

Transcriptomics combined physiology reveal the key pathway responses in *Setaria italica* L. growth exposure to different Mo concentrations

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Abstract

Molybdenum (Mo), an essential micronutrient for plant physiology, impacts plant growth by regulating physiological activities, modulating gene expression, and altering metabolite content. However, the molecular mechanisms underlying plant responses to Mo remain poorly characterized. Consequently, we utilized extensive physiological and biochemical assays, along with molecular investigations, to decipher the response pathways of *Setaria italica* to varying levels of Mo. Using physiological profiling as a foundation, RNA-seq characterized the transcriptome of foxtail millet exposed to varying Mo levels, uncovering crucial pathways such as phenylpropanoid synthesis, starch metabolism, hormone signaling, and flavonoid/carotenoid metabolism. Results showed that there were more differentially expressed genes (DEGs) at 8 mg L⁻¹ Mo compared to other concentrations, indicating that foxtail millet responded rapidly at this threshold. Compared to the 8 mg L⁻¹ treatment, the 15 mg L⁻¹ treatment inhibited starch and sucrose metabolism while enhancing phenylpropanoid and flavonoid biosynthesis. High Mo levels up-regulated key carotenoid biosynthesis genes (*NCED4*, *NCED5*, *ZSD*) and modulated hormone signaling, optimizing starch-sucrose regulation and boosting stress resilience in foxtail millet. In conclusion, these results indicate that optimal Mo concentrations enhance plant growth through metabolic coordination, whereas supraoptimal exposure induces metabolic dysregulation characterized by: carbon and nitrogen cycle imbalance, antioxidant system impairment, and ultimately growth suppression, thereby delineating key regulatory nodes response to Mo in foxtail millet.

Introduction

Molybdenum (Mo) serves as an essential trace element for plant development and agricultural productivity, participating directly or indirectly in numerous physiological and biochemical metabolic processes. Soil is the main donor of Mo assimilated by plants, when soil with less than 0.15 mg kg⁻¹ of available Mo can harm crop development (Chen Z.Q. et al., 2021). A deficiency of Mo in plants, particularly in crops, may lead to decreased N fertilizer utilization efficiency, impede crop development, reduce yields, and cause symptoms of Mo deficiency, including leaf abnormalities and stunted plant growth. Researchers observed that when Japanese lotus leaves were cultivated in a medium devoid of molybdenum, they presented phenotypes characteristic of molybdenum deficiency, like leaf discoloration and stunted growth of both main and lateral roots (Gao J.S. et al., 2016). Currently, around 4467 ha⁻¹ of Mo-deficient cultivated land are present in China (Zou C. et al., 2008), thus, it is necessary to solve this issue.

The majority of studies have shown that Mo applications can be useful in dealing with Mo deficiency in crops. However, Mo exhibits a pronounced concentration threshold effect, where both deficiency and excess detrimentally impact plants. As a micronutrient mineral, an appropriate level of Mo not only boosts the Mo content within plants but also fosters plant growth, fortifies plant resilience, raises crop yields, and enhances the quality of fruits (Xu S. et al., 2018). Adequate Mo amounts can also alleviate low temperature stress (Sun X. et al., 2009), drought stress (Wu S. et al., 2020), low nitrogen (N) stress (Kovács B. et al., 2015), and heavy metal stress (Wu M. et al., 2024) by improving plant carbon and N

metabolism and antioxidant capability. Besides, the exogenous application of low - level Mo sprays can influence seed dormancy and germination. For example, Nciizah A.D. et al. (2020) reported that the low concentration of Mo can improve the germination rate and coefficient of velocity of germination of maize seed, but the high concentration of Mo reduced the two germination parameters. Plants need only a small amount of Mo for normal growth; the Mo content in healthy plant tissues ranges from 0.2 to 300 mg·kg⁻¹ dry weight (Gupta U.C. 1997). The previous studies have found that it would alter the plants' physiological and metabolic processes at the cellular and molecular level, when the Mo content exceeded the tolerance of the plant (Rascio and Navari-Izzo, 2011). For example, the net photosynthetic rate of winter wheat would significantly decline under a Mo treatment at a concentration of 1000 mg·kg⁻¹ (Li L. et al., 2016). Excess Mo in oilseed rape initiates ROS production, upsetting the balance of AsA, soluble sugars, proteins, and enzymes (CAT, GR, DHAR). This imbalance disrupts physiological processes, causing a decline in both plant biomass and crop yield (Ul Hassan Z. et al., 2019). Although previous studies have provided insights into plant responses to high Mo, the underlying molecular mechanisms remain unclear. Thus, investigating gene expression changes and deciphering the molecular processes involved in plant responses to varying Mo levels are essential.

In the development of dry land farming agriculture, foxtail millet (*Setaria italica* L.), which originates from China, is the main crop, and efforts to develop improved elite varieties are centered in this country. Foxtail millet has been cultivated for over 10,000 years (Yang X. et al., 2012; He Q. et al., 2023), but unlike other major cereal crops such as wheat (*Triticum aestivum*), rice (*Oryza sativa*) and maize (*Zea mays*), it has not been intensively bred for grain quality and high yield (Diao, 2007). In recent years, foxtail millet industry's rapid expansion has a significant impact on the rise in farmers' income and the betterment of the Chinese people's diet. Studies on Mo in improving yields and quality of gramineous crops including rice and wheat have been revealed previously. Zakhurul et al. (2000) and Ghafarian et al. (2013) demonstrated that Mo application enhances the photosynthetic rate and increases the photosynthetic products of wheat under drought stress, leading to an improvement in the number of spikes and yield of winter wheat. In addition, Sun et al (2014) reported that Mo application to wheat under low-temperature stress regulated the expression of proteins involving in the light and dark reactions of photosynthesis, affected the carbon metabolism of photosynthesis in wheat, and increased the photosynthetic rate, the maximum net photosynthetic rate, and the carboxylation efficiency of winter wheat, which enhanced photosynthesis in winter wheat. Further studies by (Imran et al., 2019; Liu L. et al., 2017) demonstrated that that Mo not only increases the content of photosynthetic pigments and leaf -gas exchange properties in common wheat (*Triticum aestivum*) and strawberry (*Fragaria ananassa*), but also maintains the integrity of chloroplasts. Taken together, exogenous Mo exert a positive effect by promoting photosynthesis and affecting crop growth. In previous studies, exogenous Mo under adverse condition has been explore the effects in plant protection; however, it has not been reported whether exogenous Mo at different concentrations has a beneficial influence on foxtail millet growth.

Previous studies found folia spraying chelated Mo are able to effectively increase the leaf area, enhance the chlorophyll content and improve photosynthetic capacity in foxtail millet, but foxtail millet seedlings

sprayed with a high concentration of Mo have had withered leaves, which focused on the physiological analyses (Guo M.J. et al., 2023). However, until now, the molecular response of foxtail millet to Mo has to be confirmed. In this study, we aimed to examine the transcriptomic profiles of foxtail millet seedlings in response to Mo, and verified the results by analysis, using RNA-seq. Therefore, this research was designed to: (a) measure the phenotypic alterations of foxtail millet under various Mo concentrations; (b) explore physiological transformations, including those in N - associated metabolism and chlorophyll synthesis; (c) identify and detect differentially expressed genes (DEGs) along with their critical pathways; (d) uncover the molecular mechanism that regulates foxtail millet's response to different Mo levels. Clarifying foxtail millet's molecular responses to diverse Mo concentrations, our results will also forge a theoretical framework for upcoming studies.

Materials & Methods

Plant samples & growth environments

For this research, seeds of foxtail millet cultivar Jingu 21 were provided by the College of Agriculture, Shanxi Agriculture University. Seeds were disinfected with 10%(w/w) H₂O₂ for 30 min and then rinsed three times with the distilled water. Thereafter, 50 sterilized seeds were placed into plastic containers containing 30 ml of modified Hoagland's solution with different Mo concentrations. The plastic containers containing the thus-primed seeds were then placed in an incubator set at a constant 25°C for 7 days. To evaluate the foxtail millet's sensitivity to varying Mo levels, we measured its germination rate, potential, index, and vitality index. The Mo concentration were 0(control, CK), 4 mg L⁻¹(T1), 8 mg L⁻¹(T2), 10 mg L⁻¹(T3), and 15 mg L⁻¹(T4), which were continuously aerated and renewed every 3 days. Subsequently, three-leaf stage uniform seedling were grown in a growth chamber with photoperiod of 8/22°C (light/dark), temperature of 28/22°C (light/dark), light intensity of 10 klux, and relative humidity of 60 - 70%. Each treatment had three biological replicates with 10 plants per replicate, totaling 150 plants. After the 3 - week treatment, samples were collected immediately for physiological index, and transcriptome determination.

