

Integrated physiological and transcriptomic analysis revealed key genes and pathways related to continuous drought and salinity stress in *Populus*

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Research Article

Keywords: *Populus*, RNA-Seq, Salt stress, Drought stress, WGCNA

Posted Date: December 11th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-8274641/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Plant Cell Reports on March 13th, 2026.

See the published version at <https://doi.org/10.1007/s00299-026-03757-1>.

Abstract

Drought and salt stress were major abiotic factors that severely inhibited plant growth and productivity. To elucidate the molecular and genetic basis of variation in drought and salinity tolerance in *Populus*, we integrated physiological and transcriptomic analyses to investigate the response of a hybrid poplar ((*Populus simonii* × *P. nigra*) × *P. ussuriensis*) to long-term drought and salt stress, followed by a recovery phase. Physiologically, drought stress induced delayed photosynthetic inhibition primarily via non-stomatal limitations, accompanied by sustained accumulation of proline and malondialdehyde (MDA), and high peroxidase (POD) activity even after rewatering. In contrast, salt stress caused rapid stomatal closure, leading to immediate photosynthetic decline. Notably, physiological recovery from salt stress was faster than from drought. Transcriptome sequencing identified 18,860 differentially expressed genes (DEGs). Time-course analyses revealed that drought stress prioritized activation of cell wall biogenesis (e.g., cutin, suberin, and lignin biosynthesis) and UDP-glucosyltransferase activity. Salt stress, however, immediately activated genes for ion transporters involved in vacuolar sequestration and the jasmonic acid signaling pathway. In addition, weighted gene co-expression network analysis (WGCNA) identified stress-specific modules and hub genes. In summary, this study could provide valuable insight for clarifying the physiological responses and molecular mechanisms of poplar in response to drought and salt stress.

Key Messages

Integrated physiological and time-series transcriptomic analyses in poplar demonstrate drought prioritizes cell wall remodeling, while salt stress immediately activates ion transporters and JA signaling, defining distinct molecular adaptation mechanisms.

Introduction

Abiotic stresses, particularly drought and salinity, are primary factors limiting plant growth and yield productivity in the face of ongoing climate change. Currently, the frequency and duration of drought events are increasing due to irregular rainfall and global warming, exacerbating widespread plant mortality and leading to significant disruptions in the ecosystem (Gebrechorkos et al. 2025). The arid and semi-arid regions in China accounted for approximately 52.5% of the national territory. In these regions, the survival and retention rates of afforestation efforts were only around 30% in semi-arid areas and ranged from 4% to 20% in arid regions (Hou et al. 2021). Concurrently, more than 954 million hectares of land throughout the world were affected by salt, exceeding 6.5% of the world's total land area (Wang et al. 2021a), with saline-alkali conditions affecting plant growth in 7% of the total global land area and 33% of irrigated land (Chele et al. 2021). In China, the total area of saline-alkali soil exceeded $9.91 \times 10^7 \text{ hm}^2$, accounting for about 10% of the land area (Zhang et al. 2023a). More seriously, the area of saline-alkali land in the world increased year by year, which seriously hindered the sustainable development of the economy and ecology. Predictions suggested that by 2050, drought and salinity will lead to the severe salinization of over 50% of arable land, posing a significant threat to the global

ecological environment (Zhang et al. 2023b). Consequently, breeders needed to develop germplasm with enhanced tolerance to both abiotic and biotic stresses to cope with these escalating environmental challenges (Cooper & Messina 2023). This necessitated a comprehensive understanding of the underlying physiological and molecular mechanisms of salinity and drought tolerance in plants.

