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Transcriptomic Analysis of Melatonin-Mediated Salt Stress Response in Germinating Alfalfa

Zirui Liu [1], Xiangling Ren [2], Wenxuan Zhu [1], Yingao Li [1], Guomin Li [1], Caifeng Liu [1], Defeng Li [1,3,4], Yinghua Shi [1,3,4], Chengzhang Wang [1,3,4], Xiaoyan Zhu [1,3,4] and Hao Sun [1,3,4,*]

[1] Key Laboratory of Forage Processing, College of Animal Science and Technology, Henan Agricultural University, Zhengzhou 450046, China; 19545655309@163.com (Z.L.); 19839109979@163.com (W.Z.); 18338329806@163.com (Y.L.); 13353928533@163.com (G.L.); 18595328067@163.com (C.L.); leadephone@126.com (D.L.); annysyh@126.com (Y.S.); wcz7520@aliyun.com (C.W.); zxy_0512@163.com (X.Z.)
[2] School of Environmental Engineering, Yellow River Conservancy Technical Institute, Zhengzhou 450046, China; renxiangling@yrcti.edu.cn
[3] Henan Key Laboratory of Grassland Resources Innovation and Utilization, Zhengzhou 450046, China
[4] Herbage Engineering Research Center of Henan Province, Zhengzhou 450046, China
* Correspondence: sunhao@henau.edu.cn

Abstract: Salt stress poses a significant threat to crop yields worldwide. Melatonin (MT), an endogenous hormone synthesized in plants, has emerged as a crucial player in plant responses to various abiotic stresses, including drought, salinity, heat, and cold. However, the precise molecular mechanisms underlying MT-mediated abiotic stress responses remain incompletely understood. To elucidate the key genes and pathways involved in MT-mediated alleviation of salt stress, we conducted physiological, biochemical, and transcriptomic analyses on alfalfa seedlings. Our results demonstrated that alfalfa seedlings treated with melatonin exhibited higher germination rates, longer bud lengths, and greater fresh weights compared to those subjected to salt stress alone. Furthermore, the levels of malondialdehyde (MDA) and superoxide anion (O^{2-}) were reduced, while the activities and contents of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and glutathione (GSH) increased in response to melatonin treatment. Transcriptome analysis revealed 2181 differentially expressed genes (DEGs) in the salt-treated group, with 780 upregulated and 1401 downregulated genes. In contrast, the MT-treated group exhibited 4422 DEGs, including 1438 upregulated and 2984 downregulated genes. Functional annotation and pathway enrichment analysis indicated that DEGs were primarily involved in the biosynthesis of flavonoids, isoflavones, plant hormones, glutathione (GSH), soluble sugars, and other substances, as well as in ABC transporter and MAPK signaling pathways. Notably, the MT-treated group showed greater enrichment of DEGs in these pathways, suggesting that MT mitigates salt stress by modulating the expression of genes related to phytohormones and antioxidant capacity. Overall, our findings provide valuable insights into the molecular mechanisms underlying MT-mediated salt tolerance in alfalfa, with important implications for breeding salt-tolerant alfalfa and other crops.

Keywords: alfalfa; salt stress; melatonin; transcriptomics; differentially expressed genes

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1. Introduction

Soil salinization is a significant factor that impacts crop yield worldwide. Besides natural land salinization, irrigation methods and climate change can also contribute to land salinization [1]. Currently, more than 20% of cultivated land and 33% of irrigated farmland are affected by land salinization [2]. The accumulation of sodium and chloride ions in the soil reduces water availability and disrupts the ion balance in plants. This ultimately leads to osmotic stress, ionic toxicity, inhibition of photosynthesis, and alterations in metabolic processes [3–5]. Alfalfa (*Medicago sativa* L.), commonly known as the king of forage, is a perennial leguminous forage crop with strong stress resistance, high quality,

and economic value. It is widely cultivated worldwide [6]. Besides providing suitable feed quality and high nutritional value, alfalfa also improves soil structure and enhances soil fertility [7]. Although alfalfa can tolerate moderate soil salinity, it is highly sensitive to high salt concentrations. In severe cases, salt stress can cause a decline in the growth of alfalfa roots, stems, leaves, and nutritional value, possibly resulting in plant death [8,9]. Exposure to 200 mM NaCl stress, in particular, causes significant growth damage in alfalfa [9,10]. Given the limited availability of land resources and the escalating problem of soil salinization, it is crucial to develop strategies that mitigate the negative effects of salt stress on alfalfa. This can be achieved by adjusting the biological characteristics and growth patterns of alfalfa [11]. In this context, studying the molecular regulation mechanism of salt tolerance in alfalfa can accelerate the development of new salt-tolerant varieties, effectively addressing the limitations imposed by soil salinization on alfalfa cultivation areas. Consequently, this will enhance the scale and level of the forage industry and play a vital role in promoting the development of circular ecological agriculture centered around forage and animal husbandry [12–14]. Existing research has elucidated the mechanisms underlying salt–alkali tolerance in alfalfa, focusing on areas such as seed germination, plant growth and development, physiological and biochemical reactions, and molecular biology [15]. Osmotic regulation, ion balance and repair, reactive oxygen species (ROS) production, and their effects on downstream molecular targets are among the key processes investigated in the literature [2,16,17]. Moreover, it has been observed that salt stress triggers changes in gene expression, affects mRNA stability, regulates the translation process, and ultimately alters protein abundance [18].

The changes in plant hormone levels play an important role in combating salt stress [19]. Plant hormones such as gibberellin, abscisic acid, ethylene, auxin, and cytokinin play indispensable roles in different stages of plant growth, organ development, internal homeostasis, and plant response to external environmental changes [20,21]. Melatonin (MT) is a common indole heterocyclic compound found in plants and animals [22]. Previous studies have demonstrated that melatonin plays an important role in regulating plant growth and responding to biotic and abiotic stresses and is involved in regulating many physiological processes, including seed germination, flowering and fruiting, and mineral element absorption [23]. Furthermore, exogenous application of melatonin can enhance plant stress resistance and help plants maintain health under abiotic stress conditions. In particular, as an antioxidant, melatonin can activate the expression of antioxidant oxidase genes and improve enzyme activity, thereby improving the plant's tolerance to stress [24–26]. Due to the limited exploration of transcriptomics in previous studies, the role of MT in plant response to salt stress is not clear and even controversial. This study hypothesizes that the salt stress response of alfalfa may be regulated by MT. Therefore, we compared the growth performance and physiological indicators of alfalfa treated with and without MT under salt stress induced by NaCl and conducted full transcriptome sequencing analysis to identify DEGs under the influence of MT and NaCl. Furthermore, the weighted gene co-expression network analysis (WGCNA) was performed to detect key signaling pathways and hub genes regulated by MT, which might reveal the molecular mechanism of MT-mediated salt tolerance in alfalfa and provide new strategies for improving its salt tolerance.

