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Muti-omics revealed the mechanisms of MT-conferred tolerance of *Elymus nutans Griseb.* to low temperature at XiZang

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Abstract

Background Low temperature seriously limited the development of grass and crops in plateau. Thus, it is urgent to develop an effective strategy for improving the plant cold tolerance and elucidate the underlying mechanisms.

Results We found that MT alleviated the effects of cold stress on suppressing ENG growth, then improved cold tolerance of ENG. Integration of transcriptome and metabolome profiles showed that both cold exposure (TW) and MT reprogrammed the transcription pattern of galactose and flavonoids biosynthesis, leading to changes in compositions of soluble sugar and flavonoids in ENG. Additionally, TW inhibited the photosynthesis, and destroyed the antioxidant system of ENG, leading to accumulation of oxidant radicals represented by MDA. By contrast, MT promoted activities of antioxidant enzymes and flavonoid accumulation in ENG under cold condition, then reduced the MDA content and maintained normal expression of photosynthesis-related genes in ENG even under TW. Importantly, MT mainly enhanced cold tolerance of ENG via activating zeatin synthesis to regulate flavonoid biosynthesis in *vivo*. Typically, WRKY11 was identified to regulate MT-associated zeatin synthesis in ENG via directly binding on zeatin3 promoter.

Conclusions MT could enhance ENG tolerance to cold stress via strengthening antioxidant system and especially zeatin synthesis to promote accumulation of flavonoids in ENG. Thus, our research gain insight into the global mechanisms of MT in promoting cold tolerance of ENG, then provided guidance for protecting plant from cold stress in plateau.

Keywords *Campeiostachys nutans*, Low temperature stress, Multi-omics, Adaption mechanisms, Regulation network

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Introduction

Plants are often challenged by numerous adverse abiotic stresses, including drought, high salinity, and especially cold stress (i.e., low temperature stress) [1]. Of note, cold stress often determines the growth and development of plants, eventually affecting the crop yield and fruit quality [2]. Numerous crops represented by corn (*Zea mays* L.), Sorghum (*Sorghum bicolor*), rice (*Oryza sativa* L.), tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus* L.) have been documented to be adversely affected by cold stress [3]. Thus, cold stress often caused serious damages to the global economics and crop yield. Cold stress occurs frequently in early spring and late autumn, leading to a series of reactions such as freezing of plant cell membranes, which directly or indirectly inhibit plant growth and cause irreversible damage [4–6]. Long term exposure of cold condition can change the membrane permeability and cause lipid peroxidation of plant cell membrane, then leading oxidative stress to plants [7]. Additionally, cold stress could destroy the function of chloroplast and mitochondria to result in excessive accumulation of ROS in plants [8]. Moreover, numerous research found that cold exposure also affects the photosynthesis in plants, ultimately causing irreversible damage to plants [9–11]. It was found that cold stress can inhibit the synthesis and repair of D1 protein in photosystem II (PSII) reaction center, and affect the photochemical efficiency [9]. Thus, development of novel strategy for managing the damage to crops caused by cold stress is urgent and of great significance for agricultural development in future.

Plants will make a variety of physiological changes to adapt low temperature environment, such as altering metabolic compositions (glucose and flavonoid), activating antioxidant system and hormone signaling [12, 13]. Additionally, the increase of soluble sugar content also can reduce the damage caused by cold stress to plants [14]. Facing cold stress, a large amount of reactive oxygen species accumulated in plants and induce membrane peroxidation, which will destroy the integrity of the structure and function of the plant cell [15]. Malondialdehyde (MDA) is the product of membrane lipid peroxidation, and its content can reflect the damage degree of plant cells under adverse conditions, especially low temperature [16, 17]. In response, plant could deploy its antioxidant enzymes and metabolites to alleviate oxidate stress caused by cold exposure [18–20]. Melatonin (n-acetyl-5-methoxytryptamine), a small molecule belonging to the indole amines, is widely distributed in plants, where chloroplasts and mitochondria are the main sites of melatonin synthesis [21]. It was found that exogenous application of melatonin can alleviate the damage to plants caused by various abiotic stresses, especially cold stress [21–23]. It was found that melatonin could enhance plant cold tolerance via promoting the activity of antioxidant enzymes and reducing the loss of mineral elements caused by cold stress [24]. Despite these processes, the detailed mechanisms of melatonin in improving cold tolerance of plants remain unclear.

Elymus nutans Griseb. mainly distributed in cold habitat, thus investigating its responses to cold stress will contribute to our understanding about plant resistance to cold stress. In this study, we used *Elymus nutans* Griseb. to investigate the molecular mechanism of melatonin in improving plant resistance to cold stress. We performed integrated metabolome and transcriptome to systematically analyzed the changes in plants following MT and cold treatments. It was found that MT improved plant cold tolerance via improving physiological responses, and activating zeatin signal to reconstruct the metabolic composition in plants. Overall, our research provided novel insight into the mechanisms of MT-enhanced cold tolerance of plants, which contributed to the development and usage of MT in protecting crops from cold stress in filed, especially in cold plateau.

Materials and methods

Sample collection and preparation

The *Elymus nutans* Griseb. (ENG) named by Yang [25] used in this study were collected from Yangbajing (30° 28.535'N, 91° 06.246'E, altitude 4618 m), Dangxiong County, Lhasa, XiZang. The seeds were disinfected with 0.1% (W/V) sodium hypochlorite solution, and washed with distilled water. Then, the seeds were incubated in an artificial climate chamber for seedling growing to about 2 cm. The healthy plants were selected and transferred to the culture bowl, and the seedlings were cultured in the artificial climate chamber using sand culture. During the this period, the Hoagland nutrient solution was changed every 3 days. The seedlings pre-cultured for 21 days were collected for further experiments. The experiment contained 4 treatments: CK (25 °C, Hoagland nutrient solution), low temperature treatment (4 °C, Hoagland nutrient solution), CK+MT (100 μmol-1) treatment (25 °C with melatonin), low temperature+MT (100 μmol-1) (4 °C with melatonin) and 25 mg/L of zeatin (at 25 °C). The experiment was repeated every 3 times, and each time contained 3 biological replicates at least. After being treated for 5 days, all samples were collected via liquid nitrogen, and stored at -80 °C for later use.

Determination of physiological parameters

The content of malondialdehyde in ENG from different experimental treatment was extracted using 5 ml of 5% trichloroacetic acid and detected following Huang et al., (2020) [26]. After centrifugation at 3000 r/min, the supernatant was determined at 450 nm, 532 nm and 600 nm. The CAT, SOD and POD activities were analyzed

according to Du et al., (2024) [25], and the sugar content in ENG was determined following Xu [27].

RNA extraction

Total RNA of *Elymus nutans* Griseb. (ENG) with three replicates from each experimental group was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions [28]. After determination of RNA purity and integrity number, all samples were collected for Illumina RNA sequencing by Biotree company.

Transcriptome sequencing and analysis

A total amount of 1.5 μg RNA per sample was used as samples for libraries construction, and the sequencing libraries of cDNA were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations. And we used AMPure XP system (Beckman Coulter, Beverly, USA) to obtain 250 ~ 300 bp cDNA for further sequencing, and Agilent Bioanalyzer 2100 system to evaluate the library quality. Raw data (raw reads) of fastq format were firstly processed to obtain high quality clean data (clean reads). Further transcriptome assembly was performed using Trinity.