Drought and salt stress were two of the most important abiotic stress factors that suppress plant growth (Yuan et al. 2023). Drought stress could induce cellular dehydration, leading to reduced cell enlargement and division, manifesting as decreased plant height and biomass (Misra et al. 2020). Firstly, roots sensed water deficit, triggering abscisic acid accumulation. Then ABA promoted stomatal closure to minimize transpirational water loss, but restricted CO₂ uptake, suppressing photosynthesis (Takahashi et al. 2008). This suppression was initially due to stomatal limitation under mild drought, shifting to non-stomatal limitations (reduced chlorophyll content, impaired PSII photochemical efficiency, decreased photosynthetic enzyme activity) under severe stress (Yang et al. 2023). Morphologically, plants adapted via reduced leaf area, increased leaf thickness, altered mesophyll structure (increased palisade tissue, decreased spongy tissue), and prioritized root growth for deeper water access (Gowda et al. 2011). Biochemically, accumulation of osmo-protectants like proline and glycine betaine occurred for osmotic adjustment and ROS scavenging, alongside activation of antioxidant enzymes (SOD, CAT, POD), although SOD activity declined under severe/long-term stress (Yang et al. 2021a). Salinity stress imposed hyperosmotic stress similar to drought, but added the specific burden of Na⁺-induced ionic stress (Zhang et al. 2022). Excessive Na⁺ disrupted K⁺/Na⁺ homeostasis, caused ion toxicity, damaged membranes, and impaired metabolic functions like protein synthesis (Yang & Guo 2018). Critically, both stresses triggered overlapping physiological and molecular responses in plants, centered around osmotic stress, ionic imbalance, and oxidative damage, ultimately leading to growth inhibition.

Plants had evolved complex strategies to cope with drought, categorized as escape, avoidance, and tolerance (Zhang et al. 2020). Drought escape involved shortening the life cycle, often through accelerated flowering (Du et al. 2018). Avoidance strategies focused on minimizing water loss (e.g., via stomatal regulation, leaf structural adjustments) and maximizing water uptake (e.g., via root architecture optimization) (Feng et al. 2022). Tolerance involved cellular adjustments like the production of antioxidants, osmo-protectants, and chaperones to mitigate damage, driven by substantial transcriptional reprogramming. Salt stress response involved a biphasic mechanism: an initial osmotic phase causing water deficit similar to drought, followed by a late ionic phase characterized by Na⁺ and Cl⁻ accumulation leading to ion toxicity and oxidative stress (Guo et al. 2024). Both osmotic and ionic stresses disrupted cellular homeostasis, inhibited growth, and triggered excessive reactive oxygen species (ROS) production, causing oxidative damage such as chlorophyll degradation (Yang & Qin 2023). At the molecular level, responses involved the regulation of numerous genes and pathways related to stress perception, signal transduction (including key second messengers like ROS, Ca²⁺, and protein phosphorylation), ion transport (especially Na⁺/K⁺ balance), osmotic adjustment, hormone synthesis (notably ABA under water deficit), and metabolism (Zheng et al. 2015). Meanwhile, many drought-inducible genes were also induced by high salinity (Singh & Laxmi 2015). Significant cross-talk existed between drought and salt stress responses, as evidenced by the overlap in inducible genes.

Populus was both economically and ecologically valuable (Zhao et al. 2015). Currently, the widely cultivated poplar varieties used in forestry were predominantly developed through artificial crossbreeding. Hybrid poplars, prized for rapid growth and superior wood quality, exhibited heterosis (hybrid vigor) that often enhanced their resilience to environmental stresses. Crossbreeding thus played a crucial role in breeding new poplar varieties, both now and in future advancements. However, in arid, semi-arid, and salinized regions, the growth and spatial distribution of poplar were still seriously affected by drought and salinity stresses (Ding et al. 2024). Therefore, the objective of this study was to investigate the physiological and transcriptomic alterations in hybrid poplar under drought and salt stress. Specifically, we aimed to identify core sets of genes and pathways responsive to each stress type, detect associations between physiological and transcriptomic responses, and suggest key transcription factors and regulatory networks for future functional validation aimed at improving poplar performance in multi-stress environments.

Materials and Methods

Plant materials and experimental design

The superior clone 105 was used as the experimental material for this study, which was obtained through hybridization with *Populus simonii* × *P. nigra* as the female parent and *P. ussuriensis* as the male parent. The seedlings were placed in a greenhouse at Northeast Forestry University, where they received natural light and humidity. Grown for 60 days, the seedlings of uniform size with robust growth were selected and randomly divided into three groups. The first group received normal irrigation with tap water (control: CK), the second group experienced continuous drought conditions (drought stress: DS), and the third group was treated with a 150 mM NaCl solution (salt stress: SS). All seedlings were thoroughly watered before stress treatments, with the CK and SS groups receiving daily watering at consistent amounts. After 10 days of stress treatment, rehydration was conducted. The growth traits, photosynthetic parameters, and physiological indices of seedlings under different treatments were measured and analyzed at 0 d, 3 d, 5 d, 10 d, 11 d, and 13 d. The time points of 11 d and 13 d corresponded to 1 d and 3 d after rehydration, respectively. Three biological replicates were set for each treatment.

