

DNA methylation participates in drought stress memory and response to drought in *Medicago ruthenica*

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Abstract

Drought is a serious environmental problem, affecting plant growth and development and resulting in severe yield loss. Many plants exposed to drought stress can generate stress memory, which provides some advantages for resisting recurrent drought. DNA methylation is a mechanism of stress memory formation, and many plants can alter methylation levels to form stress memories; however, it remains unclear whether *Medicago ruthenica* exhibits drought stress memory, as the molecular mechanisms underlying this process have not been described in this species. Here, we conducted methylome and transcriptome sequencing to identify gene methylation and expression changes in plants with a history of two drought stress exposures (D2). Methylation analysis showed that drought stress resulted in an approximately 4.41% decrease in *M. ruthenica* genome methylation levels. CG dinucleotide contexts had the highest methylation levels, followed by CHG contexts, with CHH contexts having the lowest levels. Analysis of associations between methylation and transcript levels showed that most DNA methylation was negatively correlated with gene expression, except for methylation within CHH in gene promoter regions. Genes were divided into four categories according to correlation between methylation and gene expression; demethylated genes expression upregulation accounted for the vast majority (n = 692 genes), and included genes encoding key factors for abscisic acid (ABA) and proline synthesis. Demethylation of the promoter and body regions of these two genes induced increased gene expression levels. In conclusion, DNA methylation may contribute to drought stress memory formation and maintenance in *M. ruthenica* by increasing transcription levels of genes key for ABA and proline biosynthesis.

INTRODUCTION

Climate drought is a phenomenon that can have negative impacts on the natural environment and human wellbeing, and has become the focus of research attention in recent years, since drought events have increased [1, 2]. Drought stress can impair plant growth and development, ultimately affecting crop productivity [3, 4]. Hence, understanding the genetic and molecular mechanisms underpinning plant resistance to drought is critical [5]. Plants can undergo genetic or biochemical modifications when facing drought stress for the first time, which result in differences in their responses to future stress, a process referred to as drought stress memory [6]. Drought stress memory can be transmitted through mitosis, where plants that have been subjected to one drought will show stronger physiological responses when subjected to a subsequent drought than when they are subjected to drought treatment for the first time [7–10]. Transcription responses during repeated exposures to stress differ from those occurring during a single exposure (Ding *et al.* 2013). Exposing plants to stress-induced hormone accumulation may alter their response to subsequent stresses, improving survival rates [11, 12]. In addition to changes in physiological parameters, such as abscisic acid (ABA), after repeated exposure to stress, certain stress responsive genes exhibit transcriptional changes that differ significantly from their initial response [13, 14]. This phenomenon occurs because plant genes exhibit transcriptional memory response patterns to drought stress, which can further alter cellular responses and crosstalk between pathways by changing transcript or encoded protein levels [15–17]. The definition of transcriptional memory is that the transcript

levels produced under continuous dehydration stress reactions must be different from those in response to initial stress exposure [18]. Four types of dehydration stress memory genes have been identified in *Arabidopsis* spp. and *Zea mays*, and their transcriptional behavior under repeated stress is more complex than that in response to a single dehydration stress, mainly manifesting as differences in gene transcript levels during the first and last stress exposure [15, 16]. Hence, dehydration stress memory is a complex phenotype produced by coordinated responses of multiple signaling pathways [19].

Numerous factors influence changes in transcription levels, one of which is epigenetic modification, including DNA methylation, histone modification, miRNA, and transcription factor modification, which may be among the molecular mechanisms underlying drought stress memory. These modifications can affect gene expression by altering the state of chromatin without changing DNA sequence, and are heritable, with important roles in regulating plant growth and development. DNA methylation is a relatively stable modification, which participates in plant drought stress memory formation and maintenance [20, 21]. Particular methylation types, or increased or decreased methylation levels, occur at specific DNA locations to influence stress response-related gene response pathways, increase enzyme activity related to stress responses, and thus defend plant functional gene expression against the impact of stress, thereby protecting plant growth and development [17, 22, 23].

Medicago ruthenica is a perennial leguminous forage grass, widely distributed in grasslands, sandy land, riverbanks, and hillside wilderness with sandy soil in China, Mongolia, and Russia. *M. ruthenica* is a close relative of alfalfa (*Medicago sativa*) and has high tolerance to environmental stress, providing valuable genetic resources for understanding and improving tolerance to adversity [24, 25]. The study of epigenomic variations in plants has mainly been limited to a few model plants, such as *Arabidopsis* and rice. To date, no research into the drought stress memory of *M. ruthenica*, based on genomic methylation modification, has been reported.

The purpose of this study was to explore the drought stress memory transcriptional responses of *M. ruthenica*, based on DNA methylation, and to explore the following three issues in depth: (1) Whether *M. ruthenica* develops stress memory at the gene transcription level after repeat drought stresses; (2) What effect drought stress has on genomic methylation; and (3) What is the relationship between methylation group modifications and changes in gene transcription?

MATERIALS AND METHODS

Plant Material and Drought Treatments

Our experimental material is wild cultivated variety *Medicago ruthenica* (L.) Sojakcv. Tumote, National approved variety, registration number 379. Zhaolan Wang identified the plant materials we used. *M. ruthenica* seeds were scarified using sandpaper, then incubated in the dark at 4°C for 3 days. Next, seeds were placed in a growth chamber (temperature, 25°C ± 2°C; photoperiod, 16 h light/8 h darkness; relative humidity, 50%). After germination, seeds were transferred to pots (8 × 8 × 10 cm) filled with nutrient soil

(PINDSTRUP company) and vermiculite (1:1), with one plant in each pot under the same growth conditions. Six-week-old seedlings were randomly divided into two groups: a control group with no stress history (CK), and a group with a history of two drought stress exposures (D2) (n = 45 per group). First, D2 seedlings were first subjected to drought stress twice, then both groups were subsequently subjected to drought stress simultaneously. Drought treatment comprised 15 days in 30% soil moisture content. After exposure to drought stress, plants underwent a 3-day rehydration period, during which soil moisture content was maintained at > 80%. Details of drought stress and water recovery treatment are presented in Fig. 1. Plants phenotypic traits were observed after each stress and rehydration, and leaf samples were collected before and after the last stress exposure. Samples were immediately frozen in liquid nitrogen and then transferred to a - 80°C refrigerator for downstream processing.

Morphological Traits

M. ruthenica morphological traits, including height, stem diameter, leaf number, leaf area, internode length, aboveground biomass, underground biomass, and total biomass, were measured. Height was defined as absolute plant height measured as the straightened length from the soil surface to the top of the leaves. Leaf area was measured using a leaf area meter (LI-3000 Portable Area Meter, LI-COR Lincoln, NE, USA). The middle leaflet of the top three compound leaves of the plant was chosen for leaf area measurement; three duplicate leaves were measured and mean values calculated. Using a vernier caliper, the stem thickness of the main base, and the length of each stem node at different positions on the branch, middle, and bottom were measured, and the mean value taken as the mean internode length. For biomass measurement, individual plants were divided into aboveground and belowground parts, then dried at 65°C for 24 h and weighed (accuracy, 0.01).

Leaf Physiological Variable Measurement

Leaves were collected (weight, 0.2 g for each variable) from the same growth site from seedlings under different treatment conditions and stored in an ice box for physiological indicator measurement.

Soluble Sugar Content

Weighed samples (0.1–0.2 g) were added to 1 mL of distilled water and ground into a homogenate, poured into a covered centrifuge tube, transferred to a 95°C water bath for 10 min, allowed to cool, centrifuged (8000 g, 25°C, 10 min), and supernatants transferred into 10 ml test tubes. Samples were then diluted to 10 mL with distilled water and shaken well for later use. A spectrophotometer or enzyme reader was preheated for > 30 min, the wavelength adjusted to 620 nm, and absorbance values measured.

Malondialdehyde (MDA) Content

The Plant MDA Assay Kit with TBA (Suzhou Comin Biotechnology Co., Ltd, Suzhou, China) was used to determine MDA content in plant leaves, following the manufacturer's instructions.

Proline Content

A reagent kit (Suzhou Comin Biotechnology Co., Ltd, Suzhou, China) was used to determine plant leaf proline content, following the manufacturer's instructions for the operation and calculation methods.