RSEM was used for analyzing the gene expression levels by DESeq R package (1.10.1), and genes with an adjusted P-value < 0.05 as well as Foldchange > 2.0 was recognized as differentially expressed genes (DEGs). Further Gene Ontology (GO) and KEGG pathway enrichment analysis on DEGs was performed by GOseq R packages an KOBAS software based Wallenius non-central hyper-geometric distribution, respectively.

Metabolite extraction

Three biological replicates of ENG from different altitudes were collected for metabolite extraction to ensure the validity of altitude-induced metabolic changes in ENG. Totally, 20 mg of each ENG sample from CK, CKMT, TW and TWMT groups was used and extracted by 1000 μL extract solution (methanol: water = 3: 1, with isotopically-labelled internal standard mixture) via ultrasonic extraction method for 3 times. After centrifugation, the supernatant from quality control (QC) sample and each experimental sample was collected for further LC-MS/MS analysis [29].

LC–MS/MS analysis and data processing

LC-MS/MS analysis was performed following Du et al., (2023) [30]. In detail, UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo) was used for LC-MS/MS analysis, with 5 mmol/L ammonium acetate and 5 mmol/L acetic acid in water (A) and acetonitrile (B) as mobile phase. The temperature and injection volume were set as 4 °C and 2 μL, respectively. Information-dependent acquisition (IDA) mode was deployed to get MS/MS spectra and evaluate the full scan MS spectrum. The ESI source conditions were set as following: capillary temperature 320 °C, full MS resolution as 60,000, MS/MS resolution as 15,000 collision energy as 10/30/60 in NCE mode, spray Voltage as 3.8 kV (positive) or -3.4 kV (negative), respectively [31].

Then, the raw data of all samples were processed by an in-house MS2 database (BiotreeDB) for metabolite annotation [32]. The cutoff for annotation was set at 0.3. Typically, $p < 0.05$ and fold change > 2.0 were used to identify differential metabolites. Further metabolomics analysis was performed using Metaboanalyst 3.0, including PCA, OPLS-DA, PLS-DA algorithms and KEGG pathway enrichment analysis.

Yeast one-hybrid assay

The promoter sequence (2000-bp-long upstream sequences from the start codon) of zeatin3 was selected and cloned into pHIS2 vectors. Moreover, the coding sequences of potential TFs were fused into pGADT7 vectors. According to the instructions of the yeast one-hybrid (Y1H) system (Clontech), AD-TFs and pHIS2-zeatin3pro were co-transformed into the Y187 strains. The co-transformants were screened on the -Trp, -His, and -Leu deficient medium supplemented with 150 mM of 3-AT for 5 d. Each assay was performed in triplicate.

Luciferase assay

For the luciferase (LUC) assay, the promoter sequence of zeatin3 was amplified with the templates of pHIS2-zeatin3pro and fused with pGreenII Luc 0800 (pLUC). Then, the coding sequences of transcription factors were recombined into pGreenII 62SK. Thereafter, Agrobacterium strains (GV3101) were incubated overnight and resuspended in infiltration buffer (20 mM MES, 0.1 mM acetosyringone, and 10 mM MgCl2), and diluted to a concentration with an optical density (at 600 nm; OD600) of 0.6–0.8. The suspensions of pLUCs and pSKs were mixed in equal volumes and infiltrated into N. benthamiana leaves. After 60 h, the transformed leaves were sprayed with 0.1 M luciferin solution and placed in the dark for 7 min. A low light cooled CCD imaging apparatus (CHEMIPROHT 1300B/LND, 16 bits; Roper Scientific) was used for capturing and analyzing the acquired LUC images.

Statistical analysis

All data were analyzed using SPSS 20.0 (IBM, Inc.). The normality of distribution and homogeneity of variances were tested using Shapiro-Wilk's and Levene's tests, respectively, and differences among groups were

examined using one-way analysis of variance (Duncan). $p<0.05$ was set as significant.

Results

Melatonin (MT) treated *Elymus nutans* Griseb. (ENG) exhibited stronger physiological characteristics to cold exposure

Here, we observed that 4 °C (TW) significantly suppressed the growth of *Elymus nutans* Griseb. (ENG), compared to CK (25 °C) (Fig. 1A). Further we analyzed the effects of different concentration of melatonin (MT) in enhancing cold tolerance of ENG. The results showed that 100 μmol^{-1} of MT effectively alleviated the effects of TW exposure in affecting ENG growth, while other concentrations of MT did not exert significant effect on ENG growth under TW condition (Fig. 1A). Although 100 μmol^{-1} of MT did not affect the growth of the overground part of ENG, it observably promoted the root growth of ENG under normal condition, while low-temperature suppressed the growth of both roots and overground part of ENG (Fig. S4). To investigate the physiological responses of ENG to cold exposure (4 °C, TW), we determined numerous physiological parameters of plants from different treatments. The results showed that 4 °C (TW) did not affected the sugar contents in ENG, and significantly promoted the accumulation of MDA in *vivo* (Fig. 1B, C). By contrast, melatonin (MT) treatment reduced the accumulation of MDA in ENG under TW (Fig. 1B, C), suggesting that MT might promote the antioxidant capacity of ENG. Then, we determined the activities of antioxidant enzymes of ENG from each experiment group, including SOD, CAT and POD (Fig. 1D-F). The results showed that the activity of CAT and SOD in *vivo* was not affect by TW and MT (Fig. 1D, F). Of note, the activity of POD in TW was significantly lower than that in CK, suggesting that TW damaged the antioxidant system of ENG (Fig. 1E), then leading accumulation of MDA. Typically, MT increased the bioactivities of POD and SOD in ENG even under TW (Fig. 1E, F), suggesting that MT could enhance plant antioxidant capacity. Additionally, secondary metabolites represented by flavonoid also involved in the antioxidant system of plants [33]. Here, we found that the flavonoid content in ENG from TW was significantly higher than that in CK, whereas MT increased the flavonoid content in ENG even under TW (Fig. 1G). Taken together, TW could damage the antioxidant system of plant, while MT improved these adverse effects associated with TW, then enhanced cold tolerance of ENG.

Cold stress altered the metabolic and transcriptomic patterns of *Elymus nutans* Griseb. (ENG)