2. Materials and Methods

2.1. Experimental Materials and Design

The Zhongmu No. 3 alfalfa cultivar used in this study was obtained from the Chinese Academy of Agricultural Sciences. The seeds were sterilized by immersing them in 75% ethanol for 10 min, followed by three rinses with deionized water. The sterilized seeds were then germinated in a Petri dish (ΦA = 90 mm) containing 4 mL of either deionized water, 10 μM MT, 50 μM MT, 100 μM MT, 200 μM MT, or 300 μM MT with 200 mM NaCl. Each Petri dish contained 30 seeds, with 6 replicates per treatment group. The dishes were placed in a constant temperature incubator set at 25 °C, with a 16 h light and 8 h dark cycle. After 7 days, the germination rate, fresh weight, and root length of the seedlings

were measured. The germinated seedlings were then frozen in liquid nitrogen and stored at −80 °C. For subsequent physiological and transcriptomic analysis, the seedlings treated with 0 (also known as CK), 200 mM NaCl (also known as ZMN), or 200 mM NaCl + 10 μM MT (referred to as ZMNMT) were selected.

2.2. Measurement of the Germination Rate, Root Length, and Fresh Weight

The germination rate was assessed daily for seven consecutive days. The vertical distance from the cotyledon node to the top root was measured using a calibration ruler, and the data were recorded as root length for 10 seedlings per biological replicate. Additionally, fresh weights were determined for the same 10 seedlings per biological replicate. All collected data underwent statistical analysis using SPSS 26.0 software, and GraphPad Prism 8 software was utilized for data visualization.

2.3. Measurement of Physiological and Biochemical Indicators

The content of GSH and O^{2-} were measured using the Solarbio kits (BC1175 and BC1295, Beijing, China). SOD activity was determined using the Abbkine kit (KTB1030, Wuhan, China), while POD activity and MDA content were measured using the Abbkine kits (KTB1150 and KTB1050, Wuhan, China), respectively. Three technical replicates were performed for each physiological indicator.

2.4. Transcriptomic Analysis

Thirty alfalfa seedling samples under the same growth conditions were mixed and treated, and total RNA was isolated using the MJZol total RNA extraction kit (Shanghai Majorbio Biomedicine Technology Co., Ltd., Shanghai, China), then cDNA was synthesized using the short fragment as a template using random hexamer primers and reverse transcriptase. Then, the Biowest Agarose Kit (Biowest, Logronio, Spain) and RNA Purification Kit (Shanghai Majorbio Biomedicine Technology Co., Ltd.) were used to purify the library fragments, and bridge PCR was performed on the cBot to generate clusters. Finally, high-throughput sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, Foster City, CA, USA). After the sequencing was completed, FASTp was used to filter the quality of the original readings, removing sequencing connector sequences, low-quality reads (trim the low-quality (mass value less than 20) base groups at the end (3′ end) of the sequence. If there are still sequences with mass values less than 10 in the remaining sequences, remove the entire sequence. Otherwise, keep it), sequences with high N (remove reads with N content ratio exceeding 10%; N indicates uncertain base information) rates, and sequences with concise length (discard sequences with length less than 20 bp after removing the adapter and quality trimming). Then, mapped data (reads) for subsequent transcript assembly and expression calculation were obtained by comparing them with reference genomes [27].

2.5. Annotation Analysis of Differentially Expressed Genes GO and KEGG

The Blast2GO program [28,29] was utilized for annotating and classifying the differentially expressed genes according to their functions. The KOBAS 3.0 online program was employed to identify Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were significantly enriched among the genes responsive to salt stress [30–32]. Gene enrichment and analysis were conducted using appropriate software, with Fisher's test utilized for accuracy. The *p*-values were adjusted using the BH method, and functions with a corrected *p*-value (*p*-adjust) < 0.05 were considered significantly enriched. Differential gene expression analysis was carried out using DESeq2 R4.1.2, with the default criteria for identifying significantly differentially expressed genes set as FDR < 0.05 and $|\log2FC| \geq 1$.

2.6. 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), with the identification of modules containing closely related

genes. Once the commonly expressed gene module was obtained, it was associated with phenotypic information of interest to explore the correlation between the gene network and phenotype, as well as the identification of core genes in the network. Protein interaction network analysis utilized the interaction relationships within the STRING protein interaction database to construct the protein interaction network of differentially expressed genes, thereby highlighting the relationships between these genes.

2.7. RT-qPCR Analysis

Ten differentially expressed genes were chosen for analysis using the reverse transcription quantitative polymerase chain reaction (RT-qPCR). First, full-length complementary DNA (cDNA) was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (Cat# RR047A, TaKaRa, Tokyo, Japan). The RT-qPCR was conducted using the one-step RT-qPCR kit (Cat# RR420A; TaKaRa) following the manufacturer's instructions. Three independent RNA preparations were utilized as biological replicates. The housekeeping gene MsACTIN2 was selected as an internal control to normalize gene expression (Table S1).

3. Results

3.1. Effects of Melatonin on Alfalfa Plant Growth under NaCl Stress

In order to investigate the role of melatonin (MT) in alleviating salt stress in alfalfa, we treated alfalfa seeds with different concentrations of melatonin under salt stress and further investigated the effects of different concentrations of melatonin on seed germination and primary root length (Figure 1A). Compared with the control group, the germination rate of alfalfa was significantly inhibited under 200 Mm NaCl treatment. However, 10 μM exogenous melatonin could alleviate the adverse effects of salt stress on seed germination, and the germination rate increased from 27.78% to 52.22%. Interestingly, exogenous melatonin treatment can accelerate the germination of alfalfa seeds under salt stress. Especially at 10 μM MT concentration, the germination rate of alfalfa seeds reached 34.44% in three days. In addition, the alfalfa germination rate was improved to varying degrees with different concentrations of melatonin treatment (Figure 1B). Notably, along with the concentration of MT increases, the primary root length shows a decreasing trend (Figure 1C). Similarly, although a lower concentration of MT has a promoting effect, the fresh weight of alfalfa seedlings was suppressed at a higher concentration of MT (>100 μM) (Figure 1D). Furthermore, 10 μM MT could alleviate the adverse effects of salt stress on alfalfa seed germination. Based on the phenotype data of alfalfa seedlings between different treatments, we detected that 10 μM exogenous MT could alleviate salt stress in alfalfa. Therefore, 10 μM MT has been determined as the optimal concentration for improving salt tolerance in alfalfa, which was used for subsequent physiological and biochemical indicators detection and transcriptome analysis.

3.2. Changes in Oxidation System Indexes

In order to further elucidate the mechanism by which melatonin enhances salt tolerance in alfalfa, we conducted measurements to assess changes in oxidation systems under various treatments. Comparative analysis of the CK group revealed that NaCl stress led to a significant increase in the levels of O^{2-}, MDA, and GSH, as well as the activities of SOD and POD. Conversely, when melatonin and NaCl were combined for treatment, there was a notable decrease of 28.7% and 21.1% in the accumulation of O^{2-} and MDA, respectively (Figure 2A,B). Additionally, the activity of SOD and POD, as well as the content of GSH, exhibited significant increases of 2.5%, 43.1%, and 54.7%, respectively (Figure 2C–E).

