



# METTL4-mediated $N^6$ -methyladenine DNA modification regulates thermotolerance in *Arabidopsis thaliana*

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## ARTICLE INFO

**Keywords:**  
*Arabidopsis thaliana*  
 METTL4  
 DNA modification  
 6 mA  
 Methyltransferase  
 Heat stress

## ABSTRACT

DNA  $N^6$ -methyladenine (6 mA) is an evolutionarily conserved DNA modification in prokaryotes and eukaryotes. The DNA 6 mA methylation is tightly controlled by 6 mA regulatory proteins. DNA  $N^6$ -adenine methyltransferase 1 (DAMT-1) has been identified as a DNA 6 mA methyltransferase in animals. In plants, DNA 6 mA methylation has been found, however, the DNA 6 mA methyltransferases and their function in plants are largely unknown. In our study, we find METTL4 is a DNA 6 mA methyltransferase in *Arabidopsis thaliana*. Both in vitro and in vivo evidences support the DNA 6 mA methyltransferase activity of METTL4. *mettl4* mutant is hypersensitive to heat stress, suggesting DNA 6 mA methylation plays important role in heat stress adaption. RNA-seq and 6 mA IP-qPCR analysis show that METTL4 participates in heat stress tolerance by regulating expression of heat responsive genes. Our study find METTL4 is a plant DNA 6 mA methyltransferase and illustrates its function in regulating heat stress response.

## 1. Introduction

DNA  $N^6$ -methyladenine (6 mA), which is an evolutionarily conserved epigenetic modification, is important for embryo development (Zhang et al., 2015), transposon silencing (Wu et al., 2016), regulation of gene expression (Fu et al., 2015) and modulation of histone modification (Jia et al., 2023). DNA 6 mA methylation is performed by 6 mA methyltransferases. In *C. elegans*, DNA  $N^6$  adenine methyltransferase 1 (DAMT-1) has been identified as a 6 mA methyltransferase (Greer et al., 2015). METHYLTRANSFERASE LIKE 4 (METTL4), the mammalian homolog of DAMT-1, has also been shown to catalyze 6 mA deposition (Kweon et al., 2019). The homolog of METTL4 in *Arabidopsis*, which is also homologous to DAMT-1, functions as a  $N^6$ -2'-O-dimethyladenosine (m<sup>6</sup>Am) methyltransferase on U2 snRNA (Luo et al., 2022). DNA 6 mA methylation has been discovered in *Arabidopsis thaliana* and rice (Liang et al., 2018; Zhang et al., 2018; Zhou et al., 2018), however, plant 6 mA methyltransferases have not been characterized yet, the function of DNA 6 mA methylation in plants is largely unknown.

Abiotic stresses affect plant growth and survival. Heat stress becomes a serious threat to food security as global warming progresses (Lesk et al., 2016). Plants have developed complex mechanisms to tolerate high temperatures. Epigenetic regulators have important role in adaption to heat stress. Histone H3 lysine 4 (H3K4) methylation elevates and

functions as a heat stress memory marker under recurring heat stress conditions (Lämke et al., 2016). Demethylases of histone H3 lysine 37 trimethylation (H3K27me3) regulates heat stress adaption by activating transcription of *HEAT SHOCK PROTEIN 22* (*HSP22*) and *HSP 17.6 C* (Yamaguchi et al., 2021). *Arabidopsis thaliana* histone chaperone *ANTI-SILENCING FUNCTION 1* (*ASF1*) homologous genes *AtASF1A* and *AtASF1B* removes nucleosome to activate expression of key heat stress responsive genes in response to heat stress (Weng et al., 2014). micro-RNA (miRNA) is important for heat stress memory. miRNA156 down-regulates expression of *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) genes after heat stress which is critical for heat stress memory (Stief et al., 2014). DNA 5-methylcytosine (5mC) regulates expression of retrotransposon in *Arabidopsis* seedlings subjected to heat stress (Ito et al., 2011; Nozawa et al., 2021). In maize, 5mC buffers the effects of heat stress on transposable elements (Guo et al., 2021). In rice, the DNA 6 mA methylation level is associated with heat stress (Zhang et al., 2018). In spite of the above reports, whether and how the DNA 6 mA methylation is involved in heat stress tolerance remains elusive.

In this study, we found *Arabidopsis* METTL4 has a function similar to *C. elegans* DAMT-1. METTL4 was a nuclear localized protein and ubiquitously expressed. The DNA 6 mA methylation level decreased upon loss of function of METTL4. In vitro enzymatic result indicated METTL4 could add 6 mA methylation onto *Arabidopsis* genomic DNA. Both the in

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vivo and in vitro results suggested METTL4 functioned as a DNA 6 mA methyltransferase in *Arabidopsis*. *mettl4* mutant was hypersensitive to heat stress. RNA-seq results showed a subset of heat stress responsive genes were dysregulated in *mettl4* under heat stress. We found the upregulation of heat stress responsive genes was positively correlated with increase of DNA 6 mA methylation, suggesting that DNA 6 mA methylation regulated heat stress tolerance via promoting expression of heat stress responsive genes. In summary, we found METTL4 to be a DNA 6 mA methyltransferase in *Arabidopsis*, METTL4 regulated heat stress tolerance by controlling expression of heat stress responsive genes.

