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Transcriptomic Profiling and Functional Validation Reveal MYC2-PSK3 Mediating Salt-Alkali Tolerance in Alfalfa (*Medicago sativa* L.)

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Transcriptomic Profiling and Functional Validation Reveal MYC2-PSK3 Mediating Salt-Alkali Tolerance in Alfalfa (*Medicago sativa* L.)

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Abstract

Soil salinization severely limits alfalfa productivity; however, the molecular mechanisms governing cultivar-specific differences in salt tolerance remain largely unclear. In this study, two alfalfa cultivars (Zhongmu No.3 and WL440-HQ) were exposed to 200 mM NaCl stress, followed by integrated transcriptome sequencing, weighted gene co-expression network analysis (WGCNA), and functional validation. In total, 3,517 salt-responsive differentially expressed genes (DEGs) were identified, including 795 shared DEGs and cultivar-specific DEGs (1,336 in Zhongmu No.3 and 1,386 in WL440-HQ). GO and KEGG enrichment revealed conserved stress-response pathways, including flavonoid biosynthesis and starch and sucrose metabolism, as well as cultivar-specific patterns, with Zhongmu No.3 strongly enriched in stimulus-responsive genes. WGCNA further identified phenotype-related modules and core hub genes, notably *MsWRKY22* and *MsPSK3*. Overexpression of *MsPSK3* enhanced salt-alkali tolerance in alfalfa by activating antioxidant systems. Dual-luciferase and yeast one-hybrid (Y1H) assays verified that *MsMYC2* directly binds to and activates the MsPSK3 promoter. This study reveals the molecular regulatory network underlying alfalfa responses to salt–alkali stress and provides key candidate genes for breeding salt-tolerant alfalfa varieties.

Keywords: alfalfa; salt-alkali stress; transcriptomics; PSK3

1. Introduction

Soil salinization represents a critical threat to global agricultural sustainability, adversely affecting approximately 1 billion hectares of arable land worldwide (Van Zelm et al., 2020). Elevated salinity imposes multifaceted stresses on plants, including osmotic imbalance, ionic toxicity, and oxidative damage, thereby disrupting fundamental physiological processes such as seed germination, root architecture development, and photosynthetic efficiency (Zhou et al., 2024). Alfalfa (*Medicago sativa* L.), a perennial leguminous forage crop renowned for its superior nutritional value andbroad ecological adaptability, is extensively cultivated across diverse agroecological zones globally. However, its productivity is markedly constrained under saline-alkaline soil conditions, and substantial genetic variation in salt tolerance has been documented among different cultivars (Zhang et al., 2020; Wang et al., 2024; Zhang et al., 2025). Consequently, dissecting the molecular mechanisms governing salt tolerance and identifying key regulatory genes in alfalfa are imperative for expediting the development of salt-tolerant cultivars through molecular breeding strategies.

Transcriptomic sequencing has emerged as a powerful approach for identifying differentially expressed genes (DEGs) and elucidating stress-responsive pathways in plants under various environmental constraints (Wang et al., 2019; Li et al., 2024; Liu et al., 2024; Wang et al., 2025; Zhang

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et al., 2025). Weighted Gene Co-expression Network Analysis (WGCNA) further facilitates the identification of gene modules associated with specific phenotypic traits, thereby enabling the discovery of core regulatory genes and their interconnected networks (Langfelder & Horvath, 2008). Transcription factors (TFs), including members of the WRKY, MYC, bZIP, NF-YC and AP2/ERF families,play pivotal roles in orchestrating plant responses to abiotic stresses by regulating the expression of downstream stress-responsive genes (Yu et al., 2022; Ma et al., 2023; Wang et al., 2025). For instance, WRKY TFs participate in salt stress signaling by binding to W-box cis-elements within the promoters of target genes, thereby modulating osmotic adjustmentmechanisms and reactive oxygen species (ROS) scavengingpathways (Jiang et al., 2021). Similarly, MYC TFs are integral to jasmonic acid (JA)-mediated stress responses, coordinatingthe balance between plant growth and stress tolerance (Chen et al., 2019).

Although previous studies have identified several salt-responsive genes in alfalfa, the molecular mechanisms underlying salt tolerance divergence between different cultivars remain poorly elucidated. Moreover, the regulatory networks involving WRKY and MYC TFs in mediating alfalfa responses to salt stress have not been comprehensively characterized. In this study, two alfalfa cultivars, Zhongmu No.3 and WL440-HQ, were subjected to salt stress (200 mM NaCl), and integrated analyses including transcriptomic profiling, WGCNA, subcellular localization, yeast one-hybrid (Y1H) assay, and dual-luciferase reporter assay were performed. The objectives of this investigation were to: (1) identify common and cultivar-specific DEGs in response to salt stress; and (2) experimentally verify the interactions between candidate TFs and target gene promoters. The findings provide novelinsights into the molecular basis of salt tolerance in alfalfa and identify potential candidate genes for genetic improvementand molecular breeding programs.

2. Materials and Methods

2.1. Plant Materials and Pretreatment

Three alfalfa (*Medicago sativa* L.) cultivars, Yumu No.1, Zhongmu No.3 and WL440-HQ, were employed in this study. Yumu No.1 and Zhongmu No.3 were provided by Henan Agricultural University and Chinese Academy of Agricultural Sciences, respectively. Whereas WL440-HQ was purchased from Beijing Zhengdao Co., Ltd. (Beijing, China). Yumu No.1, Zhongmu No.3 and WL440-HQ seeds were surface-sterilized by immersion in 75% (v/v) ethanol for 10 minutes, followed by four successive rinses with double-distilled water. Seeds were germinated in Petri dishes with wet filter paper for seven days. The seedlings were transferred to vermiculite and perlite (1:1, v/v) for rearing. The alfalfa were cultivated in a greenhouse at 25 ±1.3 °C under 16 h light/8 h dark cycles with an irradiance of approximately 250 μmol quanta m^{-2} s^{-1} and 40 ±4% relative humidity. Four weeks after the seedlings were planted, seedlings that showed uneven growth was removed, and 5 seedlings remained in each pot. Seedlings were randomly divided into three treatment groups: control, saline-alkaline treatment (NaCl and NaHCO3, 200 mM Na+ molar ratio 1:1). The treatment groups received saline-alkaline stress solutions for two weeks days in addition to 1/2 ×Hoagland solution, and the control group received watering with the same solution.

Alfalfa seeds were surface-sterilized by immersion in 75% (v/v)ethanol for 10 minutes, followed by four successive rinses with double-distilled water. Based on previously optimized protocols (Zhang et al., 2020), 30 seeds per Petri dish (9 cm diameter) were germinated in 4 mL NaCl solutions at concentrations of 0 mM (control) and 200 mM (salt stress). The Petri dishes were incubated in a controlled-environmentgrowth chambermaintained at 25°C with 70% relative humidity under a photoperiod of 16 h light/8 h dark. After 7 days of cultivation, germination rate, fresh weight, and root length were measured with six biological replicatespertreatment. Seedlings treated with 0 mM NaCl (designated as ZMCK for Zhongmu No.3 and WLCK for WL440-HQ) and 200 mM NaCl (designated as ZMN for Zhongmu No.3 and WLN for WL440-HQ) were immediately flash-frozen in liquid nitrogen and stored at -80°C for subsequent transcriptomic analysis.

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2.2. Transcriptomic Analysis

Total RNA was extracted using the MJZol Total RNA Extraction Kit (Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from total RNA using random hexamer primers and reverse transcriptase. The resultingcDNA was purified using the Biowest Agarose Gel ExtractionKit (Biowest, Logroño, Spain) and the RNA Purification Kit (Shanghai Meixing Biomedical Technology Co., Ltd., Shanghai, China), followed by PCR amplification to construct sequencing libraries. Bridge PCR-based cluster generation was performed on a cBot system, and paired-end sequencing (2 × 150 bp)was conducted on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) (Chen et al., 2019).

Raw sequencing reads were subjected toqualitycontrol using fastp (v0.20.0) (Chen et al., 2018) to remove adapter sequences, trim bases with quality scores <20 at the 3′ terminus, discardreads containing >10% ambiguous bases (N), and filter outsequences shorter than 20 bp post-trimming. Theresulting clean reads were aligned to the reference genome (Zhongmu No.1) using HISAT2 (v2.2.1) (Kim et al., 2015) for subsequent transcript assembly and expression quantification. Functional annotation and classification of differentially expressed genes (DEGs) were performed using Blast2GO software (https://www.blast2go.com/) (Conesa et al., 2005). KEGG pathway enrichment analysis was conducted using the KOBAS 3.0 online tool (http://kobas.cbi.pku.edu.cn/) (Xie et al., 2011) to identify salt stress-responsive pathways.