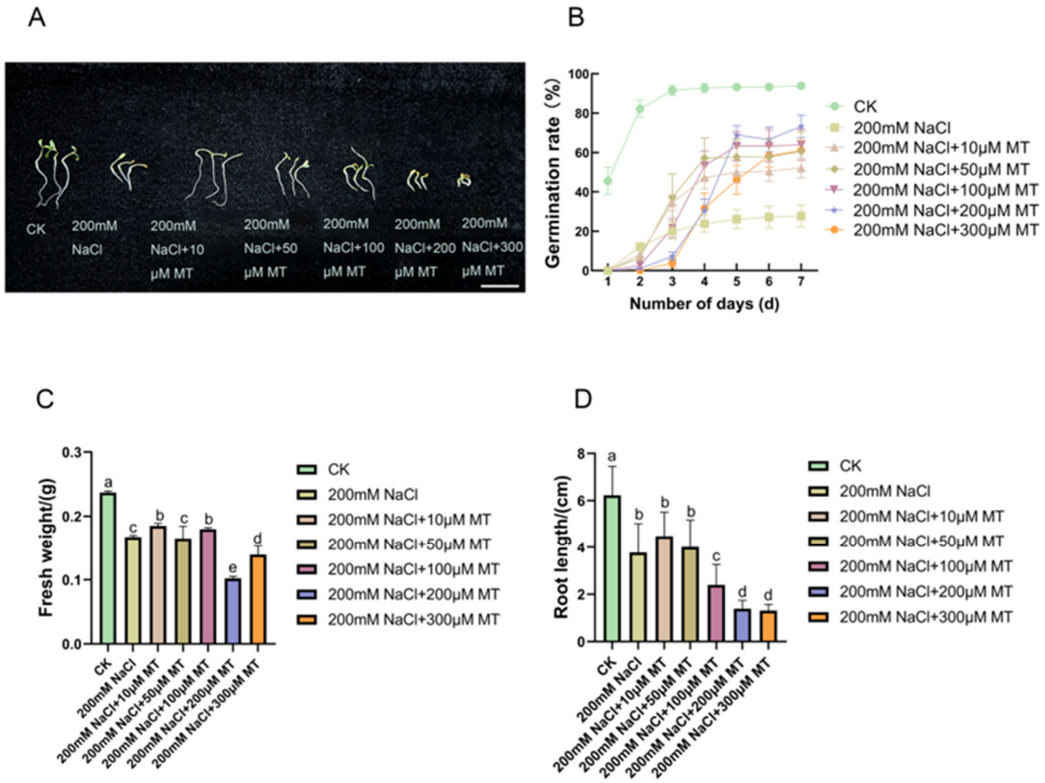

Figure 1. Melatonin alleviates the inhibitory effect of salt stress on seed germination, root length, and fresh weight. (**A**) Alfalfa seedling phenotypes. Bar = 2 cm. (**B**) Seed germination rate after 7-day treatment. Line chart represents the average of six biological replicates with standard deviation, and each replicate has 30 seeds. (**C**) Root length; columns with different lowercase letters indicate significant differences among treatments at $p < 0.05$ using Duncan's test. Data represent the average of three biological replicates with standard deviation, and each replicate has 10 plants. (**D**) Fresh weight of alfalfa seedlings; the column represents the mean of the three biological replicates. Different lowercase letters indicate significant differences between treatments by Duncan's test. CK indicates control.

3.3. Identification of Salt-Responsive Genes in Alfalfa Seedlings

In addition, transcriptomic analysis was performed on alfalfa seedlings treated with deionized water, 200 mM NaCl, and 200 mM NaCl mixed with 10 μM MT, respectively. A total of 4904 genes were identified by comparison between the two different treatments, of which 1768 genes were shared by the three treatments, accounting for 36.05% of the total genes, and 2654 genes were shared by ZMNMT vs. ZMCK and ZMN vs. ZMNMT, accounting for 54.12% of the total genes. A total of 34 genes were shared between ZMN and ZMNMT and between ZMN and ZMCK, accounting for 0.69% of the total genes; 69 genes were shared between ZMNMT and ZMCK and between ZMN and ZMNMT, accounting for 1.41% of the total genes; 379 genes were unique to ZMCK vs. ZMN, accounting for 7.73% of the total genes (Figure 3A; Table S2). Comparisons of ZMN vs. ZMCK, ZMNMT vs. ZMCK, and ZMNMT vs. ZMN consist of 780, 1438, and 79 upregulated genes and 1401, 2984, and 328 downregulated genes, respectively (Figure 3B; Table S2). Moreover, ten DEGs were detected based on the analysis of variance between different groups, which are probably related to melatonin-mediated salt tolerance in alfalfa. Meanwhile, a heat map was drawn by clustering all DEGs to show the different regulatory modes for each treatment (Figure 3D; Table S2).

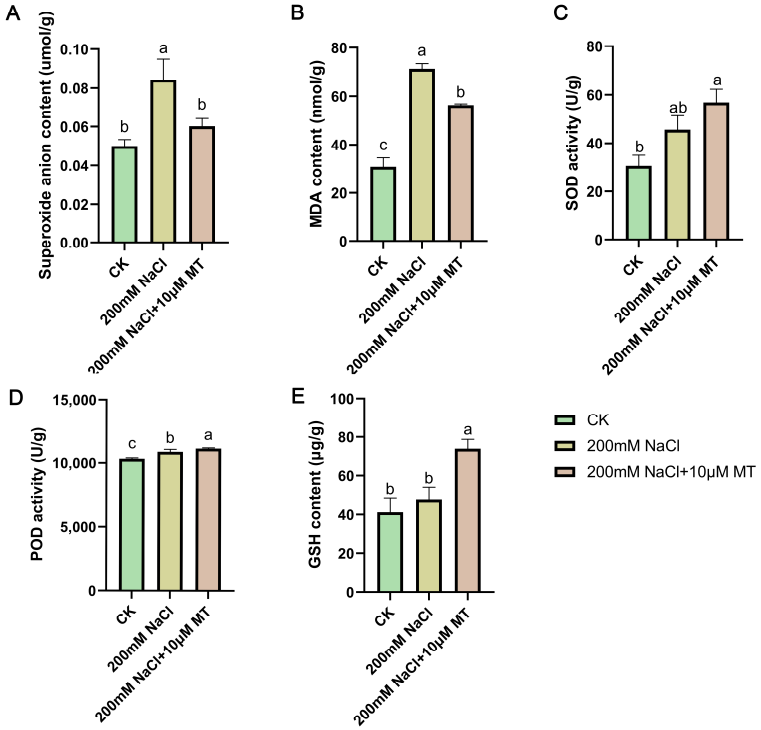

Figure 2. Exogenous melatonin can alleviate the oxidative stress caused by NaCl treatment. O^{2-} content (**A**), MDA content (**B**), SOD activity (**C**), POD activity (**D**), and GSH content (**E**). The columns represent the average of three biological replicates, each with ten individual plants. Using the Duncan test, different lowercase letters indicated significant differences between treatments.