To investigate the effects of cold stress on ENG, three biological replicates for ENG from both TW and CK treatments were collected for transcriptome sequencing. Totally, 548,970 M clean reads were generated from all samples with high-throughput sequencing, and 142,219 genes were identified in ENG. Then, we performed principal component analysis (PCA) on all transcriptome profiles to investigate the transcriptome pattern of ENG under TW (Fig. 2A). PCA plot was constructed using principal component 1 (PC1) and PC2, which explained 32% and 23% variations between all samples (Fig. 2A). The PCA plot showed that the samples from TW clearly distributed at a single region and separated with CK (Fig. 2A), suggesting that low temperature significantly altered the transcriptome pattern of ENG. Typically, the differences between TW and CK were mainly represented by PC2 (Fig. 2A). Meanwhile, both spearman and pearson correlation analysis showed an obvious cluster containing all samples of TW, and separated with CK, supporting the PCA results and the credibility of our transcriptome data (Fig. S1). Then, 12,626 DEGs were identified, with 8794 downregulated DEGs and 3832 upregulated DEGs in TW vs. CK comparison based on *FDR*<0.05 and Foldchange>2.0 (Fig. 2B). Further Go enrichment analysis on all DEGs showed that these DEGs were significantly classified into 161 Go terms (FDR<0.05), including 83 terms relevant to biological progress, 40 terms of cellular component and 39 molecular functions (Table S1). Typically, these genes mainly functioned in chloroplast, plastid, thylakoid, plastid stroma, photosynthetic membrane and plastid envelope, with numerous functions, such as chlorophyll binding, cofactor binding, oxidoreductase activity, carotenoid dioxygenase activity, linoleate 13 S-lipoxygenase activity, oxidoreductase activity and ATPase (Table S1). And these genes mainly involved in Photosynthesis, plastid organization, oxidation-reduction process, response to toxic substance, pigment biosynthetic process, tetraterpenoid metabolic process, carotenoid metabolic process and flavonoid metabolic process of ENG (Table S1). Further KEGG pathway enrichment results showed that TW treatment mainly affected the expression of genes involved in Photosynthesis, Galactose metabolism, Linoleic acid metabolism, Phenylpropanoid biosynthesis, Starch and sucrose metabolism, Peroxisome, Glutathione metabolism, hormone signaling pathway and Flavonoid biosynthesis (Fig. 2C). Typically, numerous pathways relevant to antioxidant capacity of plants were significantly enriched in TW vs. CK comparison, especially Phenylpropanoid biosynthesis, Peroxisome, Glutathione metabolism and Flavonoid biosynthesis (Fig. 2C), supporting that low temperature affected the plant antioxidant system. Although the total content of sugar was not affected by cold stress in ENG, the genes involved in soluble sugar synthesis represented by Galactose metabolism were significantly differentially expressed in ENG under cold

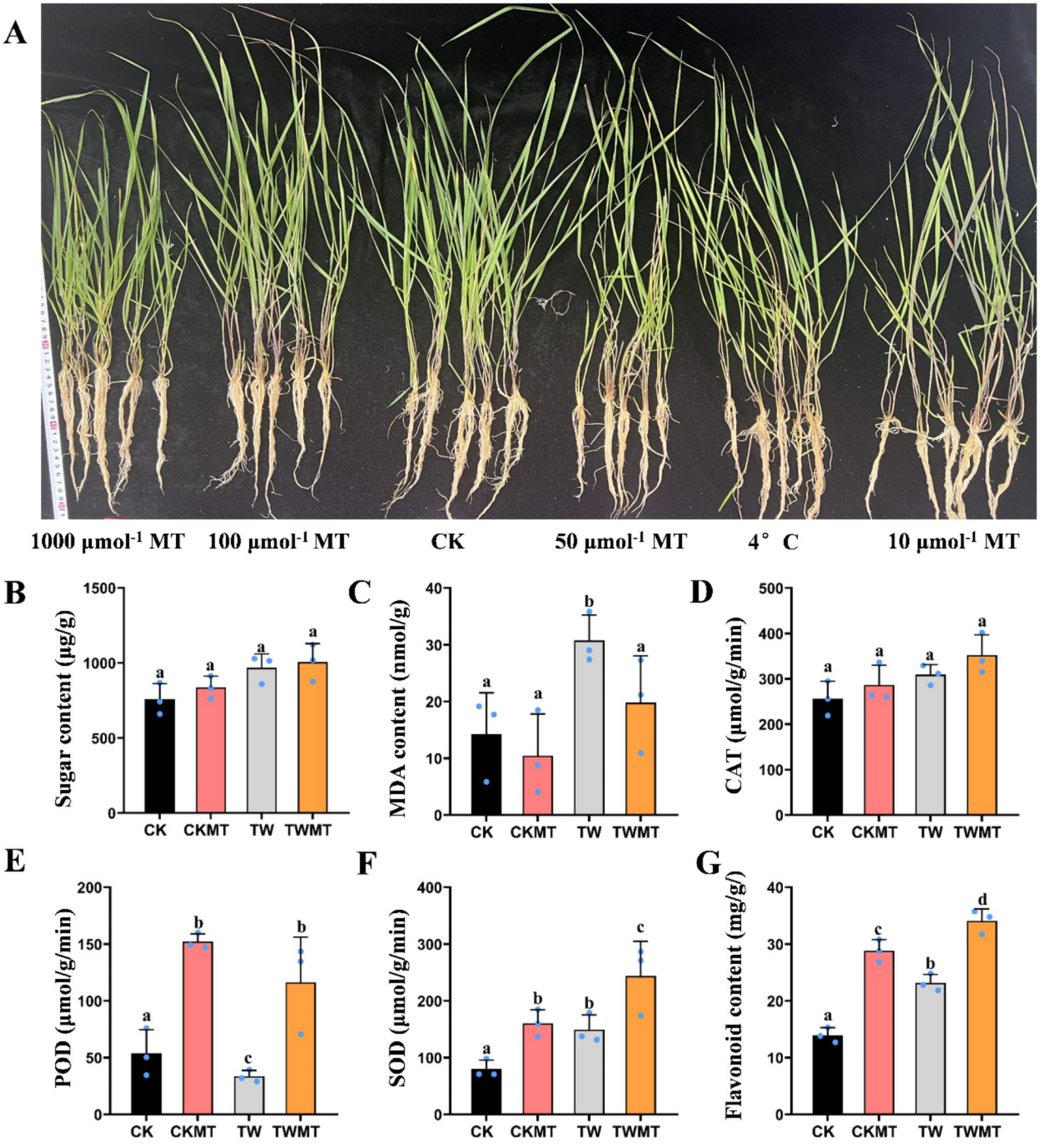

Fig. 1 Physiological characteristics of *Elymus nutans* Griseb. (ENG) under different experimental conditions. **A**: Phenotypes of *Elymus nutans* Griseb under normal and 4 °C conditions following treatment with different concentration of melatonin. (ENG). **B**: Determination of sugar contents in ENG. **C**: MDA content in ENG. **D-F**: Activity determination of antioxidant enzymes in ENG from each treatment. **G**: Total flavonoid content in ENG. Different letters indicated the significance based on $P < 0.05$

treatment (Fig. 2C). These results suggested that cold stress could altered the transcription pattern relevant to metabolisms of ENG.

Subsequently, we performed metabolomics analysis to investigate the metabolic composition of ENG under different temperature. Totally, 603 metabolites were identified in ENG, and the differentially expressed metabolites (DEMs) in TW vs. CK comparison were further filtered using $P < 0.05$ and Foldchange > 2.0 (Fig. 2E). The results showed that TW reduced the contents of 7

