µg mL⁻¹ rifampicin, and then diluted 1:100 in 100 mL of fresh LB medium to continue culturing for approximately 8 h. The agrobacteria cells were subsequently collected by centrifugation at 5,000 g for 5 min and resuspended in the infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂, and 100 µM acetosyringone) to a final OD₆₀₀ of 0.8. After being kept at room temperature for 2 h without shaking, the suspensions were injected into the octoploid strawberry fruit at large green (LG) stage by using a 1 mL syringe. The infiltrated fruits were

cultured for 5-7 days in a growth room with the following conditions: 23 °C, 80 % relative humidity, and a 16/8 h light/dark photoperiod with a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The experiment was performed with three independent biological replicates, and each group contained at least fifteen fruits. The primers used for vector constructions are listed in [Additional file 2: Table S17](#).

mRNA stability assay

For mRNA stability assay in *N. benthamiana* leaves, the coding sequence of *NCED5*, *ABAR*, and *AREBI* was amplified from the cDNAs of diploid woodland strawberry. The mutated forms of the amplified sequence in which the potential m⁶A sites were replaced by cytidine (C) or guanine (G) were constructed using the QuikChange II XL Site-directed Mutagenesis Kit (Agilent Technologies, 200518). The fragments were separately inserted into the pCambia2300 vector, which were subsequently transformed into *A. tumefaciens* strain GV3101. Then, the agrobacteria were cultured at 28 °C for 18 h in LB liquid medium containing 50 $\mu\text{g mL}^{-1}$ kanamycin, 50 $\mu\text{g mL}^{-1}$ gentamycin, and 50 $\mu\text{g mL}^{-1}$ rifampicin. After being collected by centrifugation at 5,000 g for 5 min, the agrobacteria were diluted to an OD₆₀₀ of 0.5 in the infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂, 100 μM acetosyringone), and then infiltrated into the *N. benthamiana* leaves. After 36 h of incubation, the infiltration parts in the leaves were injected with 20 $\mu\text{g mL}^{-1}$ actinomycin D (Sigma, A4262) dissolved in ddH₂O. After culture for 30 min, leaf discs were taken and considered as time 0 controls, and subsequent samples were harvested every 3 h in triplicate.

For mRNA stability assay in strawberry, the *MTA*-overexpressed fruit and the control were

sliced into ~3 mm slices, and then transferred onto plates containing 20 $\mu\text{g mL}^{-1}$ actinomycin D dissolved in ddH₂O. After incubation for 30 min, the slices were collected as the *N. benthamiana* leaf discs. The mRNA levels of genes were subsequently examined by quantitative RT-PCR as described above. All primers used for PCR amplification were listed in [Additional file 2: Table S17](#).

Translation efficiency assay

Translation efficiency was assayed according to the method described by Merchante et al (2015) [49]. Briefly, 6 g of strawberry fruits or 3 g of *N. benthamiana* leaves were ground into fine powder in liquid nitrogen. One gram of sample was used for total RNA extraction, and the rest was suspended in 15 mL of polysome extraction buffer (200 mM Tris-HCl, pH 9.0, 35 mM MgCl₂, 200 mM KCl, 25 mM EGTA, 1% Triton X-100 (v/v), 1% IGEPAL CA-630 (v/v), 5 mM DTT, 1 mM PMSF, 50 $\mu\text{g mL}^{-1}$ Chloramphenicol, and 100 $\mu\text{g mL}^{-1}$ Cycloheximide) at 4 °C for 20 min with slight shaking. The mixture was centrifuged twice at 16,000 g for 20 min at 4 °C. Then, 12.5 mL of supernatant were slowly transferred onto 13.5 mL of sucrose buffer (1.75 M Sucrose, 400 mM Tris-HCl, pH 9.0, 35 mM MgCl₂, 5 mM EGTA, 200 mM KCl, 5 mM DTT, 50 $\mu\text{g mL}^{-1}$ Chloramphenicol, and 50 $\mu\text{g mL}^{-1}$ Cycloheximide). After centrifugation at 200,000 g for 4 h at 4 °C, the supernatant was carefully removed, and the polysomes in the bottom were resuspended in 300 μL of DEPC-treated water. The polysomal RNAs, as well as the total RNAs, were isolated by using the plant RNA extraction kit (Magen, R4165-02), and used for quantitative RT-PCR analysis as described above. Translation efficiency was calculated by the abundance ratio of mRNA in

the polysomal RNA versus the total RNA using the cycle threshold (CT) $2^{(-\Delta CT)}$ method [83] with the *GADPH2* gene as an internal reference. The primers used for PCR amplification are listed in [Additional file 2: Table S17](#).