Germination trait assay

Data were measured on germination rate (GR), germination potential (GP), germination index (GI), seedling shoot lengths (SL), and seedling root lengths (RL); indices were calculated per the formulas below:

germination rate(%)=(Numbers of germinated seeds)/(Total seed number used in the test)×100

germination potential(%)=(Numbers of germinated seeds in 3 days)/(Total seed number used in the test)×100

germination index(GI)=(1.00)nd1+(0.75)nd2+(0.50)nd3+(0.25)nd4 (nd1, nd2, nd3, nd4 were the germination rates of the first, second, third and fourth days)

vitality index=GI×Sx (Sx is the average length of bud length on day 8)

Biomass and root morphology metrics of plants

Seven randomly selected plants from each treatment were rinsed with distilled water to remove contaminants, blotted dry, and weighed to record fresh biomass. Meanwhile, ImageJ software (<http://rsb.info.nih.gov/ij>) measured plant height and seedling root length.

According to the method of Guo MJ et al. (2019), root vigor was assayed. Root tip samples (0.2 g, 0.5 - 1 cm long) were weighed and transferred to a weighing vial, after which 10 mL of a 1:1 mixture of 1 % TTC solution and 0.1 mol.L⁻¹ phosphate buffer (pH 7.5) was added. The root tip samples were placed in the solution and kept in the dark at 37°C for one hour. Afterward, the reaction was stopped by adding 2 mL 1 mol.L⁻¹ sulfuric acid. Dry the sample by blotting, then grind it in a mortar along with 3–4 mL ethyl acetate and some quartz sand. Next, transfer the red extract into a 10-mL volumetric flask, dilute with ethyl acetate, and determine the absorbance at 485 nm on a Tecan Sunrise (Tecan Austria GmbH).

Photosynthetic pigment analysis

Consistent with the content of the previous section, the method of Guo MJ et al. (2019) was employed to determine the photosynthetic pigments. Select 0.05 g of the second - last leaf, mince it into small pieces, and transfer them to a glass vial containing 5 mL of 96% ethanol. Seal the vial with a rubber bung, then incubate it in a dark environment at ambient temperature for 48 hours. Agitate the vial 3 - 4 times during the incubation period until the leaves bleach completely. Measured at 470, 649, and 665 nm via a Tecan Sunrise (Tecan Austria GmbH), the extract's absorbance was determined using the formulas that follow:

$$Ca=13.95A_{665}-6.88A_{649}$$

$$Cb=24.96A_{649}-7.32A_{665}$$

$$Ccar=(1000 A_{470}-2.05 Ca-114.8 Cb)/245$$

$$\text{Pigment content (mg g}^{-1}\text{ FW)} = C \times VT \times n / (FW \times 1000)$$

The equation defines C as the pigment concentration (mg.L⁻¹), FW as the fresh weight (g), VT as the total volume of the extraction (mL), and n as the dilution ratio.

Determination of N metabolism enzymes

In line with the specified procedures, samples were collected and treated for subsequent determination. Assay kits sourced from Solarbio Biotechnology Co., Ltd. (Beijing, China) were used to assess the activities of nitrate reductase (NR), glutamate synthase (GOGAT), glutamine synthetase (GS), and glutamate dehydrogenase (GDH) in leaves. A 2400 UV - visible spectrophotometer (Sunny Heng ping Instrument, LLC., Shanghai, China) was employed to record absorbance at 540, 340, 540, and 340 nm, respectively.

The determination of soluble protein (Pro) followed the approach of Shen J. et al. (2020). A 0.1 g penultimate leaf sample was ground with 4 mL of pH 7.0 phosphate buffer, centrifuged at 4000 rpm for 10 min, filtered, and 0.3 mL of the supernatant was used to determine soluble protein content via the Coomassie brilliant G - 250 colorimetric method.

Transcriptomics analysis

Frozen samples were sent to LC-Bio Technology Co., Ltd. in Hangzhou, China, where three biological replicates per treatment underwent transcriptome sequencing: RNA was extracted from foxtail millet leaves, raw data filtered, and error rates and GC content verified to obtain clean reads for analysis. Fold change (FC) was calculated as the average FPKM ratio between two groups; differentially expressed genes (DEGs) were determined by $|\log_2 FC| \geq 1$ and $p < 0.05$, with data processed according to LC-Bio Technology cloud analysis tools (<https://www.omicstudio.cn>) (Kanehisa M. et al., 2021).

Quantitative real time PCR (qRT-PCR) validation of RNA-Seq data

Using the FlAPure Plant Total RNA Extraction Kit (Genesand Biotech Co., Ltd., Beijing, China), total RNA was isolated. This RNA was subsequently used to produce cDNA with the Union Script First Strand cDNA Synthesis Kit (Genesand Biotech Co., Ltd., Beijing, China). qRT-PCR was performed with the SYBR Green Super Mix (Mei5bio Co., Ltd., Beijing, China) on a Bio-Rad CFX96 device (Bio-Rad, USA), with SiActin (Si8g04880.2) as the internal standard. Table S1 lists the primers used in the experiment.

Statistical analysis

Via SPSS 19.0 (IBM, Chicago, USA), growth and physiological parameters were analyzed using Turkey's test (significance at $p < 0.05$). Statistical analyses were performed on treatment means obtained from three measurements ($n = 3$), with error bars denoting standard deviations of biological replicates. Origin 2021 (OriginLab, Northampton, USA) and Adobe Illustrator CC 2022 (Adobe, San Jose, USA) were employed for figure generation. Using Origin 2021 statistical software, Correlation analysis were generated from Spearman's correlation coefficients, and a principal component analysis (PCA) were used to detect loading values.. The results were visually graphically using Origin 2021.

Results

Effects of Mo application on seed germination of foxtail millet

The results showed that the concentration of Mo had a significant impact on the germination of foxtail millet. The germination rate of T1, T2 and T3 significantly increased by 1.44 %, 6.00 % and 1.44 % compared with CK, and T4 significantly decreased by 8.24 % (Figure 1). As for the germination potential, all treatments other than T2 showed no significant differences compared to the control. After a period of 7 days, with the rise of Mo concentration, the shoot length of Jingu 21 exhibited a pattern of increasing first and then decreasing, while the root length in each treatment was less than that of the control. Compared with CK, the shoot length increased significantly by 42.86 % and 20.24 % under T2 and T3

treatments. The shoot length under T4 treatment was comparable to that of the control; however, the root length was significantly decreased by 18.37%.

Plant growth performance and root morphology indicators

As the Mo concentration increased, Jingu 21's plant height increased first and then decreased, while root height declined. The result shows that root vigor also exhibited a trend of increasing first and then decreasing, with significant differences among treatments (Figure 2). Meanwhile, T1 and T2 treatments significantly boosted shoot fresh weight by 20.95% and 27.61% compared to CK, while T4 treatment sharply reduced both shoot (29.52%) and root (37.04%) fresh weights. Notably, T2 treatment enhanced root vigor by 63.02% relative to the control, while T3 and T4 treatments led to substantial decreases of 38.07% and 65.95%, respectively.

Photosynthetic pigment content and N metabolism-related enzymes

In the study, the total chlorophyll contents first increased and then decreased as the Mo concentration went up (Figure 3). Secondly, compared to the control, the total chlorophyll contents under T1, T2, and T3 treatments increased significantly to 21.23%, 51.74%, and 12.56% respectively. Additionally, regarding other pigments, compared with CK, the carotenoid content of T3 and T4 was significantly reduced by 14.83% and 25.31%. The chlorophyll a content in T3 and T4 treatments was 18.46% and 27.09% lower than that of the control, respectively. Moreover, the chlorophyll b content of T1, T3, and T4 treatments decreased significantly by 6.77%, 13.58%, and 14.53% respectively.

As shown in Figure 3, shows that while N metabolism - related enzymes in the leaves, including NR, GS, GDH, GOGAT, and Pro, were significantly regulated by Mo concentrations, their activities increased progressively with Mo concentration. Among them, after T1, T2 and T3 treatments, NR activity increased significantly by 42.70%, 75.06%, and 15.49% compared with CK, but T4 treatment did not differ significantly from CK. According to our results, the activities of GS and GOGAT, as well as the content of Pro, were significantly different between the treatments and the control. These parameters reached their maximum accumulation under T2 treatment. Notably, T2 treatment led to the highest values for all relevant indices. Specifically, compared with the control (CK), under T2 treatment, the activities of NR, GS, GDH, GOGAT, and the content of Pro increased significantly by 75.06%, 144.98%, 223.38%, 177.74%, and 489.98%, respectively.

Correlation and PCA analysis

Significant trends were noticed in seedling growth and biomass growth. PH, RH, SFW, and RFW were positively correlated with growth. Pro was positively correlated with GDH, GOGAT, GS, and NR. Similarly, root vigor was positively correlated with GDH, GOGAT, GS, NR, and Pro, indicating its importance in improving nitrate metabolism (Figure 4a).

Principal component analysis (PCA) presented in Table S3 implemented the relationships and variances among multiple foxtail millet agronomic and physiological indices. The first, second, and third

components accounted for 55.02%, 20.41%, and 16.17% of the variance, respectively. Among variables in the first component, GS, GDH, and NR had the highest factor loadings (0.286, 0.275, 0.274), suggesting it could effectively interpret foxtail millet's N metabolism. Regarding the second principal component, chlorophyll a content, root height, and root length registered relatively high factor loadings, signifying their potential as pivotal descriptors for chlorophyll metabolism and root - morphological parameters in foxtail millet. In the third principal component, the factor loadings of germination index, vitality index, germination potential, and germination rate were distinctly high, thereby serving as reliable indicators to characterize the germination traits of foxtail millet. In addition, T2 treatment clustered distinctly, with parameters Pro, GOGAT, GDH, and NR (Figure 4b).