Quality assessment of raw reads was performed using FastQC (v0.11.9) (Andrews, 2010). Gene expression levels were normalized and quantified as fragments per kilobase of transcript per million mapped reads (FPKM). Differential expression analysis was performed using the DESeq2 R package (v1.30.1) based on the negative binomial distribution model (Love et al., 2014). DEGs were identified using stringent criteria: absolute log2 fold change ($|\log2FC| \geq 1$ and false discovery rate (FDR) < 0.05. Fisher's exact test was employed for enrichment significance analysis, and the Benjamini–Hochberg method was applied for multiple testing correction; pathways with an adjusted p-value (Padj) < 0.05 were considered significantly enriched.

2.3. Weighted Gene Co-Expression Network Analysis (WGCNA) and Protein-Protein Interaction (PPI) Network Analysis

A co-expression network was constructed usingthe WGCNA package(v1.70-3) implemented in R (Langfelder & Horvath, 2008) to identify highly correlated gene modules. Modules exhibiting significant associations with phenotypic traits (includinggermination rate, fresh weight, and root length) were prioritized, enablingexploration of the correlations among gene networks, phenotypes, and core hub genes. The WGCNA package ispublicly available at https://cran.r-project.org/web/packages/WGCNA/index.html. The protein-protein interaction (PPI) network of DEGs was constructed usingthe STRING database(v11.5) (Szklarczyk et al., 2021) (https://string-db.org/) to reveal functional interactions among these DEGs. The organism was specified as Medicago sativa, and the minimum requiredinteraction score was set to 0.7 (high confidence) for network construction.

2.4. Gene Cloning and Vector Construction

Total RNA was extracted from alfalfa seedlings using the HiPure Plant RNA Plus Kit (Magen Biotech Co., Ltd., Guangzhou, China) for gene cloningpurposes.First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The coding sequences (CDSs) of MsWRKY22, MsPSK3, MsWRKY33, and MsMYC2 were amplified via polymerase chain reaction (PCR) using gene-specific primers (Supplementary Table S2) and PrimeSTAR® GXL DNA Polymerase (TaKaRa, Tokyo, Japan). The PCR amplicons were resolved by 1.5% (w/v)agarose gel electrophoresis, and the target bands wereexcised and purified using the Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA) for subsequent cloning into expressionvectors.

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2.5. Yeast One-Hybrid (Y1H) Assay

The promoter fragments of MsPSK3 werePCR-amplified and cloned into the pHis2 vector (Clontech Laboratories, Inc., Mountain View, CA, USA) to generate bait constructs. The full-lengthCDSs of MsMYC2 were inserted into the pGADT7 vector (Clontech Laboratories, Inc., Mountain View, CA, USA) to generate prey constructs. The bait and prey constructs were co-transformed intoyeast strain AH109 competent cells using the Yeast Transformation Kit (Clontech Laboratories, Inc., Mountain View, CA, USA). The transformed yeast cells were initiallyplated on SD-Leu/-Trp (SD-2) medium and incubated at 30°C for 2–3 days to select positive transformants. Single colonies were resuspended in 3 μL sterile water, and 3 μL of the suspension was spotted onto SD-His/-Leu/-Trp (SD-3) mediumsupplemented with varying concentrations of 3-amino-1,2,4-triazole (3AT). Yeast growth was monitored after incubation at 30°C for 2–3 days. The p53-His2 + pGADT7-p53 construct served as the positive control, whereas the empty pHis2 + pGADT7 construct was used as the negative control.

2.6. Dual-Luciferase Reporter Assay

Thefull-length CDSs of MsMYC2 were cloned into the pGreenII62-SK vector (Hellens et al., 2005) to generate effector constructs. The promoter fragments of MsPSK3 were amplified and inserted into the pGreenII0800-Luc vector (Hellens et al., 2005) to generate reporter constructs. The effector and reporter constructs were separately introduced into Agrobacterium tumefaciens strain GV3101. The Agrobacterium cultures harboring effector and reporter constructswere mixed at a 1:1 (v/v) ratio and co-infiltrated into distinct regions of Nicotiana benthamiana leaves using a needleless syringe. Following infiltration, the plants were maintained under standard greenhouse conditions (25°C, 16 h light/8 h dark photoperiod) for 24–48 h. Luciferase (LUC) and Renilla luciferase (REN) activities were quantified using the Dual-Luciferase Reporter Assay Kit (Yeasen Biotech Co., Ltd., Shanghai, China), and chemiluminescence signalswere detected using an automatic chemiluminescence imaging system (Tanon 5200, Tanon Science & Technology Co., Ltd., Shanghai, China). The relative LUC activity was calculated as the ratio of LUC to REN activityto normalize for transfection efficiency.

2.7. Heterologous Expression in Arabidopsis and Yeast

MsPSK3-overexpressing (*MsPSK3-OE*) Arabidopsis thaliana (Col-0) and yeast cells were generated. Plants were treated with NaCl + $NaHCO_3$ for salt-alkali stress. Yeast growth was tested on medium containing 0.5 M NaCl plus 15 or 20 mM $NaHCO_3$.

2.8. Physiological Measurements

The contents of H_2O_2, MDA, GSH, and superoxide anion, as well as the activities of POD and SOD, were measured using commercial assay kits. 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.

3. Results

3.1. Growth and Biomass Responses of Alfalfa Seedlings to Salt-Alkali Stress

Salt-alkali (SA) stress significantly inhibited the vegetative growth of all three alfalfa cultivars, with visible reductions in plant height and biomass accumulation compared to the control (CK) group (Figure 1A). Quantitatively, primary root length decreased significantly under SA stress in all cultivars (Figure 1B): YM reduced from 21.3 cm (CK) to 19.0 cm (SA, $p < 0.05$), ZM from 23.0 cm to 19.3 cm ($p < 0.05$), and WL440 from 21.0 cm to 17.7 cm ($p < 0.05$). Similarly, root fresh weight was

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significantly lower under SA stress (Figure 1C), with declines of 30.6% (YM), 35.9% (ZM), and 37.5% (WL440) relative to their respective controls ($p < 0.05$).

For above-ground traits, shoot length was significantly suppressed by SA stress in all three cultivars (Figure 1D): YM decreased from 14.3 cm (CK) to 10.7 cm (SA, $p < 0.05$), ZM from 13.7 cm to 11.7 cm ($p < 0.05$), and WL440 from 13.0 cm to 10.3 cm ($p < 0.05$). Concurrently, shoot fresh weight also showed significant reductions under SA stress (Figure 1E), with the largest relative decrease observed in WL440 (59.4%), followed by ZM (50.0%) and YM (36.4%). Notably, under SA stress, YM maintained the highest values for primary root length, root fresh weight, shoot length, and shoot fresh weight, while WL440 exhibited the lowest performance in most growth parameters. These results collectively indicate that SA stress severely impairs both root and shoot growth in alfalfa, with cultivar-specific differences in tolerance, where YM showed relatively superior salt-alkali tolerance compared to ZM and WL440 at the seedling stage. Considering that Yumu No. 1 is a novel alfalfa germplasm developed by our team and has not been widely cultivated or applied, we selected Zhongmu No. 3 and WL440-HQ for the subsequent experiments.

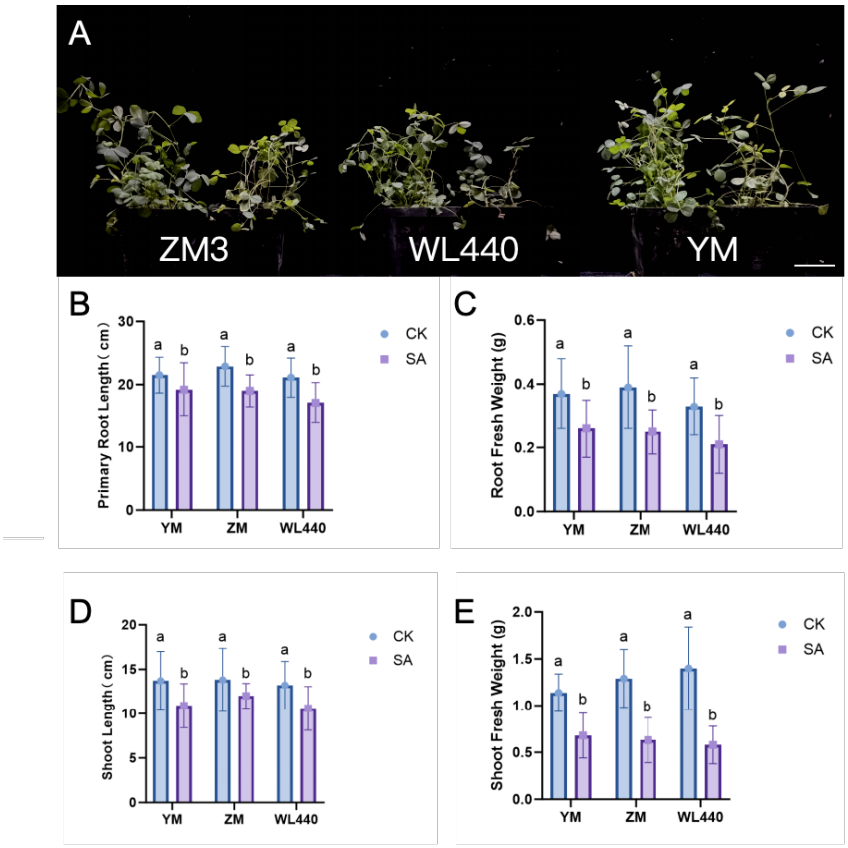

Figure 1. Effects of salt-alkali stress on the growth performance of three alfalfa (*Medicago sativa* L.) cultivars (YM, ZM, and WL440) at the seedling stage. A. Representative phenotypic images of alfalfa seedlings under control (CK) and salt-alkali (SA) stress conditions. Scale bar = 5 cm; B. Primary root length; C. Root fresh weight; D. Shoot length; E. Shoot fresh weight.Values are presented as mean± standard deviation (SD, n = 10). Different lowercase letters indicate significant differences ($p < 0.05$) between CK and SA treatments for the same cultivar.