ABA Content

ABA content was determined following previous reports [26, 27]. Briefly, plant leaf samples were ground into powder under liquid nitrogen, then placed in 2 ml centrifuge tubes and a methanol acetonitrile aqueous solution added (40:40:20). Samples were shaken, mixed for 2 min, and then 16873×g centrifuged protected from light at 4°C for 12 h (Centrifuge 5418 R, Eppendorf) and supernatants dried under nitrogen gas. A constant volume of methanol aqueous solution (50:50, v/v) was then added, followed by centrifugation (10 min, 14000 r/min), and analysis of the supernatant by chromatography. The chromatographic conditions were as follows: mobile phase, liquid A, 0.04% formic acid aqueous solution and liquid B 0.04% formic acid acetonitrile solution; column temperature, 45°C; flow rate, 400 µl per min; sample volume, 4 µl; chromatographic column, Waters, ACQUITY UPLCBEHC18 (2.1 × 100 mm, inner diameter 1.7 µm); electrospray ionization source (ESI); ion source temperature, 500°C; Agilent 1290 HPLC-MS system.

DNA Library Construction and WholeGenome Bisulfite Sequencing

Samples from CK and D2 before the last stress (CK-DB, D2-DB) were selected for methylation analysis. A Genomic DNA Extraction Kit (Tiangen Company, DP305) was used to extract genomic DNA from *M. ruthenica* leaf samples. DNA purity, concentration, and integrity were measured by spectrophotometry using NanoDrop, Qubit 2.0, Agilent 2100, or NanoPhotometer® (IMPLEN, CA, USA) instruments and by agarose gel electrophoresis. Genomic DNA was first sheared into 100–300 bp by exposure to ultrasound (Covaris, Massachusetts, USA), and then purified with MiniElute PCR Purification Kit (QIAGEN,MD,USA). Genomic DNA fragments were then ligated to a methylated sequencing adapters. Finally, DNA fragments were polymerase chain reaction (PCR) amplified and sequenced using the Illumina HiSeq™ 2500 platform by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China).

Genome Wide Methylation Level Analysis

Clean sequence reads were mapped to the species reference genome using BSMAP software [28] (version: 2.90), with default parameters. Methylated cytosine sites were identified by binomial test, which was used to exclude non-conversion errors. Methylation levels were calculated based on methylated cytosine percentage in the whole genome, in each chromosome, and in different regions of the genome for each nucleotide sequence context (CG, CHG, and CHH).

Differential DNA methylation at each locus between the two experimental groups was determined using Pearson's chi-square test (χ^2) in methylKit [29] (version: 1.7.10). To identify differentially methylated cytosines (DMCs), the minimum read coverage to call a methylation status for a base was set to 4.

Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted for DMC/differentially methylated region (DMR) -related genes. GO enrichment analysis provides a comparison of all GO terms significantly enriched in genes within genomic backgrounds, and filters out gene functions corresponding to biology. KEGG comprises publicly available molecular pathway-related data (<http://www.kegg.jp/kegg/>). Compared to the whole genome background, pathway enrichment analysis identified significantly enriched metabolic or signaling pathways in genes.

RNA Extraction and Transcriptome Sequencing

Samples from plants in the CK and D2 groups collected before the final stress exposure (CK-DB, D2-DB) were selected for transcriptome analysis. RNA was extracted from leaves using a Total RNA Extraction Kit (TianGen Company, DP424), following the manufacturer's protocol. The purity, concentration, and integrity of RNA were measured using spectrophotometers (NanoDrop, Qubit 2.0, and Agilent 2100). After total RNA was extracted, eukaryotic mRNA was enriched using Oligo(dT) beads, then the enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse transcribed into complementary DNA (cDNA) using the NEB Next Ultra RNA Library Prep Kit for Illumina. Purified double-stranded cDNA fragments were end filled, A bases added, and ligated to Illumina sequencing adapters. Ligation reactions were purified using AMPure XP Beads (1.0×) and amplified by PCR. The resulting cDNA library was sequenced using an Illumina Novaseq 6000 System by Gene Denovo Biotechnology Co. (Guangzhou, China).

Quantitative Real-Time PCR Analysis

CK and D2 samples, including CK-DB, D2-DB, CK-DB, CK-DA, were analyzed before and after the final stress exposure. Extracted RNA was used for cDNA synthesis with a reverse transcription kit (Takara, Beijing, China), and the sequence data generated used to design primers for real-time fluorescent quantitative real-time PCR (RT-qPCR), which was conducted using TB green mixture (Takara). Each experiment included three biological replicates and two technical replicates, and RT-qPCR reactions were performed using an ABI real-time fluorescence quantitative PCR machine (QuantStudio 3, Thermo Fisher Scientific).

Statistical Analysis

SPSS statistical software version 26.0 (SPSS, Chicago, Illinois, USA) was used for analysis of variance and detection of significance differences ($P < 0.05$). Edge R and Deseq2 software were used to analyze read count data obtained from analysis of gene expression levels in the CK and D2 groups, including standardization of read counts and calculation of P and false discovery rate (FDR) values. FDR and differential multiple $\log_2$ fold-change (FC) values were used to screen for differentially expressed genes (DEGs) between the CK-DB and D2-DB groups; the default threshold was $FDR < 0.05$.

RESULTS

Phenotypic Changes Reveal the Response of *M. ruthenica* to Drought Stress

After exposure to two droughts periods, plant height and leaf number in the D2 group were significantly lower than those in the CK group (Fig. 2a, b). During drought stress, CK plant height increased by 5.56%, while D2 plant height increased by 15.64% (Fig. 2a), while numbers of leaves in these two groups increased by 17.32% and 25.04%, respectively (Fig. 2b). Further, belowground biomass and root to shoot ratio were significantly higher in the D2 group than those of CK plants, with increases of 27.19% and 78.46%, respectively ($p < 0.05$) (Fig. 2f, g). In addition, internode length (Fig. 2c), stem diameter (Fig. 2d), leaf area (Fig. 2e), total biomass, and aboveground biomass (Fig. 2f) were all decreased on drought exposure and did not differ significantly between the two groups. After the final drought stress, CK group leaves exhibited early wilting and yellowing, while those in the D2 group grew normally, and plant roots in group D2 were significantly larger than those in the CK group (Fig. 2h).

Changes in Physiological Traits of *M. ruthenica* in Response to Drought Stress

Drought stress led to increases in the content of soluble sugars, proline, MDA, and ABA in *M. ruthenica* leaves (Fig. 3). Soluble sugar, proline, and MDA content were higher in the D2 group before the last drought stress (DB) than those in the CK group. In the D2 group, after the final drought stress (DA), soluble sugar content was 1.58 times (Fig. 3a) ($P < 0.001$), MDA content was 1.43 times (Fig. 3b) ($P < 0.001$), proline content was 1.07 times (Fig. 3c) ($P < 0.05$), and ABA content was 1.25 times (Fig. 3d) ($P < 0.001$) those of CK group plants.

Changes in DEGs of *M. ruthenica* Under Drought Stress

We next analyzed transcriptome data from the CK and D2 groups before the last drought (CK-DB and D2-DB, respectively). Before the last drought stress, a total of 1495 DEGs were detected between the CK and D2, including 969 upregulated and 526 downregulated genes (Fig. 4). GO enrichment analysis, indicated that the 1495 DEGs were enriched in cell metabolism and environmental information processing, among other pathways. In addition, KEGG analysis showed that DEGs were enriched in processes including the MAPK signaling pathway, plant hormone signal transduction, and secondary metabolite synthesis.

Methylation Landscape of *M. ruthenica* Under Drought Stress

We first analyzed the DNA methylation patterns in the genome of *M. ruthenica* using CK group plants. Methylcytosine was most commonly detected at CHH sites (45.79%), with fewer occurrences in CG and CHG contexts (25.12% and 29.10%, respectively) (Fig. 5a). CHH methylation levels ranged from 10–90%, while those at CG and CHG sites were mainly $> 70\%$ (Fig. 5b). Overall, the degree of methylation in gene body regions and at CG sites was highest (Fig. 5c). In CG contexts, methylation levels in intron and genomic regions were much higher than those in other regions. Methylation levels in upstream, downstream, and intron regions were highest in CHG contexts, and methylation levels in CHH contexts in various regions were similar to those in CHG contexts (Fig. 5d).

We found that drought stress led to decreased methylation levels across the entire *M. ruthenica* genome (Fig. 5e). A total of 819289 upregulated and 911822 downregulated methylation sites, as well as 1723 upregulated methylation regions and 108699 downregulated methylation regions, were detected, and these changes mainly occurred in CHH contexts (Fig. 5f, g).