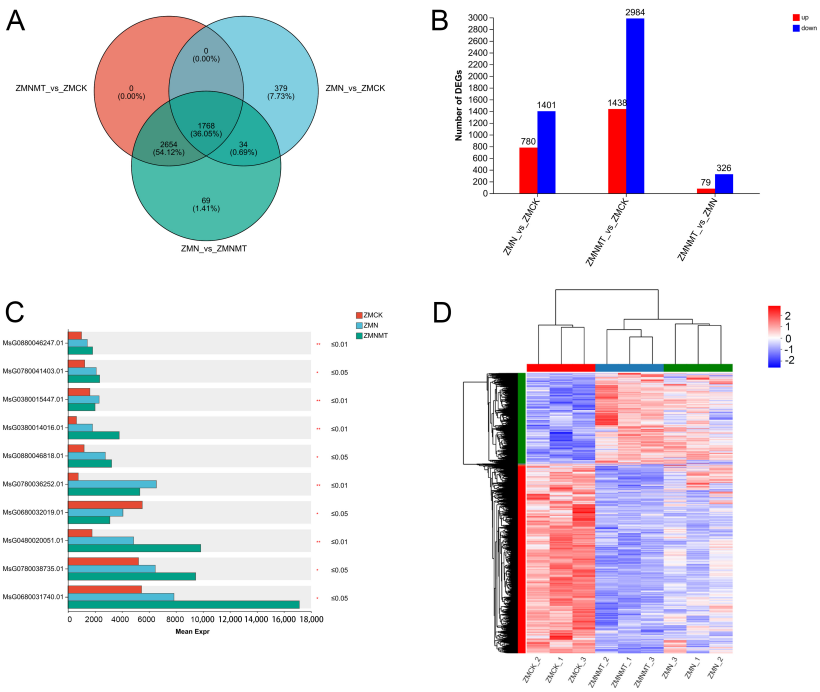

Figure 3. Comprehensive visualization of control DEGs CK, NaCl, and MT combined with NaCl (MT + NaCl). (**A**) The Venn diagram shows the unique and shared DEGs. (**B**) The number of differentially expressed genes and the number of upregulated and downregulated genes. (**C**) Analysis of variance for multiple groups. * $p < 0.05$, ** $p < 0.01$. (**D**) Hierarchical clustering analysis based on the $\log_2$ RPFK expression trends of DEGs under salt alone or with melatonin treatment.

3.4. Functional Annotation of Differentially Expressed Genes

Functional annotation of DEGs was carried out using the GO database. In the molecular function classification of GO, DEGs were assigned four high-order terms: molecular function regulator, transcriptional regulator activity, transport activity, and binding activity. The DEGs were divided into seven components: extracellular region, protein complex, organelle part, membrane, organelle part, membrane part, and cell part. Biological processes include reproductive, multi-organism, cellular component organization or biogenesis, localization, stimulus response, biological regulation, cellular processes, and metabolic processes. In most GO terms, the number of DEGs of ZMN vs. ZMNMT is higher than that of ZMN vs. ZMCK and ZMNMT vs. ZMCK (Figure 4; Table S3). KEGG pathway analysis of DEGs showed that 1234 DEGs were enriched in 124 pathways between ZMN and ZMNMT (Figure S1A; Table S4), ZMN vs. ZMCK had 591 DEGs enriched in 113 pathways (Figure S1B; Table S5), and ZMNMT vs. ZMCK had 1194 DEGs enriched in 124 pathways (Figure S1C; Table S6). In the salt treatment group, there were 10 extremely significantly enriched KEGG pathways, while there were 21 extremely significantly enriched KEGG pathways in the melatonin treatment group. It is worth noting that 10 extremely significantly enriched KEGG pathways in the salt treatment group were all reflected in the melatonin treatment group, including the synthesis pathways of flavonoids, isoflavones, and various secondary metabolites. The melatonin treatment group activated ABC transporters, plant signaling, MAPK pathway, as well as glutathione and galactose metabolism, which may be related to salt stress response.

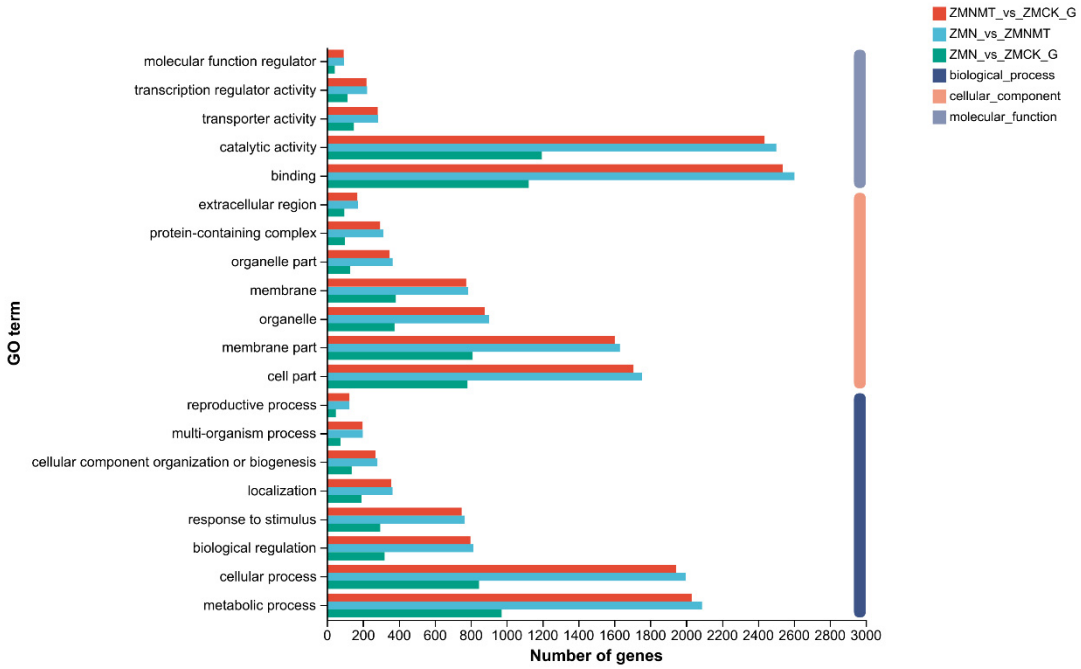

Figure 4. Functional annotation and KEGG enrichment analysis of differentially expressed genes. According to the GO classification of DEGs, DEGs are divided into metabolic classes according to biological processes, cell components, and molecular functions, and the three colors from top to bottom represent ZMNMT vs. CK, ZMN vs. CK, and ZMNMT vs. ZMN, respectively.