3.2. Growth, and Physiological Responses of Two Alfalfa Cultivars to Salt Stress

Salt stress significantly inhibited seed germination and early seedling growth in both alfalfa cultivars (Figure 2A-D). Under 200 mM NaCl treatment, the germination rate of ZM3 decreased from 91.7% (CK) to 60.0%, while that of WL440 dropped from 93.3% (CK) to 41.7% (Figure 2B). Consistently, bud length was reduced by 34.9% in ZM3 and 47.5% in WL440 under salt stress (Figure 2C). Similarly, fresh weight declined by 29.6% in ZM3 and 37.0% in WL440 compared with their

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respective controls (Figure 2D). Notably, ZM3 maintained higher germination rate, longer bud length, and greater fresh weight than WL440 under salt stress, indicating superior salt tolerance at the early growth stage.

To evaluate the physiological responses to salt-induced oxidative stress, we measured antioxidant enzyme activities and oxidative stress markers (Figure 2E-I). Under control conditions, WL440 exhibited lower GSH content (32.0 μg/g vs. 36.0 μg/g in ZM3) and MDA content (27.0 nM/g vs. 30.0 nM/g in ZM3) than ZM3 (Figure 2E-F). Upon salt stress, both cultivars showed significant increases in GSH and MDA levels. Specifically, GSH content increased by 66.7% in ZM3 and 121.9% in WL440, while MDA content rose by 133.3% in ZM3 and 107.4% in WL440 (Figure 2E-F). For antioxidant enzymes, POD activity increased in both cultivars under salt stress, with WL440 showing a larger increase (45.0%) than ZM3 (30.0%) (Figure 2G). Superoxide anion content, a marker of reactive oxygen species (ROS) accumulation, was elevated by 40.0% in ZM3 and 90.0% in WL440 under salt stress (Figure 2H). Concurrently, SOD activity increased in both cultivars, with WL440 exhibiting a higher increment (90.5%) than ZM3 (38.5%) (Figure 2I).

Collectively, these data demonstrate that salt stress induces severe growth inhibition and oxidative damage in both alfalfa cultivars. ZM3 outperforms WL440 in maintaining germination and seedling growth under salt conditions, which is associated with lower ROS accumulation and a more efficient basal antioxidant defense system. In contrast, WL440 exhibits a stronger inducible antioxidant response but fails to prevent excessive oxidative damage, resulting in poorer growth performance under salt stress.

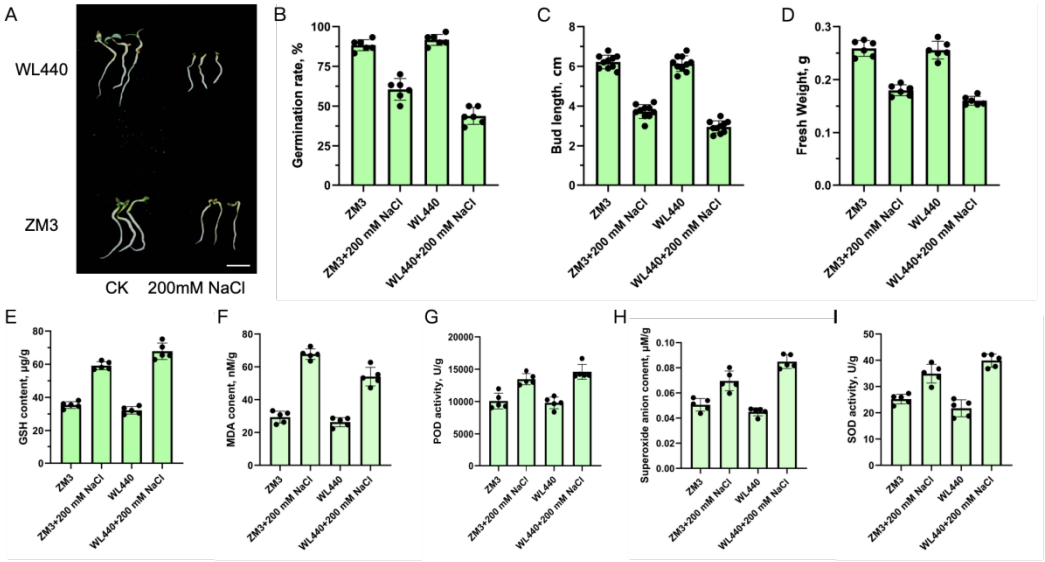

Figure 2. Physiological and biochemical responses of two alfalfa (Medicago sativa L.) cultivars (ZM3 and WL440) to salt stress (200 mM NaCl) at the germination and early seedling stage. A. Phenotypic comparison of seedlings under control (CK) and 200 mM NaCl treatments. Scale bar = 2 cm; B. Germination rate; C. Bud length; D. Fresh weight; E. Glutathione (GSH) content; F. Malondialdehyde (MDA) content; G. Peroxidase (POD) activity; H. Superoxide anion content; I. Superoxide dismutase (SOD) activity.

3.3. Identification of Salt-Alkali Responsive Genes in Alfalfa Seedlings

Transcriptomic profiling was performed on alfalfa seedlings subjected to deionized water (control) and 150 mM NaCltreatment (salt stress). Comparative analysis identified a total of 3,517 differentially expressed genes (DEGs) across both treatment conditions. Among these, 795 genes (22.6% of the total) exhibited conserved differential expression in response to salt stress in both alfalfa cultivars, reflecting shared molecular responses. In addition, 1,336 genes (37.99% of the total) were uniquely differentially expressed in the ZMN (Zhongmu No.3 under NaCl treatment) versus ZMCK

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(Zhongmu No.3 under control)comparison, whereas 1,386 genes (39.41% of the total) were uniquely differentially expressed in the WLN (WL440-HQ under NaCl treatment) versus WLCK (WL440-HQ under control)comparison, indicating cultivar-specific transcriptional reprogramming. Hierarchical clustering analysis grouped genes with similar expression patterns, and volcano plots were constructed to visualizethe magnitude and statistical significance of differential expression (Figure 3A, 3B). Statistical analysis revealed that 780 genes were significantly upregulated in the ZMN versus ZMCK comparison, while 726 genes were upregulated in the WLN versus WLCK comparison (Figure 3C). Venn diagram analysis further delineated the overlap and specificity of DEGs between the two alfalfa cultivars under salt stress conditions (Figure 3D), highlighting both conserved and divergent regulatory mechanisms.