Quantitative analysis of m⁶A level by LC-MS/MS

The global m⁶A levels in strawberry fruit were detected by LC-MS/MS as previously described [36] with minor modifications. In brief, 200 ng of mRNAs was digested at 37 °C for 6 h with 1 unit of Nuclease P1 (Wako, 145-08221) in 50 µL of reaction buffer containing 10 mM ammonium acetate, pH 5.3, 25 mM NaCl, and 2.5 mM ZnCl₂. Then, 1 unit of alkaline phosphatase (Sigma-Aldrich, P6774) and 5.5 µL of 1 M fresh NH₄HCO₃ were added, followed by incubation at 37 °C for another 6 h. After centrifugation at 15,000 g for 5 min, the supernatant was used for LC-MS/MS analysis. The digested nucleosides were separated by UPLC (Waters, ACQUITY) equipped with a ACQUITY UPLC HSS T3 column (Waters), and then detected by a Triple Quad Xevo TQ-S (Waters) mass spectrometer in positive ion mode with multiple reaction monitoring. The mobile phase was composed of buffer A (0.1% formic acid in ultrapure water) and buffer B (100% acetonitrile). Nucleosides were accurately quantified depending on the nucleoside-to-base ion mass transitions of m/z 268.0 to 136.0 (A) and m/z 282.0 to 150.1 (m⁶A). The pure commercial adenosine (A; TargetMol, T0853) and N⁶-methyladenosine (m⁶A; TargetMol, T6599) were used to generate standard curves, which were subsequently employed to calculate the contents of A and m⁶A in each sample. The global m⁶A levels were presented in the form of m⁶A/A ratio. The experiment was repeated with three independent biological replicates.

RNA immunoprecipitation

RNA immunoprecipitation (RIP) was carried out following the method of Wei et al (2018) [22] with minor modifications. Briefly, the octoploid strawberry fruit expressing the MTA-eGFP protein were sliced into ~2 mm slices, and then fixed with 1% formaldehyde under a vacuum for 30 min on ice. The fixation was terminated by the addition of 150 mM glycine, followed by incubation on ice for 5 min. The fixed fruit tissues (2 g) were homogenized in 5 mL of lysis buffer (50 mM HEPES, pH 7.5, 2 mM EDTA, 150 mM KCl, 0.5% NP-40 (v/v), 2 mM EDTA, 0.5 mM DTT, 1× cocktail protease inhibitor (Sigma, 04693132001), and 300 U mL⁻¹ RNase Inhibitor). After incubation at 4 °C for 1 h, the mixture was centrifuged at 15,000 g for 30 min. Then, 200 µL of the supernatant were taken as the input control, and the remainder was subjected to immunoprecipitation (IP) with anti-GFP monoclonal antibody or rabbit IgG at 4 °C overnight. Fifty microliters of Dynabeads Protein-A (Life Technologies, 10002A) were added to the mixture and incubated at 4 °C for 2 h. After washing four times with PBS buffer, the RNA-protein mix was catalyzed by proteinase K (Takara, 9034) at 55 °C for 1 h. The immunoprecipitated mRNAs and input mRNAs were subsequently isolated by the plant RNA extraction kit (Magen, R4165-02). Relative enrichment of individual transcript was determined by quantitative RT-PCR analysis as described above. The primers used for PCR amplification are listed in [Additional file 2: Table S17](#). The analysis was performed with three biological replicates, and each contained three technical repeats.

747 **Data access**

748 The raw sequencing data and processed peaks data in m⁶A-seq have been deposited in the
749 Gene Expression Omnibus database under the accession number GSE167183. All the other
750 data generated in this study are included in the article and the Additional files.