3.5. Gene Co-Expression Network

Multiple genes control the response of alfalfa under stress. This study revealed over 10,000 differentially expressed genes (DEGs) through transcriptomic analysis of Zhongmu No. 3 seedlings under different treatments. By WGCNA, we investigated the correlation between DEGs and physiological characteristics related to salt stress. The analysis identified 12 gene co-expression modules. Module–trait association analysis showed that phenotypic characteristics such as germination rate, root length, and fresh weight were significantly positively correlated with gene expression levels in turquoise modules, with correlation

coefficients ranging from 0.85 to 0.886. Meanwhile, germination rate, root length, and fresh weight were significantly negatively correlated with gene expression levels in blue modules, with correlation coefficients ranging from 0.783 to 0.836. This finding suggests that genes in the turquoise module may play a role in MT-mediated salt resistance. In addition, genes in the blue module were significantly positively correlated with MDA content and O^{2-} levels, indicating that genes identified in the blue module may be involved in the regulation of reactive oxygen species scavenging and oxygen free radical homeostasis. In addition, indicators of antioxidant capacity in the black modules, including SOD and POD activity, were also positively correlated with gene expression levels (Figure 5A; Table S7). The turquoise module contains 6847 DEGs, the blue module contains 3505 DEGs, and the black module has 290 DEGs. The top 30 genes based on connectivity were identified as key hub genes in these three modules, representing the main function of the corresponding module (Figure 5B; Table S7). Using CytoScape 3.9.1 software, we visualized these top 30 key hub genes and found that these key genes play important roles in flavonoid, plant hormone, soluble sugar biosynthesis, GSH metabolism, and ABA transporters (Figure 5C,D; Tables S8 and S9). Subsequently, we created clustering heat maps based on the differential expression of genes, including the synthesis and signal transduction pathways of abscisic acid (Figure 6A), gibberellin synthesis pathway (Figure 6B), flavonoid synthesis pathway (Figure 6C), glutathione synthesis pathway (Figure 6D), and MAPK signaling pathway (Figure 6E).

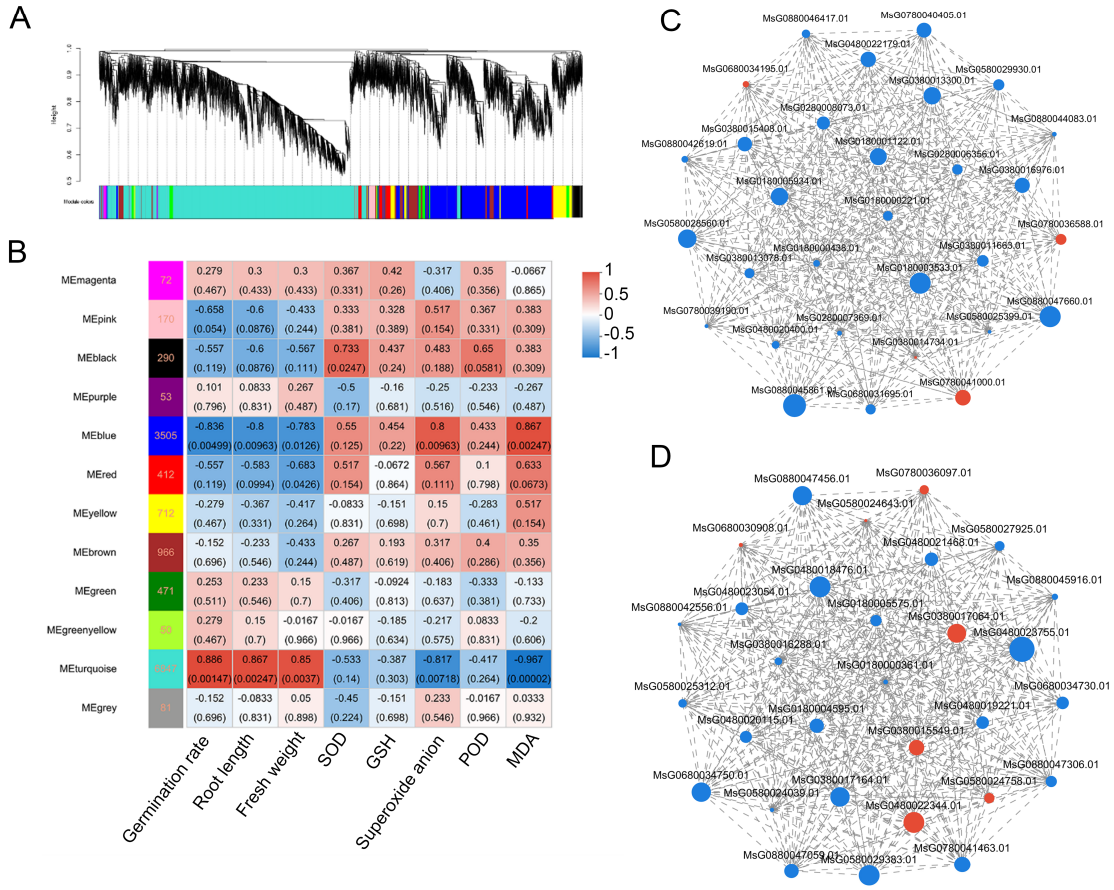

Figure 5. Weighted gene co-expression network analysis (WGCNA) revealed the correlation between salt-stress-related physiological indices regulated by melatonin and differentially expressed genes. (**A**) The heat map shows the correlation between the modules and the physiological parameters. The branches represent a gene, and one color represents a module. (**B**) Module classification tree using WGCNA hierarchical clustering. (**C**) Co-expression network of DEGs in MEturquoise. (**D**) Co-expression network of DEGs in MEblue.