Relationship between Methylation Levels and Gene Expression under Drought Stress

Focusing on the D2 group, which contained most expressed genes with difference in methylation levels, we analyzed the relationships between DNA methylation and gene expression in different contexts. DNA methylation was negatively correlated with gene expression in most contexts, except for methylation of CHH in promoter regions (Fig. 6a,c). Analysis of correlation between DMRs and DEGs showed that more hypomethylated genes had up-regulated transcription levels. Among overlapping DMRs and DEGs, 61 genes were hypermethylated with down-regulated expression levels and 692 were hypomethylated with up-regulated expression levels in CK-DB vs D2-DB; however, 195 up-regulated and 305 down-regulated genes were hypermethylated and hypomethylated, respectively (Fig. 6b).

We next conducted enrichment analysis of genes with associated DMRs and DEGs. Among the top 20 KEGG pathways, CHH context was modified in 190 enriched genes, CG context in 98 enriched genes, and CHG context in 34 enriched genes (Fig. 7). CG context methylations were mainly enriched in metabolic pathways, protein proteolysis, and starch sucrose metabolism. Notably, carotenoid synthesis and proline metabolism pathways were also enriched in CG context methylations (Fig. 7a). CHG context methylations were in genes enriched in pathways including flavonoid and flavonoid biosynthesis, MAPK signaling pathway, phenylpropanoid biosynthesis, ribosome biogenesis in eukaryotes, and cysteine and methionine metabolism (Fig. 7b). CHH context methylations were in genes enriched in metabolic pathways, plant hormone signal transduction, glutathione metabolism, and cutin suberin and wax biosynthesis (Fig. 7c).

We next focused on the proline and ABA biosynthetic pathways, ultimately focusing on two genes: *ABA2*, encoding the enzyme, zeaxanthin epoxidase, which is involved in carotenoid synthesis, and *P5CS*, encoding an enzyme involved in proline synthesis, Δ^1 -pyrroline-5-carboxylate synthase. Visualization of the methylation levels at these two genes illustrated hypomethylation of the gene and promoter regions in the D2 group before the drought, but not in the CK group. We also observed the relative expression levels of these two genes before and after the final drought. In the D2 group, the expression levels before drought were higher than those in the CK group, and rose significantly after drought ($P < 0.001$) (Fig. 8). Many other important genes were also identified, including *PRP4* and *SAMDC*, which are involved in arginine and proline metabolism; *CYP707A2*, *At2g30020*, and *MPK3*, involved in ABA biosynthesis; *ERD15*, involved in dehydration stress response; and *DRTH2*, involved in DNA methylation; indicating that these genes have important roles in regulating plant drought stress memory (Fig. 9).

DISCUSSION

M. ruthenica Plants Actively Respond to Drought Stress, due to Stress Memory

Stress memory, when plants store and retain previous stress cues and exhibit stronger and faster responses to repetitive events, is regulated by different mechanisms [26, 30]. Drought, high temperature, and salt alkali stress can induce stress memory, thereby helping plants cope with these stresses [31].

In this study, we found that *M. ruthenica* can develop stress memory after experiencing one or two drought exposures, and two drought exposures were more likely to help plants develop stress memory (data not shown). Therefore, we chose D2 for use in subsequent analyses. Stress memory can help plants respond more actively to subsequent drought stress, and is mainly manifested in changes in phenotypic responses and physiological indicators. Our results support a role for stress memory and drought tolerance in enhancing plant biomass. The aboveground leaves of plants in the D2 group exhibited less wilting, while their underground biomass was significantly higher than that of CK group plants. Further, total biomass was also higher in D2 group plants (Fig. 1, 2), which may be attributable to epigenetics-mediated mechanisms underlying stress memory. DNA methylation ‘priming’ in D2 plants did not function by increasing their biomass, but rather increased the flexibility of plants to respond to environmental stress, resulting in stronger resistance to drought stress [9, 32, 33].

DNA Methylation may contribute to Plants Stress Memory Formation

Plants can undergo epigenetic changes to adapt to stress, and such epigenetic alterations are more flexible than genetic variation. A proportion of epigenetic changes are temporary and can be restored, some of which are heritable, and this is referred to as epigenetic memory [6, 34–36]. DNA methylation is among the main epigenetic modifications commonly found in eukaryotic genomes. Methylation groups vary among different plants, and changes in methylation groups after drought stress also vary [37, 38]. In our study, genomic DNA methylation levels were highest at CG sites (> 70%), intermediate at CHG sites (60–65%), and lowest at CHH sites (< 15%), which is consistent with findings in *Arabidopsis* [39, 40]. Methylcytosine was most commonly detected at CHH sites (45.79%) and DMRs were also most abundant in CHH contexts [41], indicating that CHH contexts are more sensitive to drought stress in *M. ruthenica*. Methylation/demethylation at CHH sites may constitute a new epigenetic modification that regulates the growth performance of higher plants under stress [42]. Cytosine methylation levels in CG, CHG, and CHH contexts can affect gene expression, as well as having crucial roles in plant drought stress responses [32, 43]. Changes in DNA methylation vary among different plants under drought stress; generally, drought tolerant plants have more stable methyl groups under drought conditions, manifesting as low methylation levels [33, 44]. We found that *M. ruthenica* methylation groups were relatively stable, while drought stress caused a slight decrease in DNA methylation; similar results have been reported in *Medicago sativa*, *Lolium perenne*, and rice [33, 45–47]. DNA methylation is established to play an important role in plant responses to drought stress, and may constitute plant drought stress memory. Drought stress triggers epigenetic markers in plants, including DNA methylation, which lead to changes in gene expression, signaling pathways, and phenotypic modifications, ultimately improving adaptation to

environmental changes [6, 35, 48, 49]. In rice, 70% of drought-induced epigenetic methylation sites are demethylated, and stress exposure during the reproductive stage leads to a negative correlation between yield and methylation rate, indicating critical crosstalk between epigenetic and reproductive signals in rice plants under drought stress [50, 51]. DNA methylation in wild strawberries (*Fragaria nilgerrensis*) under drought stress may affect gene expression, regulating osmotic capacity and maintaining a balance between reactive oxygen species regeneration and clearance through ABA-dependent signaling pathways, thereby affecting plant drought tolerance [52]. In this study, we found that *M. ruthenica* genomic methylation levels decreased after two drought stress exposures, which may have triggered 'priming' stress mechanisms in the plants. Therefore, when plants were exposed to drought stress subsequently, the drought stress memory was quickly activated, helping the plant make changes to adapt to stress more rapidly.

Relationship between DNA Methylation and Gene Expression

Most often, the presence of methylation does not influence gene transcription; however, under environmental stress conditions, changes in DNA methylation may alter gene expression, leading to visible phenotypes [53]. The regulatory effect of DNA methylation on plant gene expression has been widely studied, and numerous investigations have shown that correlations between DNA methylation and gene expression are very subtle, with changes in DNA methylation status in different regions (upstream, gene body, and downstream) leading to differences in gene expression. In rice, promoter DNA methylation is associated with gene expression downregulation, while genomic methylation typically promotes transcription [54]. In apple genomes, DNA methylation of gene bodies is positively correlated with gene expression, while there was no significant correlation between promoter region methylation and gene expression [53, 55]. Interestingly, there are positive and negative correlations between gene expression and methylation in the promoter and genome regions, respectively, of wild strawberries [52, 56]. Here, we detected negative correlations between DNA methylation and gene expression, except for a positive correlation between gene expression and methylation in CHH contexts in promoter regions. Changes in promoter region methylation levels have greater impacts on gene expression variation, and methylation levels in promoter regions were highest in all three nucleotide contexts examined, which can lead to downregulation of gene expression, particularly in CG and CHG contexts; numerous genes that were specifically downregulated encoded key factors involved in drought stress memory response. In addition, we identified 38061 differential methylation and expression-related genes, which showed significant differences in both methylation and expression levels under drought stress, indicating a significant correlation between DNA methylation and gene transcription; however, our data also indicate that there are multiple types of association between DNA methylation and gene expression, rather than linear correlation alone. The number of genes upregulated by demethylation was highest, indicating that demethylation may have an important role in drought stress and a potential regulatory effect of DNA methylation on gene expression [42].

The Role of Memory Genes in Maintaining *M. ruthenica* Drought Stress Memory

Memory genes contribute to initial drought stress exposure responses, and changes in their transcription levels during subsequent stress may allow plants to fine-tune their response to sustained drought stress [57]. Transcriptional memory, similar to activation of defense genes, can provide the benefits of stronger or modified stress responses, while reducing the cost of coping with stress [16, 58].