## 2. Results

### 2.1. Identification of a DNA 6 mA methyltransferase in *Arabidopsis thaliana*

To identify potential DNA 6 mA methyltransferases in plants, we used full length *C. elegans* DAMT-1 protein sequence in BLAST searches against plant protein databases. The BLAST analysis predicted one homolog in *Arabidopsis thaliana*, which is METTL4 (AT1G19340) (Fig. 1A). MT-A70 is the catalytic domain of *C. elegans* DMAT-1 (Greer et al., 2015), METTL4 contained a MT-A70 domain at the C-terminus (Fig. 1B), the amino acid sequences of MT-A70 domain were evolutionarily conserved among *C. elegans* DAMT-1 and METTL4 (Fig. 1C). We further predicted 3D structure of MT-A70 domains of *C. elegans* DAMT-1 and METTL4, and found the MT-A70 domains had similar 3D structure (Fig. 1D). Therefore, METTL4 was a promising DNA 6 mA methyltransferase candidate in *Arabidopsis*.

### 2.2. Expression pattern and subcellular localization of METTL4

We investigated the presence of DNA 6 mA methylation in *Arabidopsis*. DNA 6 mA methylation signal was detected in all the four tissues (Fig. 2A), this result was consistent with the previous finding (Liang et al., 2018). We then studied the expression pattern of METTL4 by quantificational real-time polymerase chain reaction (qPCR). METTL4 was expressed in all tissues examined, including roots, stems, cauline leaves, rosette leaves, flowers and capsules (Fig. 2B). To study the subcellular localization of METTL4, we generated 35 S:METTL4-GFP transgenic plants. We observed nuclear localized GFP from root of 35 S: METTL4-GFP seedlings (Fig. 2C), indicating that METTL4 was nuclear localized.

### 2.3. METTL4 exhibits DNA 6 mA methyltransferase activity

To study DNA 6 mA methyltransferase activity of METTL4, we obtained two *mettl4* mutants (Fig. 3A). *mettl4-1* which was a T-DNA insertion mutant was bought from Arashare. We confirmed that *mettl4-1* was a homozygous T-DNA insertion mutant by PCR and RT-qPCR (Fig. 3B and S1A). We generated *mettl4-2* by editing METTL4, sanger sequencing result showed there was a 13 bp deletion in the fifth exon of METTL4 which caused a 4 amino acids deletion and a frame shift (Fig. S1B). Both mutants showed reduced DNA 6 mA methylation level compared to WT (Figs. 3C and 3D), suggesting METTL4 was required for DNA 6 mA methylation in living cells.

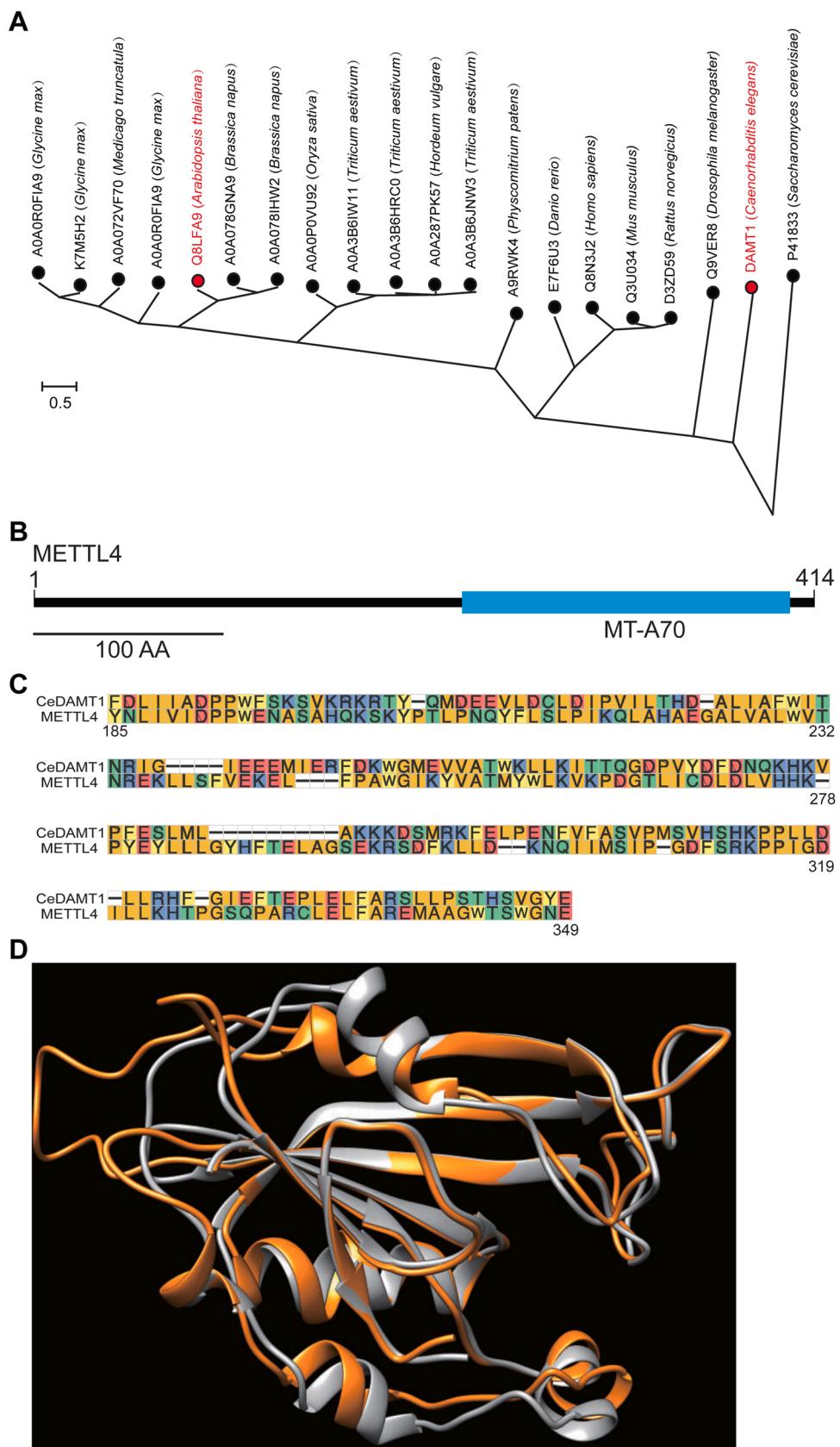
To confirm the DNA 6 mA methyltransferase activity of METTL4, we performed in vitro DNA 6 mA methylation experiment. We used genomic DNAs of *mettl4-1* as substrates. The recombinant METTL4 and GFP proteins were purified from *E. coli* (Fig. S1C). The Dot blot result showed a significant increase of DNA 6 mA methylation signal when METTL4 was added, while addition of GFP did not change the DNA 6 mA methylation signal (Figs. 3E and 3F). Taken together, our in vivo and in vitro results indicated METTL4 was a DNA 6 mA methyltransferase in plants.

