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Identification of Salt-responsive Genes in Melatonin Regulated Alfalfa Salt Tolerance by Transcriptome Analysis

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ABSTRACT

Background: Salt stress is a major limiting factor for alfalfa yield due to its relatively low salt tolerance. High soil salinity adversely impacts alfalfa growth and development, leading to reduced yield. Melatonin (MT) is known to play a significant role in enhancing plant resistance to abiotic stresses. However, the mechanisms underlying melatonin-mediated abiotic stress responses, particularly salt stress, are not well understood. To address this gap, we conducted a study focusing on germinating alfalfa seeds under salt stress.

Methods: Physiological indexes and transcriptomics analyses were carried out on the germinated seeds to investigate the effects of melatonin on alfalfa seedlings under salt stress conditions.

Result: Our results revealed that the application of melatonin led to an increase in shoot length and fresh weight of alfalfa seedlings under salt stress. Moreover, key physiological indexes such as peroxidase (POD) activity and glutathione (GSH) content were increased, while content of malondialdehyde and superoxide anions decreased. Transcriptomic analysis identified a total of 2,131 differentially expressed genes (DEGs) in the salt-treated group, with 726 up-regulated and 1,405 down-regulated genes, while the MT-treated group showed 2,896 DEGs, of which 1,097 were up-regulated and 1,799 were down-regulated. Further, KEGG enrichment analysis highlighted the enrichment of DEGs in pathways including flavonoid biosynthesis, ABC transporter, glutathione metabolism and the MAPK signaling pathway, with these pathways more significantly enriched in the MT-treated group. These findings collectively indicate that melatonin plays a crucial role in the response of alfalfa to salt stress and provide new insights into melatonin mediated gene expression of alfalfa salt tolerance.

Key words: Alfalfa, Melatonin, Salt stress, Transcriptome.

INTRODUCTION

Salt stress is a significant limiting factor in plant growth and production, with particularly adverse effects on feed crops such as alfalfa (Efe *et al.*, 2024). Saline soil covering approximately 20% of the global arable land area, resulting in substantial annual losses estimated in the tens of billions of dollars globally (Morton *et al.*, 2019). Therefore, long-term exposure to salt stress can lead to dehydration reactions in plants under high osmotic stress, which together result in a significant decrease in crop yield and quality (Tyerman *et al.*, 2019). Hence, the revelation of the molecular regulatory mechanisms of plant salt tolerance is crucial for the formulation of resistance breeding strategies and the creation of new crop germplasm, especially for the improvement of yield and quality of marginal land crops.

Alfalfa (*Medicago sativa* L.), known as the “king of forage”, due to its high yield, good nutritional quality and wide adaptability, is widely planted worldwide (Radovic *et al.*, 2009; Chen *et al.*, 2020). However, salt stress can hinder the growth and development of alfalfa and the impact of salt stress varies among alfalfa varieties in different geographical regions (He *et al.*, 2022). Therefore, it is crucial to accelerate the cultivation of new salt tolerant alfalfa varieties through the revelation of salt tolerant molecular regulatory mechanisms for the establishment and yield improvement of alfalfa in saline alkali soil. At present, research on the salt tolerance of alfalfa mainly focuses on maintaining osmotic homeostasis and ion balance, hormone regulation, changes in antioxidant enzyme activity and stress resistance gene response (Li *et al.*, 2019; Gong, 2021), revealing the basic process of alfalfa response to salt stress. In addition, exogenous plant growth regulators

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or plant hormones, especially melatonin, can enhance plant antioxidant capacity, reduce plant reactive oxygen species levels and ultimately promote plant resistance to salt stress (Guo *et al.*, 2022; Cen *et al.*, 2020). The use of such regulators has become a promising strategy for improving salinity stress (Singh *et al.*, 2024), mainly by enhancing the activity of key antioxidant enzymes, which are the first line of defense against oxidative damage (Singh *et al.*, 2025). Melatonin is a small molecule indoleamine hormone that serves as a growth regulator and potent antioxidant, with functions such as stimulating plant growth (Park and Back, 2012), promoting seed germination and enhancing plant resistance to abiotic stress (Chen *et al.*, 2018a; Zhang *et al.*, 2021). Research has shown that exogenous melatonin can activate antioxidant enzymes in maize seedlings, thereby reducing the accumulation of ROS caused by salt stress (ElSayed *et al.*, 2021). Melatonin can not only enhance plant stress resistance by directly clearing excess ROS, but also interact with other plant hormones to regulate the expression of stress resistance genes, thereby improving plant salt tolerance (Gou *et al.*, 2018). When plants are subjected to external stress, melatonin acts on the upstream signaling pathways of some defense genes to promote the synthesis of plant hormones such as jasmonic acid and salicylic acid, thereby assisting plants in resisting adverse external influences (Li *et al.*, 2012; Mukherjee *et al.*, 2014). This indicate that melatonin can directly or indirectly participate in stress response by interfering with other plant hormones, regulating the expression of its upstream and downstream genes. However, although numerous studies have shown that exogenous application of melatonin can affect plant response to salt stress, the mechanism of melatonin mediated response of alfalfa to salt stress has not been fully explored (Li *et al.*, 2017; Chen *et al.*, 2018b; Kim *et al.*, 2015). Recent transcriptomic studies in legumes such as soybean and *Medicago truncatula* have identified key salt-responsive pathways, including those involved in ion homeostasis and ROS scavenging (de Lorenzo *et al.*, 2009; Jin *et al.*, 2021), providing a valuable context for understanding melatonin-mediated responses in alfalfa.