751

Additional material

Additional file 1: Supplementary Figure S1-S10. **Figure S1.** Pearson correlation analysis of input reads in the m⁶A peak regions identified from m⁶A-seq. **Figure S2.** Pearson correlation analysis of immunoprecipitation (IP) reads in the m⁶A peak regions identified from m⁶A-seq. **Figure S3.** Validation of confident m⁶A peaks. **Figure S4.** Sequence motif identified within the m⁶A peaks by HOMER (<http://homer.ucsd.edu/homer/>). **Figure S5.** m⁶A modification and expression of genes in ABA biosynthesis and signaling pathway in octoploid cultivated strawberry. **Figure S6.** Identification of the conserved MT-A70 domain of m⁶A methyltransferases MTA and MTB in diploid woodland strawberry and octoploid cultivated strawberry. **Figure S7.** Protein sequence alignment of the MT-A70 domains in m⁶A methyltransferases. **Figure S8.** The changes in translation efficiency of ABA signaling gene and ripening genes in the MTA-overexpressed fruits. **Figure S9.** m⁶A enrichment for DNA methyltransferase genes from m⁶A-seq data. **Figure S10.** Regulatory model for the m⁶A-mediated ripening in climacteric tomato fruit and non-climacteric strawberry fruit.

Additional file 2: Supplementary Table S1-S17. **Table S1.** A summary of m⁶A-seq information in strawberry fruit at different stages. **Table S2.** Confident m⁶A peaks identified by three independent m⁶A-seq experiments in strawberry fruit at S6 stage. **Table S3.** Confident m⁶A peaks identified by three independent m⁶A-seq experiments in strawberry fruit at RS1 stage. **Table S4.** Confident m⁶A peaks identified by three independent m⁶A-seq experiments in strawberry fruit at RS3 stage. **Table S5.** m⁶A density in actively expressed transcripts. **Table S6.** Transcripts containing hypermethylated m⁶A peak in strawberry fruit at

RS1 stage compared to those at S6 stage. **Table S7.** Transcripts containing hypomethylated m⁶A peak in strawberry fruit at RS1 stage compared to those at S6 stage. **Table S8.** Transcripts containing hypermethylated m⁶A peak in strawberry fruit at RS3 stage compared to those at RS1 fruit. **Table S9.** Transcripts containing hypomethylated m⁶A peak in strawberry fruit at RS3 stage compared to those at RS1 stage. **Table S10.** Ripening-specific peaks identified in strawberry fruit at RS1 stage. **Table S11.** Differentially expressed genes between strawberry fruit at S6 and RS1 stage identified by three independent RNA-seq experiments. **Table S12.** Differentially expressed genes between strawberry fruit at RS1 and RS3 stage identified by three independent RNA-seq experiments. **Table S13.** Differentially expressed genes containing hypermethylated m⁶A peaks in strawberry fruit at RS1 stage compared to those at S6 stage. **Table S14.** Differentially expressed genes containing hypomethylated m⁶A peaks in strawberry fruit at RS1 stage compared to those at S6 stage. **Table S15.** Differentially expressed genes containing hypermethylated m⁶A peaks in strawberry fruit at RS3 stage compared to those at RS1 stage. **Table S16.** Differentially expressed genes containing hypomethylated m⁶A peaks in strawberry fruit at RS3 stage compared to those at RS1 stage. **Table S17.** A summary of primer informations.

Abbreviations

ABA: abscisic acid; *ABAR*: putative ABA receptor; AD: activation domain; ALKBH: AlkB homolog; *AREB1*: ABA-responsive element-binding protein 1; BD: binding domain; CDS: coding sequence; *CHS*: chalcone synthase; *DFR*: dihydroflavonol 4-reductase; DPA: days post-anthesis; EF: translation elongation factor; eGFP: enhanced green fluorescent protein;

796 EIF: translation initiation factor; FDR: false discovery rate; FPKM: fragments per kilobase of
 797 transcript per million fragments mapped; FR: full red; FTO: fat mass and obesity-associated
 798 protein; GO: gene ontology; HOMER: hypergeometric optimization of motif enrichment;
 799 IGB: Integrated Genome Browser; IP: immunoprecipitation; IR: initial red; LB: Luria-Bertani;
 800 LCI: luciferase complementation imaging; LG: large green; m⁶A: N⁶-methyladenosine;
 801 MAPQ: mapping quality; METTL: methyltransferase like; mRNA: messenger RNA; MTA:
 802 adenosine methyltransferase; MTB: adenosine methyltransferase B; *NCED5*:
 803 *9-cis-epoxycarotenoid dioxygenase 5*; OE: overexpression; *PGI*: *polygalacturonase 1*; RBP:
 804 RNA binding proteins; RdDM: RNA-directed DNA methylation; RIP: RNA
 805 immunoprecipitation; RNAi: RNA interference; RS1: the ripening stage 1; RS3: the ripening
 806 stage 3; RT-PCR: reverse transcription polymerase chain reaction; S6 :the growth stage 6; SG:
 807 small green; TSS: transcription start site; UTR: untranslated region; *WRKY40*: *WRKY*
 808 *DNA-binding protein 40*; Wt: white; WT: wild-type; WTAP: Wilms' tumor 1-associating
 809 protein; Y2H: yeast two-hybrid.