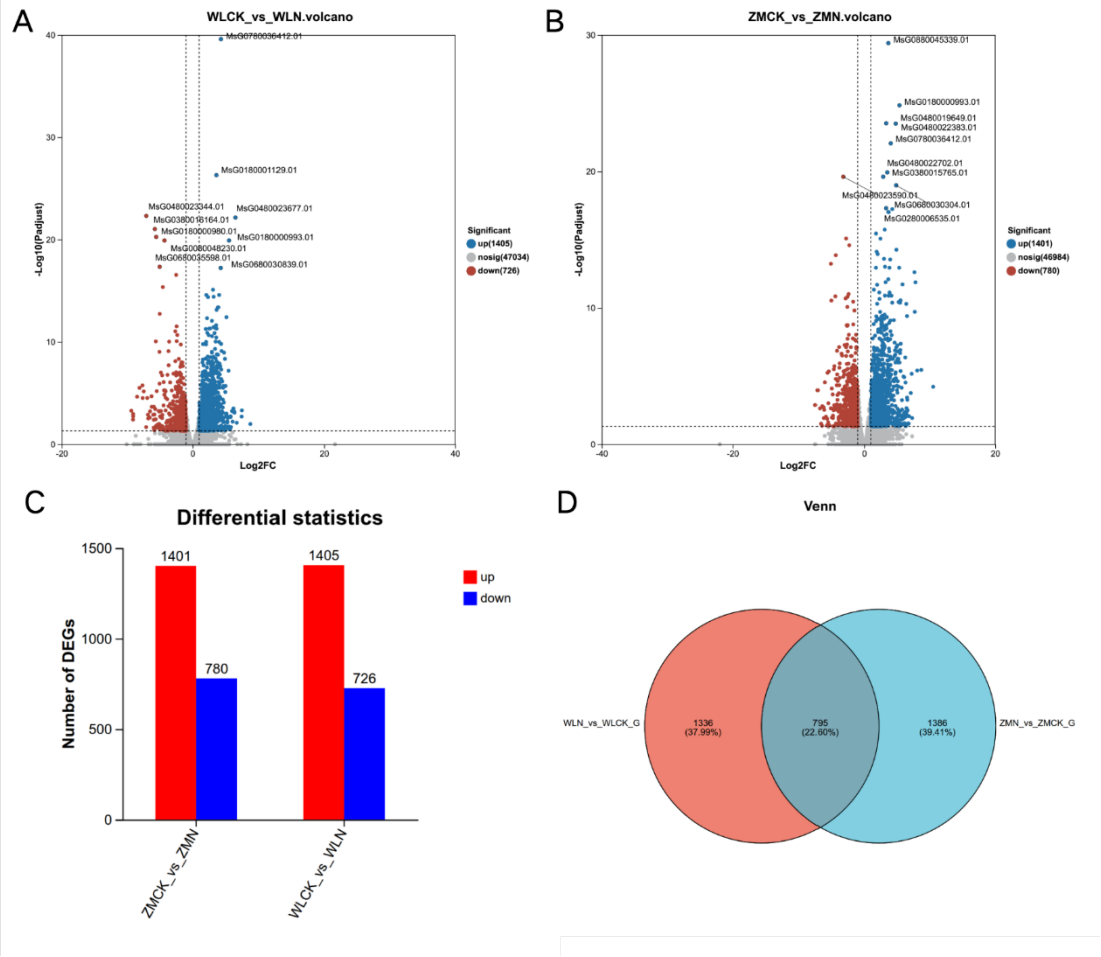

Figure 3. Identification of differentially expressed genes in alfalfa varieties with different salt stress tolerance. A. Identification of DEGs under salt stress in WL440; B. Identification of DEGs under salt stress in Zhongmu No.3; C. Statistics of DEGs; D. Venn diagram analysis of DEGs under salt stress in two alfalfa varieties.

3.4. Functional Annotation and Enrichment Analysis of DEGs

Gene Ontology (GO) annotation classified theidentified DEGs into three principal categories: molecular function(MF), cellular component (CC), and biological process (BP). At the secondary classification level, DEGs were predominantly annotated to binding activity, catalytic activity, cell part, metabolic process, and cellular process (Figure 4A). Notably, the DEGs identified in the two alfalfa cultivars exhibited nearly identical GO annotation distributions. However, within the biological process category, Zhongmu No.3 harbored a greater number of DEGs annotated to response to stimulus, biological regulation, multi-organism process, and cellular process (Figure 4B), suggesting enhanced stress perception and regulatory capacity in this cultivar. GO enrichment analysis indicated that thecommonly identified DEGs in both cultivars were significantly enriched in

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GO terms including membrane, oxidoreductase activity, transporter activity, transmembrane transporter activity, plasma membrane, and small molecule biosynthetic process (Figure 4A). Additionally, terms related to chalcone biosynthetic and metabolic processes, phenylpropanoid biosynthetic and metabolic processes, flavonoid biosynthetic and metabolic processes, as well as chalcone synthase activity and naringenin-chalcone synthase activity, were significantly enriched, suggesting that flavonoid biosynthesis may serve as a pivotal mechanism mediating alfalfa responses to salt-alkali stress.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation revealed that salt-alkali responsive DEGs in both cultivars were predominantly mapped to pathways including carbohydrate metabolism, lipid metabolism, amino acid metabolism, membrane transport, signal transduction, transport and catabolism, and environmental adaptation (Figure 4C). Interestingly, with theexception of membrane transport and transport and catabolism pathways, WL440-HQ exhibited a greater number of DEGs enriched in the aforementioned pathways compared to Zhongmu No.3. KEGG enrichment analysis revealed that DEGs in Zhongmu No.3 were significantly enriched ($P<0.05$)in 16 pathways, whereas those in WL440-HQ were significantly enriched in 20 pathways ($P<0.05$). A total of 10 pathways were commonly andsignificantly enriched in both cultivars (Figure 4D), including isoflavonoid biosynthesis, starch and sucrose metabolism, and ATP-binding cassette (ABC) transporters. Other commonly enriched pathways encompassed plant hormone signal transduction, phenylpropanoid biosynthesis, glutathione metabolism, and galactose metabolism, underscoring the conserved molecular machinery underlying salt stress adaptation in alfalfa.

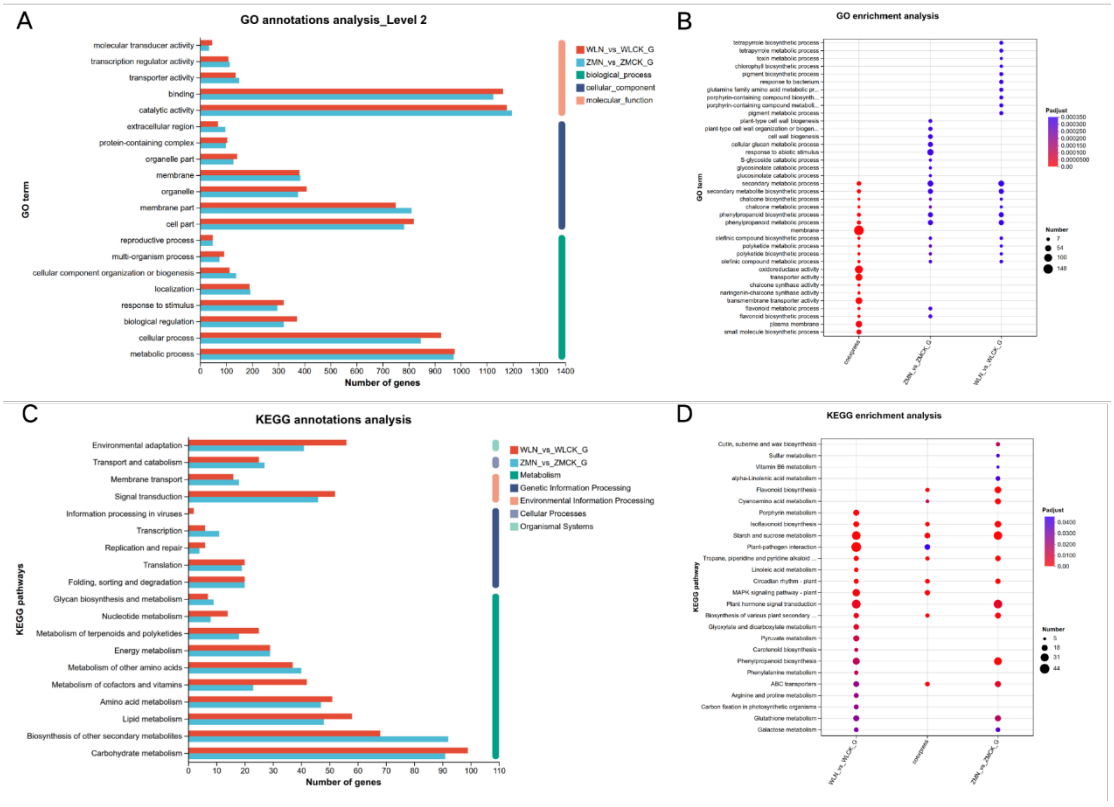

Figure 4. Functional annotation and enrichment analysis of DEGs. A. GO enrichment analysis of DEGs in two alfalfa varieties; B. Level 2 GO annotation analysis of DEGs in two alfalfa varieties; C. KEGG annotation analysis of DEGs in two alfalfa varieties; D. KEGG enrichment analysis of DEGs in two alfalfa varieties.

3.5. Gene Co-Expression Network Analysis

The response of alfalfa to salt-alkali stress is orchestrated by complex multi-gene regulatory networks. In this study, transcriptomic analysis of Zhongmu No.3 and WL440-HQ under salt-alkali treatment identified a large number of DEGs, and Weighted Gene Co-expression Network Analysis

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(WGCNA) was employed to explore the associations between DEGs and physiological traits related to salt-alkali stresstolerance (Figure 5A). A total of 12 co-expression modules were identified from the DEGdataset. Module-trait correlation analysis revealed that gene expression levels in the turquoise modulewere significantly and positively correlated with key phenotypic traits, including germination rate, shoot length, and fresh weight, with correlation coefficients ranging from 0.657 to 0.804. Conversely, gene expression levels in the pink module exhibited significant negative correlationswith the aforementioned traits, with correlation coefficients ranging from 0.702 to 0.832 (Figure 5B). These findings suggest that genes within the turquoise module may function as positive regulators of salt tolerance in alfalfa, whereas those in the pink module may act as negativeregulators or markers of stress-induced damage.