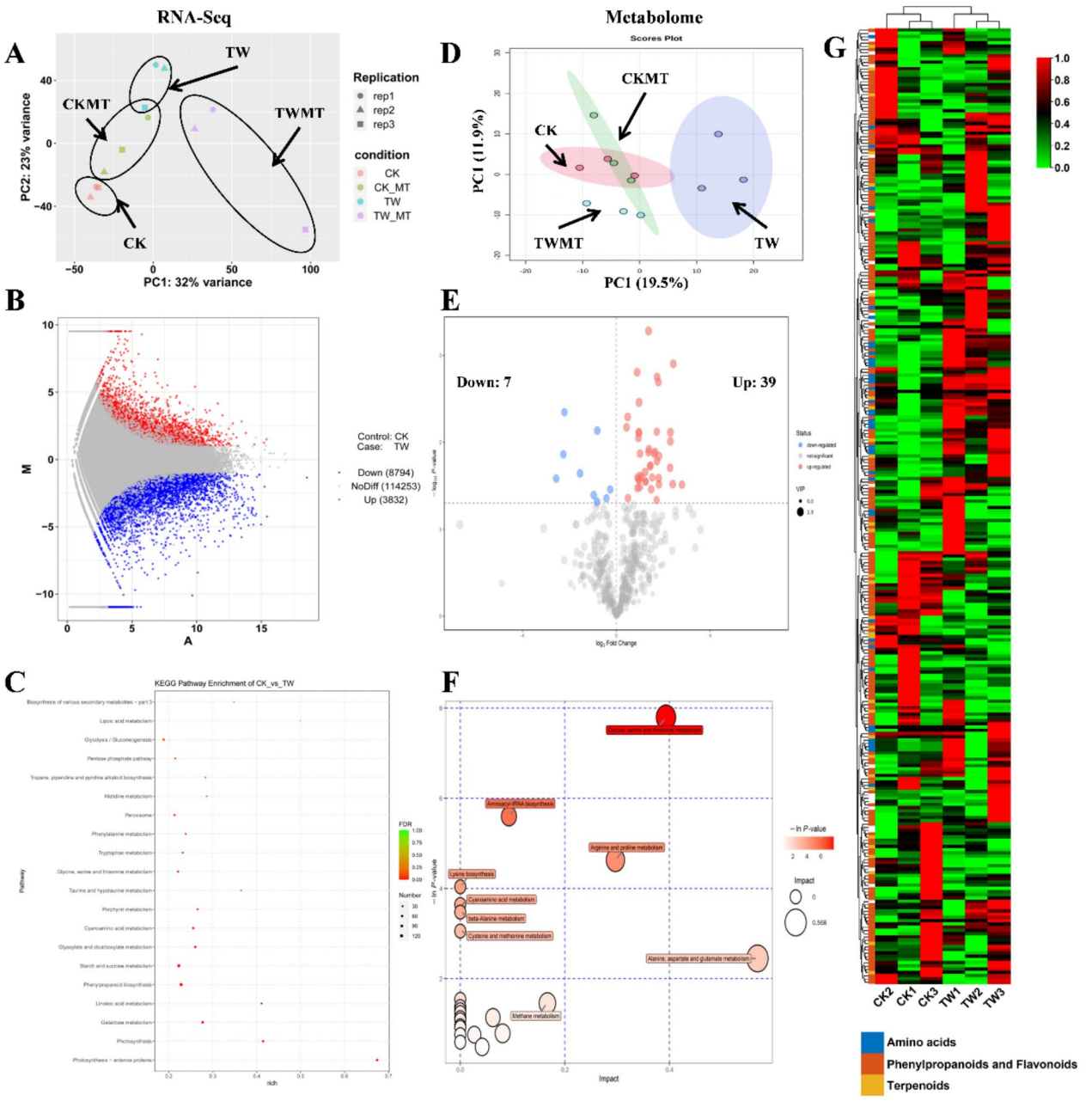

Fig. 2 Cold stress treatment altered the global metabolic and transcriptomic pattern of ENG. **A**, **D**: PCA scores plot of the samples showing distinct separation between CKMT, CK, TW and TWMT at transcriptome (A) and metabolome (D) levels. The ellipse represents the 95% confidence interval. **B**, **E**: Volcano plot shows the differential metabolites (D) and genes (A) with log2fold changed ≥ 2 and $P \leq 0.05$ in TW vs. CK pairwise comparison. The upregulated genes and metabolites are shown in red, while the downregulated ones are shown in blue. **C**, **F**: KEGG pathway enrichment analysis on DEMs (F) and DEGS (C) in TW vs. CK pairwise comparison. The various color levels displayed different levels of significance of metabolic pathways from low (green) to high (red). **G**: Heatmap displays the levels of phenylpropanoids and flavonoids in TW vs. CK comparison. The up- and downregulated metabolites were shown in red and green, respectively

metabolites, and increases 39 metabolite contents in ENG (Fig. 2E). Further PCA and OPLS-DA (Orthogonal partial least squares discriminant analysis) on metabolic profiles showed that TW significantly altered the metabolic compositions of ENG (Fig. 2D; Fig. S2), reaching a consensus with the transcriptome profiles that cold stress changed the metabolic compositions of ENG. Further KEGG enrichment analysis on these DEMs showed that these DEMs were significantly categorized in 8 pathways, including Glycine, serine and threonine metabolism, Aminoacyl-tRNA biosynthesis, Arginine and proline metabolism, Lysine biosynthesis, beta-Alanine

metabolism, Sphingolipid metabolism, Nitrogen metabolism, Glutathione metabolism, Starch and sucrose metabolism and Flavonoid biosynthesis (Fig. 2F). Of note, Phenylpropanoid and Flavonoid biosynthesis were also significantly enriched against metabolic profiles (Fig. 2F). Typically, we found that the contents of phenylpropanoids and flavonoids varied among CK and TW groups (Fig. 2G). Thus, these results suggested cold stress could alter the transcriptome and metabolic pattern of ENG, especially leading changes in compositions of flavonoids.

MT treatment altered the transcriptome response of *Elymus nutans* Griseb. to cold stress

Numerous researches documented the effects of MT in improving cold tolerance of plants [21, 22]. Here, we found that MT promoted the physiological characteristic of ENG in response to cold stress. Then, we further compared the transcriptome profiles of ENG after MT treatment under different temperature environment. The PCA results showed distinct variations in CKMT vs. CK and TWMT vs. TW comparisons (Fig. 2A), suggesting that MT altered the transcriptome pattern of ENG. Of note, the CKMT and TW samples partially overlapped with each other, suggesting that MT could affected some responses of ENG to cold challenge (Fig. 2A). Totally, 2310 (1465 downregulated DEGs and 845 upregulated DEGs) and 4902 (1856 downregulated DEGs and 3046 upregulated DEGs) DEGs were identified in CKMT vs. CK and TWMT vs. TW comparisons (Fig. 3A). Further Go enrichment analysis on DEGs from CKMT vs. CK showed that these genes significantly involved in 65 Go terms, such as NAD(P)H dehydrogenase complex assembly, cell wall modification, L-phenylalanine catabolic process, phenylpropanoid metabolic process, oxidation-reduction process, tyrosine catabolic process, ATPase inhibitor activity, dioxygenase activity, glucuronokinase activity, cell periphery, extracellular region and oxidoreductase complex (Table S1). It has been found that flavonoids accumulation and cell wall modification involved in the cold tolerance of plants [19]. Thus, these results implied that MT could alter the metabolic composition of ENG to enhance cold tolerance. In parallel, DEGs from TWMT vs. TW comparison mainly involved in photosynthesis, oxidation-reduction process, NAD(P)H dehydrogenase complex assembly, Thylakoid, photosynthetic membrane, chloroplast, plastid, hydrolase activity, oxidoreductase activity, glycerophosphocholine phosphodiesterase activity and glutathione hydrolase activity (Table S1). Cold exposure will damage the photosynthesis of plant to affect plant normal growth [9]. Of note, numerous terms relevant to photosynthesis were significantly enriched in TWMT vs. TW (Table S1), implying that MT might help plants maintain normal photosynthesis under cold condition.