810

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Availability of data and materials

The raw sequencing data and processed peaks data in m⁶A-seq have been deposited in the Gene Expression Omnibus database under the accession number GSE167183 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE167183>) with a temporary token of 'yjatoqksvribzkl'. All the other data generated in this study are included in the article and the Additional files.

Authors' contributions

GQ conceived, designed, and supervised the experiments. ST and BL provided critical discussions. LZ and RT performed the experiments and analyzed the data. LX prepared the strawberry materials. GQ and LZ wrote the manuscript with contributions from RT. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

840 Not applicable.

841

842 **Ethics approval and consent to participate**

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844

845 **Author details**

846 ¹Key Laboratory of Plant Resources, Institute of Botany, the Innovative Academy of Seed

847 Design, Chinese Academy of Sciences, No.20 Nanxincun, Xiangshan, Haidian District,

848 Beijing 100093, China. ²University of Chinese Academy of Sciences, Beijing 100049, China.

849 ³College of Horticulture, China Agricultural University, Beijing 100193, China.

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Figure legends

Figure 1 Transcriptome-wide m⁶A methylomes in strawberry fruit. **a** The representative photographs of fruit at different developmental stages. S6, the growth stage 6; RS1, the ripening stage 1; RS3, the ripening stage 3. Scale bar = 1 cm. **b** Venn diagrams depicting the overlap of m⁶A peaks from three independent m⁶A-seq experiments on fruit at the three developmental stages. Rep, replicate. **c** Percentage of the m⁶A-containing transcripts containing various m⁶A peak numbers among samples. **d** Gene Ontology (GO) enrichment for the m⁶A-modified transcripts identified in m⁶A-seq.

Figure 2 Dynamic distribution of m⁶A during strawberry fruit ripening. **a** Metagenomic profiles of m⁶A peak summit distribution along transcripts. UTR, untranslated region; CDS, coding sequence. The red arrows indicate the changes in m⁶A distribution in the CDS region adjacent to the start codon in the ripening process, while the green arrows show the changes in m⁶A distribution around the stop codon or within the 3' UTR. S6, the growth stage 6; RS1, the ripening stage 1; RS3, the ripening stage 3. **b** and **c** The percentage (**b**) and relative enrichment (**c**) of m⁶A peak summits in five non-overlapping transcript segments. TSS, transcription start site.

Figure 3 Correlation between m⁶A modification and mRNA abundance in strawberry fruit. **a** Scatter plots showing hypermethylated (red) and hypomethylated (blue) m⁶A peaks in fruit at RS1 stage compared to those at S6 stage. S6, the growth stage 6; RS1, the ripening stage 1. **b** Scatter plots depicting hypermethylated (red) and hypomethylated (blue) m⁶A

peaks in fruit at RS3 stage compared to those at RS1 stage. RS3, the ripening stage 3. **c** and **d** The distribution characteristics of the differential m⁶A peaks shown in **a** (**c**) and **b** (**d**). TSS, transcription start site; UTR, untranslated region; CDS, coding sequence. **e** and **f** Scatter plots displaying the expression of transcripts containing hypermethylated (**e**) or hypomethylated (**f**) m⁶A peaks shown in **a**. Transcripts with significantly higher and lower levels (fold change ≥ 1.5 ; P value < 0.05) in fruit at RS1 stage compared to those at S6 stage are highlighted in red and blue, respectively. **g** and **h** Scatter plots showing the expression of transcripts harboring hypermethylated (**g**) or hypomethylated (**h**) m⁶A peaks shown in **b**. Transcripts with significantly higher and lower levels (fold change ≥ 1.5 ; P value < 0.05) in fruit at RS3 stage compared to those at RS1 stage are highlighted in red and blue, respectively. **i** Gene expression ratios based on the distributions of differential m⁶A peaks between fruit at RS1 and S6 stages. **j** Scatter plots exhibiting the expression of transcripts concurrently containing hypermethylated m⁶A peaks in the CDS region and hypomethylated m⁶A peaks around the stop codon or within the 3' UTR in fruit at RS1 stage compared to those at S6 stage. Transcripts with significantly higher and lower levels (fold change ≥ 1.5 ; P value < 0.05) in fruit at RS1 stage compared to those at S6 stage are highlighted in red and blue, respectively. Gene expression analysis in **e-j** was performed by RNA-seq. FPKM, fragments per kilobase of exon per million mapped fragments.