### 2.4. METTL4 is required for heat stress adaption

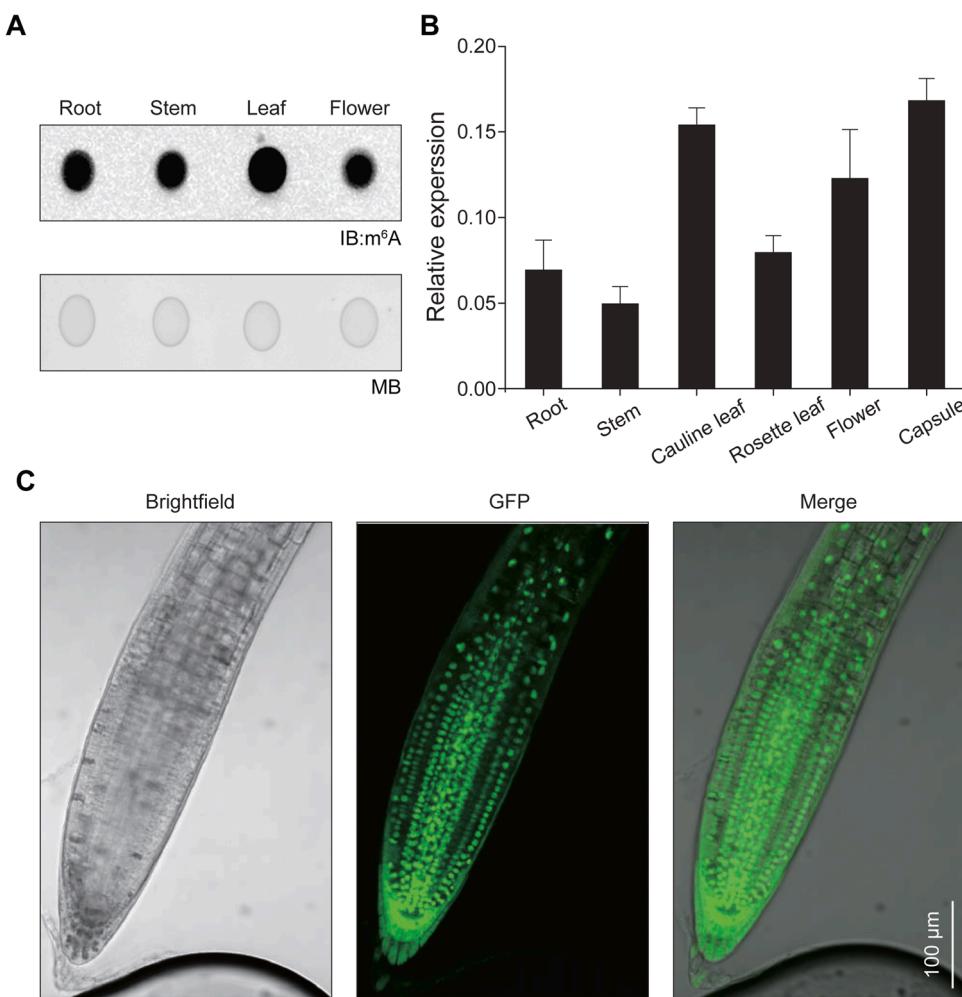
The *mettl4-1* mutant was shown to be early flowering (Luo et al., 2022), we found that *mettl4-1* seedlings did not show obvious developmental defect but displayed hypersensitivity to heat stress (Fig. S2A). We further confirmed this observation using both *mettl4-1* and *mettl4-2* mutants. After heat treatment, leaves of *mettl4-1* and *mettl4-2* became yellow while leaves of wild type (WT) was still green (Fig. 4A), the content of chlorophyll is lower in *mettl4-1* and *mettl4-2* than that in wild type (Fig. 4B). We then introduced 35S:METTL4 into *mettl4-1* mutant to generate complement lines. The complement line #5 (com #5) showed higher expression of METTL4 than wild type (Fig. S2B), the DNA 6 mA methylation level of com #5 was also higher than that of wild type (Fig. S2C), suggesting METTL4 was truly responsible for DNA 6 mA methylation. The defects of heat stress tolerance was restored in com #5 (Figs. 4C and 4D). We also used another two complement lines to analyze the DNA 6 mA methylation level, com #3 and com #8 showed higher expression of METTL4 and DNA 6 mA methylation level (Figs. S3A and S3B). Similar to com #5, com #3 rescued the defects of heat stress tolerance in *mettl4-1* (Figs. S3C and S3D), therefore, METTL4 was involved into heat stress adaption in plants.