Subsequently, we performed KEGG pathway enrichment analysis on both sets of DEGs (Fig. 3B). For DEGs from CKMT vs. CK, KEGG results showed that these DEGs mainly involved in Photosynthesis, ABC transporters, Phenylpropanoid biosynthesis, Glutathione metabolism, Plant hormone signaling, Zeatin biosynthesis and Flavonoid biosynthesis (Fig. 3B). It reached a consensus with GO results that MT could affect the transcriptome pattern of flavonoid biosynthesis in ENG under normal condition (Fig. 3B, C; Table S1). Additionally, we noted the enrichment of zeatin biosynthesis in CKMT vs. CK (Fig. 3B, C), implying that hormone zeatin might be responsible for regulating responses of ENG to cold stress. In addition, KEGG pathway enrichment analysis on DEGs from TWMT vs. TW showed that 17 biosynthesis pathways were significantly enriched, including Photosynthesis, Starch and sucrose metabolism, Galactose metabolism, Flavonoid biosynthesis, Phenylpropanoid biosynthesis, Tyrosine metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis and Zeatin biosynthesis (Fig. 3B). Of note, Flavonoid biosynthesis, Zeatin biosynthesis and Photosynthesis were also significantly enriched in TWMT vs. TW comparison (Fig. 3B, C), supporting that MT could affect these processes to enhance ENG cold tolerance. Importantly, the pathways affected by both cold stress and MT treatments were highly consistent in our transcriptome profiles of ENG (Fig. 3C). Then, we analyzed the detailed expression pattern of genes involved in Flavonoid and Zeatin biosynthesis (Fig. 3D). As shown in Fig. 3D, the expression pattern of genes relevant to flavonoid biosynthesis were reprogrammed by MT, leading to accumulation of specific metabolites in ENG. Additionally, most of genes relevant to zeatin signal were upregulated by cold and MT treatments (Fig. 3E), supporting the involvement of zeatin in regulating MT-enhanced cold tolerance of ENG.

Taken together, we proposed that MT could enhanced cold tolerance of ENG via promoting accumulation of specific flavonoids and maintaining normal photosynthesis, and this effect of MT were mediated by zeatin signal.

MT treatment altered the metabolic compositions associated with cold environment in *Elymus nutans* Griseb

Here, we further compared the metabolic profiles of ENG rom CKMT vs. CK and TWMT vs. TW comparisons. To identify the overview of changes in metabolic pattern of ENG following MT treatment under different temperature, we performed PCA analysis on all metabolic profiles of ENG (Fig. 2D). The PCA plots constructed by PC 1 and 2 showed significant variations in metabolic patterns among all groups, while PC1 and PC2 explained 19.5% and 11.9% variations associated with experimental treatments (Fig. 2D). Typically, PC1 mainly explained the variations between TWMT and TW comparison (Fig. 2D),

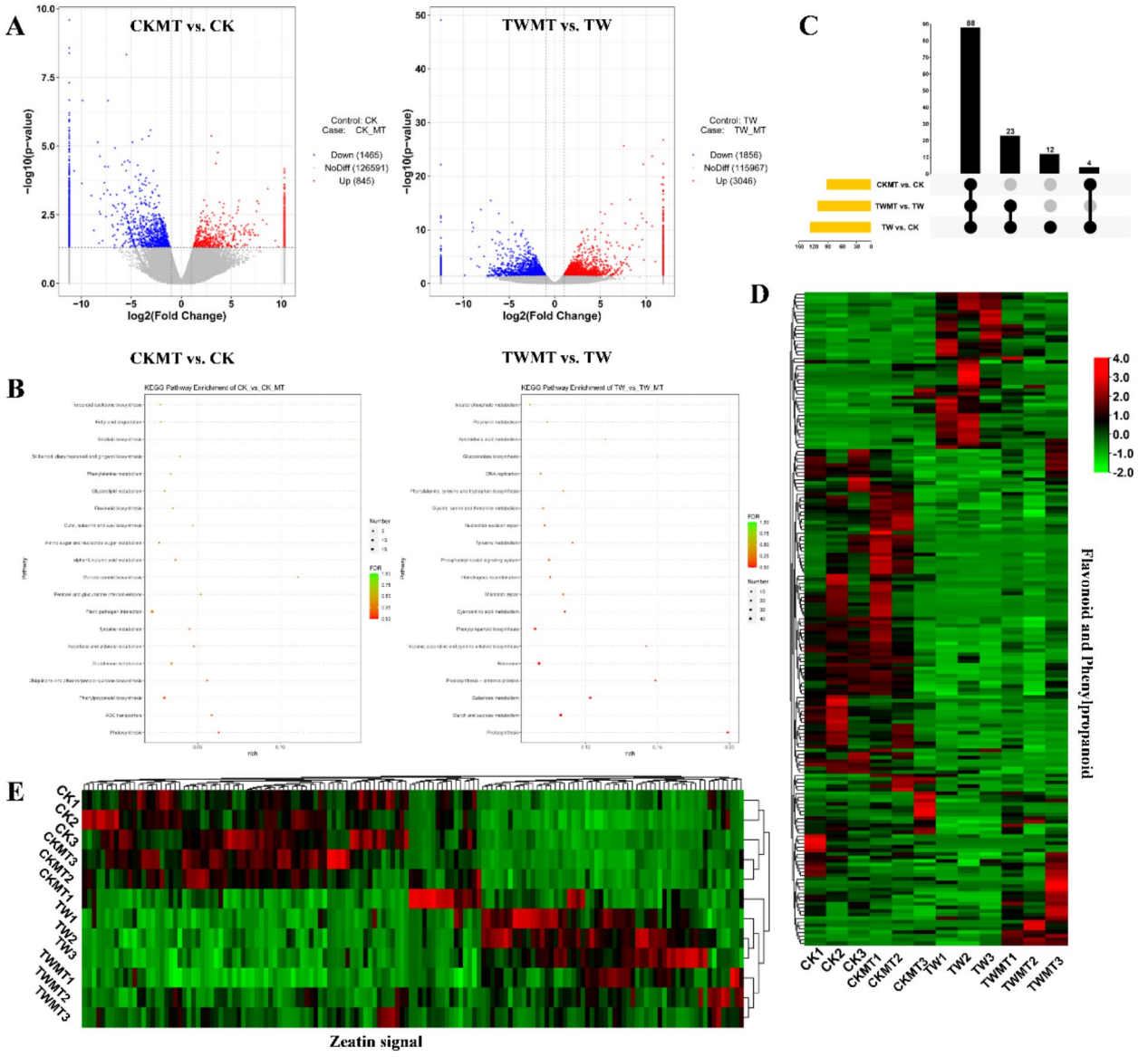

Fig. 3 MT affected the transcription responses of ENG to cold stress. **A**: Volcano plot shows the differentially expressed genes (DEGs) with Foldchange ≥ 1 and $P \leq 0.05$ in ENG from CKMT vs. CK (left) and TWMT vs. TW (right) comparisons. The up- and down-regulated genes are shown in red and blue, respectively. **B**: Scatter plot displays the pathway enrichment results based on DEGs from CKMT vs. CK (left) and TWMT vs. TW (right) comparisons. **C**: Upset plot displays the overlapped pathways associated with MT and low temperature treatments in ENG. **D**, **E**: Heatmap displays the expression levels of genes relevant to flavonoid (D) and zeatin (E) biosynthesis in ENG under different experimental conditions. The high and low expression levels of genes in each biological replicate are shown in red and green, respectively

suggesting that MT altered the metabolic composition of ENG under **cold condition**. Then, 22 (9 upregulated DEMs and 13 downregulated DEMs) and 46 (41 upregulated DEMs and 5 downregulated DEMs) DEMs were identified in CKMT vs. CK and TWMT vs. TW, respectively, based on $P<0.05$ and Foldchange > 2.0 (Fig. 4A). We cauterized all these DEMs using KEGG pathway analysis, and found that these DEMs were mainly synthesized from beta-Alanine metabolism, Citrate cycle (TCA cycle), Alanine, aspartate and glutamate metabolism, Flavonoid biosynthesis, Phenylpropanoid biosynthesis, Starch and sucrose metabolism and Zeatin biosynthesis (Fig. 4B). Of note, MT treatment also reprogrammed the compositions of flavonoids and soluble sugars in ENG (Fig. 4B). Typically, we found that numerous flavonoids were induced to increase in ENG by MT and cold treatments, especially neoglycyrol, daidzein-4'7-diglucoside, delphinidin, pelargonidin-3-O-glucoside, butein and phillyrin (Fig. 4C). Considering the effects of flavonoids in plant response to cold stress, these metabolites were identified as candidate compounds that involved in the mechanisms of MT-enhanced cold tolerance of ENG.