In this study, we investigated the effect of exogenous application of a certain concentration of melatonin on the germination characteristics of alfalfa under salt stress. Based on physiological indicator detection and transcriptomic analysis, we further identified key differentially expressed genes and metabolic pathways in response to salt stress in alfalfa, providing potential research strategies for a deeper understanding of the molecular regulatory mechanism of salt tolerance in alfalfa and salt tolerance breeding.

MATERIALS AND METHODS

Plant materials and experimental design

The experiment was conducted at the College of Animal Science and Technology, Henan Agricultural University (Zhengzhou, China) from 2024. This study selected WL440 alfalfa varieties purchased from Beijing Zhengdao Seed Industry Co., Ltd. as experimental materials. Seeds of uniform size and full grains were selected, disinfected by soaking in 75% alcohol for 10 minutes, followed by rinsing with deionized water 3-4 times. Subsequently, petri dishes (ΦA=90 mm) were prepared with 4 mL of various melatonin concentrations (0, 10, 50, 100, 200 μM) and 200 mM NaCl, with six replicates per treatment and approximately 30 seeds cultured per replicate. The petri dishes were then placed in a constant temperature incubator at 25°C for 16 hours during the day and 8 hours at night. The germination rate was assessed every 24 hours over a total period of 7 days and the measurements of bud length and fresh weight were conducted at the end of the 7-day period. Following the measurements, the samples were stored in an ultra-low temperature refrigerator at -80°C. Furthermore, the physiological and biochemical indexes of seedlings treated with 0 (WLCK), 200 mM NaCl (WLN) and 200 mM NaCl + 10 μM MT (WLNMT) were determined. Eventually, transcriptome sequencing was carried out to explore the underlying mechanisms related to the observed effects.

Measurement of germination rate, shoot length and fresh weight

The germination rate was calculated over a period of 7 consecutive days. On day 7, the vertical distance from the cotyledon node to the top bud of 10 alfalfa seedlings was measured using a calibrated ruler and recorded as the root length. At the same time, the fresh weight of the same 10 seedlings was weighed using an electronic balance.

Measurement of physiological and biochemical indices

The peroxidase (POD) activity, superoxide dismutase (SOD) activity and malondialdehyde (MDA) were measured using specific assay kits. The POD activity was determined by employing the POD activity assay kit (Abbkine, KTB1150, Wuhan, China), followed by the SOD activity determined with the SOD activity assay kit (Abbkine, KTB1030, Wuhan, China) and the MDA levels assessed with the malondialdehyde content assay kit (Abbkine, KTB1050, Wuhan, China). Furthermore, the levels of reduced glutathione (GSH) and superoxide anion ($O_2^{\cdot-}$) were measured using the GSH content assay kit (Solarbio, BC1175, Beijing, China) and the superoxide anion content assay kit (Solarbio, BC1295, Beijing, China), respectively. All measurements were conducted by the provided instructions accompanying each specific assay kit.

RNA sequencing and bioinformatic analysis

Total RNA was extracted from a pool of 30 alfalfa seedlings grown under identical conditions. The raw RNA-seq reads were processed using fastp (v0.19.5) to remove adapters and low-quality bases. Clean reads were aligned to the reference alfalfa genome using HISAT2 (v2.1.0). Gene expression levels were quantified using StringTie (v2.2.2) and RSEM (v1.3.3). Differential expression analysis was

performed using DESeq2 (v1.24.0) with thresholds set at |log2FC| ≥1 and FDR < 0.05. Library construction, transcriptome sequencing and subsequent bioinformatic analysis of sequence reads (comprising quality control, mapping, assembly and functional annotation) followed identical procedures to those described by Liu *et al.* (2024).

Weighted gene co-expression network analysis

To identify closely related genes, the genes were classified into different modules based on the relevant growth and physiological indicators of the three different treatments. Utilizing the WGCNA R software package, a co-expression network was subsequently constructed to determine the modules harboring these related genes. Following this, the analysis was visualized using the Cytoscape (3.3.0) software in order to pinpoint the hub genes within the co-expression network.