### 2.5. METTL4 regulates expression of heat responsive genes

To investigate how METTL4 regulated heat stress tolerance, we treated 5-day-old wild type and *mettl4-1* seedlings at 44°C for 150 min and collected these seedlings for RNA-seq analysis. There were only 3 significant differential expressed genes between untreated wild type and *mettl4-1* samples (Table S1-S2), which is consistent with our phenotypic analysis result (Fig. S2A). We found 1356 and 1627 genes were significantly upregulated in wild type and *mettl4-1* after heat treatment (Fig. 5A, Tables S1-S4). The upregulated genes in both wild type and *mettl4* were enriched in similar Gene Ontology (GO) terms such as “response to heat” (Fig. S4A). Of the upregulated genes, 209 genes were uniquely upregulated in wild type while 480 genes were uniquely upregulated in *mettl4* (Fig. 5B). Among the 1147 genes upregulated in both wild type and *mettl4*, 237 genes showed higher upregulation in wild type than in *mettl4*. We hypothesized that key genes regulated by METTL4 should meet the following requirements: 1) Higher upregulation in wild type than in *mettl4* after heat treatment; 2) Targeted by DNA 6 mA methylation. We combined the 209 genes and the 237 genes together and defined those 446 genes as wild type upregulated genes (Fig. S4B). In a similar way, we found 728 genes were wild type downregulated genes. In 9-day-old wild type *Arabidopsis* seedlings, 3394 protein coding genes were reported to contain 6 mA modification within gene bodies, another 5074 protein coding genes were shown to be modified by 6 mA at the promoter, 5' and 3' UTR regions (Liang et al., 2018), we chose those 8468 genes as 6 mA target genes. There were 130 and 235 overlap genes between wild type upregulated genes, downregulated genes and 6 mA target genes, respectively (Fig. 5C and Fig. S4C). Our qRT-PCR results also verified the higher upregulation of FK506 BINDING PROTEIN 65 (FKBP65) and ATJ3 which were shown to be heat responsive genes (Meiri et al., 2010; Wu et al., 2019) in wild type than in *mettl4* (Fig. 5D). FKBP65 and ATJ3 were reported to contain 6 mA modification on 3'UTR and gene body respectively (Liang et al., 2018), our result confirmed the existence of 6 mA on these two genes, moreover, we found the 6 mA DNA modification on the FKBP65 and ATJ3 genes elevated after heat treatment in wild type but not in *mettl4* (Fig. 5E). 6 mA modification on gene bodies is reported to be associated with actively expressed genes (Liang et al., 2018), consistently, our result showed that 6 mA modification on gene body and 3' UTR regions promoted gene expression. Although our result indicated that METTL4 dependent DNA 6 mA methylation affected expression of heat responsive genes to regulate heat stress tolerance, it is possible that the METTL4 dependent RNA methylation is also involved in this process.



**Fig. 1.** Bioinformatics analysis of METTL4. A: Phylogenetic analysis of METTL4. B: Protein domains of METTL4. C: Sequence alignment of MT-A70 domains of DAMT-1 and METTL4. D: Comparison of 3D structure of MT-A70 domains of DAMT-1 and METTL4.



**Fig. 2.** Expression pattern of *METTL4*. A: Detection of DNA 6 mA methylation level in different tissues by dot blot. MB, methylene blue staining. B: qRT-PCR analysis of the expression pattern of *METTL4*. Bars show the mean  $\pm$  s.e.m. of three independent replicates. C: Confocal analysis of *METTL4*-GFP expression in a root tip of 35 S:*METTL4*-GFP seedling. Scale bar: 100  $\mu$ m.

### 3. Discussion

As a novel DNA modification, 6 mA DNA methylation is reported to be tightly regulated by methyltransferases and demethylases, however, the 6 mA DNA methyltransferase in higher plants remains elusive. Here, we found *METTL4*, a homolog of *C. elegans* DAMT-1, was a 6 mA DNA methyltransferase in *Arabidopsis*. The 6 mA DNA methylation level was significantly lower in *mettl4* mutant than that in wild type (Figs. 3C and 3D). Considering *METTL4* is nuclear-localized (Fig. 2C), it is very likely the 6 mA DNA methylation on genomic DNA reduced in *mettl4*, however, we can not exclude the possibility of reduction of 6 mA DNA methylation on chloroplast and mitochondrial DNA. Interestingly, we still detected 6 mA DNA methylation signals in *mettl4* (Figs. 3C and 3D), therefore, there are other 6 mA DNA methyltransferases in plants. Identification of those 6 mA DNA methyltransferases would be helpful for comprehensive understanding of 6 mA DNA methylation in plants. *METTL4* could also methylate m<sup>6</sup>Am on snRNA, *METTL4* might need other proteins to specifically bind its substrates.

6 mA DNA methylation has been shown to be positively associated with the expression of heat stress responsive genes (Zhang et al., 2018). We find the *mettl4* mutant is hypersensitive to heat stress, *METTL4* dependent 6 mA DNA methylation promotes expression of heat stress responsive genes under heat treatment (Figs. 5D and 5E). Our results indicate 6 mA DNA methylation is important for heat stress tolerance. 5mC DNA methylation is involved in heat stress tolerance, therefore, it