Proline is an amino acid synthesized in higher plants that has an important role in response to drought stress [37, 59]. After experiencing drought stress, plants can generate more proline, to eliminate the stress-mediated harm [60]. Our results indicate that drought stress leads to an increase in proline content, consistent with the findings of previous studies [24]. Δ^1 -pyrroline-5-carboxylate synthase (*P5CS*) is a key enzyme in the proline synthesis pathway, catalyzing the conversion of glutamic acid to the intermediate compound, glutamic acid- γ -semialdehyde [61]. Accumulation of high levels of proline and elevated expression of *P5CS* in D2 group plants during the final drought stress in our experiment may be attributable to the effect of drought stress memory. Proline accumulation was also observed in rice after multiple drought stress exposures, similar to our findings [62]. In addition, increased proline induced by salt stress also exhibits a memory-related pattern, similar to the drought stress memory training observed in our study [63]. In both maize and *Arabidopsis*, many genes involved in proline synthesis and metabolism contribute to plant drought stress responses, and are defined as memory genes (Wang *et al.* 2021; Ding *et al.* 2014; Ding *et al.* 2013); In rice, *P5CS1* is involved in proline biosynthesis and has a potential role in drought tolerance, exhibiting transcriptional memory after repeated drought stress (Li *et al.* 2019; Godwin and Farrona 2020).

ABA is among the most important plant stress hormones and is involved in various important physiological processes throughout the plant life cycle, including stress response, development, and reproduction [64–66]. *Arabidopsis* responds to repeated dehydration stress by accumulating ABA at concentrations 2–3 times of those in controls [67]. We observed a similar phenomenon, where ABA content in *M. ruthenica* leaves was considerably higher after exposure to two droughts than that in the control group, suggesting that ABA may be involved in drought stress memory in this plant. Transcript levels of key regulatory genes in the *Arabidopsis* ABA biosynthesis pathway were increased, and ABA biosynthesis was active in response to drought [66, 68, 69]. *ABA2* participates in the final step of ABA biosynthesis and can catalyze the conversion of flavin to abscisic aldehyde, playing a significant role in the regulation of plant endogenous ABA levels [70, 71]. We also observed demethylation of *ABA2* gene and promoter regions in the D2 group, and the gene expression levels also showed transcriptional memory. Therefore, we speculate that *ABA2* catalysis of ABA synthesis contributes to regulation of drought stress memory in *M. ruthenica*.

CONCLUSIONS

Our genome-wide methylation and transcriptome analysis data reveal different methylation regions and genes that are potentially altered under drought stress, providing a reference for future breeding of alfalfa species. These results indicate that *M. ruthenica* plants can generate short-term stress memory after experiencing drought stress, and that this stress memory can help plants to actively cope with

subsequent drought stress exposure. Epigenetic modifications, such as downregulation of genomic methylation levels, may be a mechanism underlying formation of stress memory. Downregulation of gene methylation levels can alter gene expression levels, and affect drought response signaling pathways, such as ABA and proline synthesis pathways, in plants, helping plants respond more quickly to drought stress and improving their drought tolerance (Fig. 10). These findings expand our understanding of the mechanisms underlying short-term drought stress memory in plants that help them to adapt to environmental changes.

Declarations

Authors' contributions

NZ and WBR designed the experiments and wrote this manuscript. FY and YLL participated in part of the experiment. NZ performed the main experimental work. HQG and EF contributed to discussions and the revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The *M. ruthenica* line used in this experiment were provided by the Institute of Grassland Research of CAAS. All materials including all relevant raw data described in the manuscript are available upon request. The datasets generated and analyzed during the current study are available in the online repository: <https://www.ncbi.nlm.nih.gov/sra/PRJNA1039991>, PRJNA1039991.

Ethics approval and consent to participate

Our research did not involve any human or animal subjects, material, or data. The authors declare that all the experiments performed in this study comply with the institutional, national, or international guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

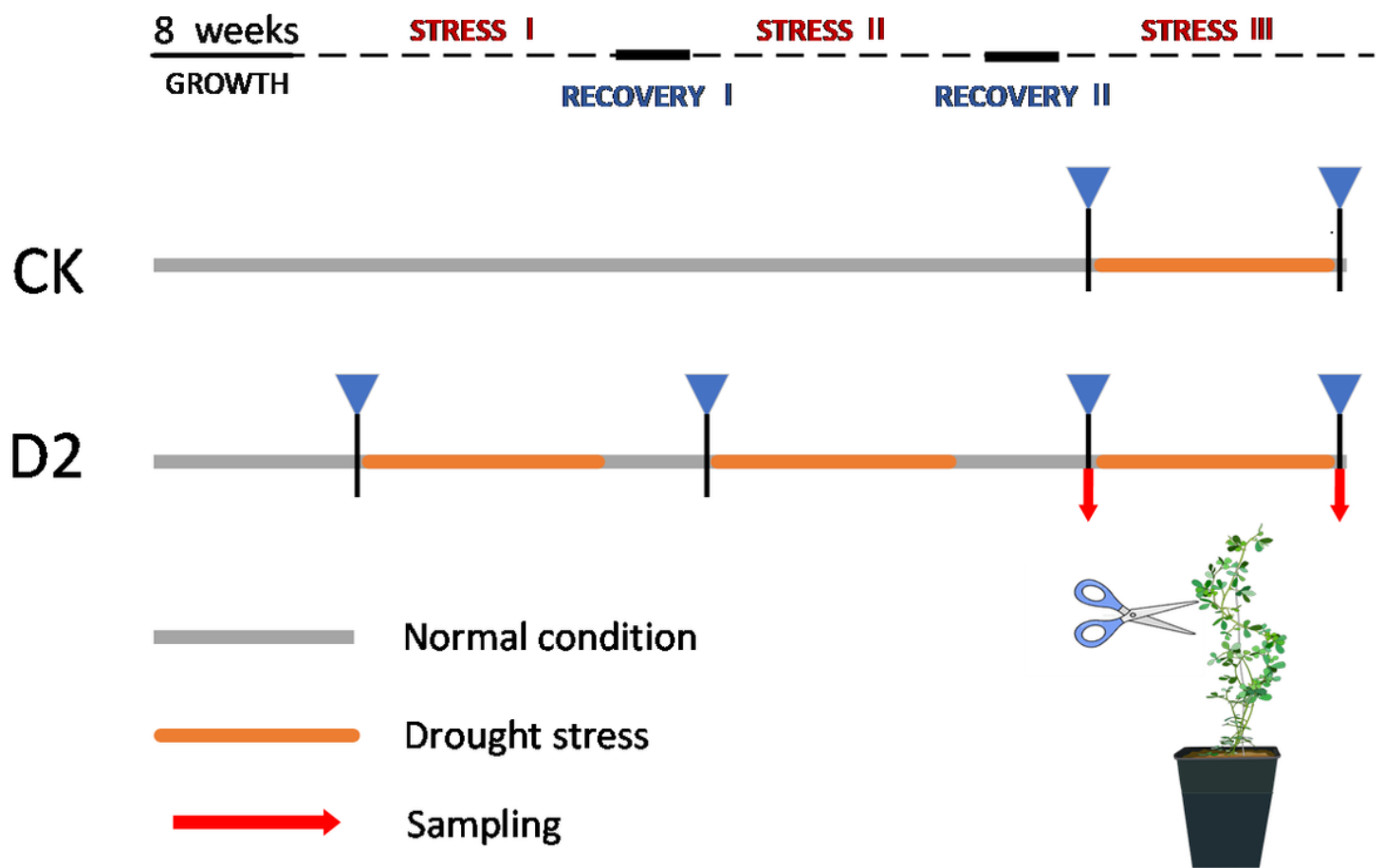


Figure 1

Drought stress treatment of *Medicago ruthenica* experimental groups in this study.

