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Transcriptomic analysis of melatonin-mediated drought stress response genes in alfalfa during germination period

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Abstract

Background Drought stress is a predominant abiotic factor contributing to reduced crop yields globally. Therefore, exploring the molecular mechanism of drought control is of great significance to improve drought resistance and ultimately achieve crop yield increase. As a plant endogenous hormone, melatonin plays a key role in the regulation of abiotic stress, but the key genes and metabolic pathways of melatonin mediated drought resistance regulation in alfalfa have not been fully revealed. Based on transcriptomics and physiological index detection, this study aimed to explore the regulatory mechanism of melatonin in alleviating drought stress during alfalfa germination.

Results The findings revealed that alfalfa seedlings treated with melatonin exhibited higher germination rates, increased shoot length, and greater fresh weight compared to those exposed solely to drought stress. Additionally, there was a reduction in the levels of malondialdehyde (MDA) and superoxide anion (O_2^-), while the activity and concentration of superoxide dismutase (SOD), peroxidase (POD), and glutathione (GSH) were enhanced to varying extents. To investigate the molecular mechanism underlying melatonin-mediated drought resistance in alfalfa, we performed a transcriptomic analysis on the seedlings. In the drought treatment group, we identified a total of 1,991 differentially expressed genes (DEGs), comprising 778 up-regulated and 1,213 down-regulated genes. Conversely, in the melatonin-treated group, we discovered 2,336 DEGs, including 882 up-regulated and 1,454 down-regulated genes.

Conclusions Through the application of GO functional annotation and KEGG pathway enrichment analysis, we discovered that DEGs were predominantly enriched in pathways related to flavonoid and isoflavone biosynthesis, plant hormone biosynthesis and signal transduction, glutathione metabolism, and MAPK signaling, and the ABC transporter signaling. Notably, the DEGs added to the MT group showed greater enrichment in these pathways. This suggests that MT mitigates drought stress by modulating the expression of genes associated with energy supply and antioxidant capacity. These findings hold significant reference value for breeding drought-tolerant alfalfa and other crops.

Keywords Alfalfa, Drought stress, Melatonin, Transcriptomics, Differentially expressed genes

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Introduction

Currently, soil drought is intensifying due to human activities and climate change. Drought stress is one of the most prevalent abiotic challenges in crop production, restricting plant growth and yield by limiting water absorption and utilization [1–3]. The impact of drought on crops is multifaceted, affecting photosynthesis, respiration, nutrient absorption, and transportation. The disruption of these physiological processes can result in delayed growth and development, and even plant death, severely impacting agricultural production and hindering local economic development [4–6].

Alfalfa (*Medicago sativa* L.) is a high-yield forage known for its substantial protein content and balanced amino acid composition. In China, it is predominantly cultivated in arid and semi-arid regions, including the northwest and northern areas. However, alfalfa in these regions frequently encounters drought, a condition that not only hampers its growth but also adversely impacts critical physiological processes such as photosynthesis. Research indicates that drought stress is a significant factor curtailing alfalfa productivity [7, 8]. Thus, exploring the molecular mechanisms underpinning drought resistance in alfalfa and enhancing its resilience is vital for increasing yield. Current research on drought stress in alfalfa primarily focuses on identifying specific drought-resistant genes, such as *MsCYP71* and *MsTHI1* [9, 10]. Nevertheless, these studies often limit themselves to the effects of one or a few genes, indicating the need for more comprehensive exploration. In combating drought, plant hormones play an essential role by regulating growth, development, and metabolism, thereby aiding adaptation to adverse environments [11, 12]. Melatonin, a multifunctional biomolecule, has recently been identified as playing a pivotal role in regulating growth, enhancing antioxidant capacity, and facilitating mineral absorption in plants [13–15]. As an antioxidant, melatonin stimulates the expression of antioxidant enzyme genes and increases enzyme activity, thereby bolstering plant stress resistance [16]. Although melatonin's role in enhancing drought resilience has been widely acknowledged across various crops, the precise transcriptomic mechanisms underlying its effects on drought tolerance in alfalfa remain largely unexplored [17, 18]. This study aims to address this critical knowledge gap by examining the gene expression changes triggered by melatonin treatment under drought stress. Previous research has demonstrated that, in *Zea mays* melatonin promotes the upregulation of antioxidant genes such as SOD and POD, thereby mitigating oxidative damage [19]. Similarly, in *Oryza sativa*, melatonin interacts with ABA signaling pathways to induce stomatal closure, improving drought tolerance [20]. These findings suggest that comparable regulatory mechanisms may also exist in alfalfa; however, they have yet to be thoroughly investigated. To bridge this gap, this study explores the gene expression changes associated with melatonin treatment during drought stress. To achieve a more comprehensive analysis, Weighted Gene Co-expression Network Analysis (WGCNA) was employed to construct gene expression-related networks. This method allows for the identification of gene modules strongly associated with specific traits, such as drought response, and facilitates the pinpointing of hub genes within the regulatory network. These insights provide a robust framework for systematically analyzing the multi-gene collaborative mechanisms involved in drought tolerance. High-throughput techniques, leveraging omics analysis, can elucidate the regulatory mechanisms of these complex plant traits. In our study, we compared the growth performance and physiological indicators of alfalfa under PEG-simulated drought stress, both with and without melatonin (MT) treatment, and performed whole-gene transcriptome sequencing to identify differentially expressed genes under the influence of MT and PEG. Furthermore, we utilized WGCNA to pinpoint key signaling pathways and hub genes regulated by MT, aiming to clarify their cellular mechanisms of action. Under drought conditions, the synthesis of vital amino acids (such as proline, tryptophan, and arginine), flavonoids (including anthocyanins, quercetin, and licorice), and plant hormones (such as abscisic acid, gibberellin, and melatonin) increases. These findings offer new insights into cultivating drought-tolerant alfalfa.

Result

Effects of melatonin on alfalfa plant growth under drought stress

Various concentrations of melatonin were tested on alfalfa seeds subjected to drought stress. In comparison to the control (CK), a 15% PEG solution significantly reduced the germination rate, seedling fresh weight, and root length of alfalfa seeds ($P < 0.05$). However, applying low concentrations of melatonin led to an increase in these metrics to varying degrees (Fig. 1 A). Conversely, a higher concentration of melatonin (> 200 μM) significantly diminished these improvements, sometimes even leading to a decline. Under treatment with a 10 μM melatonin solution, alfalfa seeds achieved a germination rate of 78.89% and a root length of 6.21 cm, showing no significant difference from the CK group. However, the fresh weight, recorded at 0.201 g, exceeded that of other concentrations (Fig. 1 B-D). Thus, 10 μM melatonin was determined to be the optimal concentration for mitigating drought stress during the germination of alfalfa seeds.