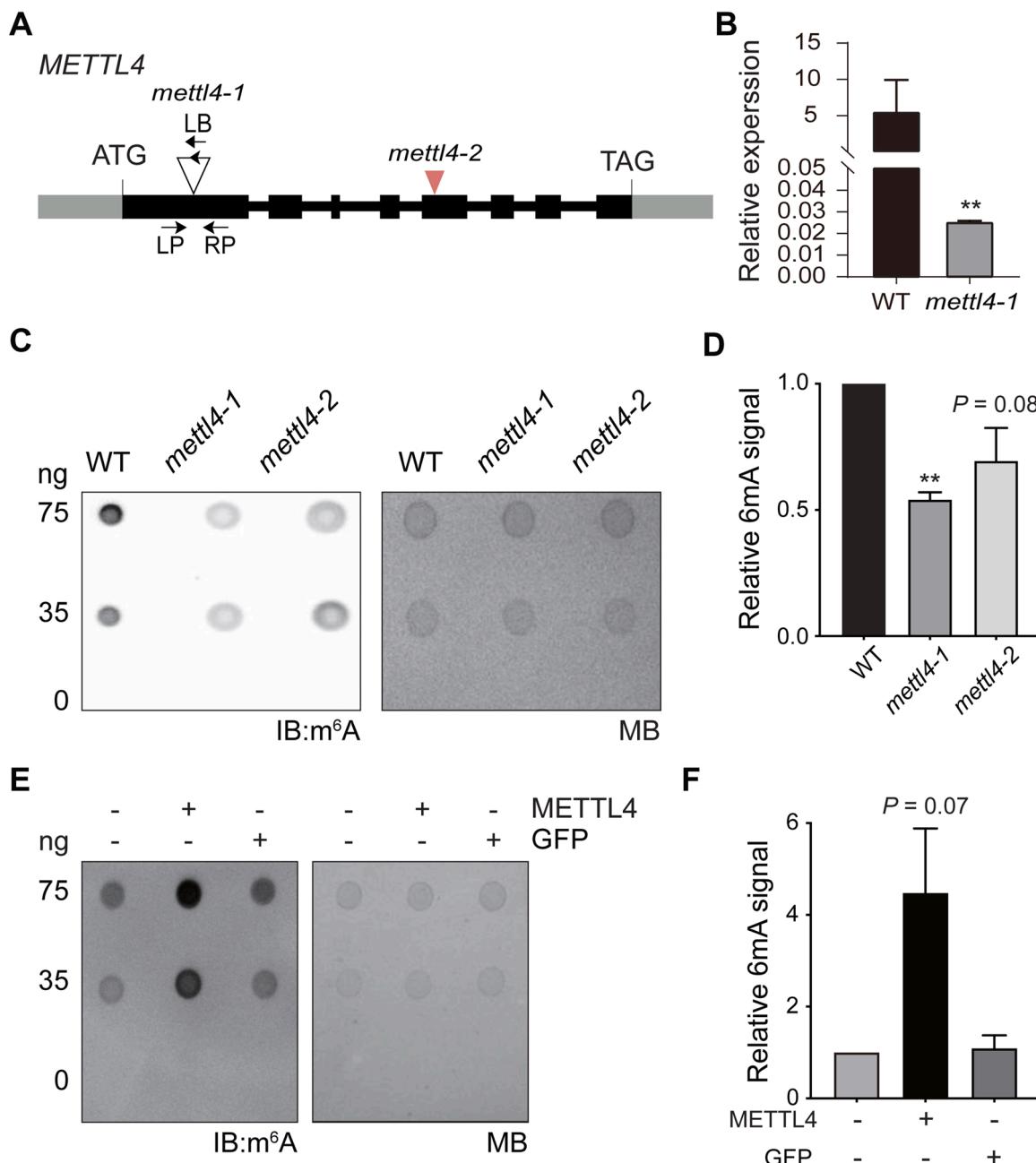
will be interesting to study how 5mC and 6 mA DNA methylation coordinately regulate heat stress tolerance in plants. *METTL4* is also essential for m<sup>6</sup>Am on U2 snRNA (Luo et al., 2022), it is possible that *METTL4* controls heat stress tolerance by regulating both 6 mA DNA methylation and m<sup>6</sup>Am on U2 snRNA.

### 4. Materials and methods

#### 4.1. Plant material and growth conditions

*Arabidopsis thaliana* plants were grown on soil or 1/2-strength Murashige and Skoog medium (1/2 MS) with 0.8% sucrose under long-day conditions (16 hr light/8 hr dark) at 21 °C  $\pm$  2 °C. The mutant *mettl4-1* (SALK-133379 C) was bought from Arashare. To edit *METTL4* (AT1G19340) to generate *mettl4-2*, synthesized sgRNA oligos (primers shown in Table S5) were introduced into the sgRNA expression cassettes. Transgenic plants were created by Agrobacterium-mediated transformation of *Arabidopsis* floral and selected by 50 mg/ml hygromycin B (SparkJade, SJ-MA0066) on 1/2 MS plates.

For heat stress analysis, 5-day-old seedlings were incubated at 44°C for 150 min. After heat stress, plants were returned to normal growth conditions for 48 h.