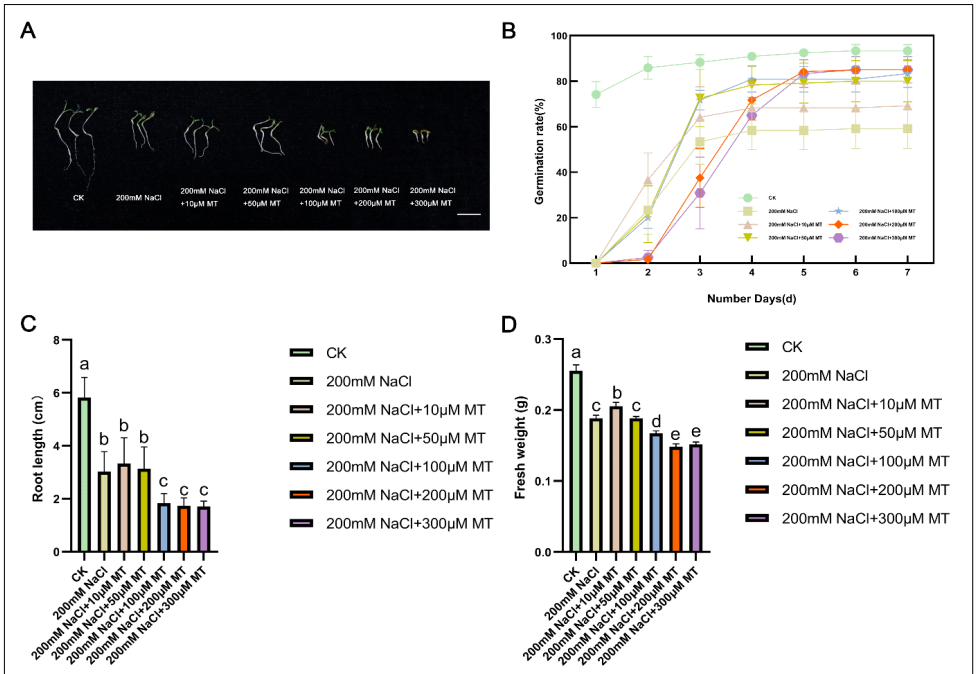
qRT-PCR analysis

To verify the accuracy of the transcriptomic data, we selected eight genes for qRT-PCR analysis. First, we used the cDNA synthesis kit PrimeScript RT Reagent kit with gDNA Eraser (Cat# RR047A, TaKaRa, Tokyo, Japan) to reverse transcribe the extracted mRNA. Subsequently, we used the qRT-PCR kit (Cat# RR420A; TaKaRa) for further analysis. All operations were performed according to the instructions of the kit and each biological replicate included three technical replicates to ensure the reliability of the results. For internal control genes, we selected *Ms-ACTIN2* as a reference gene.

RESULTS AND DISCUSSION

Effect of MT on the growth of NaCl-stressed alfalfa plants

To study the seed germination and growth under salt stress, we counted the germination rate of alfalfa sprouts and measured their root length and fresh weight. Analysis of germination rates over seven days demonstrated differences among the treatments (Fig 1A). On the seventh day, seeds treated with 200 µM and 300 µM MT exhibited higher germination rates compared to the salt stress group without MT. Among, the 300 µM MT treatment resulted in germination rates closest to the CK group (Fig 1B). In terms of fresh weight and root length, comparison between the salt stress groups treated with various concentrations of MT and the CK group revealed interesting results. The CK group had a fresh weight of 0.2553 g and a root length of 5.82 cm. The group treated with 10 µM MT closely resembled the CK group, with a fresh weight of 0.2053 g and a root length of 3.33 cm. In contrast, the salt stress group without