Meanwhile, we established PCA - developed model: $F=0.550Z_1+0.204Z_2+0.162Z_3$, which revealed that the poor comprehensive evaluation of T4 treatment suggested high Mo levels inhibited nitrogen metabolism and growth of foxtail millet. The T1 and T3 treatments, with comprehensive evaluation scores of 1.528 and 0.0773 respectively (Table S4), exhibited positive values for the third principal components. This indicates that they had a beneficial effect on the germination characteristics of foxtail millet. As a result, all three - factor scores in the T2 treatment were relatively high, indicating the overall impact of Mo on foxtail millet.

Effects of Mo application on transcriptomes of foxtail millet

Transcriptome sequencing & DEGs identification

To investigate the molecular mechanisms of foxtail millet's reaction to diverse Mo concentrations, we performed transcriptome sequencing on Jingu 21 seedlings. Following read trimming, we got 33.71 - 51.66 million top - quality reads, with a Q30 base proportion >97.01% and a GC content of roughly 53%, suggesting the data quality was adequate for further study (Table S5). By employing qRT - PCR to assess 8 DEGs, we discovered that the gene expression profiles were in agreement between the two techniques, verifying the correctness of the transcriptome data (Figure S1).

We gauged gene expression levels using fragments per kilobase of transcript per million mapped reads (FPKM), founded on normalized read counts, to appraise the global gene expression profiles of various samples. After excluding genes with FPKM values of 0 in all samples, 30,242 genes were identified based on expression, and genes with $FPKM > 1$ were categorized into five groups by expression level: extremely low ($FPKM < 10$), low (10 - 30), medium (30 - 100), high (100 - 300), and very high ($FPKM > 300$) (Figure S2). For all the DEGs that were expressed, we performed hierarchical cluster analysis and classified them according to KEGG pathways, as depicted in Figure 5c and 5d.

Furthermore, pairwise comparisons of T1, T2, T3, and T4 against the control identified 397 (95 up, 302 down), 2066 (403 up, 1663 down), 1474 (515 up, 959 down), and 1252 (622 up, 630 down) DEGs, respectively (Figure 5a). There were more DEGs at T2 than other treatments, suggesting that foxtail millet response rapidly to Mo. Analyzing the differential genes across different comparison combinations using a Venn diagram, we found 74 common differential genes (Figure 5b).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of DEGs

To further assess the biological roles of DEGs and examine the alterations under distinct Mo treatments, we carried out GO and KEGG enrichment analyses. In the T2 treatment, upregulated genes showed 72 enriched GO terms, categorized into 47 biological processes, 9 cellular components, and 16 molecular functions (adjusted $p < 0.05$), as per GO analysis. T4 also showed enrichment in those terms by 69, 10, and 41 (Figure 6c, 6d, 6g, 6h). The foxtail millet plant system mainly enhanced secondary metabolite biosynthetic process, abscisic acid metabolic process, signal transduction, and DNA-binding transcription factor activity to improve plant tolerance to Mo.

Bubble plots were utilized in Figure 6 to display the KEGG pathway enrichment analyses of all DEGs, aiming to explore how Mo exposure impacts the enrichment pathways of these genes. The KEGG pathway analysis showed that 2066 annotated DEGs in T2 were assigned to 12 pathways (adjusted p -values < 0.05) (Table. S6). In T4, 1252 DEGs were assigned to 11 pathways (Table. S6), and in T3, 1474 DEGs were assigned to 10 pathways (Table. S6), and in T1, 397 DEGs were assigned to 5 pathways (Table. S6). Phenylpropanoid biosynthesis, plant hormone signal transduction, starch and sucrose metabolism, along with other pathways, were recognized by KEGG ontology analysis as the most enriched and significantly impacted in the T2 treatment. Significantly enriched pathways including tryptophan metabolism, plant hormone signal transduction, carotenoid biosynthesis, N metabolism, and more were identified by KEGG ontology analysis of the T4 treatment. The consolidation of previous research outcomes led to the filtration of five pathways: 'phenylpropanoid biosynthesis', 'starch and sucrose metabolism', 'plant hormone signal transduction', 'flavonoid metabolism', and 'carotenoid metabolism'.

Genes enriched in the hormone signaling pathway exposure to different Mo concentrations

Our results identified 99 genes involved in phytohormone signaling, including those associated with auxin, CTK, GA, ABA BR, JA, and SA pathways (Figure S3). Transcriptome data revealed that, among these, 23 *IAA* genes, along with several *ARF* and *SAUR* genes, were significantly enriched under T3 and T4 treatments. Significantly contributing to auxin signaling, the *GH3s* family also takes part in the plant defense response systems (Fu J. et al., 2011; Hui S. et al., 2019). In the case of T4 treatment, two *GH3s* genes were highly up-regulated, whereas the expression of two *GH3s* genes in T2 treatment showed a gradual decrease. Several crucial genes' expression under varying Mo concentrations is notably affected by cytokinin. Our study identified eight members of the response regulator type-A/B family, notably two *A-ARR* genes, which exhibited high expression levels under T2 treatment. GA signaling is a complex pathway involving four GA receptor *GID1* genes, three *DELLA* protein genes, and one TF gene. Additionally, we detected two genes from the ABA receptor *PYR/PYL* family, five protein phosphatase *PP2C* genes, and two *ABFs* genes that serve as binding factors for the ABA-responsive element. Following Mo exposure, nine ETH-related genes were significantly altered, including four genes encoding *SIMKK*, *EIN3*, and *ERF1/2*, which were upregulated under T4 treatment. The identification of a total of eight BR-related genes, fourteen JA-related genes, and four SA-related genes revealed that they

encompass various functional categories, such as receptor kinases, hydrolases, protein kinases, and TFs. Among these, four *BR1* genes, three *JAZ* genes, and two *MYC2* genes were upregulated under T2 treatment. These data suggest that Mo regulates key genes in hormone signaling pathways via a dose - dependent mechanism, thereby influencing the development of foxtail millet seedlings.

Sucrose and starch metabolism in foxtail millet exposure to different Mo concentrations

Sugars not only serve as carbon and energy compounds but also regulate and integrate signals that manage all plant processes throughout their life cycle, from germination to senescence (Sun M. et al., 2024). This study identified 57 genes associated with starch and sucrose metabolism (Figure 7). The T2 treatment increased the expression of *HK7* and *TPP11* genes, which promoted the phosphorylation of fructose to fructose - 6P and enhanced glycolysis for energy. Meanwhile, the T3, and T4 treatment led to the upregulation of *SUS2*, *EG*, and *GUS* - related genes, but caused the downregulation of *AMY2*, *BMV1*, and *BMV2* genes. These results indicated that high Mo concentrations likely inhibit starch degradation while activating trehalose synthesis pathways, thereby inducing trehalose accumulation to enhance stress resistance.

Expression profile of DEGs involved in phenylpropanoid biosynthesis, carotenoid metabolism, and flavonoid metabolism

Through appropriate Mo treatment, 47 genes in the 'phenylpropanoid biosynthesis' pathway showed differential regulation, as opposed to 22 genes regulated by high Mo exposure (Figure 8a). DEGs encoding *PAL*, *HCT*, *C4H*, *CCR*, *CAD* and *POD* were identified in T2 and T4 treatments. Among them, *PAL*, *C4H*, and *CCR* were upregulated, while *CCR1*, *CCR2*, and *CCR3* were downregulated in T2 treatment. Additionally, *HCT*, *CAD* and *POD* expression were higher in T4 treatment than in T2 treatment. With their higher expression, *CAD* and *POD* promote lignin synthesis and cell wall stress resistance, consequently improving the antioxidant capacity of the plants. We found 18 genes related to the flavonoid biosynthesis pathway, and among them, two *PAL*, one *C4H*, four *HCT*, and one *CHS2* genes had the highest levels of expression when treated with T2 but lower levels when treated with T4. Versus the CK treatment, the initiation of the 'flavonoid metabolism' pathway by T2 treatment, as proposed by this, could give rise to compounds utilized for advancing plant growth (Figure 8b). Furthermore, genes related to carotenoid synthesis such as β -carotene 3-hydroxylase (*CrtZ*), 9-cis-epoxycarotenoid dioxygenase (*NCED4*, and *NCED5*), and zeaxanthin synthase (*ZSD*) were significantly upregulated at T4 treatment (Figure 8c). These results imply that Mo enhances antioxidant pathways and ABA homeostasis by regulating key genes, thereby optimizing the carotenoid metabolic network to improve stress resistance and photoprotection in seedlings.