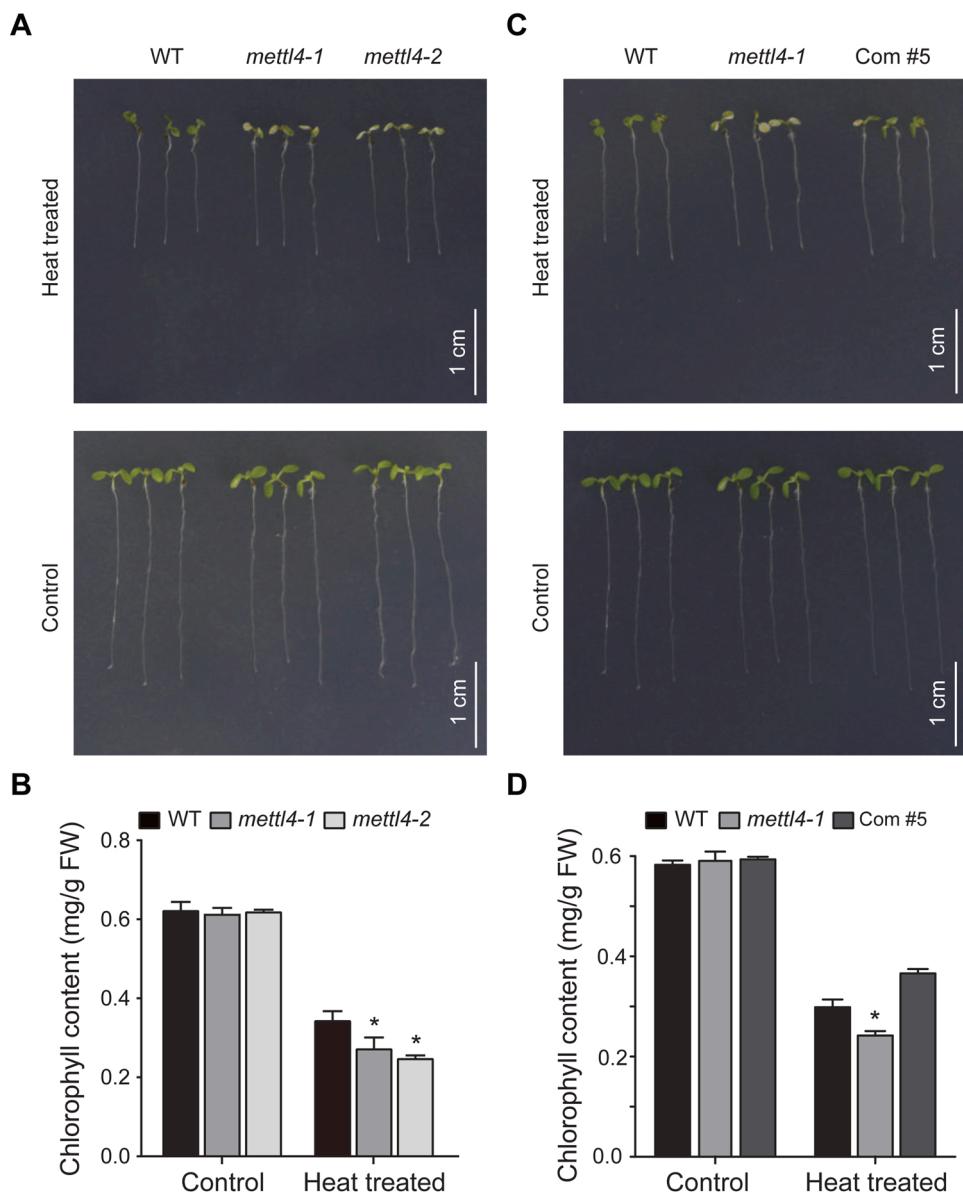
**Fig. 3.** DNA 6 mA methyltransferase activity of METTL4. A: Gene structure of *METTL4* and position of *mettl4-1* and *mettl4-2*. B: qRT-PCR analysis of the expression of *METTL4* in wild type and *mettl4-1*. Bars show the mean  $\pm$  s.e.m. of three independent replicates. \*\* $P < 0.01$ , Student's t-test. C: Dot blot analysis of DNA 6 mA methylation levels in wild type, *mettl4-1* and *mettl4-2*. MB, methylene blue staining. D: Quantification of 6 mA signal in (C). Quantification was performed using ImageJ (1.53a), 6 mA signal was normalized to that of wild type. Bars show mean  $\pm$  s.e.m. of three independent replicates. \*\* $P < 0.01$ , Student's t-test. E: Dot blot analysis of DNA 6 mA methylation levels in control, METTL4 and GFP treated samples. MB, methylene blue staining. F: Quantification of 6 mA signal in (E). Quantification was performed using ImageJ (1.53a), 6 mA signal was normalized to that of control. Bars show mean  $\pm$  s.e.m. of three independent replicates. P value was from Student's t-test.

#### 4.2. Plasmid construction

To construct 35 S:*METTL4*, the coding sequence of *METTL4* were amplified from *Arabidopsis* cDNA using Super-Fidelity DNA Polymerase (Vazyme, P515-01) and inserted into pCAMBIA1300 containing a 35 S promoter and an C-terminal GFP-tag by One Step Cloning Kit (Vazyme, C112-01). Primers were shown in Table S5.

#### 4.3. Phylogenetic reconstruction, sequence alignment and 3D structure prediction

*C.elegans* DAMT-1 amino acid sequence was used for BLAST searches (blastp). Trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2021). The amino acid sequences of MT-A70 domain of DAMT-1 and METTL4 were used for multiple sequences alignment by MEGA. 3D structure of MT-A70 domain of DAMT-1 and METTL4 were predicted by Robetta (Baek et al., 2021) and visualized using UCSF Chimera (Pettersen et al., 2004).



**Fig. 4.** Phenotypic analysis of *mettl4* mutants and the complement line #5. A: Wild type, *mettl4-1* and *mettl4-2* seedlings after heat stress treatment. B: Chlorophyll content of control and heat treated wild type, *mettl4-1* and *mettl4-2* seedlings. \* $P < 0.05$ , Student's t-test. C: Wild type, *mettl4-1* and com #5 seedlings after heat stress treatment. D: Chlorophyll content of control and heat treated wild type, *mettl4-1* and com #5 seedlings. \* $P < 0.05$ , Student's t-test.

#### 4.4. Recombinant protein expression and purification

The protein-coding regions of *METTL4* were cloned from *Arabidopsis* cDNA and inserted into the pET28a vector (with a His-MBP tag at the N-terminus) to generate His-MBP-METTL4 protein. Construct was transformed into *E. coli* (BL21) followed by induction with 0.1 mM IPTG at 16 °C overnight. Cells were lysed by sonication in lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl). The supernatant was incubated with Ni-NTA beads (Qiagen, 30210) at 4 °C for 2 h. The Ni-NTA agarose beads were washed three times with wash buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole), and recombinant protein was eluted using elution buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 50 mM imidazole). Primers were shown in Table S5.