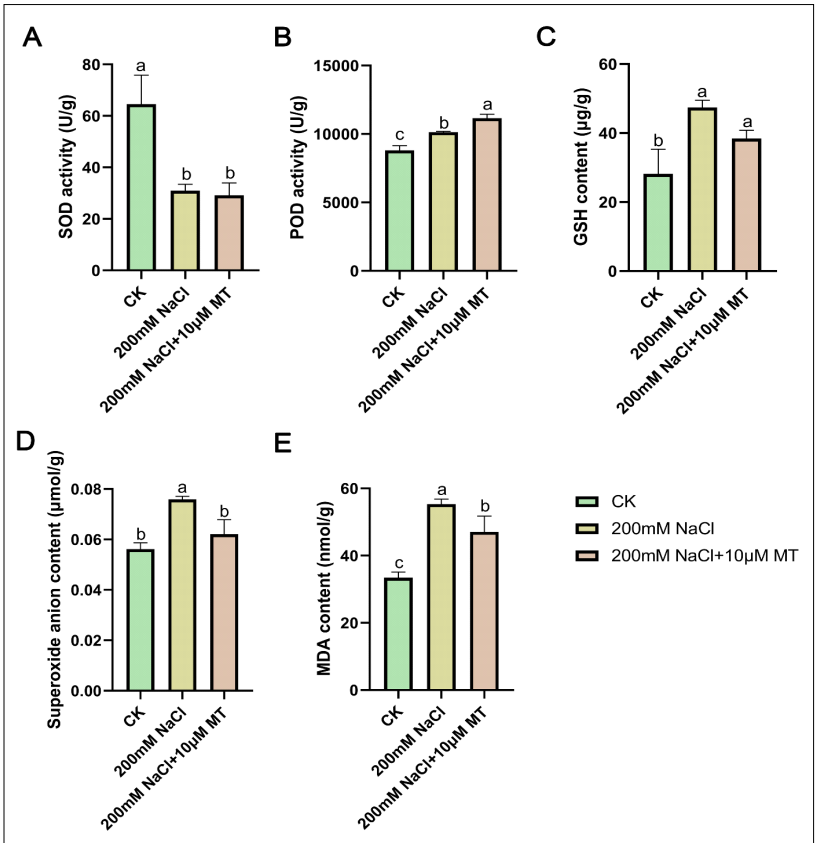
(A) Alfalfa seedling phenotypes. Bar = 2 cm. (B) Seed germination was evaluated after a 7-day treatment. The line graph displays the average germination rate of six biological replicates, each consisting of 30 seeds, along with the standard deviation. (C) The shoot lengths of alfalfa seedlings were measured after 7 days of treatment. The values in each column represent the mean of three biological replicates, with different lowercase letters indicating significant differences between treatments ($p < 0.05$). (D) The fresh weight of alfalfa seedlings was measured after 7 days of treatment. The values in each column represent the mean of three biological replicates. Different lowercase letters indicate significant differences as determined by Duncan's test. CK serves as the control group.

Fig 1: Effect of melatonin (MT) application on seed germination and young shoot growth.

MT exhibited a fresh weight of 0.1884 g and a root length of 3.02 cm (Fig 1C, 1D). These findings suggest the efficacy of 10 μM MT in ameliorating the growth of salt-stressed plants, as evidenced by superior fresh weight and root length outcomes compared to other MT concentrations.The observed improvement in germination and growth parameters under MT treatment aligns with previous studies showing that exogenous melatonin enhances plant tolerance to abiotic stresses by promoting growth and mitigating stress-induced damage (Chen *et al.*, 2018a; Zhang *et al.*, 2021). This indicates that melatonin may act as a growth regulator to counteract the inhibitory effects of salt stress on alfalfa seed germination and seedling development.

Changes of indicators in oxidation system

To investigate the mechanism by which melatonin enhances alfalfa salt tolerance, we analyzed oxidative stress indices under various treatments. Our examination of different concentrations of MT in alfalfa growth conditions under salt stress revealed that the group treated with 10 μM MT under salt stress exhibited the most favorable outcomes. Consequently, we assessed the physiological indexes in the control group (CK), the 200 mM NaCl treatment group and the group treated with 10 μM MT under NaCl stress. It was found that SOD enzyme activity was reduced in the salt-stressed group, while POD, MDA, $O_2^{\cdot-}$ and GSH contents were increased in the salt-stressed group compared to the CK group. Interestingly, when 10 μM MT was added to the salt stress group, the activity of POD increased significantly by 10.17%, while the levels of MDA, $O_2^{\cdot-}$ and GSH decreased by 14.91%, 18.22% and 18.95%, respectively, with minimal change in SOD activity (Fig 2A-2E). These results indicate that melatonin alleviates salt-induced oxidative damage by modulating the antioxidant system. Under salt stress, plants accumulate reactive oxygen species (ROS) such as $O_2^{\cdot-}$, leading to lipid peroxidation (reflected by elevated MDA levels) and oxidative stress (Tyerman *et al.*, 2019). The melatonin-enhanced POD activity and reduced ROS accumulation align with previous findings that melatonin activates antioxidant enzymes to scavenge excess ROS (ElSayed *et al.*, 2021). Furthermore, the decrease in GSH levels in MT-treated groups may reflect its utilization in ROS detoxification, as GSH is a key antioxidant involved in maintaining redox homeostasis (Gill *et al.*, 2013). Collectively, these findings



(A) SOD activity. (B) POD activity. (C) MDA content. (D) $O_2^{\cdot-}$ content. (E) GSH content. Each column presents the mean value and standard deviation of three biological replicates, with each replicate comprising three plants. Significant differences (p<0.05) are denoted by distinct lowercase letters based on the results of Duncan's test.