Multiple TFs involved in different Mo concentrations in foxtail millet

For the application of Mo in plants, the regulation of gene expression at the transcription level matters significantly, with 583 TFs grouped into 25 gene families. As shown in Figure S4a, bZIPs (197 members) were the predominant differentially expressed TFs, followed by MYBs (89), AP2/ERFs (37), GeBPs (36),

and bHLHs (26), with other TFs like CPPs, DOFs, etc. also detected. Notably, at T2 treatment, 104 bZIPs, 60 MYBs, 16 GeBPs, 14 NACs, and 13 bHLHs showed significant changes, followed by T4 treatment (Figure S4b). In addition, we found that 50 transcription factors were up - regulated in T2 treatment. These mainly included members of the MYB family, ERF family, bHLH family, etc (Figure S5).

Discussion

The metabolic processes of carbon, N, and sulfur in plants, as well as the synthesis of hormone - signaling substances, are both related to Mo, an essential trace element for plant growth (Huang X.Y. et al., 2022). With the overuse of multi - elemental fertilizers, the application of trace elements has been gradually neglected. Consequently, Mo deficiency is increasingly emerging as a critical limiting factor in agricultural production. One of China's most vital crops, foxtail millet acts as both a substantial energy provider for the human body and the principal means of mineral nutrient assimilation.

Changes on the Growth of Jingu 21 Plants

To tackle Mo deficiency issues in China's key foxtail millet - growing regions, we gathered 25 foxtail millet varieties in this study. The preliminary analysis revealed that the Mo content of the grains among different foxtail millet varieties can vary up to 7 - fold, which suggests that the capacity of Mo uptake and transportation may differ among the various foxtail millet (Table S2).

For this research project, the impacts of assorted Mo concentrations on the seed germination and growth attributes of Jingu 21 seedlings were evaluated. Our study's results showed that Mo at T2 concentration promoted the development of seedlings. The roots of the seedlings responded more strongly to the stimulation of Mo than the leaves, while the inclusion of Mo led to divergent results (Figure 1d). For example, shoot length, seedling height, and biomass increased then decreased with rising Mo levels (Figure 1b, Figure 2b, d, g), suggesting that moderate Mo concentration benefits seedling growth, while root vigor of T2 seedlings significantly differed from others. Similarly to these observations, exogenous Mo treatment resulted in seed germination and stimulated the growth of barley seedlings, but the higher Mo concentrations did not stimulate root growth (Batyrschina Z. et al., 2018), which indicated this effect might be associated with toxic effects of the metal. Besides, upon being cultured in a 1 mM H_2MoO_4 nutrient solution for 14 days, bush bean plants evidenced slight yellowing symptoms (Wallace A. et al., 1977). Altogether, the results of our investigation suggest that the obstructive effect of Mo took place in a concentration-dependent manner.

Nitrate Metabolism and Chlorophyll content

The pigments utilized in photosynthesis are critical components of the photosynthetic mechanism. The impact of Mo on plant chlorophyll has been widely reported. Zheng Y. et al. (2006) observed a positive correlation between chlorophyll content and Mo application rates within a certain range in Chinese cabbage. Liu P. & Yang Y. (2003) reported that Mo application maintains chlorophyll instability, increases leaf surface area and photosynthetic area, ultimately enhancing photosynthetic efficiency. Yu M. et al.

(2006) and Sun X. et al. (2003) explored the role of Mo in the mechanism of chlorophyll formation, which indicated that Mo deficiency impedes the conversion of δ -aminolevulinic acid (ALA) to urobilinogen III (UroIII) in wheat leaves, thereby affecting chlorophyll synthesis and reducing chlorophyll content. In addition, carotenoids, within the realm of photosynthesis, have a wide range of roles. Acting as light - collecting entities and important antioxidants, they reduce the occurrence of photodamage and counteract photoinhibition (Kreslavski V.D. et al., 2018; Simkin A.J. et al., 2022). In our study, carotenoid and chlorophyll contents were significantly elevated under T2 treatment, while the T4 treatment exhibited the opposite tendency (Figure 3c, d). Furthermore, there were more DEGs at T2 than at other treatments, and T4 treatment statistically up-regulated the expression of four genes in the pathway of 'carotenoid biosynthesis'. The potential mechanisms by which a surfeit of Mo dampens photosynthesis in Jingu 21 consist of reducing the leaf - chlorophyll levels, attenuating the activity associated with LCH, hampering the utilization of solar radiation and the energy - transfer capability, and blocking the electron - transfer pathway in PSII (Song X. et al., 2019). Our past research findings have revealed that photosynthetic carbon assimilation was enhanced when Mo was supplied at the T2 optimal rate. Similarly, in the case of Jingu 21 exposed to high Mo concentrations, P_n , G_s , and Tr diminished, while C_i escalated. This evidence suggests that the elevated Mo concentration potentially disrupted the photosynthetic machinery (Guo M.J. et al., 2023). Like-wise to these observations, studies have shown that augmented Mo availability heightens the gas exchange attributes of leaves, the concentrations of photosynthetic pigments, the intactness of chloroplasts, and their shape in common wheat (Li L. et al., 2017). Besides, the hindrance of photosynthesis due to high - level Mo treatments impacts the buildup of photosynthetic products.

Mo is essential for N metabolism processes like fixation, reduction, and assimilation, and its deficiency can cause nitrate accumulation (Mendel and Schwarz, 2011). Studies show that adequate Mo levels boost NR and GS activities, enhancing nitrate uptake, ammonium conversion, and organic N synthesis (Li L. et al., 2017). Agreeing with prior investigations, we determined that the activities of enzymes involved in N metabolism (NR, GS, GDH, GOGAT) and the amount of soluble protein were significantly greater under the T2 treatment. The transcriptomic data also showed that there were more DEGs at T2 than at other treatments, and two genes encoding GS (*Seita.9G485600*, *Seita.1G311400*) were significantly upregulated under T2 compared to other treatments (Table S7). Moreover, qRT-PCR was employed to verify key genes involved in N metabolism, such as *NRT2.1* (*Seita. 1G218500*, *Seita. 1G218600*), nitrate reductase (*Seita. 1G334700*), and molybdate transporter (*Seita. 9G190100*) (Figure S1). These findings further demonstrated that Mo is indispensable for maintaining the stability and activity of nitrate reductase. Liu L. et al. (2017) obtained similar data in a non-soil culture system by cultivating strawberry seedlings sprayed with different Mo concentrations. Based on his findings, strawberry seedlings treated with 135 g Mo ha⁻¹ exhibited relatively elevated activities of N - metabolic enzymes, as well as up - regulated expressions of nitrate uptake genes (*NRT1.1*; *NRT2.1*) and nitrate - responsive genes. Collectively, these results indicate that suitable Mo concentrations promote N metabolism, enabling the prompt conversion of inorganic N into amino acids, which are subsequently assembled into proteins for the plant's utilization (Liu C. et al., 2024).

Transcriptional response mechanism of foxtail millet to different Mo concentrations

In GO functional category, the “peroxidase activity”, “signal transduction”, and “response to oxidative stress” functional categories increased after T3 and T4 treatments (Figure 6e,g), suggesting that high Mo concentration can cause metabolic problems or phytotoxic effects in plants. In the set of enriched KEGG pathways, a large number of DEGs were concentrated in the pathways of phenylpropanoid biosynthesis, starch and sucrose metabolism, plant hormone signal transduction, flavonoid metabolism, and carotenoid metabolism. It is our speculation that these are intricately linked to exposure to Mo. Therefore, in the following discussion, we further analyzed the DEGs that were enriched in these pathways.

When plants face abiotic stress, secondary metabolism is crucial, with phenylpropanoids like flavonoids, phenolic acids, and coumarins serving as key protectants that are essential for plant growth, metabolic regulation, and stress resilience (Yin Q. et al., 2025). Flavonoids, produced via the phenylpropanoid pathway, efficiently scavenge ROS generated by biotic and abiotic stresses, protecting plant cells from oxidative damage (Nabavi S.M. et al., 2020; Dong N.Q. et al., 2021). Earlier investigations have shown that plants subjected to severe or successive drought stress accumulate more phenylpropanoids, which reinforces their ability to adapt to long-term stress (Shen X. et al., 2022; Shao C. et al., 2023). The expression changes of key genes in the phenylpropanoid biosynthesis pathway (such as *PAL* and *C4H*) under high Mo treatment affect the changes in related metabolites in the plant, thereby strengthening the plant's stress response ability. In the phenylpropanoid pathway, *PAL* deaminates phenylalanine to trans-cinnamic acid, which *C4H* further modifies to coumarin coenzyme A, leading to the synthesis of phenolics, lignins, and flavonoids (Tomás-Barberán & Espín, 2001; Blushan B. et al., 2015). Also, our research findings demonstrated that a majority of the *CAD* and *POD* genes were notably up-regulated by the T3 and T4 treatments. In a similar fashion, flavonoid-related biosynthetic pathways were enriched in Jingu 21, which contributed to the improvement of antioxidant functions and the alleviation of oxidative stress. In the flavonoid biosynthesis pathway, *CHS* and *F3H* are essential enzymes, with *CHS* serving as the primary rate-limiting enzyme for flavonoid synthesis (Liu W. et al., 2021). Our study demonstrated that the expression of *CHS* and *F3H* was suppressed in the T4 treatment relative to the T2 treatment. This implies that, as compared with the CK treatment, the T2 treatment stimulated the activation of the ‘flavonoid metabolism’ pathway, and the synthesized products could be used to promote the growth and development of plants.