Genes within the blue module displayed significant positive correlationswith malondialdehyde (MDA) and superoxide anion ($O2^-$) levels, indicating potential involvement in reactive oxygen species (ROS) homeostasis and oxidativestress responses. Genes in the green module were significantly positively correlated with peroxidase (POD) activity, whereas genes in the pink module showed positivecorrelations with glutathione (GSH) content, highlighting their roles in antioxidant defense mechanisms. Three key hub genes were identified in the blue module (Figure 5C): MsG0680034750.01 (zinc finger homeodomain protein 11, ZHD11), MsG0580027925.01 (zinc finger homeodomain protein 2, ZHD2), and MsG0480019221.01 (YABBY transcription factor 1, YABBY1). Four key candidate hub genes were identified in the turquoise module (Figure 5D): MsG0580058560.01 (WRKY transcription factor 22, WRKY22), MsG0280007369.01 (WRKY transcription factor 6, WRKY6), MsG0280009717.01 (NAC transcription factor 22, NAC22), and MsG0380015408.01 (SCARECROW-LIKE protein 1,SCL1). Additionally, several key genes implicated in salt-alkali response and ROS scavenging were also identified, including MsG0480023517.01 (phytosulfokine 3, PSK3), MsG0680030908.01 (UDP-glycosyltransferase 2, UGT2), MsG0580024758.01 (peroxidase 51, PER51), and MsG0180004879.01 (glutathione S-transferase F, GSTF), collectively constituting a core regulatory network for salt stress adaptation.

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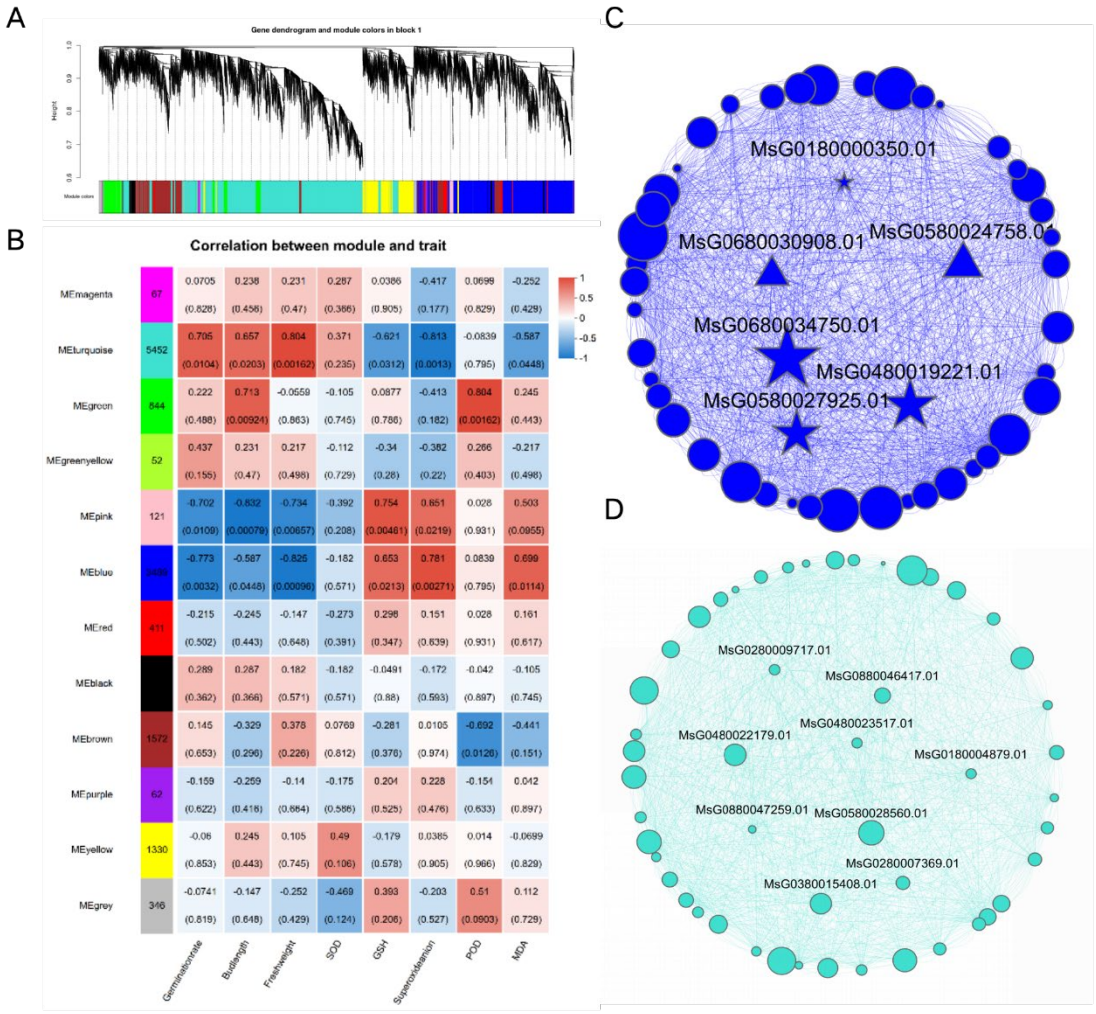

Figure 5. WGCNA identified correlations between salt stress-related physiological indicators and DEGs. A. Module classification tree with colors representing different modules; grey indicates unassigned genes; B. Module-phenotype correlation heatmap showing the relationships between modules and physiological parameters; C. Co-expression network of DEGs in the blue module (MEblue); D. Co-expression network of DEGs in the turquoise module (MEturquoise).

3.6. Overexpression of MsPSK3 Confers Enhanced Salt-Alkali Tolerance in Arabidopsis and Saccharomyces cerevisiae

As a member of the phytosulfokine (PSK) peptide hormone family, PSK3 participates in plant growth and development through phytosulfokine receptor (PSKR)-mediated signaling pathways, while also playing critical roles in defense responses, particularly in conferring tolerance to drought and salt stress (Ma et al., 2025; Nagar et al., 2020; Tang et al., 2024). Notably, WGCNA in this study identified *PSK3* as a key hub gene within salt-alkali stress-responsivemodules, underscoring their potential functional significance in alfalfa salt tolerance. To investigate the functional role of *MsPSK3* in salt-alkali stress tolerance, we generated MsPSK3-overexpressing (*MsPSK3-OE*) Arabidopsis thaliana plants and evaluated their performance under salt-alkali stress. Under normal growth conditions (CK), both wild-type (Col-0) and *MsPSK3-OE* plants exhibited similar growth phenotypes (Figure 6A). However, upon exposure to salt-alkali (SA) stress, Col-0 plants showed severe growth inhibition, leaf chlorosis, and wilting, while *MsPSK3-OE* plants maintained relatively healthier growth with less visible damage (Figure 6A).

To further verify the protective role of MsPSK3 against salt-alkali stress, we performed a bacterial heterologous expression assay. Under control conditions, both empty vector (EV)-transformed and *MsPSK3-OE Saccharomyces cerevisiae* showed robust growth (Figure 6B). In contrast,

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when exposed to media supplemented with 0.5M NaCl plus increasing concentrations of $NaHCO_3$ (15 mM and 20 mM), EV-transformed cells displayed drastically reduced or completely inhibited growth, whereas *MsPSK3-OE Saccharomyces cerevisiae* retained significantly better growth capacity (Figure 6B).

To assess the physiological basis of enhanced tolerance, we measured oxidative stress markers and antioxidant enzyme activities in Arabidopsis plants. Under salt-alkali stress, wild-type plants accumulated significantly higher levels of H_2O_2 (5.0 μmol/g) and MDA (30 nmol/g) compared to control conditions (H_2O_2: 2.1 μmol/g; MDA: 10 nmol/g; $p < 0.05$) (Figure 6C, D). In contrast, MsPSK3-OE plants exhibited substantially lower H_2O_2 (2.6 μmol/g) and MDA (19 nmol/g) contents under stress, indicating reduced oxidative damage ($p < 0.05$) (Figure 6C, D). Concurrently, both POD and SOD activities were significantly higher in *MsPSK3-OE* plants than in wild-type plants under salt-alkali stress (Figure 6E, F). Specifically, POD activity increased to 4000 U/g in *MsPSK3-OE* plants compared to 1200 U/g in wild-type plants ($p < 0.05$), and SOD activity reached 42 U/g in *MsPSK3-OE* plants versus 24 U/g in wild-type plants ($p < 0.05$). These data suggest that *MsPSK3* overexpression alleviates salt-alkali-induced oxidative stress by enhancing antioxidant enzyme activities, thereby reducing reactive oxygen species (ROS) accumulation and lipid peroxidation.

Collectively, these results demonstrate that MsPSK3 overexpression confers enhanced salt-alkali tolerance in both Arabidopsis and bacterial cells, likely through the activation of antioxidant defense systems to mitigate oxidative damage.