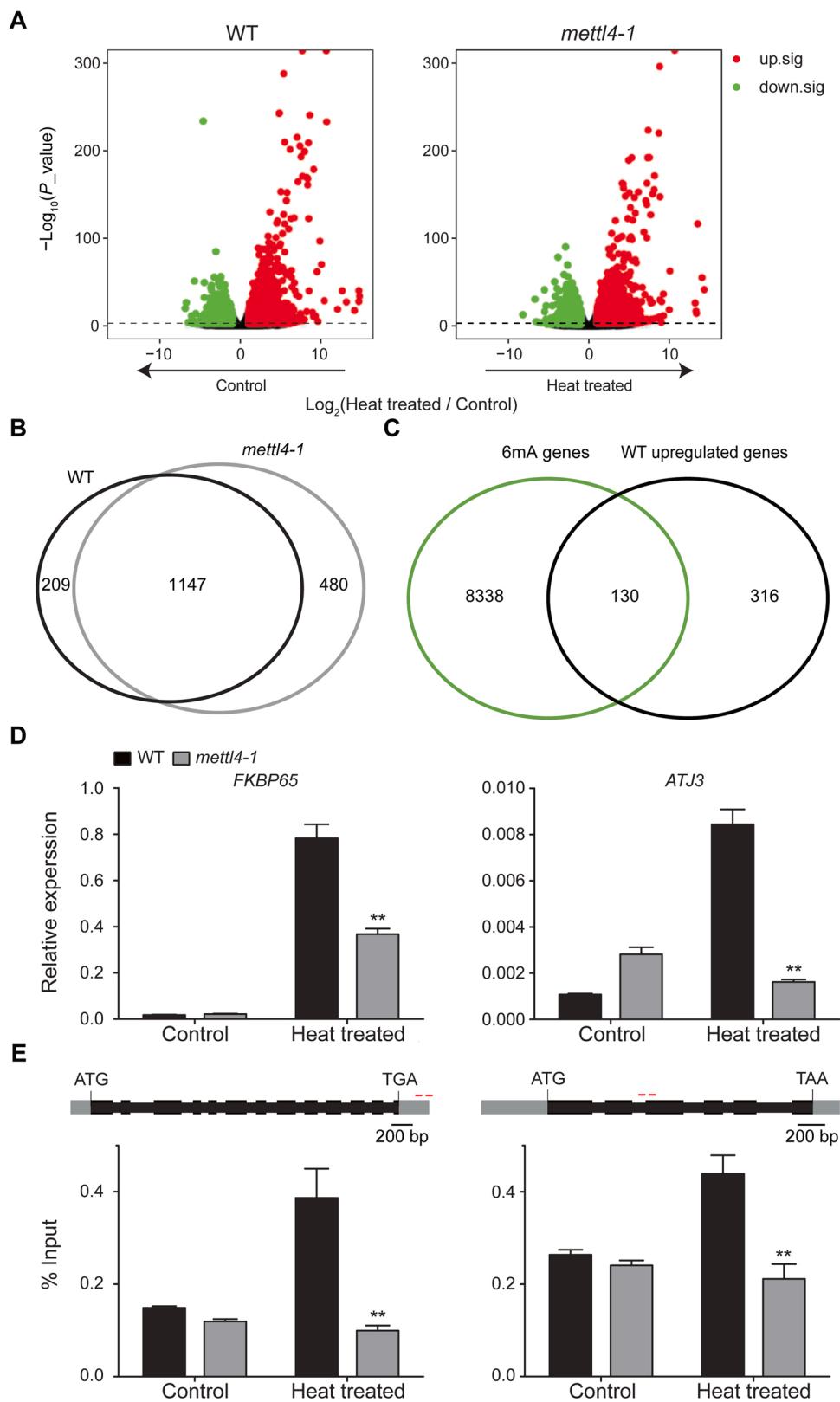
#### 4.5. Genomic DNA purification

*Arabidopsis* seedlings were homogenized in liquid nitrogen and incubated in DNA lysis buffer (100 mM Tris-HCl pH 8.0, 1 M KCl,

10 mM EDTA) at 60°C for 20 min 2 x CTAB buffer (50 mM CTAB, 1.4 M NaCl, 0.5 M EDTA, 1 M Tris-HCl pH 8.0) with 3% PVP-40 was added to extract genomic DNA. Genomic DNA was purified by phenol/chloroform and precipitated by ethanol. RNA was removed using RNase I (Thermo, EN0601), to completely remove RNA, we treated genomic DNA with RNase I twice.

#### 4.6. DNA dot blot

Genomic DNAs (gDNAs) were diluted to equal concentrations, diluted gDNAs were heated at 95°C for 5 min and quickly transferred into ice. gDNAs were spotted on nitrocellulose membranes. Nitrocellulose membranes were air-dried and baked at 80°C for 2 h followed by blocking in TBST buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.1% Triton X-100) with 5% milk at room temperature for 30 min. The membranes were incubated in TBST buffer with 3% BSA and 1:1000 diluted m<sup>6</sup>A antibody (ABclonal, A19841) at 4°C overnight. After washing with TBST, the membranes were incubated in TBST with 5%



**Fig. 5.** METTL4 regulates expression of heat responsive genes. A: Differential expression analysis for genes in control and heat treated 5-day-old wild type and *mettl4* seedlings. Estimated (DESeq2) fold changes are plotted on the x axis and the log-converted *P*-value on the y axis. Green and red dots highlight genes that pass an adjusted *P*-value cutoff of 0.001 and are downregulated or upregulated in the heat treated wild type or *mettl4* seedlings compared to untreated control (Fold Change  $>2$ ), respectively. B: Overlap of upregulated genes in heat treated wild type and *mettl4* seedlings compared to untreated control. C: Overlap of upregulated genes in heat treated wild type compared to untreated control and 6 mA target genes. D: qRT-PCR results showing the relative expression level of *FKBP65* and *ATJ3* in control and heat treated wild type and *mettl4* seedlings. Bars show the mean  $\pm$  s.e.m. of three independent replicates. \*\**P* < 0.01, Student's t-test. E: 6 mA-IP-qPCR results showing *FKBP65* and *ATJ3* 6 mA modification in control and heat treated wild type and *mettl4* seedlings. Upper panel show the gene structure of *FKBP65* and *ATJ3*, red lines indicate position of primers for 6 mA-IP-qPCR, bars show the mean  $\pm$  s.e.m. of three independent replicates. \*\**P* < 0.01, Student's t-test.

milk and 1:5000 diluted HRP-conjugated anti-rabbit IgG secondary antibody (Jackson Immuno Research, 111–035–045). The membranes were further washed and imaged.