Figure 4 m⁶A modification facilitates mRNA stability or translation of genes in ABA pathway. **a** A brief model of the two core ABA signaling pathways in plants. **b** Integrated Genome Browser (IGB) tracks showing the distribution of m⁶A reads in transcripts of the

1131 *9-cis-epoxycarotenoid dioxygenase 5 (NCED5)*, *putative ABA receptor (ABAR)*, and
1132 *ABA-responsive element-binding protein 1 (AREB1)*. The hypermethylated m⁶A peaks (fold
1133 change ≥ 1.5 ; P value < 0.05) in fruit at RS1 stage compared to those at S6 stage are
1134 indicated by shadow boxes. S6, the growth stage 6; RS1, the ripening stage 1. Rep, replicate.
1135 **c** m⁶A enrichment for *NCED5*, *ABAR*, and *AREB1* from m⁶A-seq data. **d** Validations of the
1136 m⁶A enrichment by m⁶A-immunoprecipitation (IP)-qPCR. **e** and **f** Transcript levels of
1137 *NCED5*, *ABAR*, and *AREB1* determined by RNA-seq (**e**) and quantitative RT-PCR (**f**). FPKM,
1138 fragments per kilobase of exon per million mapped fragments. The *ACTIN* gene served as an
1139 internal control in **f**. **g** Schematic diagram of the expression cassettes used for mRNA stability
1140 assay. The intact (WT) or mutated (MU) cDNA fragments of *NCED5*, *ABAR*, and *AREB1*
1141 were separately cloned into the pCambia2300 vector driven by the CaMV 35S promoter. The
1142 potential m⁶A sites identified in m⁶A-seq were mutated from adenosine (A) to cytidine (C) or
1143 guanine (G) using site-directed mutagenesis kit. **h** Determination of mRNA stability for
1144 *NCED5*, *ABAR*, and *AREB1*. The intact (WT) or mutated (MU) cDNA fragments were
1145 expressed in *Nicotiana benthamiana* leaves. After actinomycin D treatment at an indicated
1146 time point, the total RNAs were extracted and submitted to quantitative RT-PCR assay with
1147 the *N. benthamiana ACTIN* gene serving as an internal control. **i** m⁶A-IP-qPCR assay
1148 showing the relative m⁶A enrichment in the intact or mutated transcripts. **j** Brief workflow for
1149 analysis of translation efficiency. **k** Translation efficiency of *NCED5*, *ABAR*, and *AREB1*.
1150 Translation efficiency was expressed as the abundance ratio of mRNA in the polysomal RNA
1151 versus the total RNA. Data are presented as mean $\pm$ standard deviation ($n = 3$). Asterisks
1152 indicate significant differences ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$; Student's t test).