Sugar is one of the crucial substances for plant growth and stress resistance (Liu R. et al., 2025). In our study, low concentrations of Mo enhance *HK7* activity, facilitating the conversion of fructose phosphate to fructose-6-phosphate and thereby promoting the glycolytic process. Findings from the transcriptome demonstrate that the T2 treatment increased the expression levels of three sucrose-synthase-related genes, *Seita.8G142600*, *Seita.5G390300*, and *Seita.3G109600*, in the ‘starch and sucrose metabolism’ pathway. This pathway is responsible for supplying energy and the carbon framework necessary for plant growth. Concurrently, it activates UTP-glucose pyrophosphorylase. This activation stimulates UDP-glucose production, which is then utilized for cell wall synthesis or glycogen storage. Conversely, high

Mo concentrations inhibit the enzymatic activities of *AMY* and *BMV*, suppressing starch degradation. As a major carbon reserve in plants, starch metabolism is of great significance for maintaining plant functions and homeostasis. *AMY* is essential for hydrolyzing and solubilizing starch (Damaris et al., 2019), and our results showed *AMY* genes were down-regulated under T4 treatment, resulting in trehalose accumulation as a stress-responsive adaptation. Notably, the significant upregulation of *BBE18* under high Mo exposure suggests its regulatory role in orchestrating carbon partitioning to enhance stress resistance through this pathway. Furthermore, starch and sucrose metabolism also were influenced by hormone metabolism, which, in turn, affects plant development. Based on the results of the comprehensive transcriptome analysis, we focused on the pathways related to hormone synthesis.

Plant hormones such as ABA, auxin (IAA), GA, CTK, and Jasmonic acid (JA) could regulate plant growth process, including cell division and enlargement, seed development, and leaf development (Li Y. et al., 2023). Based on plant hormone signal transduction pathway in the KEGG map, we found that genes related to *PYR/PUL*, *PP2C* and *ABF* were up-regulated in ABA signaling cascade, which may indicate that the high Mo application induced oxidative damage. In our study, under T2 treatment, the expression of genes related to *AUX1*, *GH3* and *SAUR* were significantly up-regulated during IAA signaling, *DELLAs* and *GID1* were significantly up-regulated during GA signaling, and *JAR* and *MYC* were significantly down-regulated during JA signaling. Recent research indicates that there is crosstalk between GA, ABA, IAA, and other signaling hormones. This crosstalk forms a coordinated regulatory network designed for plant development and adaptation (Sun M. and Shen Y., 2024). Moreover, low - concentration Mo - derived ROS act as signaling molecules for various hormones, namely GA, ABA, IAA, and other phytohormones. Consequently, this interplay culminates in increased plant growth and heightened stress tolerance (Maity D. et al., 2022). Hence, we speculated Mo could fine-tune the plant hormonal balance to improve Jingmu 21 resistance in different situations (Chagas F.O. et al., 2018).

Numerous TFs play a regulatory role in plant development, and a significant number of these TFs have been thoroughly studied (Chowdhary et al., 2023; Yuan H.Y. et al., 2024). In the present study, various types of TFs (including bZIP, MYB, AP2/ERF, GeBP, bHLH, and ZFB, etc). were highly expressed in the T2 and T4 treatments. In addition, we found that most of the TFs changed significantly at T2 treatment. They mainly include the MYBs family, ERFs family, bHLHs family, etc. For instance, we found strong up - regulation in ERFs from our RNA - seq data under T2 treatment. This upregulation may modulate sugar transporters to promote foxtail millet growth, and these results clarify the molecular mechanisms of physiological effects while highlighting the need for further study of TF functions.

Conclusions

In conclusion, the exogenous application of Mo significantly affected gene expressions and enhanced the growth of foxtail millet compared with the control group. The results of RNA - seq analysis manifested that 5189 genes presented unique differential expression in the group treated with Mo as compared to the control group. Subsequently, we implemented transcriptome analysis to uncover the dynamic mechanisms involved in the pathways of TFs, phenylpropanoid biosynthesis, starch and sugar

metabolism, plant hormone signaling, carotenoid metabolism, and flavonoid biosynthesis pathways exposure to different Mo concentrations in foxtail millet. The findings revealed that, in the T4 treatment as opposed to the T2 treatment, the metabolic processes of starch and sucrose were suppressed, while the biosynthesis of phenylpropanoids and the metabolism of flavonoids were promoted. Moreover, a high Mo treatment upregulated *NCED* and *ZSD*, modulating plant hormone signal transduction to enhance starch - sucrose balance regulation and stress tolerance in foxtail millet. Physiological analyses showed the optimum Mo concentration can enhance N metabolic enzyme activities, promote chlorophyll biosynthesis and increase the content of soluble protein. These results elucidated the mechanism of biological responses to exposures to Mo, providing new insights for trace element risk assessment. Furthermore, our findings suggest that the application of 8 mg L^{-1} Mo has the potential to boost the germination and early seedling growth of Jingu 21, highlighting its efficacy and safety in foxtail millet cultivation.

Declarations

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contribution

Conceptualization, M.J. Guo and M.M. Sun; methodology, Y.Q. Bai; software, M.M. Sun; validation, L.T. Lan and Y.F. Wang; formal analysis, L.T. Lan and W.M. Yang; writing—original draft preparation, M.J. Guo; writing—review and editing, M.M. Sun; visualization, Y.Q. Bai; supervision, P.Y. Ji and Y.Z. Wu; funding acquisition, M.J. Guo and Y.J. Yang. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA1271431.

Supplementary Material

The following supporting information can be downloaded at: Supplementary files.

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Figures

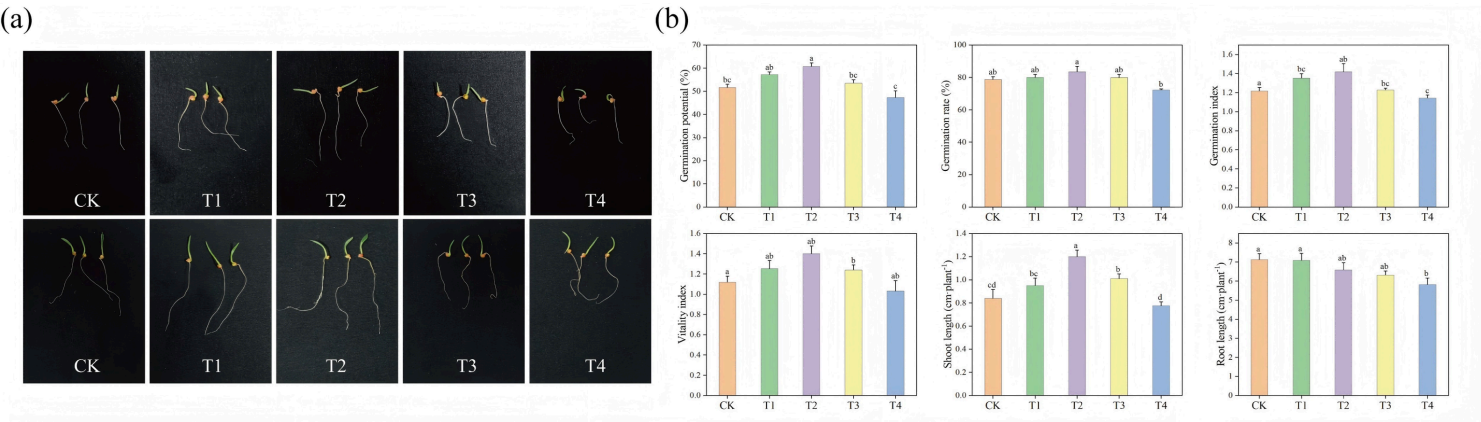


Figure 1

Effects of various concentration of exogenous Mo on seed germination of Jingu 21. (a) foxtail millet seedlings growth, (b) germination index. Seedlings grow on Hoagland’ s nutrient solution with a series of Mo concentration (0, 4, 8, 10, 15 mg L⁻¹) for 7 days. Data are presented as the mean±standard error (for germination rate, n=3; shoot length, n=10; root length, n=10). Different lowercase letters denote significant differences at *p*<0.05.