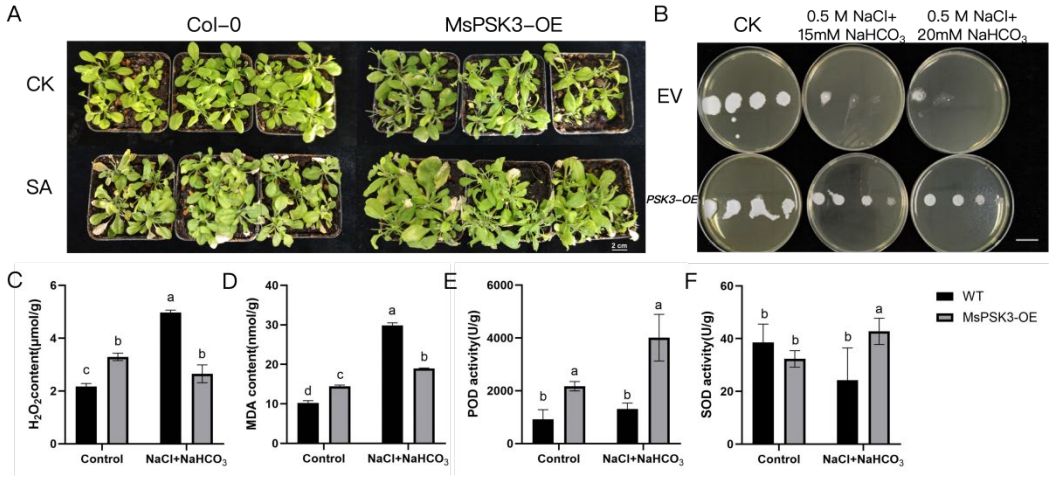

Figure 6. Overexpression of *MsPSK3* enhances salt-alkali stress tolerance in *Arabidopsis thaliana* and *Saccharomyces cerevisiae*. A. Phenotypic comparison of wild-type (Col-0) and *MsPSK3*-overexpressing (*MsPSK3-OE*) Arabidopsis plants under control (CK) and salt-alkali (SA, NaCl + $NaHCO_3$) treatments. Scale bar = 2 cm; B. Growth of empty vector (EV)-transformed and *MsPSK3*-overexpressing (*PSK3-OE*) *Saccharomyces cerevisiae* on LB medium supplemented with 0.5 M NaCl plus 0, 15, or 20 mM $NaHCO_3$. Scale bar = 2 cm; C. Hydrogen peroxide (H_2O_2) content; D. Malondialdehyde (MDA) content; E. Peroxidase (POD) activity; F. Superoxide dismutase (SOD) activity. Values are presented as mean ± standard error (SE, n=3). Different lowercase letters indicate significant differences ($p < 0.05$) between groups.

3.2. Growth, and Physiological Responses of Two Alfalfa Cultivars to Salt Stress

Through systematic analysis of online transcription factor-binding site databases and in-depth mining of our transcriptomic datasets, we identified MsMYC2 as a potential upstream transcription factor targeting MsPSK3, respectively. Previous studies have established that MYC2, a basic helix-loop-helix (bHLH) transcription factor, functions as a negative regulator of salt stress responses in Arabidopsis by directly binding to the 5′ untranslated region (5′UTR) of P5CS1 (delta-1-pyrroline-5-carboxylate synthase 1, a rate-limiting enzyme in proline biosynthesis), thereby repressing proline accumulation. The expression of MYC2 is bidirectionally regulated by the mitogen-activated protein

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kinase (MAPK) cascade pathway. This gene integrates diverseintracellular and extracellular signals to participate in salt stress regulatory networks, and the MYC2-mediated modulation of proline biosynthesis represents a critical node for plants to maintain normal physiological metabolism under salt stress conditions (Verma et al., 2020).

To investigate the transcriptional regulatory mechanism of *MsPSK3*, we first performed a dual-luciferase reporter assay in tobacco leaves. Co-expression of the MYC2 effector with the *proMsPSK3*:LUC reporter resulted in significantly enhanced luminescence intensity compared to the control (Figure 7A). Consistent with the imaging results, quantitative analysis showed that the LUC/REN ratio was significantly higher in the MYC2 co-infiltrated group than in the control ($p < 0.05$, Figure 7B). These data indicate that MYC2 activates the transcription of *MsPSK3* in planta.

To further validate the direct interaction between MYC2 and the *MsPSK3* promoter, a yeast one-hybrid (Y1H) assay was conducted. Under non-selective conditions (0 mM 3AT), both the negative control (PSK3-phis2+AD) and the experimental group (PSK3-phis2+MYC2-AD) showed normal growth on SD/-Leu/-His medium (Figure 7C). However, with increasing concentrations of 3AT (15-80 mM), the negative control yeast cells failed to grow, while the cells co-expressing MYC2-AD and the *MsPSK3* promoter maintained robust growth, even at the highest 3AT concentration (80 mM, Figure 7C). These results confirm that MYC2 directly binds to the promoter of *MsPSK3*.

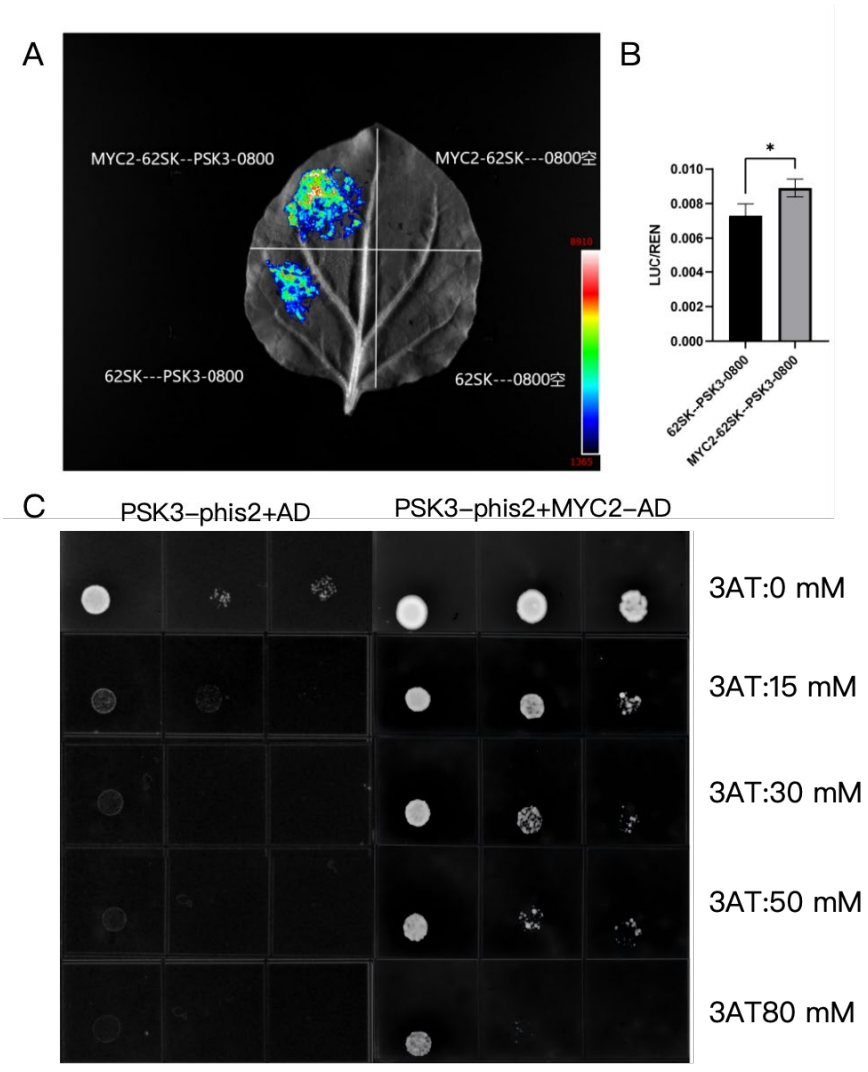

Figure 7. ranscriptional activation and protein interaction analysis of MsPSK3 with transcription factor MYC2. A. Visualization of firefly luciferase (LUC) activity in tobacco leaves after co-infiltration with *proMsPSK3*:LUC reporter and 35S::MYC2 effector constructs. The color scale indicates luminescence intensity (blue to red, low to high); B. Quantitative analysis of LUC/REN ratio in dual-luciferase reporter assay. Values are means+SE (n = 3),

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*$p < 0.05$ (Student's *t*-test); C. Yeast one-hybrid (Y1H) assay verifying the interaction between MYC2 and the MsPSK3 promoter. Yeast cells were grown on selective SD/-Leu/-His medium supplemented with increasing concentrations of 3-amino-1,2,4-triazole (3AT, 0–80 mM). PSK3-phis2 + AD serves as the negative control, while PSK3-phis2 + MYC2-AD represents the experimental group.Theserial dilution factors of the yeast suspension in each channel, from left to right, are 10-fold, 100-fold, and 1,000-fold, respectively.