1153

1154 **Figure 5 The m⁶A methyltransferase MTA interacts with MTB in strawberry. a** The
 1155 working model for m⁶A installations mediated by the methyltransferase complex in mammals.
 1156 The m⁶A methyltransferases METTL3 and METTL14 interact and function as the stable
 1157 catalytic core for internal m⁶A installations in the form of heterodimer. **b** Phylogenetic
 1158 analysis of eukaryotic m⁶A methyltransferases. The phylogenetic tree was generated by
 1159 MEGA (version 5.2). Bootstrap values from 1000 replications for each branch are presented.
 1160 Species names are abbreviated as follows: Hs, *Homo sapiens*; Ms, *Mus musculus*; At,
 1161 *Arabidopsis thaliana*; Os, *Oryza sativa*; Zm, *Zea mays*; Sl, *Solanum lycopersicum*; Nb,
 1162 *Nicotiana benthamiana*; Fa, *Fragaria × ananassa*; Fve, *Fragaria vesca*. **c** Transcript levels
 1163 of the m⁶A methyltransferase genes *MTA* and *MTB* in diploid woodland strawberry at
 1164 different developmental stages revealed by RNA-seq. S6, the growth stage 6; RS1, the
 1165 ripening stage 1; RS3, the ripening stage 3. **d** Representative images of octoploid cultivated
 1166 strawberry fruit at various developmental stages. SG, small green; Wt, white; IR, initial red;
 1167 FR, full red. Scale bar = 1 cm. **e** Transcript levels of *MTA* and *MTB* in octoploid strawberry
 1168 fruit determined by quantitative RT-PCR. The *ACTIN* gene was used as an internal control.
 1169 Data are presented as mean ± standard deviation (n = 3). Asterisks indicate significant
 1170 differences (**P* < 0.05, ***P* < 0.01, ****P* < 0.001; Student's *t* test). **f** Y2H assay revealing the
 1171 interactions between MTA and MTB. The MTA fused with the activation domain (AD) of
 1172 GAL4 (AD-MTA) and the MTB fused with the binding domain (BD) of GAL4 (BD-MTB)
 1173 were co-expressed in yeast. The transformants were grown on SD/-Leu/-Trp (-LW), and further
 1174 selected on SD/-Leu/-Trp/-His (-LWH) and SD/-Leu/-Trp/-His/-Ade (-LWHA) with or

without X- α -gal. **g** LCI assay revealing the interactions between MTA and MTB. The MTA fused with the N-terminus of LUC (MTA-nLUC) was co-expressed with the MTB or its MT-A70 domain fused with the C-terminus of LUC (cLUC-MTB or cLUC-MTB^D) in *N. benthamiana* leaves. **h** and **i** Subcellular localization (**h**) and colocalization (**i**) of MTA and MTB. The MTA-mCherry or/and MTB-eGFP fusion proteins were transiently expressed into *N. benthamiana* leaves. The *N. benthamiana* leaves expressing eGFP or/and mCherry were used as the negative control. Scale bar = 10 μ m.

Figure 6 m⁶A methyltransferases positively regulate the ripening of strawberry fruit. a

Ripening phenotypes of MTA/MTB RNA interference (RNAi-MTA/RNAi-MTB) and overexpression (OE-MTA/OE-MTB) fruits. Strawberry fruit agroinfiltrated with empty plasmid were used as controls. The representative images are shown. Scale bar = 1 cm. **b** Transcript levels of *MTA* and *MTB*, as well as the two important ripening genes *CHS* and *PGI*, in the RNAi and overexpression fruits determined by quantitative RT-PCR. The *ACTIN* gene served as an internal control. **c** LC-MS/MS assay revealing the changes in global m⁶A methylation levels in the MTA-overexpressed fruits. **d** RNA immunoprecipitation (RIP) assay revealing the binding of MTA protein to the transcripts of *NCED5*, *ABAR*, and *AREB1*. The protein-RNA complexes were extracted from strawberry fruit expressing the MTA-eGFP fusion protein and subjected to immunoprecipitation with anti-GFP monoclonal antibody or mouse IgG (negative control). **e-h** The changes in relative m⁶A enrichment (**e**), gene expression (**f**), mRNA stability (**g**), and translation efficiency (**h**) of *NCED5*, *ABAR*, and *AREB1* in the MTA-overexpressed fruits. The relative m⁶A enrichment and gene expression

were determined by m⁶A-IP-qPCR and quantitative RT-PCR, respectively. For mRNA stability assay, the total RNAs were extracted after actinomycin D treatment at an indicated time point and submitted to quantitative RT-PCR assay. Translation efficiency was expressed as the abundance ratio of mRNA in the polysomal RNA versus the total RNA. Data are presented as mean $\pm$ standard deviation (n = 3). Asterisks indicate significant differences (**P* < 0.05, ***P* < 0.01, ****P* < 0.001; Student's *t* test). NS, no significance.