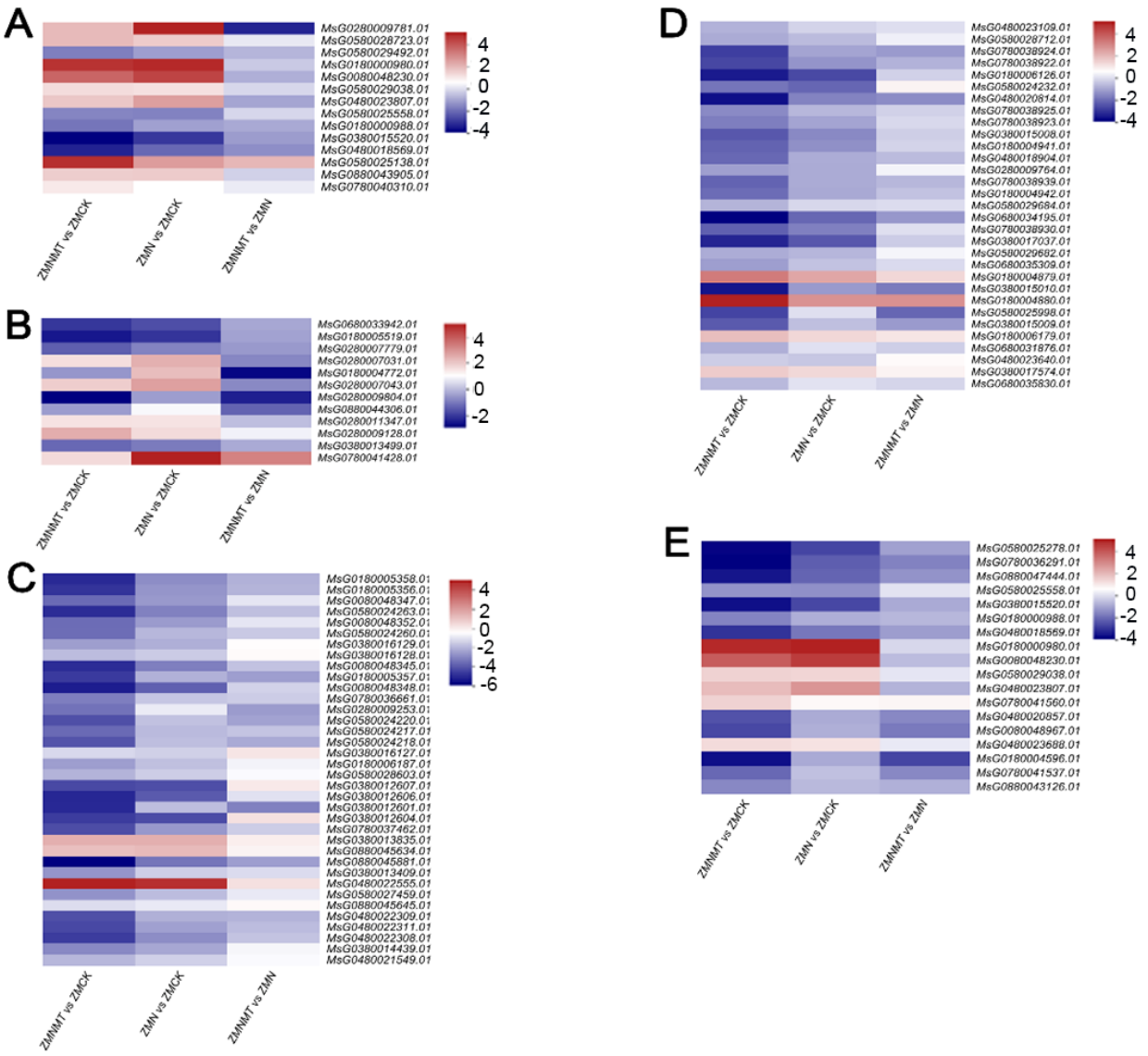

Figure 6. According to KEGG and the related literature, the $\log_2$FC of essential genes for the synthesis of important metabolites under three different treatments was plotted for cluster analysis. Italics represent gene IDs of labeled enzymes or transcription factors, including essential genes involved in abscisic acid synthesis and signal transduction (**A**), gibberellin (**B**), flavonoid biosynthesis (**C**), GSH biosynthesis (**D**), and MAPK signaling pathway (**E**).

3.6. RT-qPCR Verified the Results of RNA-seq

Genes involved in ABA and GA synthesis pathways (*MsG0180001827.01*, Figure 7A and *MsG0180005519.01*, Figure 7B), flavonoid synthesis pathway (*MsG0180005358.01*, Figure 7C), key genes involved in proline synthesis and degradation (*MsG0780040428.01*, Figure 7D and *MsG0780036812.01*, Figure 7E), and key genes involved in melatonin synthesis (*MsG04800020076.01*, Figure 7F). The relative expression of 10 genes was validated through RT-qPCR (Table S10). The results are consistent with the relative expression level trend obtained from sequencing data, indicating that the sequencing results have a certain degree of reliability and can be used for subsequent transcriptomic analysis.

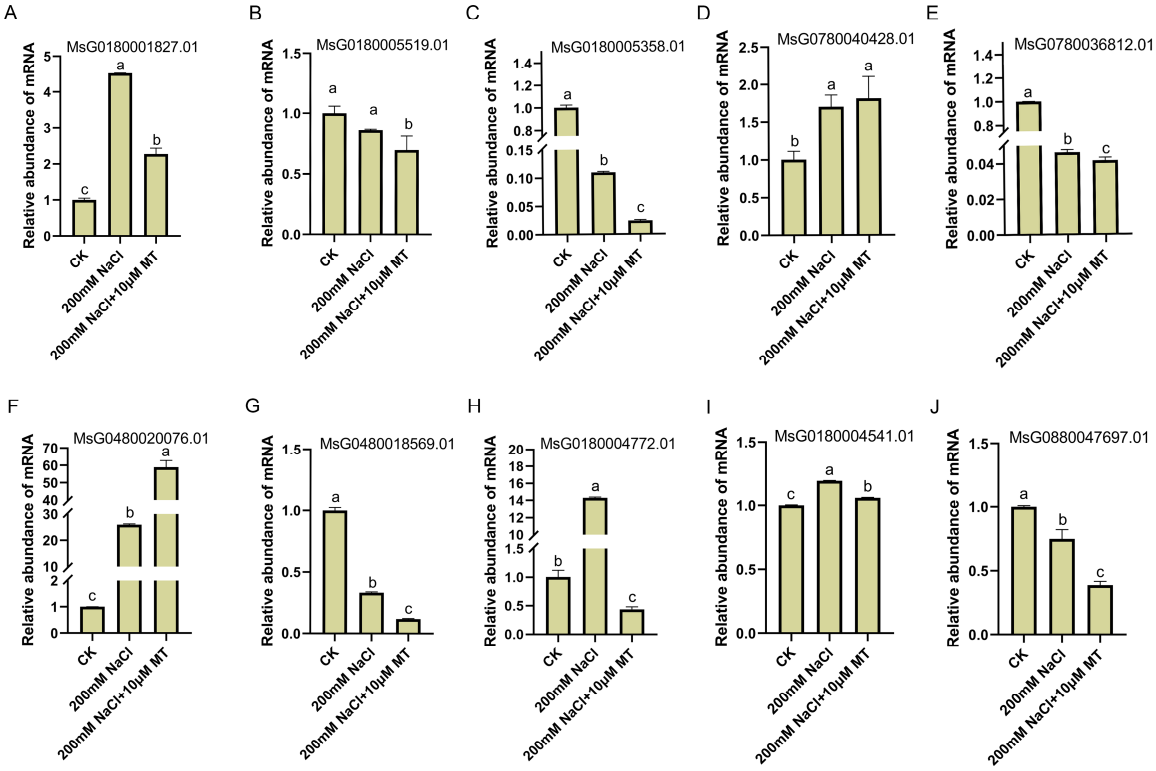

Figure 7. The results of RT-qPCR involved ten key genes, including genes synthesized by *NCED* (**A**), *GA20ox* (**B**), *CHS* (**C**), *proC* (**D**), *PRODH* (**E**), *ASMT* (**F**), *PYL* (**G**), *GA2ox* (**H**), *G1D1* (**I**), and *ACCO* (**J**). To ensure reproducibility and reliability, RT-qPCR analysis was performed on three independent biological replicates. The relative expression level was calculated according to the $2^{-\Delta\Delta CT}$ method. The columns represent the average of three biological replicates. Using the Duncan test, different lowercase letters indicated significant differences between treatments.