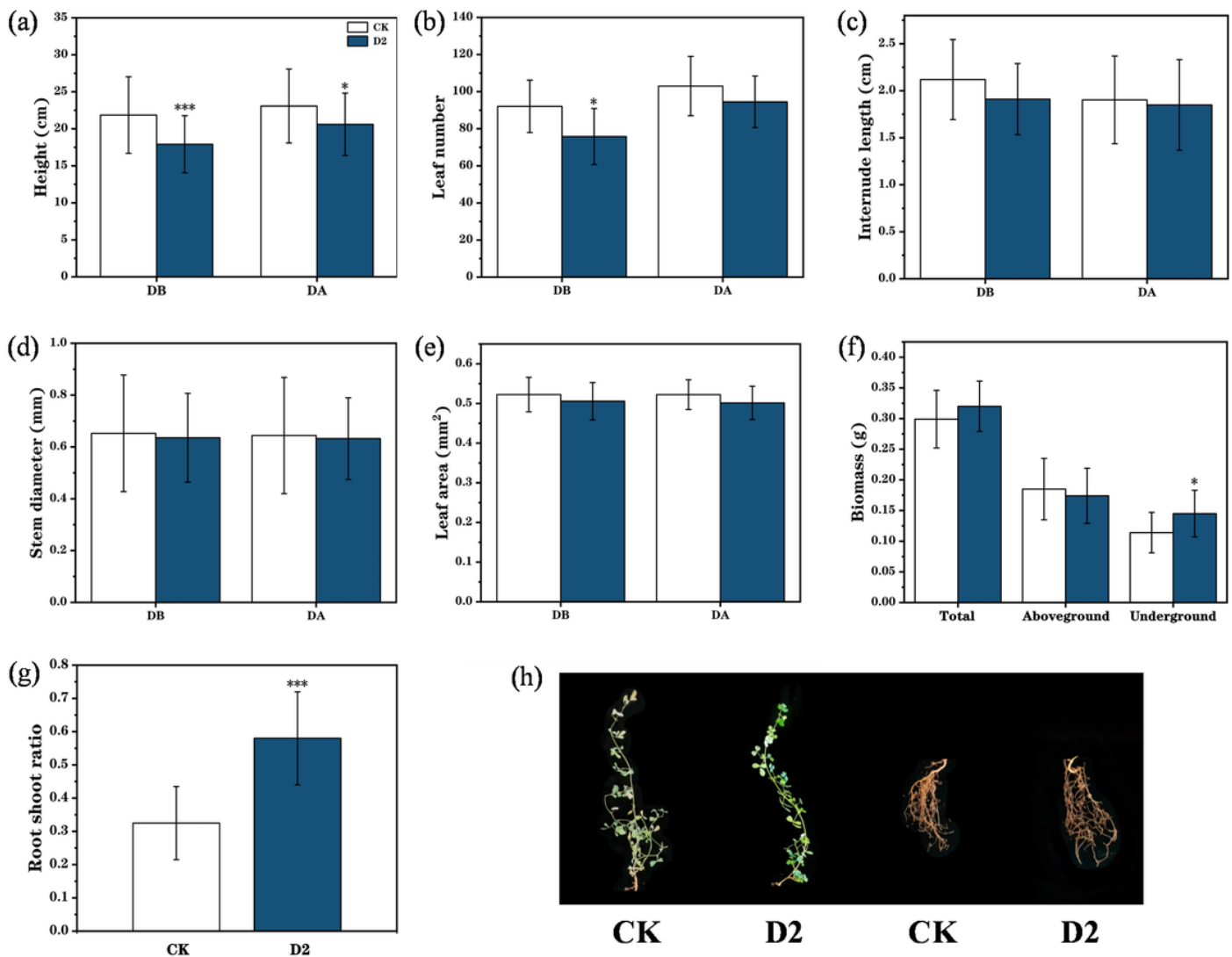


Figure 2

Differences in phenotypic traits between control (CK) and stress memory (D2) *Medicago ruthenica* before (DB) and after (DA) drought stress. (a) Height. (b) Leaf number. (c) Internode length. (d) Stem diameter. (e) Leaf area. (f) Biomass. (g) Root shoot ratio. (h) Representative photographs showing phenotypic changes after the final drought stress. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. Error bars indicate standard error of the mean.

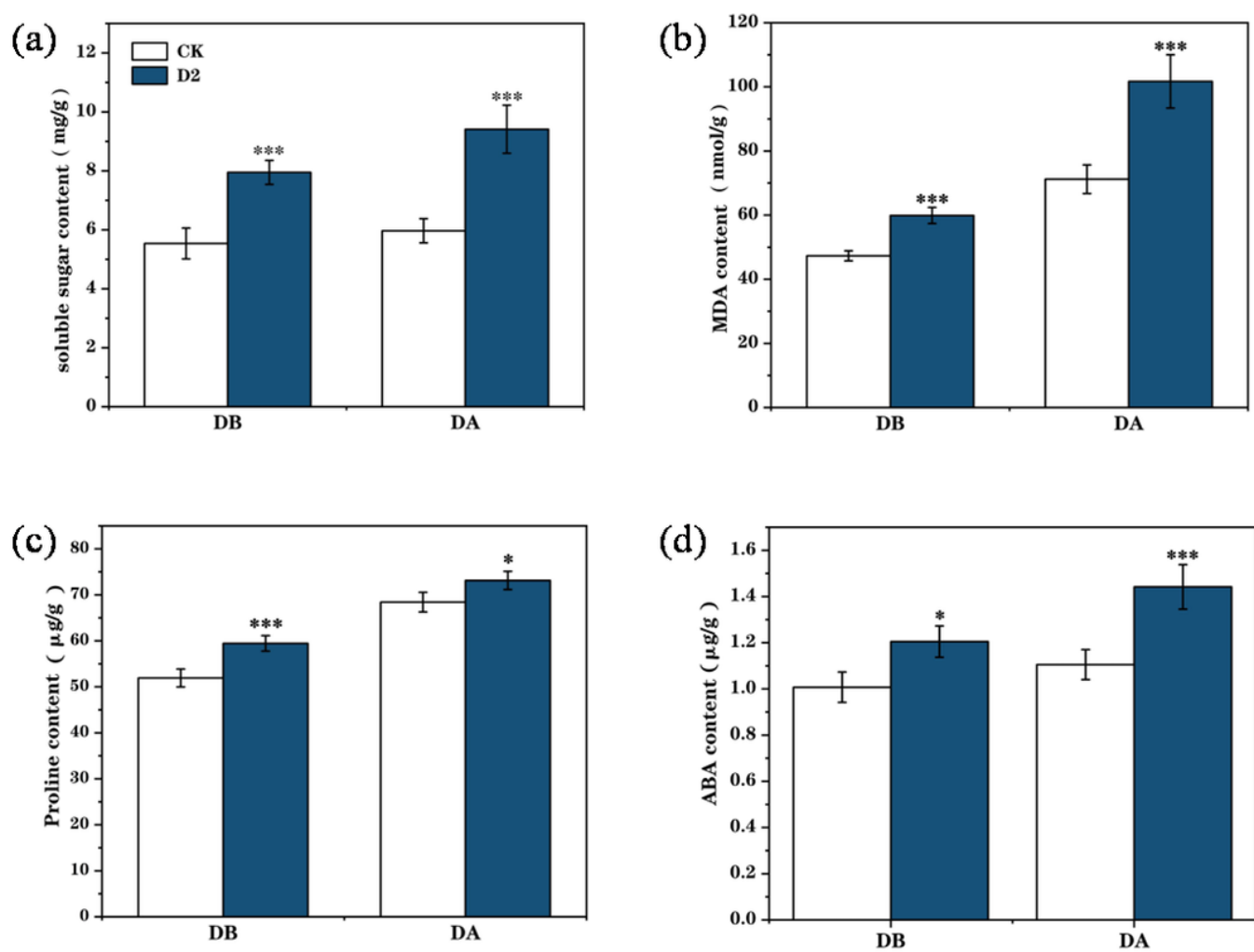


Figure 3

Changes in physiological indices of control (CK) and two drought-exposed (D2) *Medicago ruthenica* before (DB) and after (DA) drought stress. ***P < 0.001; **P < 0.01; *P < 0.05. Error bars indicate standard error of the mean.