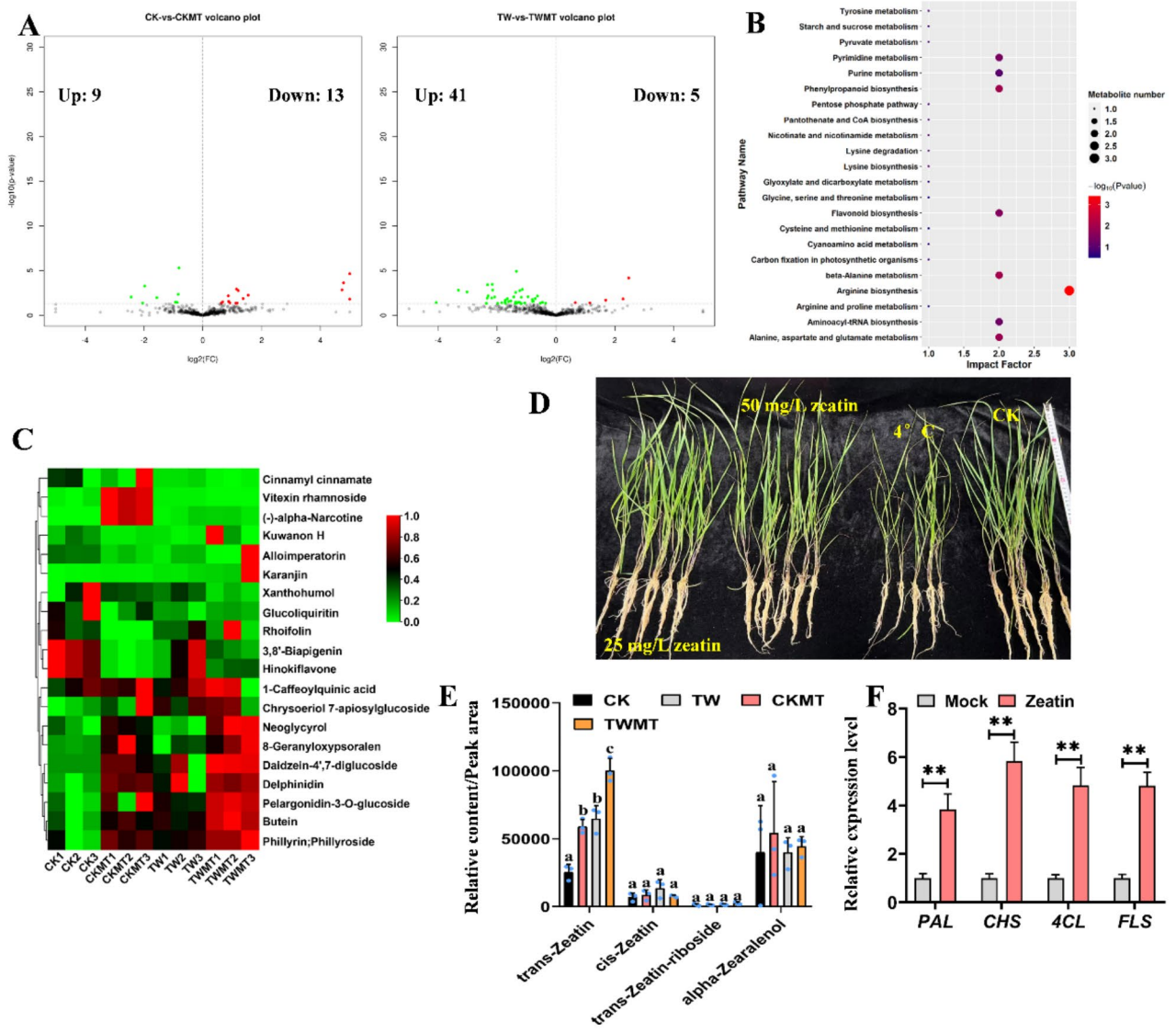

Fig. 4 Zeatin regulated the MT-induced accumulation of flavonoid in ENG in response to cold stress. **A**: Volcano plot shows the DEMs in ENG from CKMT vs. CK (left) and TWMT vs. TW (right) comparisons. The up- and down-regulated proteins are shown in green and red, respectively. **B**: Scatter plot of the most enriched KEGG pathways of all DEMs associated with MT treatment in ENG under different temperature. **C**: Heat maps of the relative contents of flavonoids in ENG following different treatment. The scale bar showed the normalized peak area values of metabolites in each biological samples. **D**: Phenotypes of *Elymus nutans* Griseb under normal and 4 °C conditions following treatment with different concentration of zeatin. **E**: Content of zeatin in ENG from different experimental groups. Different letters indicated the significance based on $P < 0.05$. **F**: RT-qPCR detects the expression levels of genes relevant to flavonoid biosynthesis in ENG after exogenous zeatin treatment. "**": $P < 0.01$ based on Student's T-test"

Additionally, we noted the accumulation of zeatin in TW, and its content were further induced to higher levels in ENG by MT (Fig. 4E). Thus, we proposed that zeatin might involve in regulating MT-enhanced cold tolerance of ENG. To test this possibility, we exogenously applied zeatin to ENG under cold condition, and found that zeatin effectively alleviated the suppression effects of cold stress on ENG growth, especially 25 mg/L of zeatin (Fig. 4D). Then, we detected the expression levels of numerous genes relevant to flavonoid biosynthesis in ENG after exogenous zeatin treatment using RT-qPCR (Fig. 4F). The results showed that zeatin treatment significantly induced the expression levels of *PAL*, *CHS*, *4CL* and *FLS* (Fig. 4F), suggesting that the MT- and cold-induced accumulation of flavonoids in ENG were mediated by zeatin signal. Overall, these results suggested that MT mainly promoted the cold tolerance of ENG via increasing flavonoid content, which was mediated by zeatin signal.