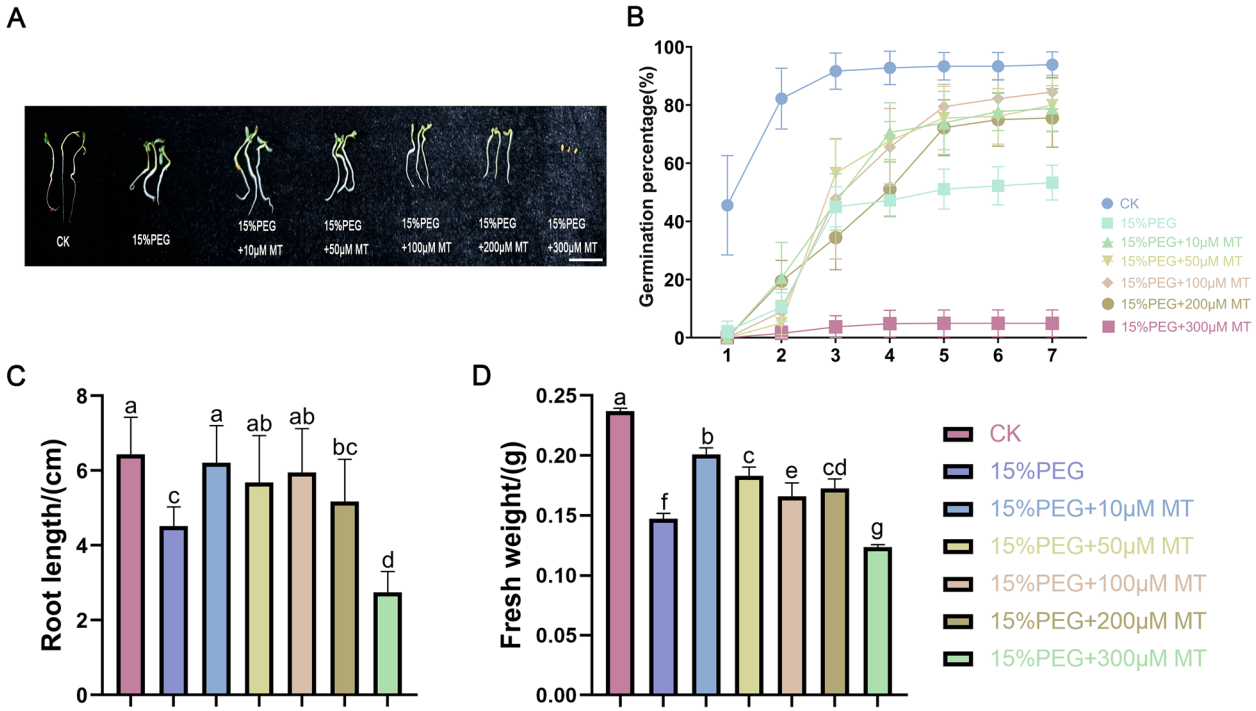

Fig. 1 **A** Phenotypic display of alfalfa seedlings, with a scale of 2 cm. **B** Statistics of seed germination rate after 7 days of treatment. The line graph displays the mean and standard deviation of six biological replicates, each containing 30 seeds. **C** Root length measurement results; The columns with different lowercase letters in the figure represent the significant differences between treatments at the $p < 0.05$ level according to Duncan's test. The data is presented as the average and standard deviation of three biological replicates, each replicate consisting of 10 plants. **D** Fresh weight of alfalfa seedlings; This column of data represents the average of three biological replicates. Different lowercase letters indicate significant differences between the treatments identified through Duncan's test. CK represents the control group

Effects of exogenous melatonin pretreatment on antioxidant properties of alfalfa seedlings under drought stress

To further investigate the physiological changes associated with melatonin-mediated drought resistance in alfalfa seedlings, we examined various antioxidant indicators, including superoxide anion, malondialdehyde (MDA), glutathione (GSH), peroxidase (POD), and superoxide dismutase (SOD). Our findings indicate that osmotic stress led to an increase in both superoxide anion and MDA accumulation (Fig. 2 A-E). However, when treated with exogenous melatonin, these levels were reduced by 27.8% and 26.6%, respectively (Fig. 2 A-B). Additionally, while GSH content decreased by 21.8% under osmotic stress, the introduction of exogenous melatonin not only restored but also increased GSH content by 18.2% compared to the control group (Fig. 2 E). Furthermore, under osmotic stress, POD and SOD activities exhibited slight increases, which were further enhanced with exogenous melatonin treatment (Fig. 2 C-D).

Identification of drought-responsive genes in alfalfa seedlings

A transcriptome analysis was performed on alfalfa seedlings using deionized water, 15% PEG, and a combination of 10 μM MT with 15% PEG as treatments. Through comparison of these treatment methods, a total of 3,134 genes were identified. Only six genes (0.19% of total) were common across all treatments. Additionally, 28 genes were shared between ZMPMT vs. ZMCK and ZMPMT vs. ZMP, comprising only 0.83% of the overall gene count. Eighteen genes were common among ZMPMT, ZMP, and ZMCK, amounting to just 0.57% of the total gene count. In contrast, a significant 1,215 genes were found in all three treatments (ZMPMT, ZMCK, and ZMCK), accounting for approximately 38.77% of the total. Furthermore, 764 genes were unique to the comparison between ZMP and ZMCK, making up about 24.38% of the total gene count (Fig. 3 A). Analyze differentially expressed genes across three treatments, categorize genes with similar or identical expression patterns, and create volcano plots. (Fig. 3 B). The Venn diagram

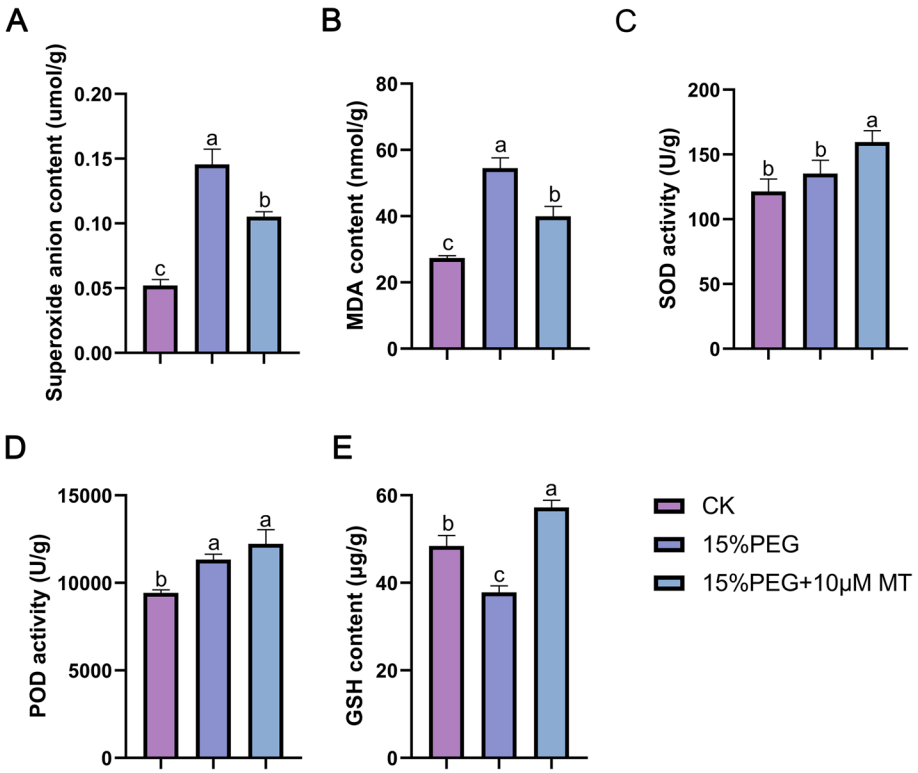

Fig. 2 Exogenous melatonin can alleviate oxidative stress caused by drought. Specific indicators include O_2^- level (**A**), MDA concentration (**B**), SOD activity (**C**), POD activity (**D**), and GSH content (**E**). Each set of data underwent three biological replicates, with each replicate containing ten independent plant samples. Duncan's test was used for data analysis, with different lowercase letter markers indicating significant differences between treatments