4. Discussion

4.1. Plant Hormone Synthesis and Signal Transduction

Plants have the ability to effectively regulate the production, transport, and decomposition of hormones in response to adverse conditions like salt stress [33]. Under salt stress, plants can synthesize abscisic acid (ABA), which serves various functions, including controlling leaf stomata size, restricting lateral root growth, participating in seed germination, hastening fruit ripening, and promoting the production of intracellular protective proteins [34–36]. In our experiment, we observed that in the salt-treated group, the expression of *NCED* (*MsG0180001827.01*), a key gene involved in ABA synthesis, was significantly upregulated. Furthermore, the key gene *crtB*, responsible for catalytic ABA synthesis, was also upregulated, while the key gene *CYP707A*, involved in the ABA degradation pathway, was downregulated. These findings suggest that these alterations in gene expression may be a response of alfalfa seedlings to high levels of salt stress, as they reduce nutrient loss by limiting lateral root elongation and controlling stomatal size. Chuong et al. [37] discovered that 2C protein phosphatase (PP2Cs) play a crucial role as protein phosphatases in plants, and they are also involved in regulating ABA signal transduction pathways. This indicates that members of the *PP2C* family play an important role in helping plants cope with adversity. In our experiment, we observed an increase in the expression of ABRE binding factors (ABFs) and *PP2C* genes in ABA signal transduction. Based on this, we hypothesized that the upregulation of *ABF* and *PP2C* genes accelerates the action of ABA, thus regulating seed dormancy and enhancing plant resistance to salt stress, as reflected in our measured phenotypic indicators like root length and fresh weight (Figure 1A–D). Melatonin has been shown to inhibit the expression of ABA synthesis genes, leading to a decrease in ABA content [38]. In our experiment, we observed a decrease in the expression

of *NCED* and *crtB* when comparing melatonin with salt treatment. This indicates that melatonin counteracts stress by regulating ABA synthesis. However, the exact mechanism behind the interaction between melatonin and ABA is not yet fully understood. Further research in this area would greatly benefit the cultivation of salt-tolerant alfalfa.

Under salt stress, the concentration of gibberellin (GA) in plants decreases. This decrease is correlated with the reduced expression of the key gene for gibberellin synthesis, gibberellin 20 oxidase (*GA20ox*, *MsG0180005519.01*) [39]. Salt stress inhibits plant growth by lowering GA levels. However, the application of exogenous MT can stimulate the synthesis of endogenous GA in plants and promote plant growth [40]. In the gibberellin signaling process, GA regulates the interaction between the DELLA protein and the *PIF3* and *PIF4* genes, influencing the elongation of cotyledon cells [41]. In our study, the expressions of the key genes Ent-kaurenoic acid oxidase (*KAO*) and *GA20ox*, involved in GA synthesis, were significantly downregulated under NaCl stress. This suggests that algae suppress plant growth by reducing GA content, allowing them to better adapt to stressful environments. However, in the gibberellin signaling pathway, the addition of MT upregulates gene expression for synthesizing the DELLA protein, which is consistent with previous research results [42–44]. These results confirm the reliability of our phenotype data (Figure 1A–D). Consequently, we speculate that GA and MT have a synergistic effect in promoting plant growth, providing a theoretical basis for alfalfa growth in saline–alkali soil.

4.2. The Roles and Connections between Physiological Indicators

High concentrations of ROS have significant toxic effects on plant cells. They cause oxidative damage to cell membranes and, in severe cases, damage RNA and DNA, leading to cell death. However, plants have their own antioxidant system that effectively neutralizes ROS and reduces oxidative damage to cells [45]. In our physiological analysis, the content of MDA and O^{2-} significantly increased under salt stress. MDA is produced when free radicals interact with lipids in lipid peroxidation reactions. The concentration of MDA is a crucial indicator of the antioxidant capacity of organisms. It not only reflects the rate of lipid peroxidation in the body but also indirectly indicates the extent of tissue damage caused by peroxidation [46]. During the reduction process, oxygen can generate various reactive oxygen species depending on the number of electrons it receives. Accepting a single electron for reduction produces superoxide anions, and excessive production can lead to tissue damage [47]. Conversely, under salt stress, the activity of reactive oxygen species scavenging enzymes, such as SOD and POD, increases. These enzymes play a vital role in scavenging free radicals and maintaining the stability of the cellular environment [48]. Flavonoids are secondary metabolites found widely in plants and play an important role in antioxidant capacity by scavenging free radicals and inhibiting oxidase activity [49]. Exogenous melatonin alleviates stress by regulating antioxidant capacity and flavonoid biosynthesis [50]. We hypothesize that the increase in SOD and POD activity is closely related to the synthesis of flavonoids. Previous studies have indicated that flavonoids can influence the activity of SOD and POD [51]. In our transcriptome analysis, when comparing salt treatment and melatonin treatment, we found that the expression abundance of DEGs related to flavonoid biosynthesis, such as chalcone synthase (*CHS*, *MsG0180005358.01*), chalcone-flavanone isomerase-2 (*CFI2*), *CYP73A*, *CYP75B1*, caffeoyl-CoA O-methyltransferase (*CAMT*), and *CYP75A*, was higher. Additionally, upregulating the anthocyanidin reductase (*ANR*) gene in *tobacco* can enhance the accumulation of flavan-3-ols (catechins and epicatechins) and directly boost the antioxidant capacity of *tobacco* plants [52]. In the MT treatment group, the *ANR* gene involved in epicatechin synthesis was significantly upregulated, enhancing the antioxidant system's ability. This observation is consistent with the physiological indicators we measured (Figure 2C,D). In the KEGG pathway, it is noteworthy that *CHS* is associated with isoliquiritin synthesis. Isoliquiritin is converted to liquiritigenin by the action of *CFI2*, which is linked to naringin synthesis. Both liquiritigenin and naringin are involved in isoflavone biosynthesis. Moreover, melatonin can promote the synthesis of isoflavones [53]. Therefore, we have a reason to speculate

that melatonin can counteract salt stress by regulating the synthesis of flavonoids and subsequently modulating the activity of SOD and POD. This finding provides new genetic insights into improving the antioxidant system's ability and offers a direction for the cultivation of salt-tolerant alfalfa.

When plants are exposed to salt stress, oxidative stress and cell damage are often caused [54], and glutathione (GSH) is an essential non-enzymatic antioxidant in cell defense [55]. Glutathione-s-transferase (*GST*) plays a vital role in the metabolic pathway of glutathione. *GST* can promote the binding of GSH to electrophilic substrates; at the same time, *GST* can catalyze the reaction of hydrogen peroxide (H_2O_2) and GSH, thereby reducing cell oxidative damage [56,57]. Studies have shown that melatonin can stimulate the synthesis of GSH [58]. In this study, 18 and 41 differentially expressed genes were enriched in the glutathione pathway in the NaCl and MT treatment groups, among which 16 and 27 genes were related to *GST*, respectively, which have been proven to play a key role in combating salt stress [59–61]. In the melatonin-treated group, genes related to *GST* were significantly upregulated, which may be because melatonin promotes the expression of *GST*, thereby catalyzing GSH to neutralize reactive oxygen species. Studies have shown that oxidized glutathione (*GSSG*) can be reconverted to GSH through the ascorbate–glutathione cycle to help maintain the reduced state within cells. This process helps protect cell membranes from oxidative damage [62]. In the glutathione biosynthesis pathway, we found that *DHAR*, an essential gene involved in the ascorbate–glutathione (ASA-GSH) cycle, was significantly upregulated in the MT treatment group, which may accelerate the process of *GSSG* conversion to GSH to achieve the purpose of antioxidant, and the physiological indicators we measured also support this hypothesis (Figure 2E). This provides a new basis for MT to enhance the salt tolerance of alfalfa. Subsequent research can cultivate salt-tolerant alfalfa by introducing this key gene.