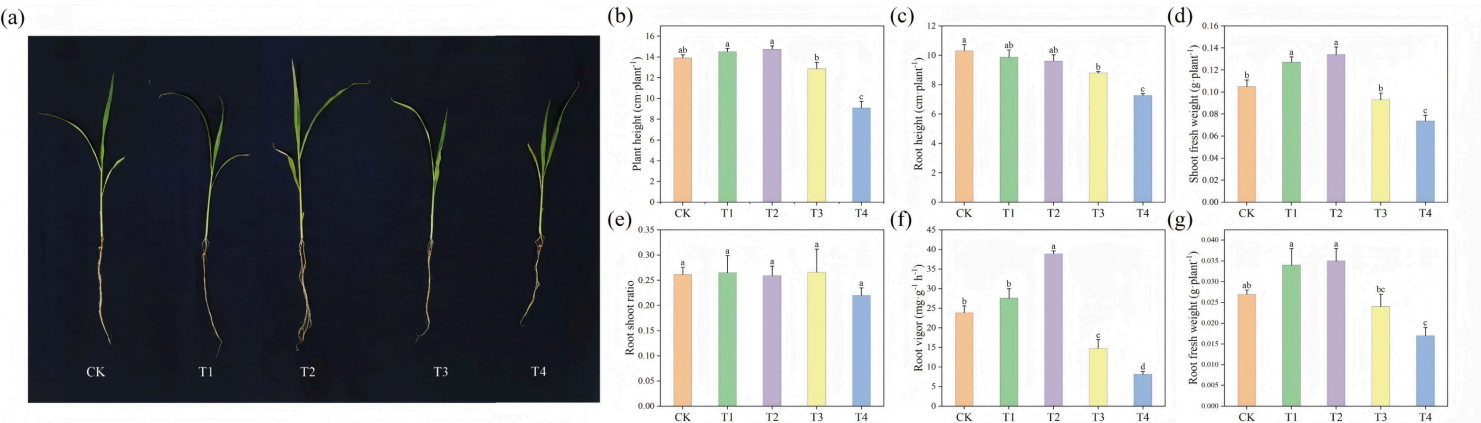


Figure 2

Effects of various concentration of exogenous Mo on plant growth performance and root morphology indicators of Jingu 21. (a) foxtail millet morphology, (b) plant height, (c) root height, (d) shoot fresh

weight, (e) root shoot ratio, (f) root vigor and(g) root fresh weight. Different lowercase letters denote significant differences at $p<0.05$.

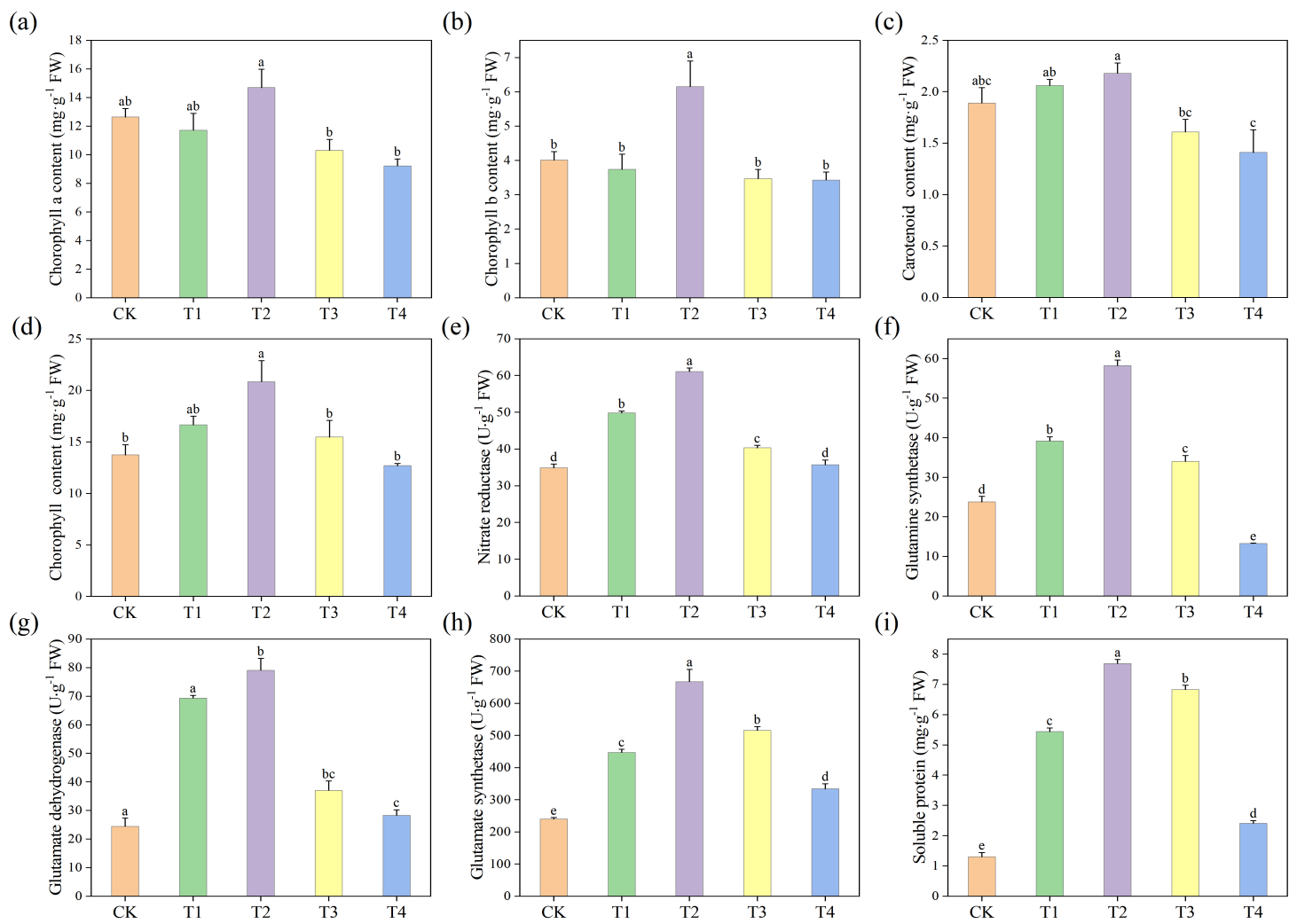


Figure 3

Effects of various concentration of exogenous Mo on the photosynthetic pigment content nitrogen metabolism-related enzymes of Jingu 21. (a) chorophyll a content, (b) chorophyll b content, (d) chorophyll content, (c) carotenoid content, (e) nitrate reductase, (f)glutamine synthase, (g)glutamate dehydrogenase, (h)glutamate synthetase, and (i)soluble protein. Different lowercase letters denote significant differences at $p<0.05$.

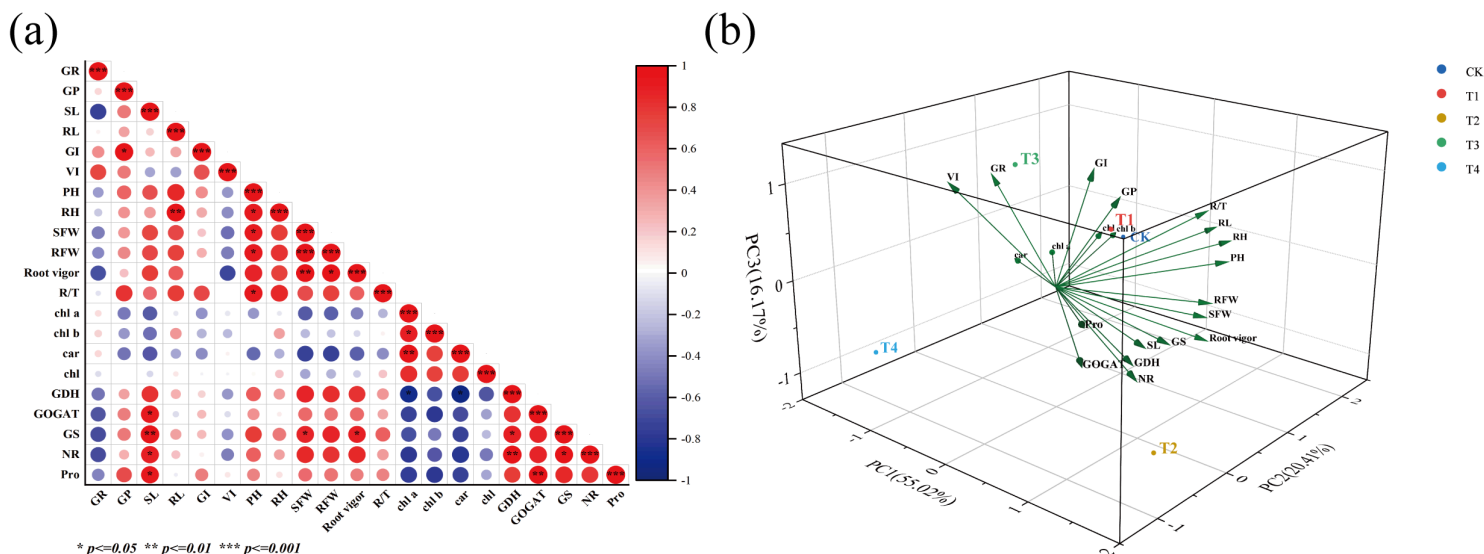


Figure 4

Correlation and PCA analysis. (a) Heatmap of the Pearson correlation coefficients obtained from variables, (b) Three-dimensinal principal component analysis (3D-PCA) of variables and treatments.

GR: Germination rate, GP:Germination potential, SL: Shoot length, RL: Root length, GI: Germination index, VI:Vitality index, PH: Plant height, RH: Root height, SFW: Shoot fresh weight, RFW: Root fresh weight, R/T: Root shoot ratio; chl a: Chlorophyll a content, chl b: Chlorophyll b content, car: Carotenoid content, chl: Chlorophyll content, GDH: Glutamate dehydrogenase, GOGAT: Glutamate synthase, GS: Glutamine synthetase, NR: Nitrate reductase, Pro: Soluble protein.