analysis revealed that 778 genes were upregulated in the ZMP vs. ZMCK comparison. In the ZMPMT vs. ZMCK comparison, 882 genes were upregulated, while the ZMPMT vs. ZMP comparison showed a minimal difference, with only 28 genes upregulated (Fig. 3 C). Additionally, variance analysis identified ten differentially expressed genes potentially related to drought tolerance mediated by the melatonin hormone in alfalfa (Fig. 3 D).

Enrichment analysis of differentially expressed genes

In the primary classification of GO, DEGs are assigned to three major categories: molecular function, cellular component, and biological process. In the secondary classification, DEGs are more frequently annotated with terms such as binding activity, catalytic activity, cell-binding entity, metabolic process, and cellular process. Notably, the number of DEGs in the ZMP and ZMPMT treatment groups exceeds that in the ZMCK control group (Fig. 4 A). KEGG pathway analysis of DEGs reveals 15 significantly enriched pathways ($P < 0.05$) in the ZMP versus ZMCK treatment group and 14 significantly enriched pathways in the ZMPMT versus ZMCK treatment group (Fig. 4 B-C). Shared pathways include phenylpropanoid biosynthesis, flavonoid and isoflavone biosynthesis, starch and sucrose metabolism, glutathione metabolism, and MAPK signaling (Fig. 4 D). It is noteworthy that the arginine and proline metabolism pathways are unique to the ZMP versus ZMCK treatment group, whereas the ABC transporter protein pathway is exclusively observed in the ZMPMT versus ZMCK treatment group.

Gene co-expression network

The response of alfalfa to osmotic stress is regulated by multiple genes. In this study, we performed a transcriptome analysis on Zhongmu-3 under various treatments, identifying numerous differentially expressed genes (DEGs). We utilized Weighted Gene Co-expression Network Analysis (WGCNA) to explore the correlation between DEGs and physiological traits linked to drought stress (Fig. 5 A). We identified twelve co-expressed modules and found that correlation analysis of module traits indicated a significant positive relationship between gene expression levels in the green module and phenotypic characteristics such as germination rate, root length, and fresh weight, with correlation coefficients ranging from 0.767 to 0.867.

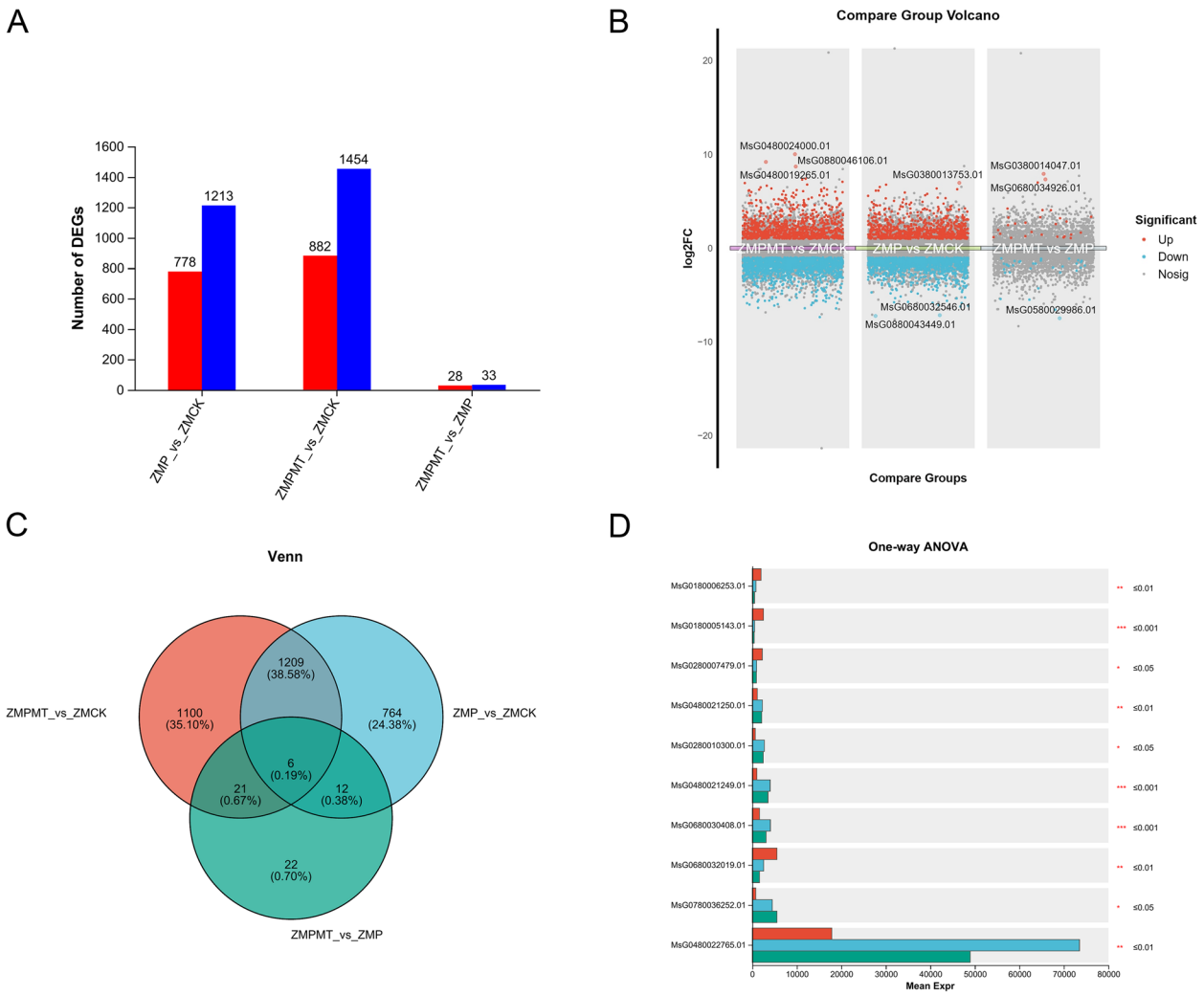

Fig. 3 Control the comprehensive visualization of PEG, CK, PEG, and MT combined with NaCl (MT + PEG). **A** The Venn diagram reveals the uniqueness and sharing of DEGs. **B** The scatter plot illustrates the upregulated and downregulated characteristic genes across each differential group. **C** Multiple inter group analysis of variance was conducted* Indicates *P*-value < 0.05, * * indicates *P*-value < 0.01. **D** Hierarchical clustering analysis of log2 RPKM expression trends of DEGs treated with PEG or melatonin alone