#### 4.7. In vitro DNA 6 mA methylation assay

About 3 µg genomic DNAs were diluted in 50 µl reaction buffer (10 mM Tris-HCl pH 7.0, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 160 µM SAM (NEB B9003S), 1 mM DTT), 4 µg purified recombinant proteins were added. Reaction was performed for 2 h at 37 °C, and chill at 4 °C for 5 min. DNA was purified by phenol/chloroform and precipitated by ethanol.

#### 4.8. Measurements of chlorophyll

5-day-old seedlings were treated at 44°C for 150 min and recovered at 22 °C for 48 h. The weight of each sample was recorded (W). Seedlings were immersed in 95% ethanol solution (v/v) at room temperature for 48 h in the dark, the volumes were recorded (V). The absorbance at 645 nm (A645) and 663 nm (A663) was measured, and the chlorophyll content was calculated by the formula  $(8.02 \times A663 + 20.21 \times A645) \times V/W$ .

#### 4.9. mRNA library construction

5-day-old seedlings were treated at 44°C for 150 min and collected for RNA extraction. Total RNA was extracted using TRIzol (Thermo-fisher 15596026). Oligo(dT)-attached magnetic beads (Thermofisher 61002) were used to purify the mRNA. Purified mRNA was fragmented into small pieces with fragment buffer (NEB E6150S) followed by first-strand cDNA synthesis using SuperScript III (Thermo Fisher 18080051) by random hexamer-primed reverse transcription. After the second-strand cDNA was generated, the reaction product was purified by Ampure XP Beads (Beckman A63880) followed by addition of A-Tailing Mix and RNA Index Adapters. The cDNA fragments with adapters were amplified by several rounds of PCR, and the product was purified by Ampure XP Beads. The library was quality and paired-end sequenced on an Illumina HiSeq 4000 platform (Annoroad Gene Tech. (Beijing) Co., Ltd).

#### 4.10. Differential expression and GO analysis

Reads were aligned to the *Arabidopsis* TAIR10 genome annotation using STAR (v 2.5.3a) (Dobin et al., 2013) to assign multi-mapping reads in an unbiased, random way. Reads were assigned to gene models using the R package DEGseq (Wang et al., 2009). All assigned reads were imported into the R package DESeq2 (Love et al., 2014) for differential expression analyses. Genes with significantly differential expression levels were defined as fold change > 2 or fold change < -2 and FDR < 0.001. GO enrichment analysis was performed using the database for annotation, visualization and integrated discovery (DAVID) (Huang et al., 2009).

#### 4.11. qRT-PCR analysis

5-day-old seedlings were treated at 44°C for 150 min and collected for RNA extraction. Reverse transcription was performed using Exon-Script First-Strand Synthesis System (Exogen A501–01) according to the manufacturer's instructions. Real-time PCR was performed using gene-specific primers (Table S5) and the results were normalized against those produced by ACTIN primers to represent the relative expression levels. Three biological replicates were analyzed, and each was tested by three technical replicates.

#### 4.12. 6 mA IP-qPCR

Genomic DNAs were extracted from *Arabidopsis* seedlings followed by sonication to generate 200–300 bp fragments. Fragmented DNAs were incubated for 2 h at 4 °C with m<sup>6</sup>A-specific antibody (Synaptic Systems 202003) in IP buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.01% NP40) supplemented with BSA (0.5 µg/µl). The mixture was then incubated with protein-G beads (Thermofisher 10003D) for 1 h at 4°C. After extensive washing, bound DNA was eluted in elution buffer (50 mM Tris-HCl pH 7.5, 75 mM NaCl, 1% SDS, 6.25 mM EDTA) with proteinase K (Thermofisher 25530049) at 37°C for 30 min. Eluted DNAs were purified using CTAB and used for real-time PCR. The primers used for real-time PCR are listed in Table S5.

#### 4.13. Statistics

Sample size and statistical tests are indicated in the figure legends when necessary. Unless otherwise noted all statistical tests were two-sided. All replicates were obtained by measuring distinct samples (biological and/or experimental replicates).

#### 4.14. Data accessibility

mRNA-seq data have been deposited in the NCBI GEO with accession number GSE237262 (reviewer access token, ktqliwimvhonpod). The raw sequence data reported in this paper have also been deposited in the Genome Sequence Archive (Chen et al., 2021) in National Genomics Data Center (Xue et al., 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA011780) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa>.

#### Funding

This work was funded by the National Natural Science Foundation of China (32070613, 32270623), The Science and Technology Innovation Program of Hunan Province (2021RC3045) and the Natural Science Foundation of Hunan Province of China (2023JJ30123).

#### CRediT authorship contribution statement

C. H. conceived the project; L. C. performed the experiments with the help of A. M. and J. L.; C. H. and L. C. analyzed the sequencing data; C. H. designed the experiments and interpreted the results; C. H. and L. C. wrote the manuscript.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111916.

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