Fig 2: Melatonin from external sources reduces oxidative stress caused by NaCl treatment in alfalfa seedlings.

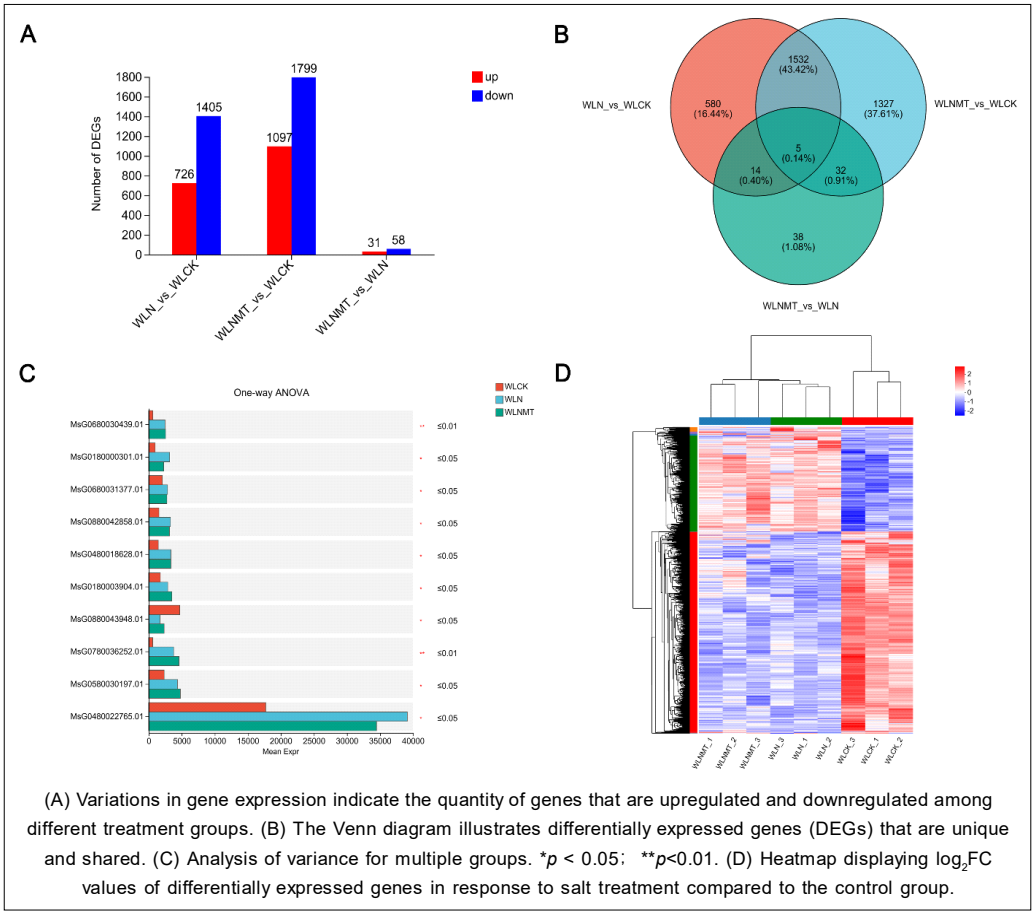
suggest that melatonin improves salt tolerance by enhancing the antioxidant capacity of alfalfa seedlings.

Differential expression analysis and cluster analysis of genes in alfalfa seedlings

Transcriptomic analysis of WLCK, WLN and WLNMT identified 3,547 differentially expressed genes (DEGs). Compared to WLCK, WLN had 726 up-regulated and 1,405 down-regulated genes, while WLNMT had 1,097 up-regulated and 1,799 down-regulated genes. Only 31 up-regulated and 58 down-regulated genes were found in WLNMT vs. WLN (Fig 3A). A Venn diagram showed minimal overlap (0.14%) among all three groups, with most DEGs being unique to WLNMT vs. WLCK (37.61%) (Fig 3B). ANOVA and heatmap clustering confirmed significant expression differences across treatments (Fig 3C, 3D). The large number of DEGs in WLNMT vs. WLCK suggests melatonin modulates a broad range of genes under salt stress. This aligns with studies showing melatonin regulates stress-responsive gene expression by interacting with signaling pathways or hormones (Gou *et al.*, 2018). The limited DEGs between WLNMT and WLN indicate melatonin primarily modulates genes already affected by salt stress, rather than activating entirely new pathways, supporting its role in enhancing existing stress responses.