Conversely, in the blue module, we noted a significant negative correlation, with coefficients ranging from 0.683 to 0.85, among gene expression levels related to similar features. This indicates that genes within the green module may contribute to MT-mediated drought resistance, while those in the blue module may be involved in regulating reactive oxygen species clearance and maintaining oxygen free radical balance, given their significant positive correlation with MDA content and O_2^- levels, respectively. Furthermore, indicators of antioxidant capacity, such as SOD and POD activity, showed positive correlations with gene expression levels in the yellow module. The gene expression level in the black module is negatively correlated with GSH content. The green module includes 564 DEGs, the blue module comprises 2,748 DEGs, the yellow module contains 970 DEGs, and the black module includes 132 DEGs (Fig. 5 B). In the blue, green, yellow, and black modules, we identified thirty key genes representing corresponding functions (Fig. 5 C-F). These central genes were visualized using CytoScape software version 3.9.1, highlighting their critical roles in flavonoid biosynthesis pathways, plant hormone signaling pathways, soluble sugar metabolism pathways, glutathione metabolism pathways, and ABA transporter regulation.

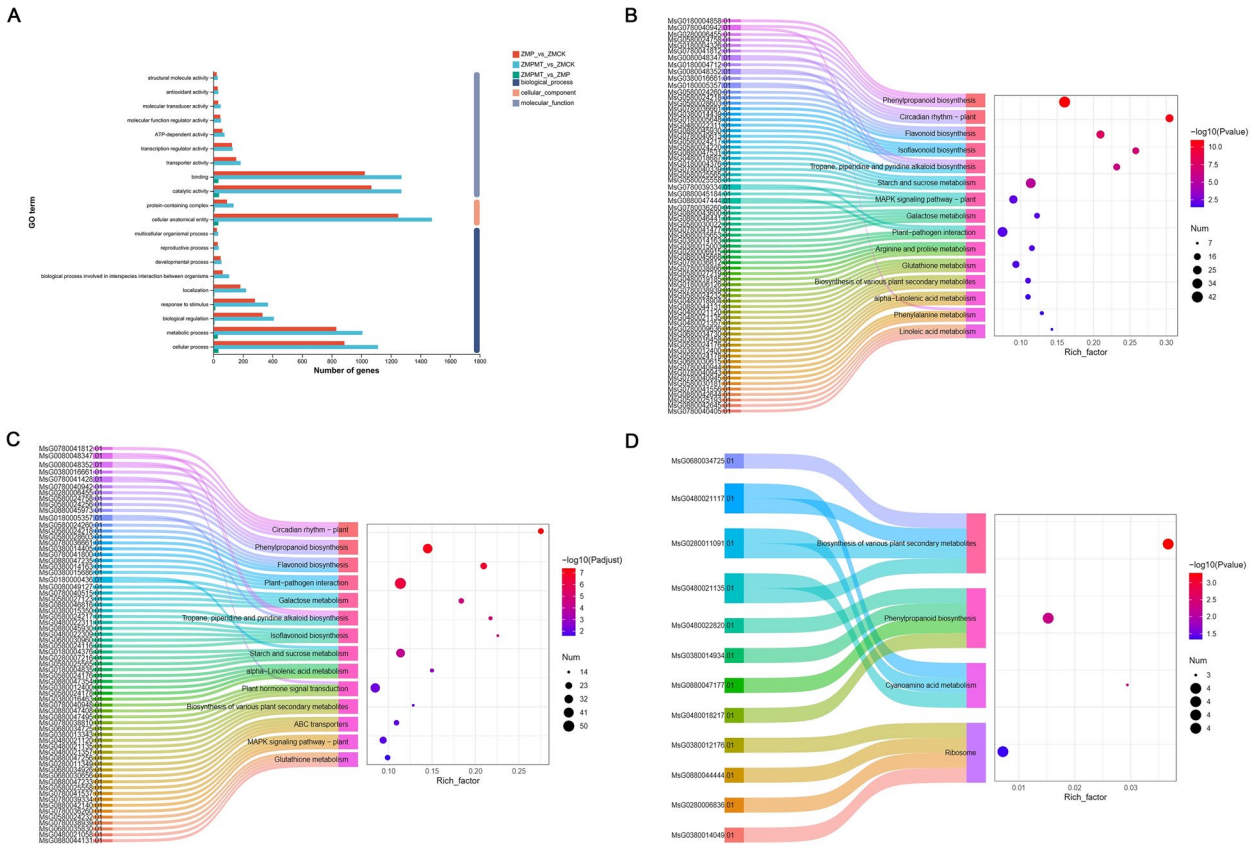

Fig. 4 Functional annotation and KEGG enrichment analysis of DEGs were conducted. **A** The GO annotation analysis categorized the differentially expressed genes into biological processes, cellular components, and molecular functions, represented by distinct colors for group differences. **B** Enrichment analysis revealed functional classification differences in the comparison between ZMP and ZMCK. **C** The functional classification and enrichment analysis described the differentially expressed genes between ZMPMT and ZMCK. **D** Functional classification enrichment analysis was presented for the differentially expressed genes between ZMPMT and ZMP

Transcriptional analysis of several differentially expressed genes

To further confirm the trend of changes in differentially expressed genes (DEGs), qRT-PCR was performed. The eight selected genes were primarily involved in flavonoid synthesis (*MsG0180005358.01*), ABA biosynthesis and signal transduction (*MsG0180001827.01*), GA biosynthesis (*MsG0180005519.01*), and melatonin synthesis (*MsG04800200076.01*). As illustrated in Fig. 6, the trend of transcript changes in these eight DEGs was consistent with the RNA-seq data, indicating that the data is reproducible and suitable for further analysis. To ensure the reliability of the transcriptome data, we validated it by calculating the linear regression coefficient between the RNA-seq data (log2 (FC)) and the qRT-PCR results (ΔΔCt method). The results showed that for the ZMP vs. ZMCK group, $R^2 = 0.61$, and for the ZMPMT vs. ZMCK group, $R^2 = 0.70$, indicating the consistency between our transcriptome data and the fluorescence quantitative data. The correlation has been illustrated in the attached Figure S1.