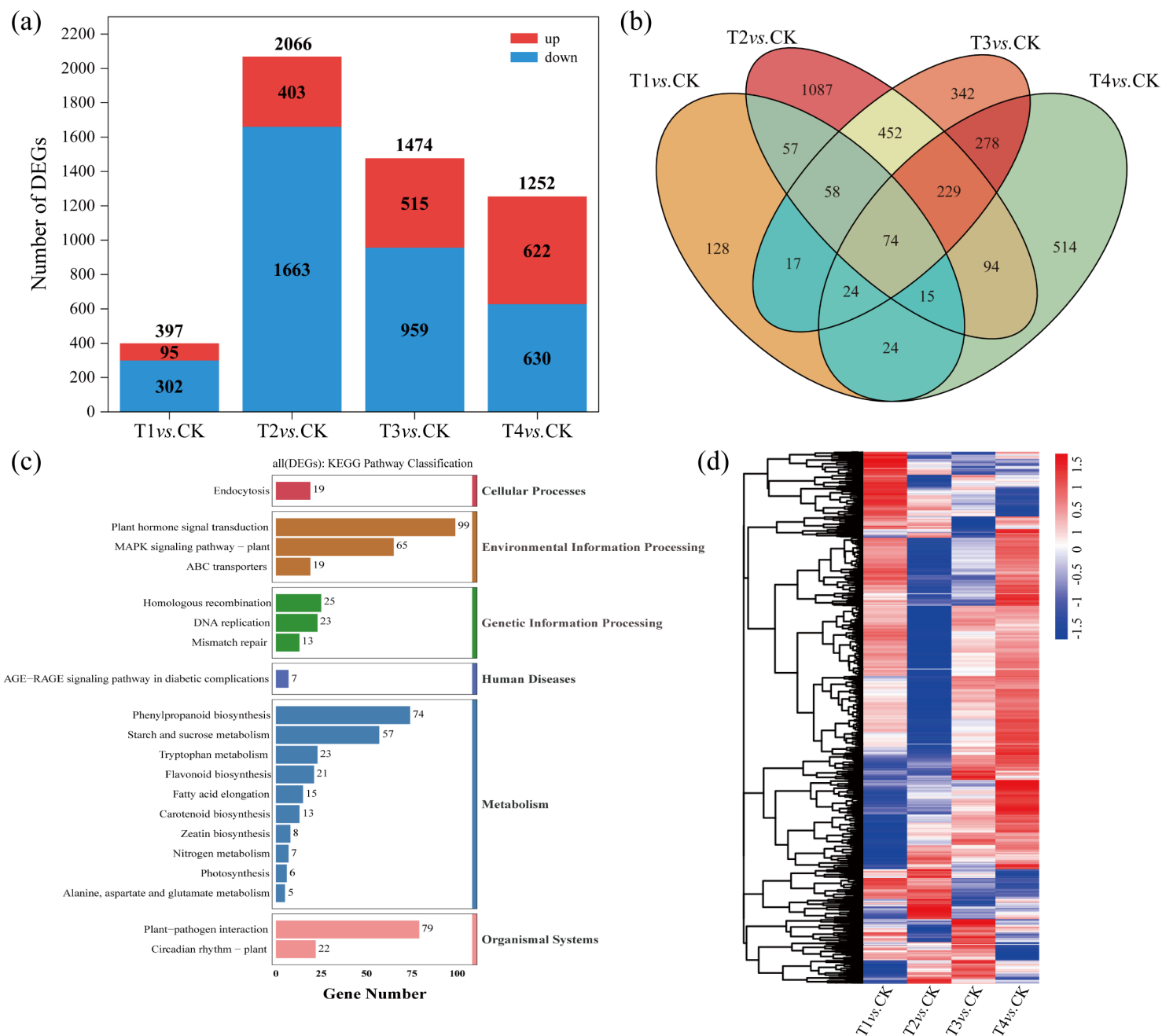


Figure 5

Global gene expression profiling and KEGG pathway analysis. (a) number of up-regulated and down-regulated differentially expressed genes (DEGs) between treatment vs. control, (b) Venn diagram showing the overlap of DEGs treatment vs. control under hydroponic culture condition, (c) KEGG pathway classification of the all DEGs, (d) Hierarchical cluster analysis of all DEGs.

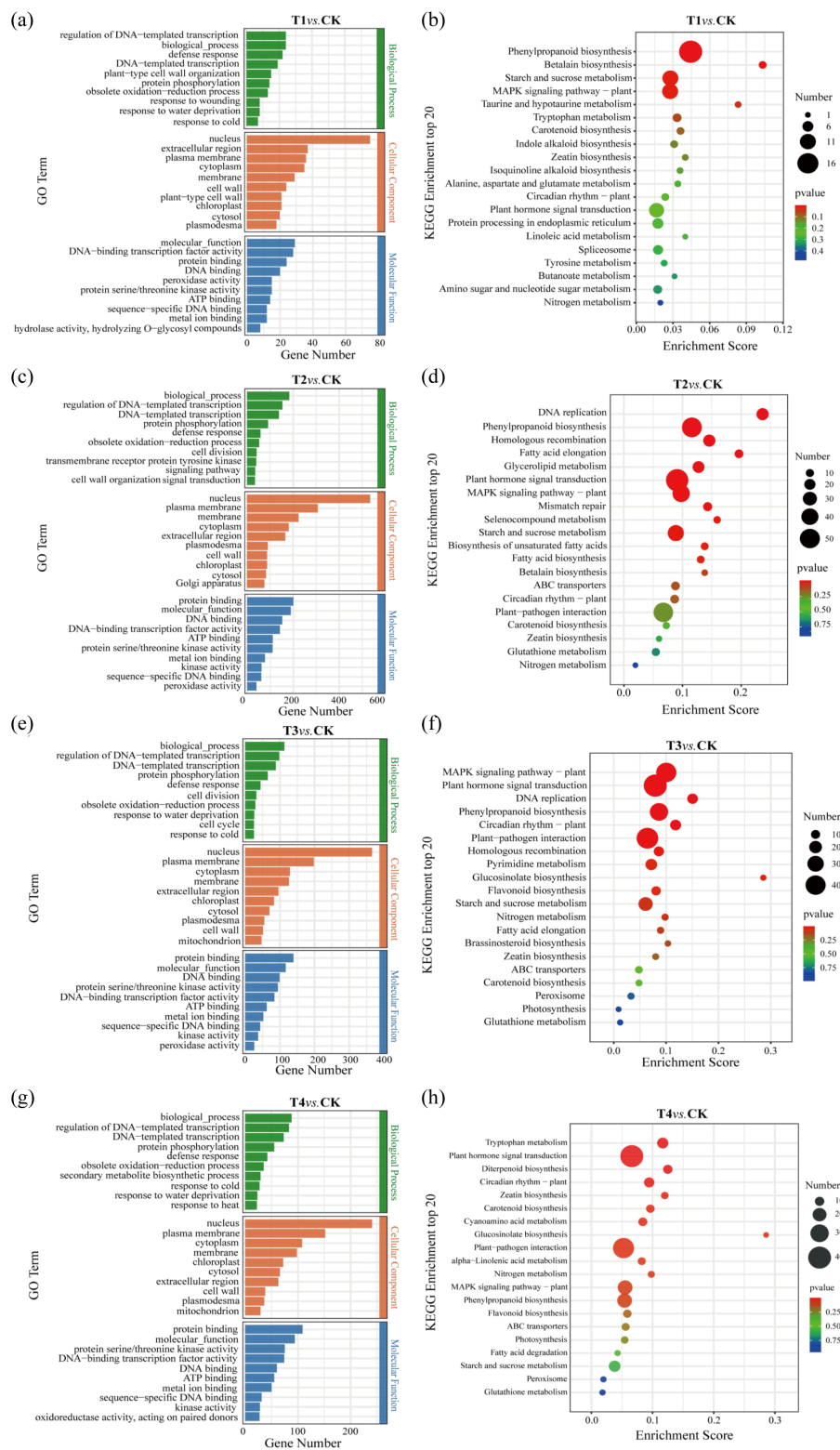


Figure 6

Effects of various concentration of exogenous Mo on the global transcriptomic profiles of Jingu 21 seedling. (a), the top 30 of Gene Ontology (GO) terms enrichment analysis by T1 vs. CK. (b), top 20 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for DEGs by T1 vs. CK. (c), the top 30 of Gene Ontology (GO) terms enrichment analysis by T2 vs. CK. (d), top 20 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for DEGs by T2

vs.CK. (e), the top 30 of Gene Ontology (GO) terms enrichment analysis by T3 vs.CK. (f), top 20 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for DEGs by T3 vs.CK. (g), the top 30 of Gene Ontology (GO) terms enrichment analysis by T4 vs.CK. (h), top 20 significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for DEGs by T4 vs.CK.