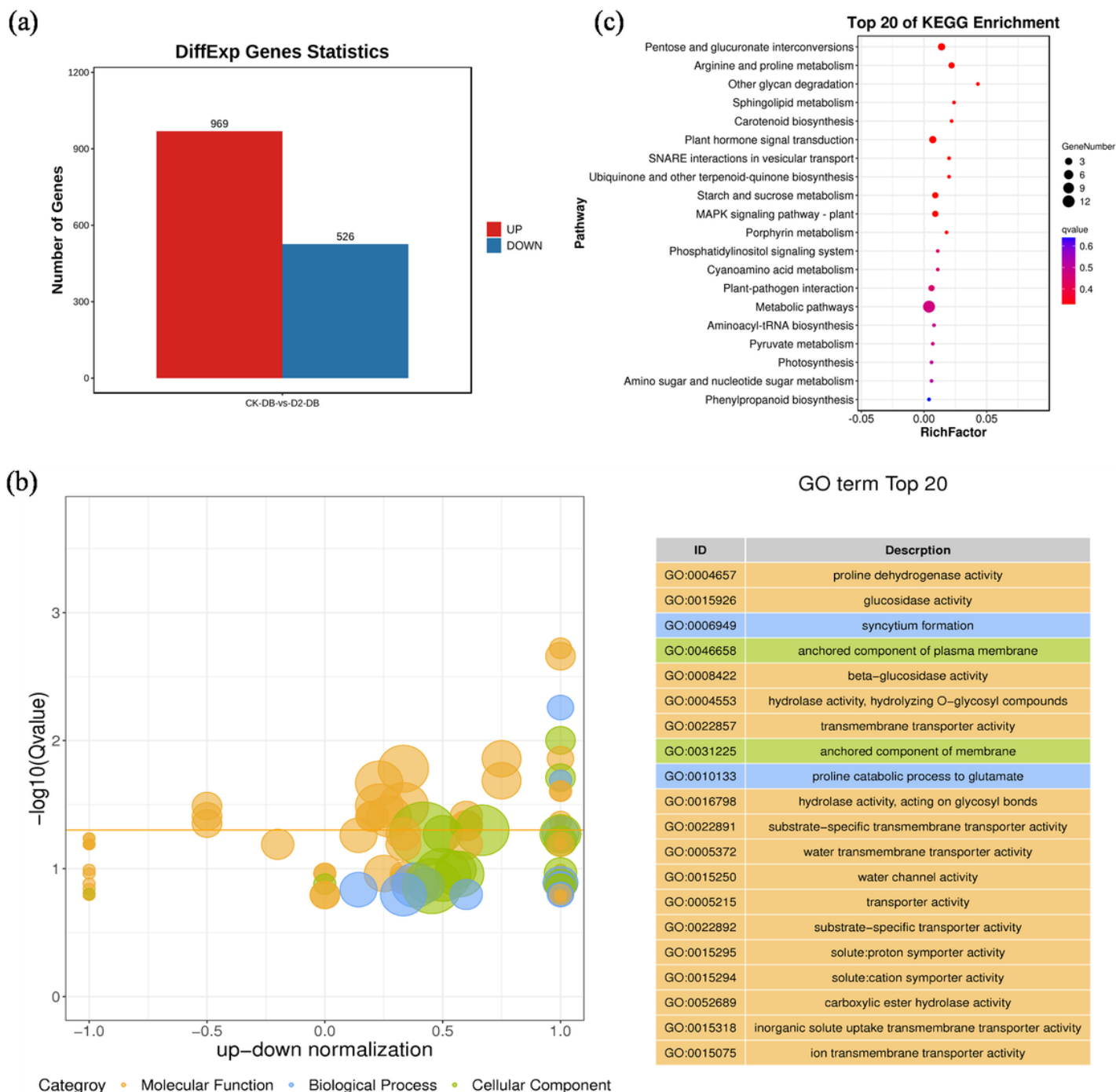


Figure 4

Differences between CK and D2 plants at the transcriptome level. (a) Number of differentially expressed genes (DEGs) between the CK and D2 groups. (b) Gene Ontology (GO) enrichment map of DEGs. (c) Kyoto Encyclopedia of Genes and Genomes (KEGG) DEGs enrichment map.

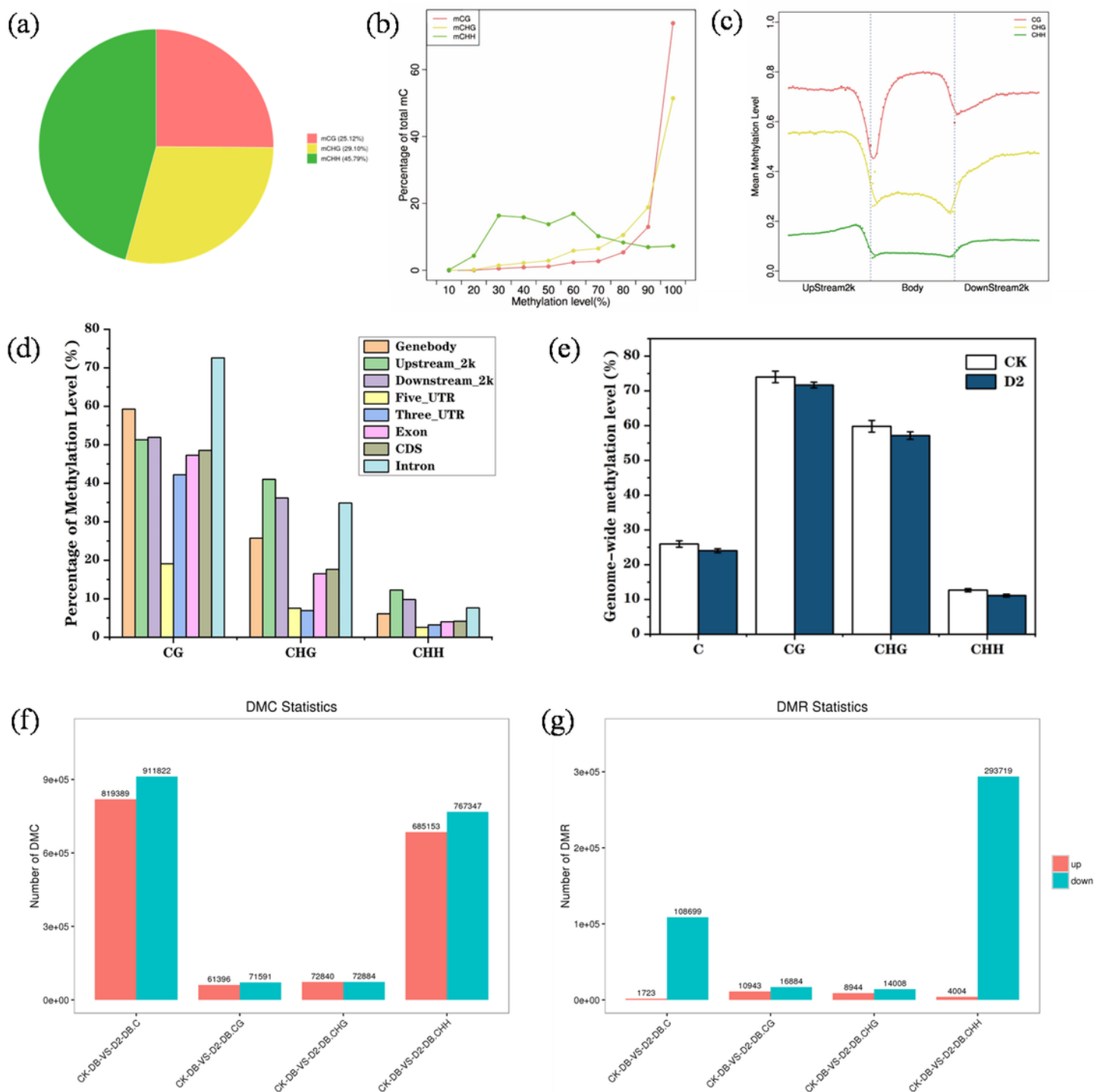


Figure 5

The *Medicago ruthenica* epigenome. (a) The relative proportion of methylcytosines (mCs) in three sequence contexts: CG, CHG, and CHH. (b) C-site methylation level distribution. (c)(d) DNA methylation patterns in different genomic regions. (e) Whole genome methylation levels in the CK and D2 groups. (f) (g) Distribution of differentially methylated sites and regions in the CK and D2 groups.