Discussion

Removal of ROS

In plants, resistance to drought stress is mediated through a complex network of genes, metabolic pathways, and other molecules [21–23]. When plants encounter drought stress, they produce a substantial amount of reactive oxygen species (ROS) and harmful substances like malondialdehyde (MDA), leading to increased membrane lipid peroxidation [24, 25]. The plant's elimination of ROS is primarily accomplished through intricate non-enzymatic and enzymatic antioxidant systems [26–28]. Non-enzymatic antioxidants mainly include reduced glutathione and osmolytes such as proline, arginine, and soluble sugars, while enzymatic antioxidants include superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, and peroxidase [29–31]. Glutathione

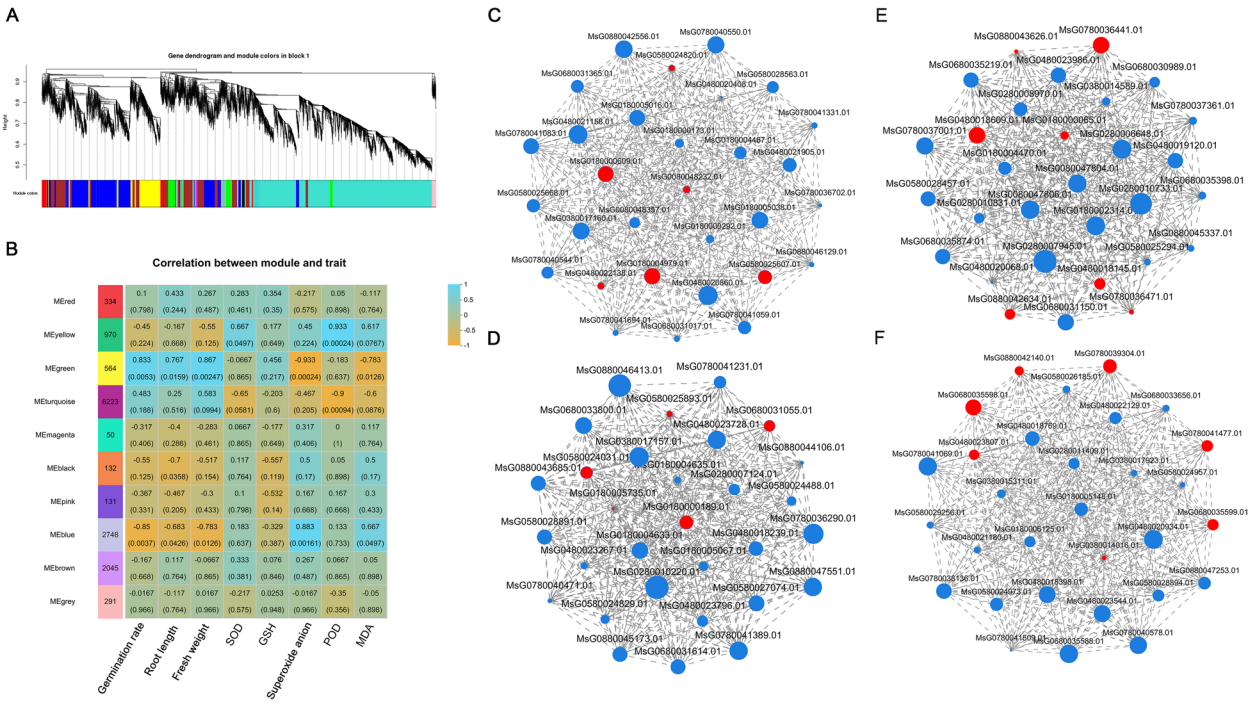

Fig. 5 The WGCNA analysis revealed the correlation between DEGs and physiological traits related to drought stress regulated by melatonin. **A** The heatmap clearly shows the correlation between gene expression modules and physiological traits related to drought stress regulated by melatonin. **B** A module classification tree was constructed through hierarchical clustering analysis of WGCNA, demonstrating the similarity and difference between gene expression modules. **C** In the MEblue module, a detailed co-expression network of DEGs is depicted. **D** In the MEgreen module, a detailed co-expression network of DEGs is depicted. **E** In the MEblack module, a detailed co-expression network of DEGs is depicted. **F** In the Meyellow module, a detailed co-expression network of DEGs is depicted

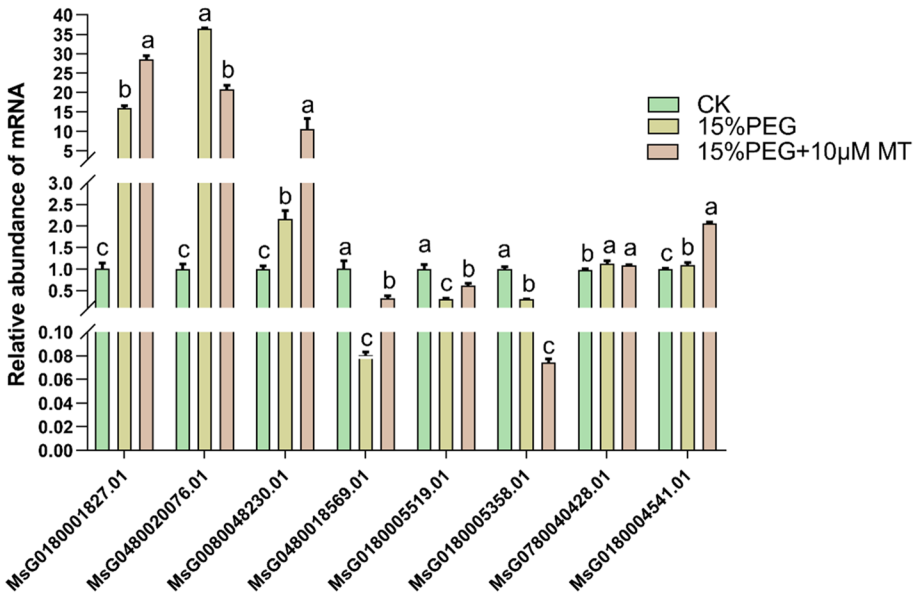

Fig. 6 Transcriptional analysis of 8 differentially expressed genes. To ensure reproducibility and reliability, qRT-PCR analysis was performed on three independent biological replicates. Calculate the relative expression level using the $2^{-\Delta\Delta Ct}$ method. These columns represent the average of three biological replicates. Using Duncan's test, different lowercase letters indicate significant differences between treatments ($p < 0.05$)