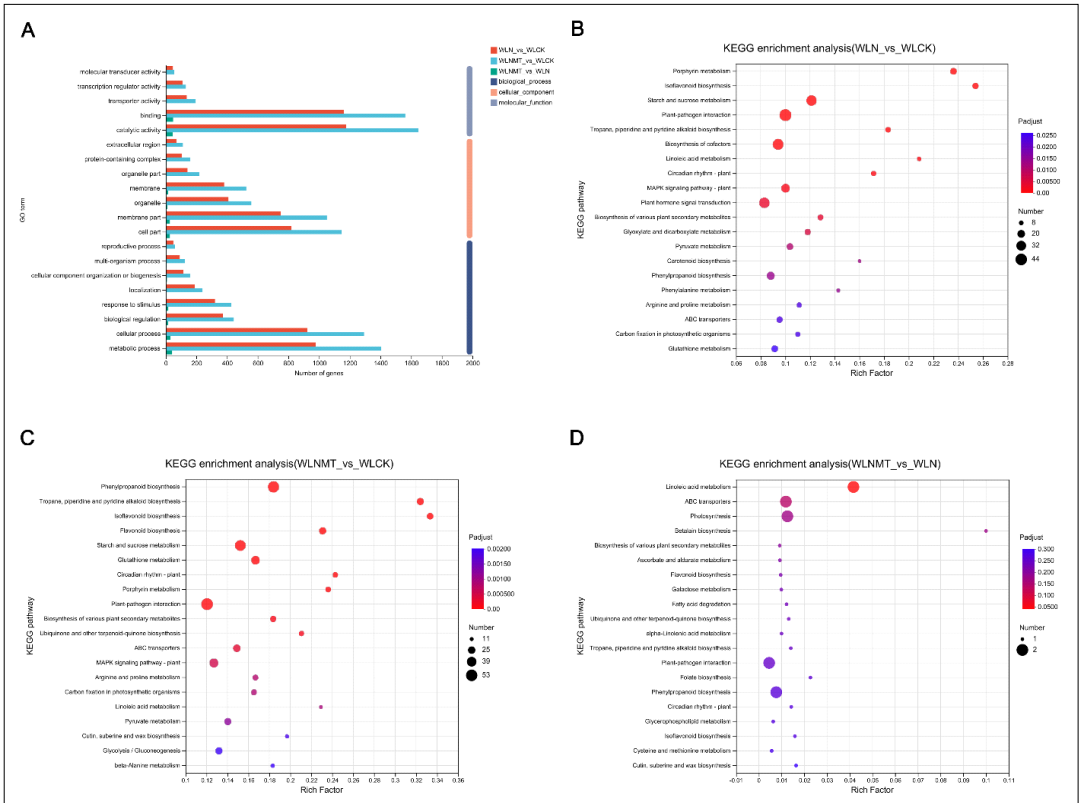
GO annotation analysis and KEGG enrichment pathway analysis of DEGs

GO annotation revealed DEGs in all comparisons were enriched in biological processes (metabolic processes, cellular processes), cellular components (cell parts, membrane parts) and molecular functions (binding, catalytic activity), with stronger enrichment in WLNMT vs. WLCK (Fig 4A). KEGG analysis showed that DEGs of WLN vs. WLCK were enriched in porphyrin metabolism and linoleic acid metabolism pathways; The DEGs of WLNMT vs. WLCK are enriched in the biosynthesis pathways of isoflavones and alkaloids; The DEGs of WLNMT vs. WLN are enriched in ABC transporters and photosynthetic pathways (Fig 4B-4D). The enrichment of metabolic and cellular processes in GO terms suggests salt stress and melatonin primarily affect basic physiological functions. KEGG pathways related to secondary metabolism (*e.g.,* isoflavones, flavonoids) and transporters (ABC) highlight key mechanisms. Isoflavonoids and flavonoids are known to enhance antioxidant capacity and stress tolerance (Back,



(A) Variations in gene expression indicate the quantity of genes that are upregulated and downregulated among different treatment groups. (B) The Venn diagram illustrates differentially expressed genes (DEGs) that are unique and shared. (C) Analysis of variance for multiple groups. *$p < 0.05$; **$p<0.01$. (D) Heatmap displaying log_2FC values of differentially expressed genes in response to salt treatment compared to the control group.

Fig 3: Control differentially expressed genes (DEGs), CK (WLCK), NaCl (WLN) and MT with NaCl (WLNMT) were visualized in combination.



(A) Based on the Gene Ontology annotation analysis of Differentially Expressed Genes (DEGs), the DEGs were categorized into biological processes, cellular components and molecular functions. These categories are represented by colors from top to bottom corresponding to the WLN_vs_WLCK, WLNMT_vs_WLCK and WLNMT_vs_WLN comparisons. (B-D) Bubble plots representing KEGG pathway enrichment analyses. (B) KEGG functional classification of DEGs in WLN_vs_WLCK. (C) KEGG functional classification of DEGs in WLNMT_vs_WLCK. (D) KEGG functional classification of DEGs in WLNMT_vs_WLN.