Active regulation network of zeatin synthesis involved in regulating MT-conferred cold tolerance of ENG

Considering the effects of zeatin in regulating MT-enhanced cold tolerance of ENG, we further constructed the co-expression network of genes relevant to zeatin synthesis (*zeatin1*, *zeatin2*, *zeatin3* and *UGT73C3*) using WGCNA based on all transcriptomic profiles of ENG (Fig. 5). The expression data of all transcription factors of ENG were selected via Nr annotation for WGCNA construction. We set the soft threshold to 22 (R^2=0.75) to construct a scale-free network (Fig. 5A). Then, 14 modules were identified by hierarchical clustering and the dynamic branch cutting (Fig. S3A, B), each module was assigned a unique color as an identifier (Fig. 5B; Fig. S3A, B). Then, the modules highly correlated with the related traits were filtered out for further construction of regulation network of zeatin synthesis in ENG (Fig. 5B). Based on the WGCNA results, we identified that MEbrown, MEmagenta, MEyellow and MEturquoise were highly related to genes relevant to zeatin synthesis (Fig. 5B). Among genes from these modules, we constructed the co-expression regulation networks for zeatin synthesis (Fig. 5C). Totally, we identified that 170 transcription factors were responsible for positively regulating zeatin synthesis in vivo (P<0.05; Fig. 5C; Table S3). As shown in Fig. 5E, NAM, GT-3a, GHD7, WRKY17, WRKY11, MYB59 and RAP2 (Accession number: TRINITY_DN1111_c0_g2, TRINITY_DN17254_c0_g1, TRINITY_DN20832_c0_g1, TRINITY_DN25531_c0_g1, TRINITY_DN30587_c0_g1, TRINITY_DN37908_c0_g1 and TRINITY_DN5309_c0_g1) were the hub genes involved in the regulation network of zeatin synthesis in ENG, and it was positively correlated with the expression of zeatin-related genes (Fig. 5C; Table S3). Subsequently, to investigate the binding capacity of these transcription factors to zeatin promoters, we expressed these transcription factors represented by *WRKY11*, *MYB59* and *WRKY17* fused to AD in a Y1H system to challenge it with the *zeatin3* promoter fused to the HIS2 reporter (Fig. 5E). The induction effect of WRKY11 on yeast growth compared to the control indicated that WRKY11 were able to regulate *zeatin3* expression by directly binding to their promoters (Fig. 5E). Furthermore, the LUC assay was carried out by co-transfecting protoplasts with reporter and effector constructs to assess the effect of WRKY11 on the promoter activity of *zeatin3*. A DNA fragment corresponding to the promoter region of zeatin3 was fused to the firefly luciferase (LUC) coding sequence to generate the luciferase reporter plasmid with renilla luciferase (REN) reporter driven by the 35 S promoter as an internal control. Co-expression of SK::WRKY11 effector with the prozeatin3::LUC in *N. benthamiana* leaves resulted in a significant increase in luminescence (Fig. 5D), revealing the ability of WRKY11 to activate the transcription of zeatin3. Typically, as shown in Fig. 5F, most of these genes were upregulated to higher expression levels in ENG by MT and cold treatments, suggesting that MT could activate the regulation network of zeatin synthesis to increase zeatin content in ENG. Further RT-qPCR assay confirmed that transcription factors including WRKY17, WRKY11 and MYB59 were activated to upregulate in ENG by MT and cold treatments (Fig. 5G). Taken together, these results suggested that MT could activate zeatin signal to promote flavonoid accumulation in ENG, then promoted the cold tolerance of ENG.

Discussion

In the cold regions, especially plateau, low temperature disaster has become a nonnegligible obstacle to the development of grass. Thus, it is of great scientific value and practical significance to develop an effective management strategy of improving the cold tolerance of plants for guiding the development of grass industry. MT is known to promote plant growth and improve plant resistance to adapt abiotic stress in field [21, 22]. Presently, we found that cold stress reprogrammed the expression pattern of genes involved in galactose and flavonoids biosynthesis, leading to dramatic changes in the metabolic compositions of ENG, typically altering the composition of soluble sugar and flavonoids. Additionally, cold stress inhibited the photosynthesis, and destroyed the antioxidant system of ENG, leading accumulation of oxidant radicals represented by MDA in ENG. Of note, we found that MT treatment effectively promoted cold tolerance of ENG via increasing the activity of antioxidant system of ENG even under cold condition, leading to decreases in MDA content. Moreover, transcriptome profiles showed that MT altered the transcription responses of ENG to cold stress, especially maintaining normal expression of photosynthesis-related genes and reprogramming the synthesis pattern of flavonoid and soluble sugar. Further metabolome showed that MT promoted the accumulation of specific flavonoids and soluble sugars. Importantly, we noted that MT mainly enhanced cold tolerance of ENG via activating zeatin signal to regulate flavonoid biosynthesis in *vivo*. Meanwhile, coexpression network analysis showed that MT could activate expression of numerous transcription factors (WRKY17, WRKY11 and MYB59) to promote zeatin synthesis in ENG. Importantly, WRKY11 was identified to regulate MT-activated zeatin synthesis in ENG via directly binding on *zeatin3* promoter. Overall, our research investigated the detailed global mechanisms of MT-promoted cold tolerance of ENG, which provided guidance for usage of MT in managing damage to plant caused by cold stress in plateau.

Cold stress always caused excessive accumulation of reactive oxygen species (ROS) in plants [34]. ROS