S-transferase (*GST*) plays a pivotal role in glutathione metabolism and in maintaining cellular redox homeostasis, contributing significantly to plant resistance against abiotic stress [32]. In our study, 14 differentially expressed genes (DEGs) were identified as enriched in *GST*. Under drought conditions, these genes showed higher expression levels in the melatonin (MT) treatment group compared to the polyethylene glycol (PEG) treatment group. We hypothesize that melatonin promotes *GST* expression, accelerates glutathione (GSH) synthesis, and neutralizes ROS. Research indicates that oxidized glutathione (*GSSG*) can be reverted to GSH through the ascorbic acid-glutathione cycle, maintaining a reduced cellular state and protecting cell membranes from oxidative harm [33, 34]. The upregulation of *DHAR*, a pivotal gene in the ASA-GSH cycle, observed under melatonin treatment aligns with the increased GSH content (Fig. 2E). This suggests that melatonin enhances the regeneration of glutathione, a critical component for neutralizing ROS and preserving cellular redox balance. Such a mechanism plays a significant role in mitigating oxidative damage caused by drought stress.. Additionally, amino acids and soluble sugars act as accumulated osmolytes and free radical scavengers during stress responses [35, 36]. In our study, the expression levels of crucial genes for proline synthesis, such as *ProA* and *ProB*, as well as *argF* for arginine synthesis, were significantly upregulated in the MT group. Conversely, the main gene for proline degradation, *PRODH*, was notably downregulated, indicating that melatonin aids in proline accumulation to counter drought stress. In galactose metabolism, we noticed a downregulation of important genes *GLA* and *INV* during drought stress. However, their expression was significantly upregulated upon melatonin addition, suggesting that exogenous melatonin may mitigate drought stress by regulating osmotic balance. Furthermore, the expression of genes encoding ROS scavenging enzymes like superoxide dismutase, glutathione peroxidase, ascorbate peroxidase, peroxidase, and catalase, was markedly upregulated under drought conditions. This has significant implications for maintaining ROS homeostasis and enhancing plant drought resistance [37, 38]. Flavonoids, widely present as secondary metabolites in plants, play a crucial role in antioxidant capacity by scavenging free radicals and inhibiting oxidase activity. Studies show that flavonoids can influence the activity of SOD and POD [39–41], with upregulation of the anthocyanin reductase (*ANR*) gene in tobacco enhancing catechin and epicatechin accumulation, thereby boosting the plant's antioxidant capacity [42]. Our transcriptome analysis revealed that under melatonin treatment compared to drought treatment, DEGs associated with flavonoid biosynthesis, including *HCT* (responsible for catalyzing the synthesis of lignin precursors) and *ANR* (involved in producing antioxidant catechins), were upregulated in response to melatonin treatment. These metabolites play a pivotal role in scavenging ROS and stabilizing cellular membranes, thereby synergistically enhancing the activities of SOD and POD (Fig. 2 C-D). This combined effect significantly contributes to protecting against oxidative stress caused by drought conditions. This may contribute to increased SOD and POD activity, consistent with our enzyme activity assay results.

Plant hormone regulatory network

Plant hormones are subtle signaling molecules produced within plants, crucial for adapting to adverse environments [43, 44]. Under drought stress, plants synthesize abscisic acid (ABA), which performs multiple functions such as regulating leaf stomatal size, restricting lateral root growth, aiding seed germination, and expediting fruit ripening [45, 46]. In our experiment, we noted a significant upregulation of the key gene *NCED* (*MsG0180001827.01*), which is involved in ABA synthesis when subjected to drought stress. This finding suggests that the ABA biosynthesis pathway is activated, enabling plants to manage drought stress by restricting lateral root elongation and regulating stomatal size. Notably, *NCED* expression diminished following the addition of melatonin. This may occur because melatonin modulates ABA synthesis by inhibiting ABA synthesis gene expression, thus counteracting drought stress [47, 48]. The *SnRK2* kinase can induce the expression of the transcription factor *ABF2* in alfalfa's ABA signaling pathway. Protein phosphatase *PP2C* (*MsG0080048230.01*) inhibits *SnRK2* kinase activity via dephosphorylation, while the receptor protein *PYL* (*MsG0480018569.01*) counters *PP2C* activity through ABA. Research indicates that overexpression of the transcription factor *ABF2*, responsible for synthesizing ABA in alfalfa, can elevate the expression of stress response genes, thereby enhancing the plant's drought resistance [49]. In this study, under PEG stress conditions, *PYL* and *SnRK2* gene expressions were downregulated, whereas *ABF2* and *PP2C* gene expressions increased. This suggests that alfalfa can boost the expression of downstream resistance genes in the root system by enhancing the expression of the transcription factor *ABF2*, thereby improving the plant's response to drought stress.

Gibberellin (GA) is a trace endogenous signaling molecule synthesized by plants, known for promoting plant cell and stem elongation, leaf enlargement, accelerating growth and development, advancing crop maturity, and increasing yield [50, 51]. Under drought stress, the expression of *GA2ox*, a key gene responsible for GA degradation, is upregulated [52]. Concurrently, GA

modulates the interaction between the DELLA protein and the *PIF3* and *PIF4* genes, influencing hypocotyl cell elongation. Excessive GA results in the degradation of DELLA protein and inhibition of *PIF4* activity [53, 54]. In this study, under PEG stress, the mRNA abundance of *GA2ox* increased while *GA20ox* expression decreased under osmotic stress, leading to a reduction in the biosynthesis of biologically active gibberellin and ultimately inhibiting hypocotyl elongation in alfalfa seedlings, which contradicted the observations in Fig. 1C. Furthermore, the expression of *PIF4* was significantly upregulated, indicating a direct interaction between GA and *PIF4*. Upon the addition of melatonin, *GA2ox* expression was notably downregulated, promoting GA accumulation. This explains why the root length in the melatonin-treated group exceeded that observed under drought stress, highlighting its significance in drought response.

Melatonin is a significant class of indole compounds prevalent in plants. It serves multiple physiological roles, such as protecting chlorophyll, removing excess reactive oxygen species, and bolstering stress resistance in plants [55–57]. Numerous studies suggest that the increase in melatonin content in plant tissues following the application of exogenous melatonin may be due to the enhancement of endogenous melatonin synthesis. This involves enzymes like tyrosine decarboxylase (*TDC*) and acetylserotonin O-methyltransferase (*ASMT*), which promote the accumulation of endogenous melatonin [58–60]. In our experiment, transcriptomic analysis revealed that the key genes responsible for synthesizing *ASMT* (*MsG0480020076.01*) were significantly upregulated under drought stress and further intensified with the addition of melatonin. This aligns with findings from studies on other plant species, such as rice, where exogenous melatonin application improved salt tolerance by enhancing the antioxidant defense system [61]. Additionally, in wheat, similar treatments were shown to bolster drought resistance by increasing leaf water content [62]. Research indicates that exogenous melatonin application can mitigate plant damage under adverse conditions and improve their tolerance to various abiotic stresses [63, 64]. Our findings showed that alfalfa seedlings treated with exogenous melatonin exhibited significantly reduced wilting and increased fresh weight under drought conditions compared to those not treated with melatonin. This effect is likely due to melatonin enhancing water absorption and organic matter accumulation [65, 66], which is crucial for alfalfa's ability to withstand drought stress. Previous studies indicate that melatonin plays a role in the regulatory network of plant hormone synthesis signals [67]. Furthermore, it can increase the level of GA in plants and significantly boost the expression of ABF genes during drought stress [68, 69]. Therefore, it can be inferred that GA, ABA, and melatonin are all involved in alfalfa's defense response to drought stress, with melatonin potentially being a key hormone in this regulatory network.