Fig 4: Functional annotation and KEGG enrichment analysis of differential gene expression.

2021), while ABC transporters mediate the transport of stress-related compounds (Kang *et al.*, 2011). The stronger enrichment in WLNMT vs. WLCK indicates melatonin amplifies these pathways to improve salt tolerance.

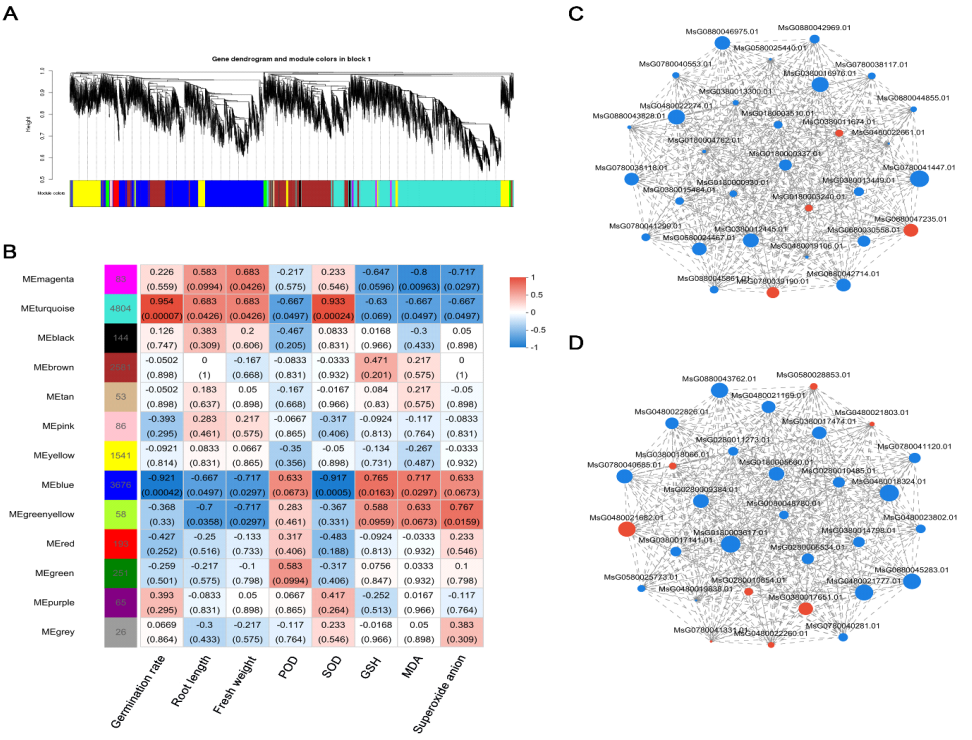
Gene co-expression networks

WGCNA identified 13 co-expression modules. The turquoise module (4,804 DEGs) correlated positively with germination rate, root length, fresh weight and SOD (correlation coefficients 0.683-0.954), while genes in the blue module (3,676 DEGs) exhibited positive correlations with POD, GSH, MDA and $O_2^{\cdot-}$ (0.633-0.765) (Fig 5A, 5B). Genes in these modules were associated with flavonoid biosynthesis, glutathione metabolism, phytohormone signaling, MAPK pathways and ABC transporters (Fig 5C, 5D). These modules link gene expression with physiological characteristics. The turquoise module likely contributes to growth recovery under MT treatment, while the blue module is involved in oxidative stress responses. The association with flavonoid and glutathione pathways supports their roles in ROS scavenging (Gill *et al.*, 2013; Back, 2021). MAPK signaling and phytohormone pathways further indicate melatonin regulates stress signaling networks (Kumar *et al.*, 2020; Waadt *et al.*, 2022).