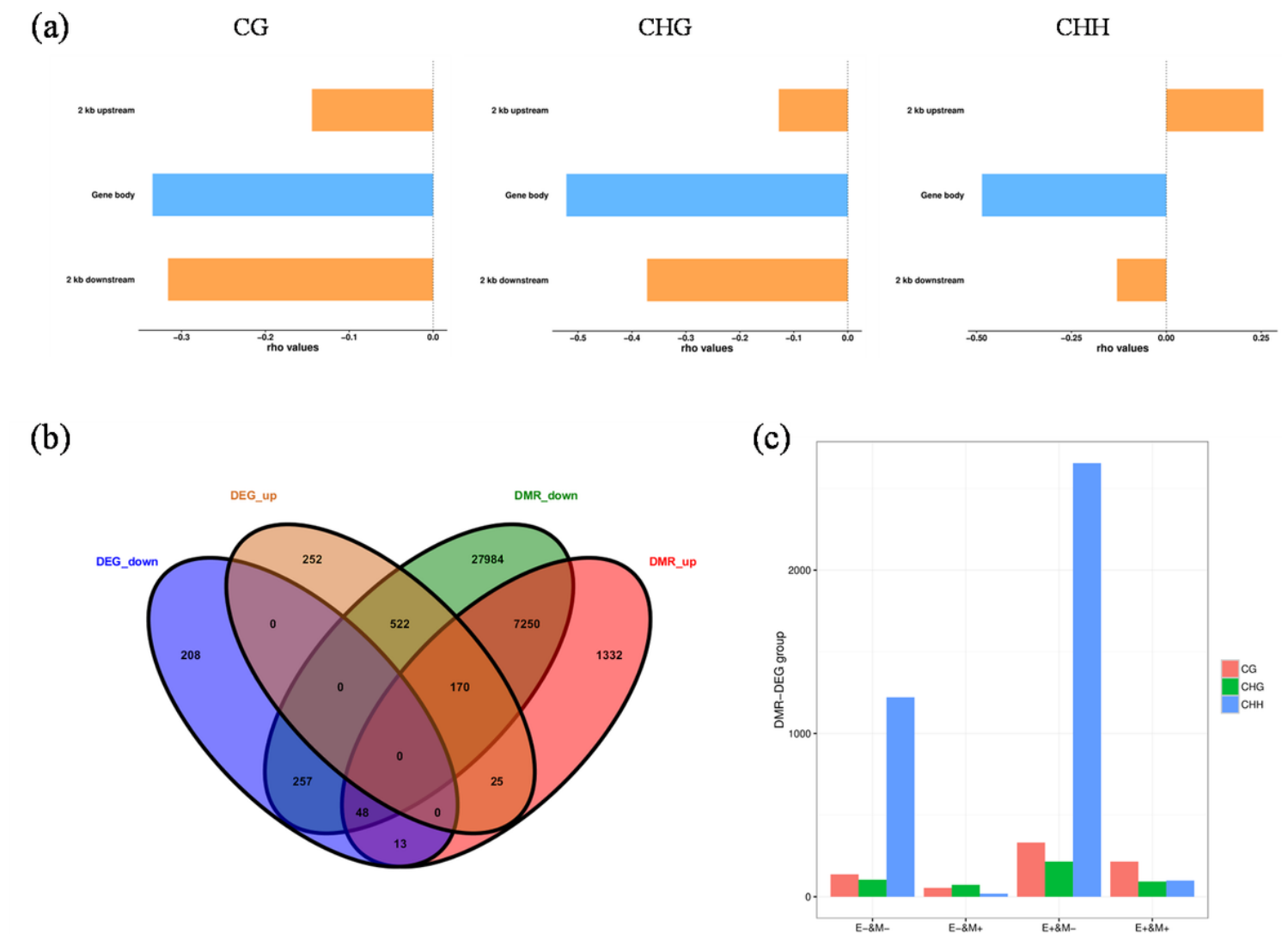


Figure 6

Analysis of correlations between methylation groups and transcriptome data in the D2 group. (a) Spearman correlation analysis of DNA methylation and gene expression levels in upstream and downstream 2 kb regions of coding genes in the D2 group. Rho > 0 indicates a positive correlation, while rho < 0 indicates a negative correlation. (b) Venn diagram of DMRs and DEGs. (c) Histogram showing changes in expression levels of shared DMR-related genes and DEGs. E+/- indicates up-/down-regulation of gene transcript level. M+/- indicates the up-/down-regulation of gene methylation level.

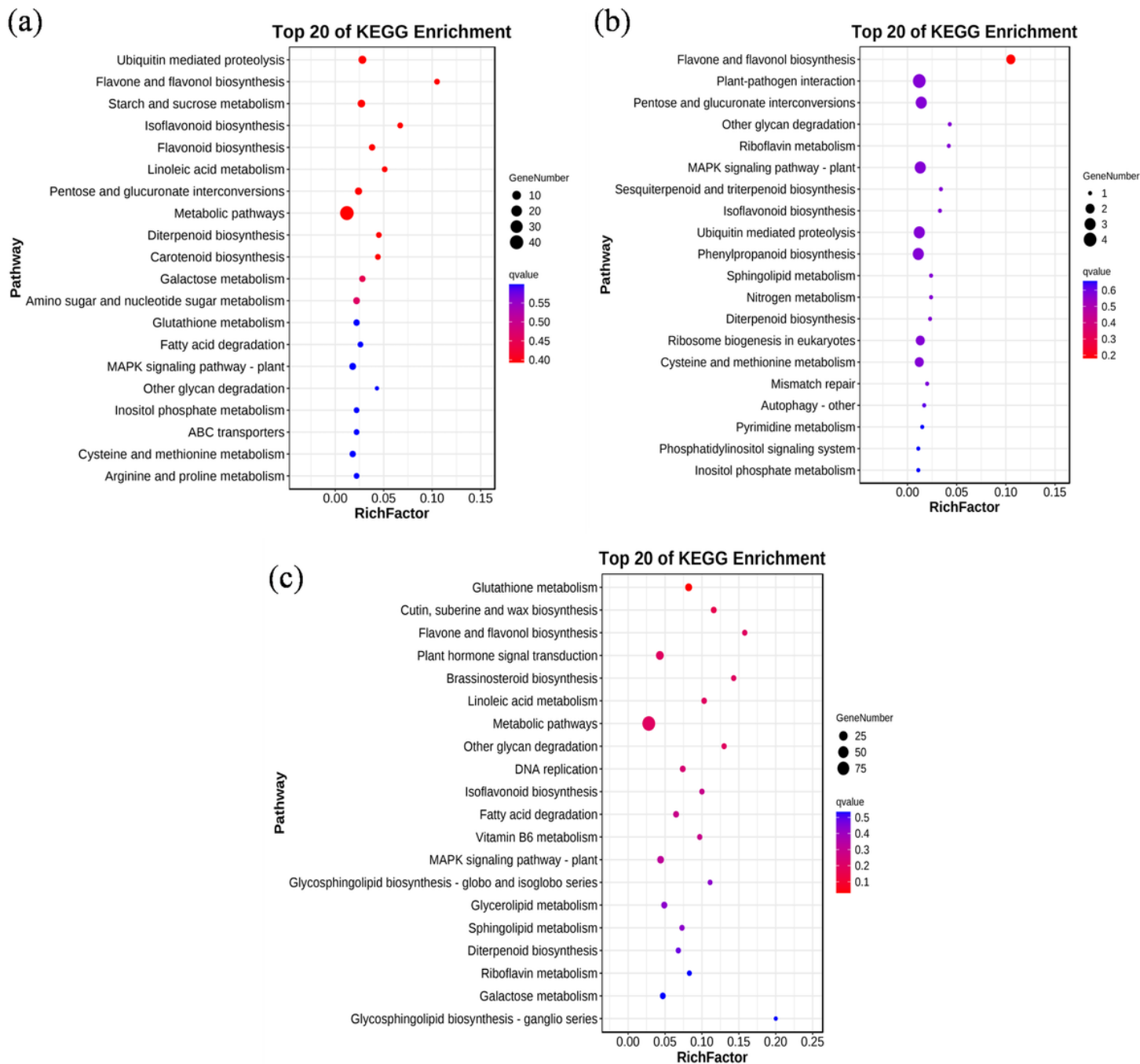


Figure 7

Enrichment analysis of DMR- and DEG-related genes in different sequence contexts. Enrichment analysis of DMR- and DEG-related genes in (a) CG, (b) CHG, and (c) CHH contexts. Copyright permission has been granted for related KEGG images.