Figure 7 Influence of m⁶A on translation initiation factors or elongation factors in strawberry. **a** Integrated Genome Browser (IGB) tracks displaying the distribution of m⁶A reads in transcripts of genes encoding translation initiation factors (*EIF2*, *EIF2B*, *EIF3A*, and *EIF3C*) and elongation factors (*EF1A*). The hypermethylated m⁶A peaks (fold change $\geq$ 1.5; *P* value < 0.05) in fruit at RS1 stage compared to those at S6 stage are indicated by shadow boxes. S6, the growth stage 6; RS1, the ripening stage 1. Rep, replicate. **b** m⁶A enrichment for *EIF2*, *EIF2B*, *EIF3A*, *EIF3C*, and *EF1A* from m⁶A-seq data. **c** Transcript levels of *EIF2*, *EIF2B*, *EIF3A*, *EIF3C*, and *EF1A* determined by RNA-seq. FPKM, fragments per kilobase of exon per million mapped fragments. **d** and **e** Relative m⁶A enrichment (**d**) and gene expression (**e**) for *EIF2*, *EIF2B*, *EIF3A*, *EIF3C*, and *EF1A* in the octoploid strawberry fruit at the white (Wt) and initial red (IR) stages. The relative m⁶A enrichment and gene expression were determined by m⁶A-IP-qPCR and quantitative RT-PCR, respectively. The *ACTIN* gene served as an internal control. **f** RNA immunoprecipitation (RIP) assay revealing the binding of MTA protein to the transcripts of *EIF2*, *EIF2B*, *EIF3A*, *EIF3C*, and *EF1A*. **g-i** The changes in relative m⁶A enrichment (**g**), gene expression (**h**), and mRNA stability (**i**), of *EIF2*, *EIF2B*,

1219 *EIF3A*, *EIF3C*, and *EF1A* in the MTA-overexpressed fruits. For mRNA stability assay, the
 1220 total RNAs were extracted after actinomycin D treatment at an indicated time point and
 1221 subjected to quantitative RT-PCR assay. Data are presented as mean $\pm$ standard deviation (n =
 1222 3). Asterisks indicate significant differences (* P < 0.05, ** P < 0.01, *** P < 0.001; Student's
 1223 t test).
 1224

Figure 1 Transcriptome-wide m⁶A methylomes in strawberry fruit. **a** The representative photographs of fruit at different developmental stages. S6, the growth stage 6; RS1, the ripening stage 1; RS3, the ripening stage 3. Scale bar = 1 cm. **b** Venn diagrams depicting the overlap of m⁶A peaks from three independent m⁶A-seq experiments on fruit at the three developmental stages. Rep, replicate. **c** Percentage of the m⁶A-containing transcripts containing various m⁶A peak numbers among samples. **d** Gene Ontology (GO) enrichment for the m⁶A-modified transcripts identified in m⁶A-seq.

Figure 2 Dynamic distribution of m⁶A during strawberry fruit ripening. **a** Metagenomic profiles of m⁶A peak summit distribution along transcripts. UTR, untranslated region; CDS, coding sequence. The red arrows indicate the changes in m⁶A distribution in the CDS region adjacent to the start codon in the ripening process, while the green arrows show the changes in m⁶A distribution around the stop codon or within the 3' UTR. S6, the growth stage 6; RS1, the ripening stage 1; RS3, the ripening stage 3. **b** and **c** The percentage (**b**) and relative enrichment (**c**) of m⁶A peak summits in five non-overlapping transcript segments. TSS, transcription start site.

Figure 3 Correlation between m⁶A modification and mRNA abundance in strawberry fruit. a Scatter plots showing hypermethyalted (red) and hypomethyalted (blue) m⁶A peaks in fruit at RS1 stage compared to those at S6 stage. S6, the growth stage 6; RS1, the ripening stage 1. **b** Scatter plots depicting hypermethyalted (red) and hypomethyalted (blue) m⁶A peaks in fruit at RS3 stage compared to those at RS1 stage. RS3, the ripening stage 3. **c** and **d** The distribution characteristics of the