4. Discussion

4.1. Conserved and Cultivar-Specific Salt Stress Responses

Soil salinity imposes complex transcriptional reprogramming in plants, and the systematic identification of differentially expressed genes (DEGs) constitutes a fundamental step toward elucidating stress adaptation mechanisms (Wang et al., 2019). In this study, a total of 3,517 DEGs were identified in two alfalfa cultivars subjected to salt stress, among which 795 common DEGs reflected conserved molecular responses to salt stress, such as osmotic adjustment, ionic homeostasis, and reactive oxygen species (ROS) scavenging pathways. The identification of cultivar-specific DEGs (1,336 in Zhongmu No.3 and 1,386 in WL440-HQ) underscores divergent molecular strategies between the two cultivarsin coping with salt stress. Notably, Zhongmu No.3 harbored a greater number of DEGs enriched in stimulus response and biological regulation processes, which may contribute to its superior salt tolerance phenotype compared to WL440-HQ, as evidenced by growth-related parameters including germination rate, fresh weight, and root length. These findings are consistent with previous studies demonstrating that cultivar-specific DEGs serve as key determinants of stress tolerance divergence among genotypes (Zhang et al., 2020).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses further elucidated the functional roles of identified DEGs in mediating salt stress responses. The contrasting KEGG enrichment profiles between the salt-tolerant alfalfa cultivar Zhongmu No.3 (ZM) and the salt-sensitive cultivar WL440 (WL) reveal key molecular mechanisms underlying cultivar-specific salt tolerance, with both genotypes sharing conserved stress-responsive pathways but exhibiting divergent activation of specialized adaptive processes. While both cultivars showed significant enrichment in core salt-response pathways including starch and sucrose metabolism, plant hormone signal transduction, phenylpropanoid biosynthesis, and MAPK signaling, which reflecting conserved strategies for osmotic adjustment, signal transduction, and basal oxidative stress mitigation. However, ZM displayed notably stronger and more diverse enrichment in pathways linked to enhanced stress resilience, such as isoflavonoid biosynthesis, cutin/suberine/wax biosynthesis, and glutathione metabolism, whereas WL was uniquely enriched in pathways associated with stress-induced damage, including porphyrin and linoleic acid metabolism. The robust activation of isoflavonoid and secondary metabolite biosynthesis in ZM supports a more potent antioxidant defense and ROS-scavenging system, consistent with its lower MDA and H_2O_2 accumulation under salt stress, while the enrichment of cutin and suberine biosynthesis further suggests enhanced structural barriers that limit Na^+ influx and water loss, complemented by more efficient glutathione-mediated detoxification. In contrast, WL's enrichment of pathways linked to lipid peroxidation and photodamage, alongside its weaker activation of specialized secondary metabolic and structural defense pathways, indicates insufficient stress mitigation, leading to greater oxidative damage and impaired growth under salt conditions. Collectively, these divergent enrichment patterns demonstrate that ZM's superior salt tolerance stems from a multi-layered adaptive strategy integrating enhanced secondary metabolism, structural adaptation, and robust antioxidant defense, whereas WL relies primarily on core stress responses without activating these specialized protective pathways, resulting in greater sensitivity to salt-induced stress.

Soluble sugars accumulated via enhanced starch and sucrose metabolism can effectively reduce cellular osmotic potential, thereby sustaining water uptake and preventing cellular dehydration under saline conditions (Gao et al., 2019). Pathways involved in plant external barrier formation, such as cuticle biosynthesis and cellwall modification, may enhance structural integrity and reduce

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excessive salt ion influx into plant tissues. Fatty acid degradation pathways provide essential energy reserves and metabolic intermediates for the biosynthesis of stress-related metabolites, including antioxidants, osmoprotectants, and signaling molecules. These conserved pathways collectively constitute the molecular foundation of alfalfa salt tolerance, while cultivar-specific pathway enrichment patterns may account for observed differences in tolerance levels between cultivars.

Notably, the tryptophan metabolic pathway, a critical branch of amino acid metabolism intimately linked to plant abiotic stress adaptation, was also implicated in ourenrichment analysis. Within this pathway, two key genes encoding N-acetylserotonin O-methyltransferase (ASMT), namely MsG0480020072.01 and MsG0480020076.01, exhibited significant upregulation under salt stress conditions. ASMT functions as the rate-limiting enzyme catalyzing the terminal step ofmelatonin biosynthesis, converting N-acetylserotonin to melatonin (Arnao & Hernández-Ruiz, 2019). Melatonin, an indoleamine molecule initially identified in animalsystems, has been increasingly recognized as a pivotal regulator of plant abiotic stress tolerance, including responses to salt stress, owing to its multifaceted roles in antioxidant defense, osmotic adjustment, membrane stabilization, and modulation of stress-related gene expression (Li et al., 2022; Wang et al., 2023).

In the specific context of *Medicago sativa*, accumulating experimental evidence supports the functional involvement of melatonin in mediating salt tolerance mechanisms. Li et al. (2022) demonstrated that exogenous melatonin application to alfalfa seedlings subjected to 150 mM NaCl stress significantly enhanced the activities of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), while concomitantly reducing the accumulation of reactive oxygen species (hydrogen peroxide, H_2O_2; superoxide anion, O_2^-) and thelipid peroxidation product malondialdehyde (MDA), thereby markedly improving root elongation, biomass accumulation, and photosynthetic efficiency. Similarly, Wang et al. (2023) reported that melatonin pretreatment upregulated genes involved in proline and soluble sugar biosynthesis in alfalfa, thereby reinforcing osmotic adjustment capacity and mitigating salt-induced cellular dehydration. Although direct functional characterization of MsASMT genes in alfalfa remains limited, studies conducted in closely related legumes and model plant species provide compelling mechanistic insights: overexpression of ASMT homologsin *Glycine max* (soybean) and *Arabidopsis thaliana* resulted in elevated endogenous melatonin levels, enhanced salt tolerance, and improved ion homeostasis through transcriptional upregulation of Na^+/H^+ antiporter genessuch as NHX1 and SOS1 (Zhang et al., 2021; Zhao et al., 2024). The pronounced upregulation of MsASMT genes observed in our study strongly suggests that alfalfa actively promotes endogenous melatonin biosynthesis via the tryptophan metabolic pathway under salt stress conditions, which likely represents a conserved adaptive strategy to mitigate salt-induced oxidative damage, maintain ionic homeostasis, and stabilize cellular osmotic potential—consistent with the concurrent upregulation of genes encoding antioxidant enzymes, osmolyte biosynthetic enzymes, and ion transporters within our DEG dataset.

4.2. Key Co-Expression Modules and Core Genes

Weighted Gene Co-expression Network Analysis (WGCNA) has proven to be a highly effective bioinformatics approach for identifying phenotype-associated gene modules and core regulatory hub genes within complex transcriptomic datasets (Langfelder & Horvath, 2008). The turquoise module, which exhibited significant positive correlationswith growth-related traits such as germination rate, shoot length, and fresh weight, likely harbors genes that actively promote growth maintenance and stress adaptation under salt stress conditions. Core hub genes within this module may be functionally involved in sustaining fundamental cellular processes, including cell division, cell elongation, and photosynthetic efficiency, which are critical for maintaining plant growth and productivity under adverse environmental conditions. Conversely, the blue module, which displayed significant positive correlations with malondialdehyde (MDA) and superoxide anion (O_2^-) levels, is likely enriched in genes regulating reactive oxygen species (ROS) homeostasis and oxidative stress responses.Given that MDA serves as a well-established biomarker of lipid peroxidation and O_2^- represents a primary reactive oxygen species, genes within this module may encode ROS-generating enzymes(e.g.,

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NADPH oxidases) as well as ROS-scavenging enzymes (e.g., superoxide dismutases, SODs) (Zhu, 2016). The green module's significant positivecorrelation with peroxidase (POD) activity suggests its involvement in hydrogen peroxide (H_2O_2) detoxification, as POD constitutes a key enzymatic component of the plant antioxidant defense system. The pink module's positive correlation with glutathione (GSH) content indicates a functional role in non-enzymatic antioxidant defense, given that GSH is a major cellular antioxidant and redox buffer. Collectively, these modules coordinate growth maintenance, ROS homeostasis, and antioxidant defense mechanisms, thereby forming a comprehensive and integrated molecular network underlying alfalfa salt stress responses. Notably, the upregulated MsASMT genes may functionally interact with genes within the blue or green modules (e.g., genes encoding antioxidant enzymes such as SOD, POD, and CAT) to synergistically enhance ROS scavenging capacity, as melatonin has been demonstrated to cooperatewith glutathione and antioxidant enzymes to maintain cellular redox homeostasis under stress conditions (Arnao & Hernández-Ruiz, 2019; Zhao et al., 2024). Additionally, the three MsWRKY33 genes (MsG0380014920.01, MsG0580028560.01, and MsG0480018500.01) identified within the mitogen-activated protein kinase (MAPK) signaling pathway were clustered within stress-responsive modules (e.g., blue or turquoisemodules), implying their function alintegration into the core regulatory network thatlinks upstream signal transduction cascades with downstream stress adaptation mechanisms.