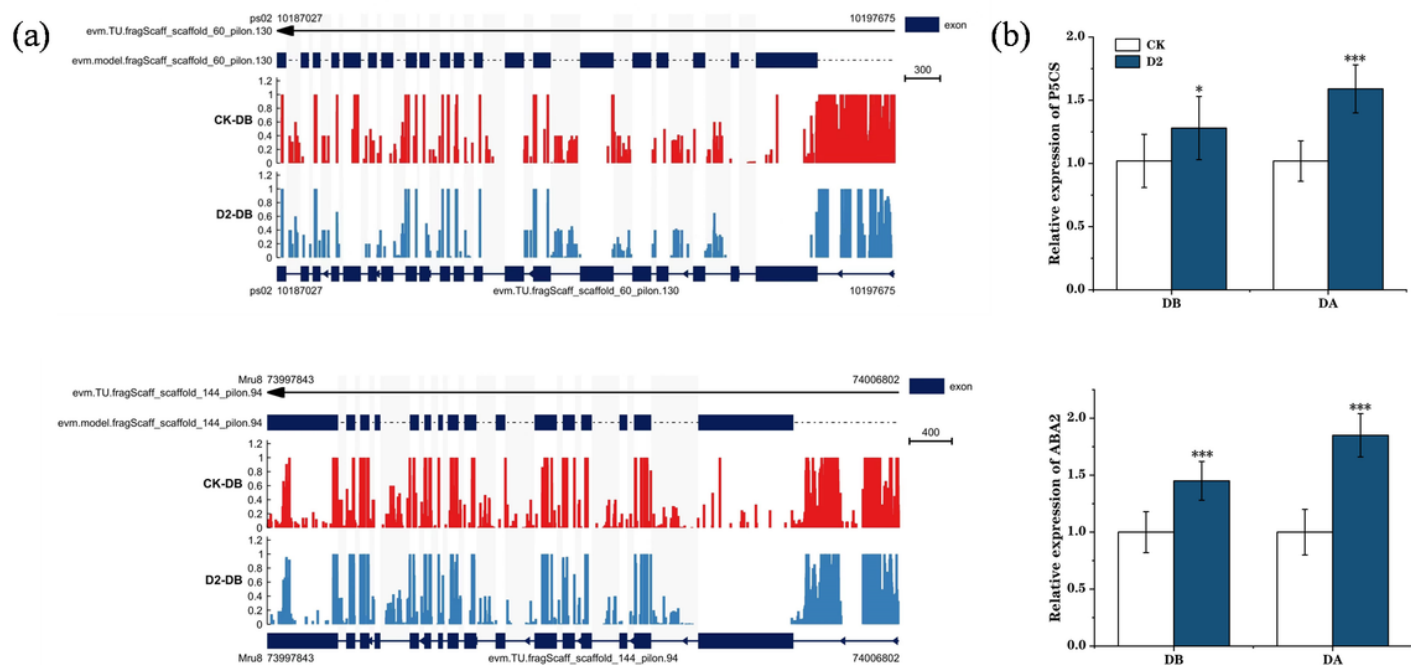


Figure 8

Visualization of methylation and relative expression levels of *P5CS* and *ABA2* before and after the final drought stress. (a) IGV software analysis of stress-induced demethylation of the *P5CS* and *ABA2* promoter and gene regions. (b) Relative expression levels of *P5CS* and *ABA2*.

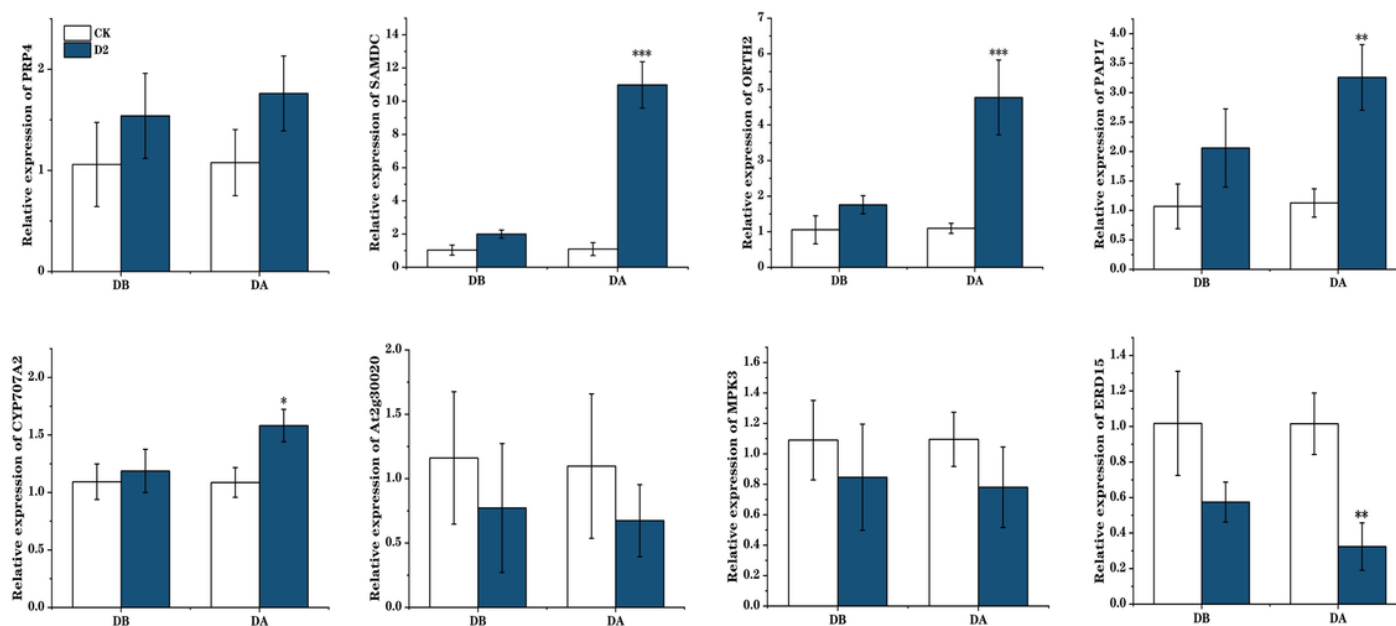


Figure 9

The expression level of response genes under drought stress was validated by real-time quantitative PCR. Bars represented means $\pm$ SD from three biological replicates.

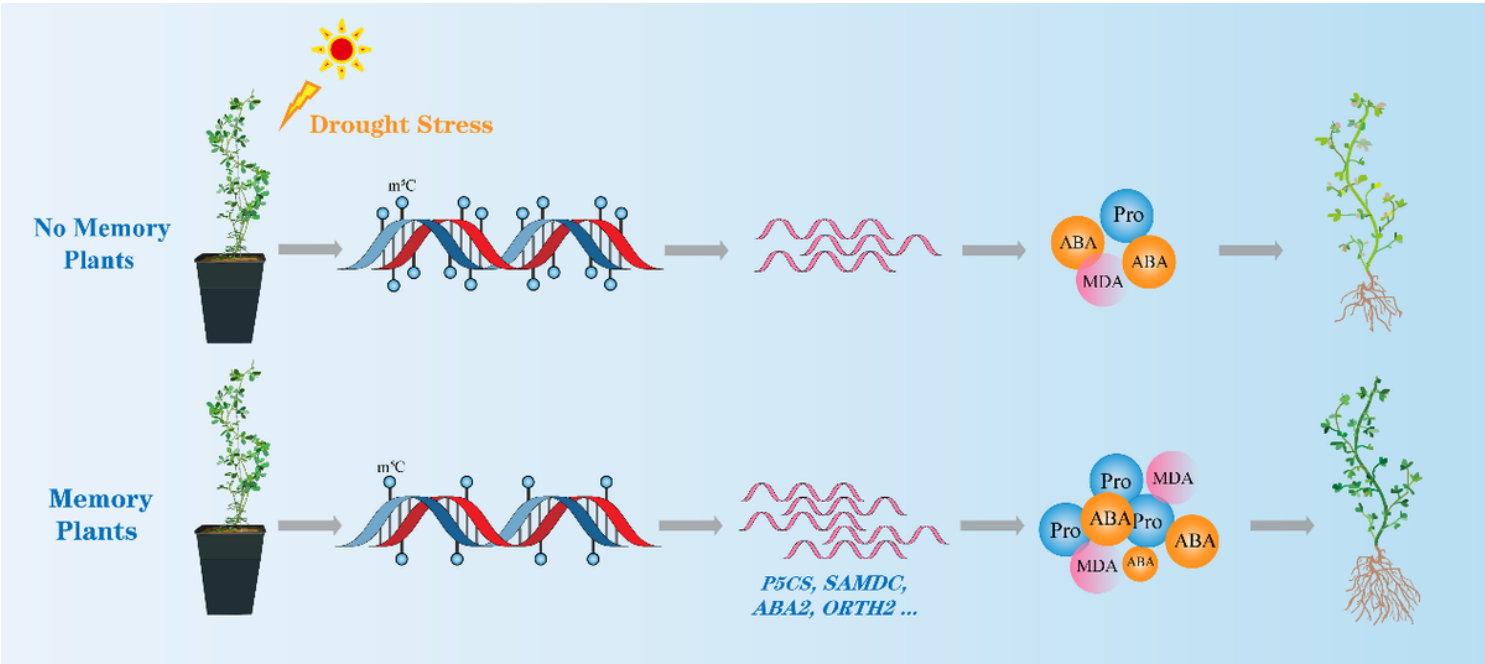


Figure 10

Conceptual diagram of Drought Stress Memory of *M. ruthenica*.