differential m⁶A peaks shown in **a** (**c**) and **b** (**d**). TSS, transcription start site; UTR, untranslated region; CDS, coding sequence. **e** and **f** Scatter plots displaying the expression of transcripts containing hypermethylated (**e**) or hypomethylated (**f**) m⁶A peaks shown in **a**. Transcripts with significantly higher and lower levels (fold change ≥ 1.5 ; P value < 0.05) in fruit at RS1 stage compared to those at S6 stage are highlighted in red and blue, respectively. **g** and **h** Scatter plots showing the expression of transcripts harboring hypermethylated (**g**) or hypomethylated (**h**) m⁶A peaks shown in **b**. Transcripts with significantly higher and lower levels (fold change ≥ 1.5 ; P value < 0.05) in fruit at RS3 stage compared to those at RS1 stage are highlighted in red and blue, respectively. **i** Gene expression ratios based on the distributions of differential m⁶A peaks between fruit at RS1 and S6 stages. **j** Scatter plots exhibiting the expression of transcripts concurrently containing hypermethylated m⁶A peaks in the CDS region and hypomethylated m⁶A peaks around the stop codon or within the 3' UTR in fruit at RS1 stage compared to those at S6 stage. Transcripts with significantly higher and lower levels (fold change ≥ 1.5 ; P value < 0.05) in fruit at RS1 stage compared to those at S6 stage are highlighted in red and blue, respectively. Gene expression analysis in **e-j** was performed by RNA-seq. FPKM, fragments per kilobase of exon per million mapped fragments.

Figure 4 m⁶A modification facilitates mRNA stability or translation of genes in ABA pathway. a A brief model of the two core ABA signaling pathways in plants. **b** Integrated Genome Browser (IGB) tracks showing the distribution of m⁶A reads in transcripts of the 9-cis-epoxycarotenoid dioxygenase 5 (*NCED5*), putative ABA receptor (*ABAR*), and ABA-responsive element-binding protein 1 (*AREB1*).

The hypermethylated m⁶A peaks (fold change ≥ 1.5 ; P value < 0.05) in fruit at RS1 stage compared to those at S6 stage are indicated by shadow boxes. S6, the growth stage 6; RS1, the ripening stage 1. Rep, replicate. **c** m⁶A enrichment for *NCED5*, *ABAR*, and *AREB1* from m⁶A-seq data. **d** Validations of the m⁶A enrichment by m⁶A-immunoprecipitation (IP)-qPCR. **e** and **f** Transcript levels of *NCED5*, *ABAR*, and *AREB1* determined by RNA-seq (**e**) and quantitative RT-PCR (**f**). FPKM, fragments per kilobase of exon per million mapped fragments. The *ACTIN* gene served as an internal control in **f**. **g** Schematic diagram of the expression cassettes used for mRNA stability assay. The intact (WT) or mutated (MU) cDNA fragments of *NCED5*, *ABAR*, and *AREB1* were separately cloned into the pCambia2300 vector driven by the CaMV 35S promoter. The potential m⁶A sites identified in m⁶A-seq were mutated from adenosine (A) to cytidine (C) or guanine (G) using site-directed mutagenesis kit. **h** Determination of mRNA stability for *NCED5*, *ABAR*, and *AREB1*. The intact (WT) or mutated (MU) cDNA fragments were expressed in *Nicotiana benthamiana* leaves. After actinomycin D treatment at an indicated time point, the total RNAs were extracted and submitted to quantitative RT-PCR assay with the *N. benthamiana ACTIN* gene serving as an internal control. **i** m⁶A-IP-qPCR assay showing the relative m⁶A enrichment in the intact or mutated transcripts. **j** Brief workflow for analysis of translation efficiency. **k** Translation efficiency of *NCED5*, *ABAR*, and *AREB1*. Translation efficiency was expressed as the abundance ratio of mRNA in the polysomal RNA versus the total RNA. Data are presented as mean $\pm$ standard deviation ($n = 3$). Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Student's t test).

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 1333 RT-PCR assay. Translation efficiency was expressed as the abundance ratio of mRNA in the
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 1351 MTA protein to the transcripts of *EIF2*, *EIF2B*, *EIF3A*, *EIF3C*, and *EF1A*. **g-i** The changes in relative
 1352 m⁶A enrichment (**g**), gene expression (**h**), and mRNA stability (**i**), of *EIF2*, *EIF2B*, *EIF3A*, *EIF3C*,
 1353 and *EF1A* in the MTA-overexpressed fruits. For mRNA stability assay, the total RNAs were extracted
 1354 after actinomycin D treatment at an indicated time point and subjected to quantitative RT-PCR assay.
 1355 Data are presented as mean ± standard deviation (n = 3). Asterisks indicate significant differences (**P*
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