4.3. Biosynthesis of Physiological Regulatory Substances

Certain amino acids, such as phenylalanine, tryptophan, tyrosine, and other aromatic amino acids, serve as precursors for natural products like alkaloids, flavonoids, plant auxin, and cell wall components, playing a crucial role in plant growth and response to environmental stress [63]. Previous research has examined the metabolic response and adaptation mechanism of wheat to salt–alkali stress. It has been found that salt stress increases sugar content in wheat, and the metabolic process tends to respond to osmotic stress through gluconeogenesis. Various differential metabolites involved in the tricarboxylic acid (TCA) cycle, glycolysis, and amino acid metabolism were detected in the roots and leaves of wheat seedlings under salt stress [64]. Through KEGG pathway analysis, we observed significant upregulation of the expression levels of *P5CS* and *proC* (*MsG0780040428.01*), key genes for proline synthesis, as well as downregulation of the expression levels of *PRODH* (*MsG0780036812.01*), a key gene for proline degradation, resulting in a substantial accumulation of proline. The addition of exogenous melatonin can promote the synthesis of endogenous melatonin in plants, playing a crucial role in combating salt stress [26,65]. It is worth noting that under salt stress, the expression level of the key gene *ASMT* (*MsG0480020076.01*) for melatonin synthesis in the tryptophan synthesis pathway is significantly upregulated, and the addition of exogenous MT further increases the upregulation of *ASMT* expression. This suggests that in cases of insufficient endogenous melatonin secretion, applying exogenous melatonin can enhance the plant's response to stress. Additionally, in the gluconeogenic pathway, the expression of the gene glucose-6-phosphate 1-epimerase (*AAPC*), responsible for synthesizing fructose-6-phosphate, was significantly increased. Furthermore, we observed upregulation of the expressions of genes *AKR1A1* and *ADH5* involved in the synthesis of fructose-6-phosphate in both glycolysis and gluconeogenesis in the MT group. This finding aligns with the study conducted by Guo et al. [63].

4.4. ABC Transporter

The ABC transporter family is widely distributed in the biological world and is currently recognized as the largest and most functional transmembrane transporter. It plays a crucial role in the transmembrane transport of substances between eukaryotes and prokaryotes [66]. Research has indicated that the expression level of ABC transporter in plant leaves increases under salt stress, suggesting that ABC protein may be integral to regulating internal and external balance in plants. This helps plants adapt to salt stress through internal regulation [67]. The majority of ABCB subfamily members are mainly involved in transporting and regulating hormone-like substances such as auxin [68]. When multiple phosphatase inhibitors were applied to inhibit the activity of the ABCG transporter, the use of PDR transporter inhibitors significantly decreased the secretion of flavonoids and design by soybean roots. The analysis of the EST database revealed an abundance of *PDR* transporter coding genes in roots. Consequently, PDR transporters are speculated to be key proteins in the secretion of flavonoids by roots [69]. Moreover, the ABCC2 transporter is involved in ABA transport, which is, in turn, involved in flavonoid transport [70]. Our experiment showed that the expression of ABCB1 was significantly upregulated, the expression of the *PDR5* gene was downregulated, and the expression of ABCC2 was upregulated under salt stress. This suggests that the synthesis and transport of flavonoids and plant hormones are vital mechanisms for alfalfa to adapt to salt stress. Studies have revealed that melatonin participates in the transport of plant hormones by regulating key transport proteins, thus alleviating stress on plants [71]. After the addition of MT, the expression of the ABC transporter gene was upregulated, indicating that the transport of flavonoids and plant hormones plays a significant role in combating salt stress. Furthermore, melatonin may act on transport proteins to expedite the transport of flavonoids and plant hormones.

4.5. MAPK Signal Path

The Mitogen-Activated Protein Kinase (MAPK) signaling pathway is a vital signal transduction system in eukaryotic cells. It consists of three core protein kinase components: MAPK (also known as MPK), MAPK kinase (also known as MAPKK, MKK, or MEK), and MAPKK kinase (also known as MAPKKK, MKKK, or MEKK). This pathway facilitates the transfer of signals from upstream sensors of cells to downstream effector molecules through continuous phosphorylation [72–74]. MAPK plays a crucial role in mediating the response of plants to salt stress. It senses the external high salt signal and transmits it to the interior of the cell, thereby improving the salt stress tolerance of plants [75]. The MAPK signaling pathway is intricately involved in the plant's response to salt stress, and its function depends on the ABA signaling pathway [76]. Under salt stress, the expression of differentially expressed genes involved in the PYL-PP2C-SnRK2 signaling pathway in alfalfa seedlings significantly changes. Most of these genes are upregulated, suggesting their involvement in response to salt stress. Genes that do not show upregulation may not play a role in the salt stress response. *SnRK2* can activate *MAPKKK17_18*, followed by *MKK3*, *MPK1_2*, and downstream stress adaptation genes through a series of phosphorylation reactions [77]. Additionally, the downregulation of the *CALM* gene, which synthesizes *MAPK8*, helps maintain a balance between ROS production and clearance [78]. Treatment with melatonin results in the downregulation of genes such as *MAPK3* and *WRKY22*, which are associated with cell death and H_2O_2 production [79]. This indicates that the clearance of ROS is not solely dependent on the antioxidant system but may also involve specific genes.

5. Conclusions

In summary, our comprehensive physiochemical and transcriptomic analyses have elucidated the mechanisms underlying MT-mediated salt tolerance in alfalfa. MT supplementation enhances salt tolerance by promoting plant growth and bolstering antioxidant capacity. Additionally, MT treatment under salt stress conditions induces significant transcriptomic changes, particularly in genes associated with flavonoid biosynthesis, plant

hormone signaling, and MAPK signaling pathways. Through weighted gene co-expression network analysis (WGCNA), potential hub genes linked to salt-responsive traits have been identified, suggesting their involvement in MT-regulated salt tolerance. These findings shed light on the molecular mechanisms underlying MT-mediated salt tolerance in alfalfa and offer valuable insights for breeding salt-tolerant varieties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14050661/s1. Figure S1: The functional classification of KEGG is that the spot size represents the number of genes and the color represents the g-value (A–C). Table S1: Primers designed for RT-qPCR analysis. Table S2: All differentially expressed genes identified in the transcriptomic data through pairwise comparison. Table S3: GO functional annotation of differentially expressed genes through pairwise comparison of gene sets. Table S4: The KEGG pathway enrichment of ZMN vs. ZMNMT gene concentration was analyzed using R script. Table S5: The KEGG pathway enrichment of ZMN vs. ZMCK gene concentration was analyzed using R script. Table S6: The KEGG pathway enrichment of ZMNMT vs. ZMCK gene concentration was analyzed using R script. Table S7: All differentially expressed gene modules were identified by weighted gene co-expression network analysis (WGCNA). Table S8: Top 30 hub genes in the turquoise module. Table S9: Top 30 hub genes in the blue module. Table S10: The raw data of RT-qPCR.

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