4.3. Regulatory Role of the WRKY33-Centered Signaling Network in Salt Tolerance

The mitogen-activated protein kinase (MAPK) signaling pathway represents an evolutionarily conserved phosphorylation cascade in plants that transduces abiotic stress signals from the plasma membrane to the nucleus, thereby regulating downstream stress-responsive gene expression programs (Zhang et al., 2023). WRKY33, a well-characterized substrate of MAPK kinases (e.g., MPK3and MPK6), is frequently activated via phosphorylation in response to diverse abiotic stresses, includingsalt, drought, and oxidative stress (Mao et al., 2020). In this study, three MsWRKY33 homologs (MsG0380014920.01, MsG0580028560.01, and MsG0480018500.01) were identified within the MAPK signaling pathway and exhibited significant transcriptional upregulation under salt stress conditions. This observation aligns with extensive experimental evidence demonstrating the pivotal role of WRKY33in mediating plant salt tolerance across diverse species. For instance, in Arabidopsis, *wrky33*-knockout mutants exhibit markedly increased salt sensitivity, characterized by elevated reactive oxygen species (ROS) accumulation and impaired ionic homeostasis, whereas WRKY33 overexpression lines display enhanced salt tolerance (Jiang et al., 2021; Zhang et al., 2021). In rice, OsWRKY33 regulates salt tolerance by transcriptionally activating genes involved in abscisic acid (ABA) biosynthesis and ROS detoxification pathways (Chen et al., 2022). In the specific context ofalfalfa, a recent study by Liu et al. (2024) confirmed that MsWRKY33 enhances salt stress tolerance by modulating ROS scavenging capacity through direct transcriptional activation of MsERF5, which is consistent with our observation of MsWRKY33 upregulation and the significant enrichment of ROS-related metabolic pathways in our transcriptomic dataset.

Notably, our transcriptomic profiling also revealed significant upregulation of 1-aminocyclopropane-1-carboxylate oxidase (ACCO), a rate-limiting enzyme catalyzing the terminal step of ethylene biosynthesis, along with three ETHYLENE RESPONSE FACTOR 1 (ERF1) genes (MsG0180003836.01, MsG0180001804.01, and MsG0180004079.01). Ethylene functions as a key phytohormone intricately involved in mediating plant responses to salt stress, orchestrating diverse physiological processes including stomatal closure, root growth modulation, and activation of antioxidant defense systems (Yang et al., 2023). The observed upregulation ofACCO expression suggests enhanced ethylene biosynthesis under salt stress conditions, which may serve as a critical signaling molecule to activate downstream ERF1 transcription. ERF1 belongs to the AP2/ERF transcription factor superfamily, whose members function as direct downstream effectors of ethylene signaling cascades (Xu et al., 2022). Intriguingly, WRKY33 and ERF family transcription factors frequently form synergistic regulatory cascades in orchestrating stress responses: in Arabidopsis,

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WRKY33 directly binds to GCC-box or W-box cis-regulatory elements within the promoters of ERF genes (e.g., ERF5, ERF104) to enhance their transcriptional activity, thereby amplifying stress-responsive geneexpression programs (Mao et al., 2020; Zhang et al., 2021). The coordinated upregulation of MsWRKY33, ACCO, and MsERF1 genes in our study strongly suggests the existence of a potential MAPK-WRKY33-Ethylene-ERF regulatory axis mediating alfalfa salt tolerance: salt stress activates theupstream MAPK signaling cascade,which phosphorylates and activates MsWRKY33; activated MsWRKY33 may dually promote ethylene biosynthesis (via transcriptional regulation of ACCO expression) and directly induce ERF1 gene transcription; ethylene further amplifies ERF1 expressionthrough hormone-mediated signaling, and collectively, these transcription factors synergistically regulate downstream genes encoding ROS-scavengingenzymes (e.g., SOD, POD, CAT), iontransporters maintaining ionic homeostasis (e.g., Na^+/H^+ antiporterssuch as NHX and SOS1), and osmolyte biosynthetic enzymes (e.g., proline synthases, P5CS). This proposed regulatory hypothesis is substantiated by Liu et al. (2024), who experimentallydemonstrated that MsWRKY33 directlyactivates MsERF5 to enhance ROS scavenging capacity in alfalfa, and by Yang et al. (2023), who reported that ethylene-induced ERF1 overexpression improves salt tolerance in *Medicago truncatula* by transcriptionally upregulating antioxidant enzyme genes.

Furthermore, the potential crosstalk between the WRKY33-centered regulatory network and melatonin metabolic pathways warrants particular attention. Zhang et al. (2021) demonstrated that Arabidopsis WRKY33 directly binds to W-box elements within the ASMT promoter to enhance melatonin biosynthesis, and melatonin, in turn, promotes WRKY33 gene expression via a positive feedback regulatory loop, thereby amplifying stress tolerance responses. In our study, the concurrent transcriptional upregulation of MsWRKY33, MsASMT, and genes encoding ROS-scavenging enzymes strongly suggests the existence of a functionally analogous feedback mechanismoperating in alfalfa: MsWRKY33 may promote melatonin biosynthesis by transcriptionally activating MsASMT expression, and the accumulated melatonin may further reinforce MsWRKY33-mediated transcriptional activation of ERF1 genesand antioxidant enzyme genes, thereby forming a multi-layered, self-amplifying regulatory network that robustly enhances salt stresstolerance.This intricate regulatory network integrating signal transduction (MAPK cascade), transcription factors (WRKY33, ERF1), phytohormones (ethylene), and antioxidant metabolites (melatonin) represents a sophisticated molecular strategy evolved by alfalfa to cope with salt-induced osmotic, ionic, and oxidative stresses.

4.4. The MYC2–MsPSK3 Module Positively Regulates Salt–Alkali Tolerance in Alfalfa

The functional validation of the MYC2–MsPSK3 regulatory module in mediating salt-alkali tolerance provides critical mechanistic insights into how leguminous plants integrate hormone signaling and peptide-mediated stress responses to combat environmental adversity. Overexpression of MsPSK3 in both Arabidopsis thaliana and *Saccharomyces cerevisiae* significantly enhanced tolerance to combined NaCl and $NaHCO_3$ stress, as evidenced by healthier plant phenotypes, improved bacterial growth, reduced accumulation of reactive oxygen species (H_2O_2) and lipid peroxidation (MDA), and elevated activities of antioxidant enzymes (POD and SOD), demonstrating that *MsPSK3* acts as a positive regulator of salt–alkali tolerance by reinforcing cellular antioxidant defense and mitigating oxidative damage, consistent with the established roles of phytosulfokines (PSKs) in promoting abiotic stress resilience through the modulation of redox homeostasis and stress signaling pathways (Yang et al., 2022; Ma et al., 2025). Dual-luciferase reporter assays and yeast one-hybrid experiments further confirmed that the transcription factor MYC2 directly binds to the promoter of *MsPSK3* and activates its transcription, revealing a key regulatory link between jasmonic acid (JA) signaling and PSK-mediated stress responses, where MYC2, as a core component of the JA signaling cascade (Chen et al., 2019), transduces stress signals to upregulate *MsPSK3*, thereby triggering downstream antioxidant defense mechanisms to counteract salt–alkali-induced oxidative stress. Collectively, these results suggest a conserved and functionally conserved regulatory module in which MYC2-mediated activation of *MsPSK3* enhances salt-alkali tolerance in alfalfa by orchestrating

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the antioxidant system to alleviate oxidative damage, providing novel molecular targets for breeding stress-tolerant forage crops and expanding our understanding of the crosstalk between hormone signaling and peptide-mediated stress responses in plants.

5. Conclusion

This study systematicallyelucidates the conserved and cultivar-specific molecular responsemechanisms of two alfalfa cultivars to salt stress, identifying a total of 3,517 differentially expressed genes (DEGs) and establishing a key regulatory modules, MsMYC2-MsPSK3, which play pivotal roles in mediating salt tolerance. Conserved DEGs were significantly enriched in pathways involved in osmotic adjustment, ion homeostasis, and reactive oxygen species (ROS) scavenging, whereas cultivar-specific DEGs contributed to tolerance divergence, with Zhongmu No.3 exhibiting enhanced stress response capacity through enrichment of stimulus-related DEGs. Melatonin biosynthesis via the tryptophan metabolicpathway, mediated by upregulated ASMT genes, represents a conserved adaptive strategy employed by alfalfa to mitigate salt-induced oxidative damage and maintain cellular redox homeostasis. The MsWRKY33-centered regulatory network, intricately interacting with ethylene signaling and melatonin metabolism, orchestrates a multi-layered molecular framework that coordinates ROS detoxification, ionic homeostasis, and osmotic adjustment mechanisms. These findings unveil the molecular architecture underlying alfalfa salt tolerance and identify core candidate genes, including MsWRKY33, MsMYC2, and MsASMT, for targeted genetic improvement. Future functional validation of these genes and systematic dissection of their regulatory networks will substantially accelerate the development of salt-tolerant alfalfa cultivars through precision molecular breeding strategies, thereby alleviating salinization-induced yield losses and enhancing forage production sustainability in salt-affected agricultural ecosystems.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author: Contributions: R.Y., Y.Z., C.W., Y.S., X.Z., D.L., H.Y., Y.C. and H.S. Conceptualization the idea and methodology; H.S. developed the plant material; R.Y., Y.Z., Y.Z., H.Y., Y.C. and H.S. participated in growth characterization; R.Y., Y.Z. expresses the data collection, analysis, and writing - original draft manuscript; Y.C., H.S. and H.Y. acquired funding for the study and supervision during the experiments. All authors have read and agreed to the published version of the manuscript.

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