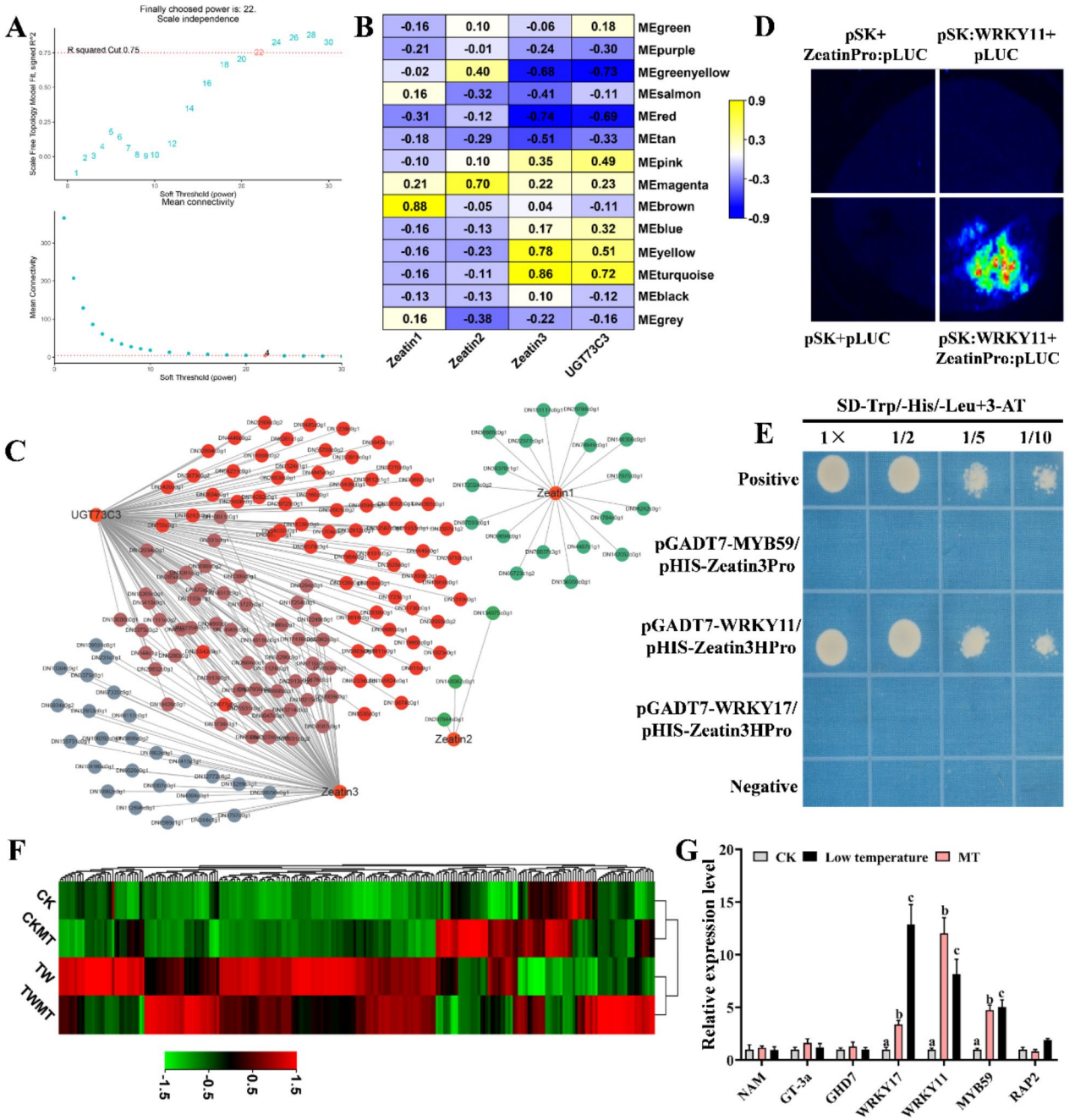

Fig. 5 MT activated the regulation network for zeatin synthesis in ENG to enhance its cold tolerance. **A**: Scale independence and mean connectivity analysis of WGCNA construction. **B**: Heatmap displaying the Module-trait relationships. Each row represents a module eigengene, and column represents a trait. **C**: The weight network of the significant genes involved in the module labeled by MEblack. The color and the size of the circle represented the weight value of each gene in the network. The circle sizes displayed the degree value of corresponding genes in the network, which showed the gene importance. **D**: Yeast one-hybrid assay was used to detect the interaction between WRKY11 and Zeatin3 promoter. Yeast cells grew in SD/−Leu/−Trp/-His + 3-AT plates after transformation with WRKY11 and Zeatin3 promoter. **E**: Luciferase assay was used to detect the binding activities of WRKY11 to Zeatin3 promoter. Zeatin3 promoter-LUC and WRKY11-SK were co-expressed in *N. benthamiana*. **F**: Heatmap showing the expression of genes encoding hub transcription factors that involved in regulating zeatin synthesis of ENG. The scale represented the normalized FPKM values of genes from each sample. The genes with high expression abundance were shown in red. **G**: RT-qPCR detects the expression levels of genes encoding hub transcription factors in ENG following MT and cold treatment. "**": $P < 0.01$ based on Student's T-test"

accumulation aggravated membrane lipid peroxidation and destroyed the membrane integrity, then negatively affected plant development [35]. In response, plants deployed ROS scavenging systems consisted with SOD, CAT and POD enzymes to scavenge ROS in vivo [36]. Here, we found that these antioxidant enzymes of ENG were inhibited by cold stress, leading to accumulation of oxidant radical, especially MDA in vivo. Interestingly, MT effectively increases the activity of antioxidant enzyme of ENG even under cold conditions, leading to decreases in MDA content. Additionally, numerous research found that cold stress significantly destroyed photosystem II (PSII) and photosystem I (PSI), leading to the degradation of chloroplasts in severe cases [37, 38]. Chloroplast degradation reduces the APX activity, then blocking the scavenging of H2O2 and causing changes in plant metabolism [35]. Moreover, disruption of photosynthesis leads to a decrease in soluble sugars of plants. Contents of soluble sugar are important for plants under cold stress, and their accumulation enhanced plant cold resistance through increasing the concentration of cell fluid to reduce the freezing point of cytoplasm [39–43]. Here, our transcriptome profiles also showed downregulation of photosynthesis-related genes in ENG following cold challenge. This phenomenon has been documented in a range of plants under cold stress [41, 42, 44]. However, MT treatment increased their expression levels in ENG under cold conditions, suggesting that MT could alleviate the obstacle of photosynthesis in plants, then maintaining normal growth of plant under cold conditions. Additionally, we found that genes involved in biosynthesis of flavonoid and soluble sugars were also significantly affected by cold stress. Flavonoids and their derived anthocyanins have antioxidant activities and could scavenge ROS in vivo [45]. It was found that the reprogrammed pattern of flavonoids in vivo was a response of plant to cold stress [46, 47]. Typically, accumulation of flavonoids will enhance the cold tolerance of plants [46, 47]. Here, our integrated transcriptome and metabolome of ENG showed that MT effectively reprogrammed the biosynthesis of flavonoids in ENG under cold condition, leading to accumulation of numerous metabolites, including neoglycyrol, daidzein-4'7-diglucoside, delphinidin, pelargonidin-3-O-glucoside, butein and phillyrin. Thus, these results supported our hypothesis that MT could enhance cold resistance in ENG through preventing decreases in related metabolites to promote capacity of antioxidant system in plants. Importantly, we found that MT mainly triggered zeatin synthesis to promote flavonoid biosynthesis in ENG. Numerous transcription factors represented by WRKYs were identified to be responsible for regulating MT-triggered zeatin synthesis in ENG. It has been well documented that WRKYs transcription factors performed important function in plant resistance to cold stress [48–51]. Here, we found that WRKY11 could directly bind with the promoters of genes involved in zeatin synthesis. Typically, LUC assay showed that WRKY11 mainly triggered the expression of zeatin-related genes in vivo. Thus, its upregulation will promote the expression of genes encoding enzymes for zeatin synthesis. Meanwhile, the accumulation of zeatin could further activate the expression of genes involved in flavonoid biosynthesis, especially *CHS*, *4CL*, *FLS* and *PAL*, which has been well documented in encoding enzymes in synthesizing flavonoids. Moreover, these flavonoids always exerted antioxidant activity and will protect ENG from cold stress, especially related oxidant damage. Thus, we conclude that MT mainly enhanced ENG cold tolerance via increasing antioxidant system and promoting zeatin-mediated flavonoid biosynthesis via deploying WRKY11. Although the function of these WRKYs were not investigate in present work, we fully demonstrated that MT could be applied to improve cold resistance of crops in plateau.

Conclusion

In the present study, we deciphered the global mechanisms of MT-enhanced cold tolerance of ENG via integrating metabolome and transcriptome. We found that exogenous MT could promoted the cold resistance in ENG. Typically, MT mainly enhanced ENG cold tolerance via increasing antioxidant system, maintaining photosynthesis and promoting zeatin-mediated flavonoid biosynthesis. Moreover, our MT-cold regulation network identified that numerous transcription factors represented by WRKY11 were identified to involve in regulating MT-triggered zeatin synthesis for high cold tolerance of ENG through directly binding on promoters of genes involved in zeatin synthesis. Overall, the present study provides a novel and valuable strategy for protecting ENG from cold stress in plateau through the application of MT.

Abbreviations

C. nutans	*Campeiostachys nutans*
PSII	Photosystem II
RuBisCO	Ribulose bisphosphate carboxylase oxygenase
MDA	Malondialdehyde
ROS	Reactive oxygen species
CAT	Catalase
MT	CK + melatonin
SOD	Superoxide dismutase
POD	Peroxidase
GO	Gene Ontology
DEGs	Differentially expressed genes
IDA	Information-dependent acquisition
PCA	The Principal Component Analysis
KEGG	Encyclopedia of Genes and Genomes
TCA cycle	Citrate cycle

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions
Conceived and designed the experiments: PBZM and DJTD Performed the experiments: PBZM, TDQP and DS. Analyzed the data: PBZM and JJF. Contributed reagents/materials/analysis tools: TDQP. Wrote the paper: PBZM and DJTD. All authors have read and approved the final manuscript.

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Data availability
The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA014891) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Declarations

Ethics approval and consent to participate
The collection of plant materials used in our study complied with permission of related institutions, and complied with national or international guidelines and legislation. The experiments did not involve endangered or protected species.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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