Interactions between flavonoids, plant hormones, and ABC transporters

Plant hormones and growth regulators play a crucial role in modulating several physiological processes, including development, metabolism, and aging, by interacting with nucleic acids, proteins, and enzymes in plants [70]. These elements also influence the synthesis of secondary metabolites such as flavonoids and terpenoids. Research has demonstrated that plant hormones can regulate the synthesis of flavonoid metabolites like quercetin, anthocyanins, and glycyrrhizin, key components in flavonoid biosynthesis [68]. In this study, transcriptomic analysis disclosed that several DEGs, primarily involved in flavonoid biosynthesis (such as *CHS*, *CYP73A*, and *HCT*), were induced under osmotic stress, with the transcriptional level of *HCT* notably enhanced with additional melatonin (MT). Furthermore, ATP-binding cassette (ABC) transporters are known to transport entities including plant hormones, terpenes, and alkaloids [71]. This research, under drought conditions, revealed through transcriptome analysis that the levels of gibberellins (*GAs*, *MsG0180005519.01*), MT, and certain flavonoids increased in alfalfa roots. The enrichment of ABC transporter genes (ABCB and ABCC) suggests that melatonin may contribute to regulating the transport of stress-related metabolites, including flavonoids and abscisic acid (ABA), across cellular membranes. This regulatory mechanism potentially strengthens root-to-shoot signaling and reinforces systemic antioxidant defenses, forming a coordinated strategy for adapting to drought conditions., particularly involving the ABCB and ABCC transporter proteins. Additionally, the genes responsible for anthocyanin synthesis within the flavonoid synthesis pathway showed significant upregulation. The presence of *ABF2*, *PP2C*, and *PYL* genes in the ABA signaling pathway also saw substantial enrichment in DEGs. ABA regulates stress responses by modulating stomatal closure and scavenging ROS. Its interaction with flavonoid biosynthesis genes likely enhances antioxidant defenses under drought stress. We propose that anthocyanin synthesis is closely linked to the *ABF2*, *PP2C*, and *PYL* genes [72]. In this process, ABA is detected by the *PYL* receptor, which inhibits the protein phosphatase PP2C and activates the kinase *SnRK2*. This activation triggers the phosphorylation of the transcription factor *ABF2*, facilitating its translocation to the nucleus, where it binds to ABA-responsive elements in the promoters of target genes—including those involved in flavonoid biosynthesis, such

as *HCT* and *ANR*. Flavonoids, such as epicatechin, are crucial for neutralizing ROS during drought stress, while ABA minimizes water loss by reducing stomatal apertures. The combined action of flavonoid-mediated ROS scavenging and ABA-driven water conservation significantly boosts drought tolerance in alfalfa. Moreover, these findings indicate that ABC transporters may contribute to the movement of plant hormones, further regulating flavonoid synthesis and underpinning a complex, interconnected stress response system (Fig. 7).

Conclusions

Our findings demonstrate that melatonin (MT) significantly enhances the drought tolerance of alfalfa by harmonizing physiological responses and transcriptomic adjustments. From a physiological standpoint, the treatment group exposed to exogenous MT exhibited superior performance in root length and fresh weight compared to the control group, effectively mitigating the adverse effects of drought stress on alfalfa. This improvement is largely attributable to MT's ability to boost the activity of antioxidant enzymes, such as superoxide dismutase (SOD), thereby minimizing oxidative damage. At the molecular level, transcriptome sequencing combined with Weighted Gene Co-expression Network Analysis (WGCNA) revealed that MT regulates the expression of genes involved in flavonoid biosynthesis. Specifically, MT upregulated key genes, including *PAL*, *CHS*, and *FLS*, which facilitated the accumulation of flavonoids that play a critical role in scavenging reactive oxygen species (ROS). In addition, MT plays a pivotal role in the abscisic acid (ABA) and gibberellin (GA) hormone signaling pathways. MT enhances the expression of ABA-responsive genes, such as *ABF2* and *PYL4*, while suppressing the activity of GA synthesis genes, like *GA20ox*. This dual regulation achieves an optimal balance between stomatal closure and growth recovery, ultimately contributing to alfalfa's improved drought tolerance. In conclusion, our study revealed a melatonin mediated regulatory network in alfalfa, which involves flavonoid biosynthesis, ABA/GA signaling pathway and ABC transporters. These findings provide key insights into the molecular mechanism of melatonin in alleviating drought stress in alfalfa, and provide operational target genes such as *ABF2* and *ASMT* for improving crop drought resistance.

Materials and methods

Experimental materials and pretreatment

The *Medicago sativa* L. alfalfa cultivar was utilized in this study, provided by Professor Qingchuan Yang from the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences. Alfalfa seeds were soaked in 75% ethanol for 10 min and then rinsed four times with double-distilled water. We germinated Thirty seeds in a Petri dish (with a diameter of 9 cm) containing 4 mL of melatonin-polyethylene glycol (PEG) solution at concentrations of 0, 10, 50, 100, 200, and 300 μM, based on previous research [73, 74]. The seeds were cultivated in a growth chamber at 25 °C with a relative humidity of 70% under a 16-h light and 8-h darkness cycle. The

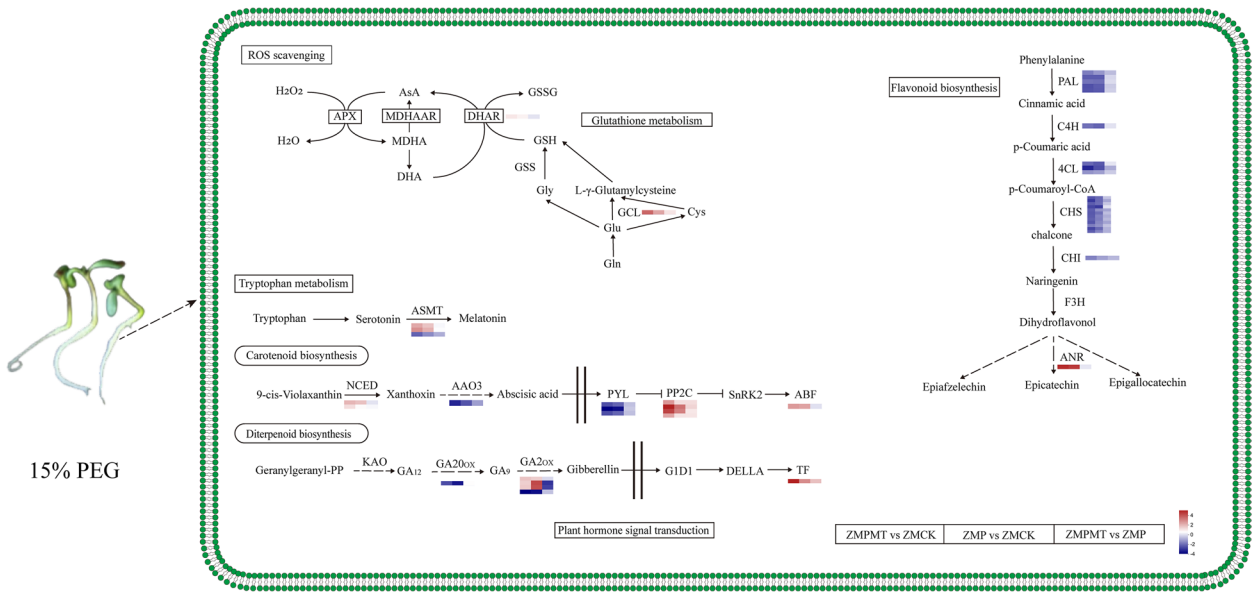

Fig. 7 A pathway map depicting key genes and metabolites associated with various treatment groups was generated using KEGG enrichment pathway analysis

germination rate, fresh weight, and root length were measured for six biological replicates after seven days. For subsequent physiological and transcriptomic analyses, seedlings treated with 0 (designated as CK), 15% PEG (designated as ZMP), or 15% PEG +10 μM melatonin (designated as ZMPMT) were collected, immediately frozen in liquid nitrogen, and stored at −80 °C.