Transcriptome analysis

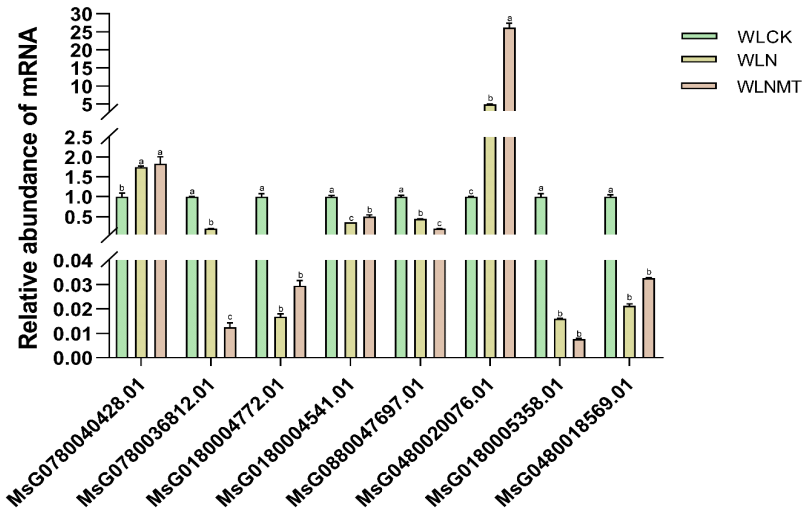
Transcriptomic analysis revealed up-regulation of flavonoid biosynthesis genes (HCT, DFR, CYP81E9) in WLN vs. WLCK and WLNMT vs. WLCK. Among them, the MT-treated groups showed greater up-regulation than salt-only groups. Flavonoids are critical antioxidants and their accumulation enhances salt tolerance by scavenging ROS (Back, 2021). DFR up-regulation stimulates anthocyanin synthesis, which improves stress tolerance in crops like rapeseed (Kim *et al.*, 2017). Melatonin's promotion of these genes suggests it enhances flavonoid synthesis to mitigate salt stress damage.

Genes involved in glutathione metabolism (speE, DHAR, GST, APX) were up-regulated in WLN vs. WLCK and more so in WLNMT vs. WLCK. DHAR enhances GSSG-to-GSH conversion, while GST accelerates glutathione-mediated detoxification (Reinemer *et al.*, 1992; Chen *et al.*, 2022). Glutathione is central to ROS scavenging (Gill *et al.*, 2013). Up-regulation of these genes under MT



(A) A module classification tree uses colors to represent different modules, with grey indicating genes that have not been assigned to a specific module. (B) Module-phenotype correlations heatmap depicting relationships between modules and physiological parameters. (C) Co-expression network of DEGs in MEturquoise. (D) Co-expression network of DEGs in MEblue.

Fig 5: WGCNA identified correlations between melatonin-regulated salt stress-related physiological indicators and differentially expressed genes.



The bars with the same superscript letter indicated that the relative expression of the gene was not significantly different. The data were expressed as 3 replicates ±SE. The results were statistically analyzed using SPSS 27 software. Perform one-way ANOVA to determine any significant differences between groups with a significance level of $p<0.05$. The post hoc Duncan test is used to determine significant differences between groups.

Fig 6: The mRNA relative abundance of WLN, WLN and WLNMT was calculated by qRT-PCR quantitative analysis.

treatment indicates enhanced glutathione recycling and detoxification, reducing oxidative damage. This aligns with physiological data showing lower MDA and $O_2^{\cdot-}$ in MT-treated plants, confirming glutathione's role in melatonin-mediated salt tolerance.

This study confirms the potential of melatonin to enhance salt tolerance in alfalfa under controlled conditions. However, its efficacy may be influenced by variations in temperature, light and soil properties and these findings may not extrapolate directly to field environments. Further validation through field trials is essential to assess melatonin's effectiveness under real-world conditions, where complex interactions among environmental variables occur.

qRT-PCR validation

qRT-PCR analysis of eight selected genes showed mRNA relative abundance trends consistent with RNA-seq results (Fig 6), validating transcriptome reliability. These genes, involved in pathways like flavonoid biosynthesis and hormone signaling, likely play key roles in salt stress responses.

CONCLUSION

Transcriptomics was employed in this study to analyze the impact of NaCl stress on gene transcription levels in alfalfa plants. It was observed that flavonoids, glutathione and various phytohormones are crucial for plant adaptation under salt stress. Under salt stress conditions, genes related to oxidoreductase activity and secondary metabolism demonstrated decreased expression. However, supplementation with melatonin was found to enhance the expression levels of genes inhibited by salt stress, which ultimately led to an increase in the synthesis and metabolism of flavonoids, glutathione and phytohormones, as well as the activation of ABC transporter proteins and the MAPK signaling pathway, thereby mitigating the negative effects of salt stress on gene expression in alfalfa plants. Consequently, the ability of plants to handle salt stress improved. The study elucidated the potential molecular mechanisms of salt stress in alfalfa plants and identified several key genes, paving the way for a more comprehensive examination of salt tolerance in alfalfa plants.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

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Writing - review and editing: Hao Sun, Xiaoyan Zhu, Chengzhang Wang, Defeng Li, Yinghua Shi.

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Data availability

The following information was supplied regarding data availability:
The raw data are available in the Supplemental Files.

DNA deposition

The following information was supplied regarding the deposition of DNA sequences:
Sequences are available at NCBI GenBank: PRJNA1099414.

Conflict of interest

The authors declare there are no competing interests.

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