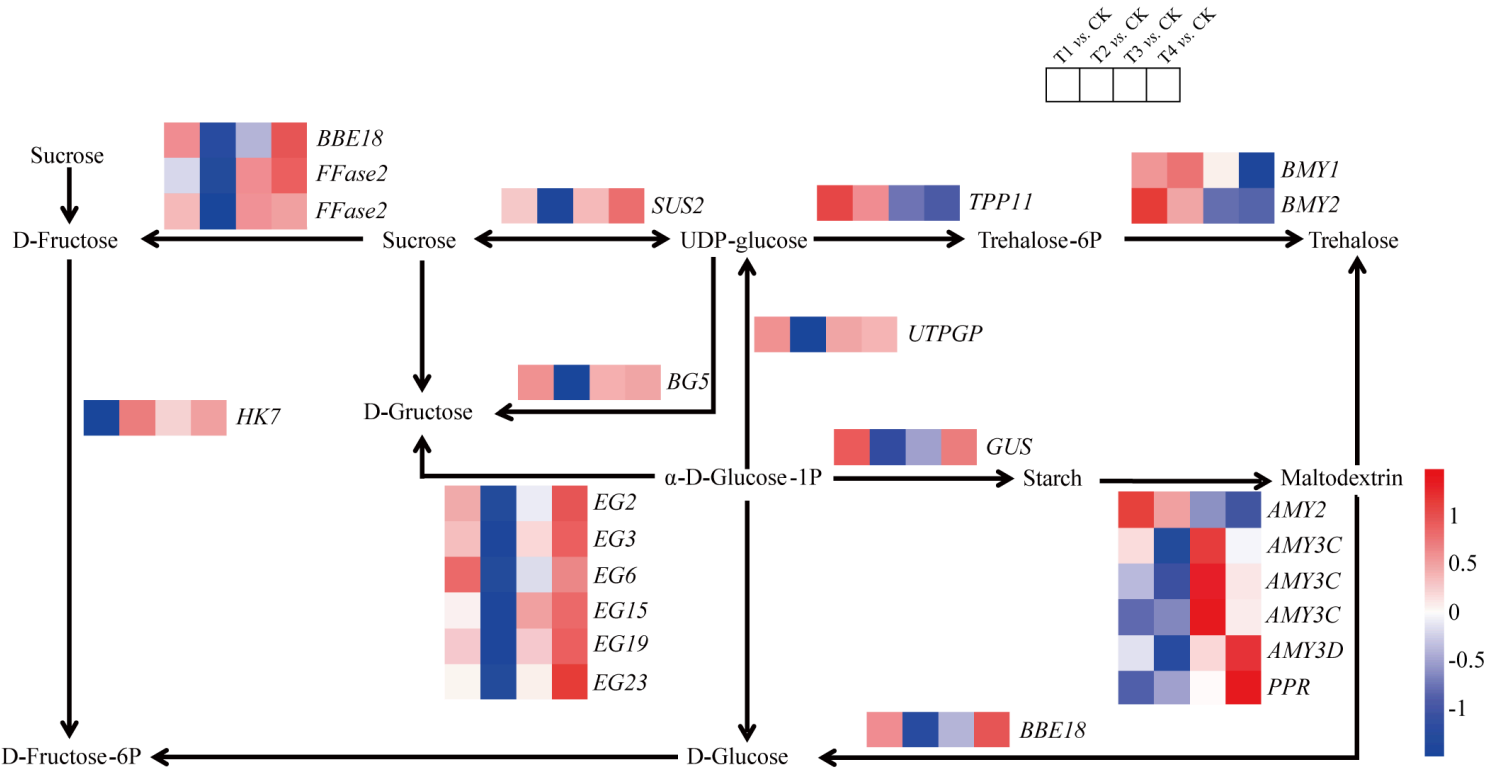


Figure 7

Analysis of expression patterns of genes in the sucrose-starch metabolism pathway under various concentration of exogenous Mo. Colored boxes represent the log2 fold change. BBE, nitrate reductase; FFase, β-fructofuranosidase; SUS, sucrose synthase; TPP, glutamate synthase; HK, glutathione S-transferase; BG, gamma-glutamyl transferase; EG, aromatic-L-amino-acid/L-tryptophan decarboxylase; GUS, β-glucuronidase; UTPGP, UTP glucose-1-phosphate uridylyltransferase; AMY, alpha-amylase; BMY, Beta-amylase; PPR, pentatricopeptide repeat.

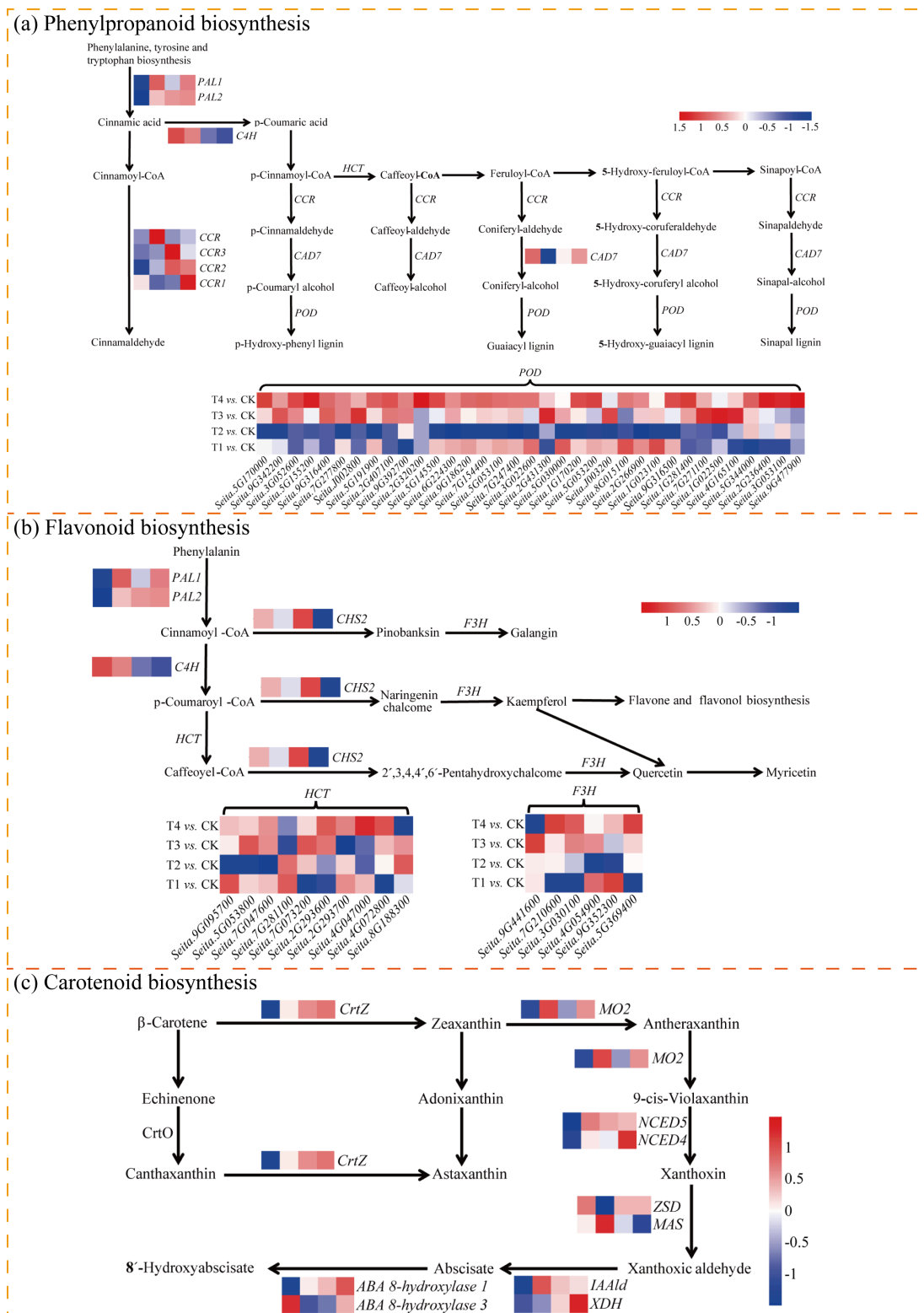


Figure 8

Major metabolism pathway of Jingu 21 after 21 days of treatment with different concentration of Mo. (a) DEGs in the phenylpropanoid biosynthesis pathway under Mo treatment, (b) DEGs in the flavonoid biosynthesis pathway under Mo treatment, (c) DEGs in the carotenoid biosynthesis pathway under Mo treatment.

Colored boxes represent the $\log_2$ fold change. PAL, phenylalanine ammonia-lyase; C4H, p-coumarate 4-hydroxylase; CCR, cinnamoyl-CoA reductase; POD, peroxidase; HCT, Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; CHS, chalcone synthase; F3H, flavanone-3-Hydroxylase; CrtZ, β -carotene hydroxylase; MO2, monooxygenase 2; NCED, 9-cis-epoxycarotenoid dioxygenase; ZSD, zerumbone synthase; MAS, momilactone A synthase; XDH, Xanthine Dehydrogenase; IAA, indole-3-acetic acid; ABA 8-hydroxylase, abscisic acid 8'-hydroxylase.

Supplementary Files

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