Measurement of physiological indicators

The levels of GSH and O_2^- were determined using Solarbio kits (BC1175 and BC1295) from Beijing, China. SOD activity was assessed with the Abbkine kit (KTB1030) from Wuhan, China, whereas POD activity and MDA content were evaluated using Abbkine kits (KTB1150 and KTB1050), respectively. Each physiological index measurement was conducted in triplicate to ensure accuracy and reliability.

Transcriptomic analysis

Total RNA was extracted using the MJZol total RNA extraction kit (Shanghai Meiji Biomedicine Technology Co., Ltd., Shanghai, China). Complementary DNA (cDNA) was synthesized from the short RNA fragments, utilizing random hexamer primers and reverse transcriptase. The library fragments were subsequently purified with the Biowest agarose kit (Biowest, Logroño, Spain) and the RNA purification kit (Shanghai Meizhao Biomedicine Technology Co., Ltd.). Bridge-PCR clusters were generated on cBot. High-throughput sequencing was conducted on the Illumina NovaSeq 6000 platform (Illumina, USA). Upon completion of sequencing, the FASTp tool filtered the sequence quality. This involved removing the sequencing link, low-quality reads (trimming bases at the sequence's 3'end with quality scores below 20). If remaining sequences contained bases with a quality score below 10, they were discarded entirely. Reads with a high N-content ratio (exceeding 10%, where N represents uncertain bases) and sequences shorter than 20 base pairs, post adapter removal and quality pruning, were also eliminated. Mapping these data (reads) to a reference genome (Alfalfa Zhongmu No.1) enabled subsequent transcript assembly and expression calculations [75]. Differential gene expression was functionally annotated and classified using the Blast2GO program (https://www.blast2go.com/) [76, 77]. The KOBAS 3.0 online tool (http://kobas.cbi.pku.edu.cn/) identified drought stress response genes, assessing their enrichment in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (http://www.genome.jp/kegg/.) [78–80]. The raw sequencing reads underwent quality assessment using FastQC, followed by genome alignment carried out with HISAT2 (v2.2.1). Gene concentration was enriched and analyzed through software, with Fisher's exact test ensuring accuracy. The Benjamini–Hochberg method adjusted the *p*-values, and functions were deemed significantly enriched at an adjusted *p*-value (*P*-adjust) of less than 0.05. Differential expression analysis was conducted using DESeq2 R4.1.2(v1.30.1), a normalization approach grounded in the negative binomial distribution. The significance criteria were defined as |log2 FC|> 1 and FDR < 0.05.

Weighted gene co-correlation network analysis and protein interaction network analysis

The co-expression network was constructed using Weighted Gene Co-expression Network Analysis (WGCNA), identifying modules of closely related genes. Among the frequently used expression gene modules, those associated with phenotypic information are highlighted to explore gene networks and the correlation between phenotype and core network genes. The relevant functions of WGCNA are provided by the WGCNA package (https://cran.r-project.org/web/packages/WGCNA/index.html).

Protein interaction network analysis utilized the STRING (v11.5; https://string-db.org) database to construct a network of differential genes, illustrating the relationships among these differential genes [81].

qRT-PCR analysis

Eight differentially expressed genes were chosen for qRT-PCR analysis. Initially, full-length complementary DNA (cDNA) was synthesized using the PrimeScript RT kit (Cat# RR047A, TaKaRa, Tokyo, Japan), incorporating gDNA Eraser. The one-step qRT-PCR kit (Cat# RR420A; TaKaRa) was utilized following the manufacturer's guidelines. To ensure reliability, three independent RNA preparations served as biological replicates. The housekeeping gene *MsACTIN2* was employed as an internal control to normalize gene expression. The primers were designed using Primer-BLAST (NCBI) and Oligo 7 software. The design parameters included a primer length of 18–24 bp, GC content of 40–60%, an annealing temperature (Tm) of 58–62 °C, and a product length of 80–200 bp. Primer specificity was thoroughly validated through melting curve analysis and sequencing. The detailed primer sequences are presented in Table 1.

Data processing

The data were organized and computed using Microsoft Excel 2021, while statistical analyses were conducted with SPSS 26.0. Data visualization was performed with GraphPad Prism 8.

Table 1 Primers designed for qRT-PCR analysis

Gene	Forward primers (5′−3′)	Reverse primers (5′−3′)
MsG0180001827.01	AACCTGGATTTCGCGGTGAT	TACACCCAACTTCCTCCCCT
MsG0180005519.01	TAGGGCAGTGGTAAACAGCG	CAGGGTAGATCCTTGGGTTGT
MsG0180005358.01	GAAAGCCACCAGAGAAGTGC	CCCACTCAAGTCCTTCTCCC
MsG0780040428.01	CCGGTAAAATGGCCGAAAGC	GAGCGGGGTTAGAGTGGATG
MsG0780036812.01	TGCCATCATCGGTAACAACCA	TGGGACGTGCGAAAAGAGTT
MsG0480020076.01	CAAGGTATGGTTTGCACCGC	GAGATCCTCCGATACCCCCA
MsG0480018569.01	CTGTGACCAGCACCATTCCT	CTTGTGGTTTGTCGAAGCGG
MsG0080048230.01	CGGTGTCAACCATAGGCTGT	CTCTCTCTACACGACACCGC
Ms-ACTIN2	CAAAAGATGGCAGATGCTGAGGAT	CATGACACCAGTATGACGAGGTCG

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06665-w.

Supplementary Material 1

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Authors' contributions
XLR, XYZ, YHS, CZW, DFL, HS: Conceptualization and methodology; XXZ, WXZ, ZRL, GML, YAL: Resources; XXZ, WXZ, ZRL, GML, JZW, YAL: Investigation; XXZ, WXZ, ZRL: Data curation, Formal analysis, and writing – original draft; HS: Supervision and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability
The data in this study can be requested from the corresponding author. The raw RNA-seq data has been deposited in the NCBI SRA database under the accession number PRJNA1113790.

Declarations

Consent for publication
We declare that all experimental consumables and plants are collected with permission from the local agricultural department, and all plant experimental research complies with the requirements of relevant institutions, national and international standards, and legislation.

Competing interests
The authors declare no competing interests.

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