Phenotyping, photosynthetic, and physiological measurements

The tree height (H) was measured using a tower ruler, and three seedlings in each group were randomly selected. The 5th to 7th leaf from the top of each plant was used for measuring photosynthetic parameters using a portable photosynthetic instrument (Yaxin-1102G, Beijing Yaxinlily Science and Technology Company, China), including net photosynthetic rate (P_n), transpiration rate (Tr), stomatal conductance (G_s), and intercellular CO_2 concentration (C_i). The assimilatory chamber was controlled to maintain the leaf temperature, CO_2 concentration, and photosynthetic photon-flux density at 25 ± 1 °C, $400 \mu mol \cdot mol^{-1}$, and $1400 \mu mol \cdot m^{-2} \cdot s^{-1}$, respectively. The 8th to 10th leaves were used for

physiological indices analysis. The detection kits (Jiangsu Aidisheng Biological Technology Co., Ltd, China) were used to determine and analyze the levels of superoxide dismutase activity (SOD), peroxidase activity (POD), malondialdehyde content (MDA), and proline content (Pro) in leaves in accordance with the guidelines provided by the manufacturer. There were 3 trees were randomly selected from each treatment, and each indicator included three technical replicates.

RNA-sequencing

The RNA-seq libraries of 33 samples were constructed using 3 biological replicates at 0 d, 3 d, 5 d, 10 d, 11 d, and 13 d after drought and salt treatment and rehydration, including CK (0d), DS3, DS5, DS10, DS11, DS13, SS3, SS5, SS10, SS11, and SS13, and the sequencing was performed on the Illumina sequencing platform by Metware Biotechnology Co., Ltd. (Wuhan, China). After removing the low-quality reads, adapter sequences, and poly-N sequences from each read, the clean reads were mapped to the *P. trichocarpa* genome v.4.1 (https://phytozome-next.jgi.doe.gov/info/Ptrichocarpa_v4_1) using HISAT2 software (Kim et al. 2015)

Identifying differentially expressed genes

The fragments per kilobase of transcript per million mapped reads (FPKM) value was calculated to compare the expression differences of genes among samples. Differentially expressed genes (DEGs) were identified by DESeq2 software (Love et al. 2014), and genes with the false discovery rate (FDR) < 0.05 and $|\log_2\text{Fold Change}| \geq 1$ were considered as DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to annotate the biological functions of DEGs, those pathways with $\text{FDR} \leq 0.05$ were defined as significantly enriched pathways. GO, KEGG, principal component analysis (PCA), and weighted gene co-expression network analysis (WGCNA) were performed using the online platform (<https://cloud.metware.cn/>). Cytoscape (v3.9.1) software was used to visualize the co-expression networks (Doncheva et al. 2019). All of the DEG protein sequences were uploaded to PlantTFDB (<https://planttfdb.gao-lab.org/>) for TF analysis. The sequencing data were submitted to NCBI under the accession number (PRJNA1344462).

RNA extraction and qRT-PCR

To validate the RNA-seq results, the same RNA-seq samples were used for qRT-PCR analysis. Total RNA extraction and cDNA synthesis were performed using a RNeasy Plant Mini Kit. (BioTeke, Beijing) and PrimeScript™ RT reagent Kit with gDNA Eraser kit (TaKaRa). The quality of the purified RNA was determined using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The quality and quantity of the constructed libraries were assessed using an Agilent 2100 Bioanalyzer and an ABI StepOnePlus Real-Time PCR System. qRT-PCR was performed in triplicate on an Agilent Mx3000P Real-Time PCR System using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TaKaRa). The relative expression levels of

nine common DEGs were calculated using the $2^{-\Delta\Delta C_t}$ method, with internal control of Actin gene. Primer details were shown in Table S1.

Statistical analysis

The statistical analysis of the data was conducted using the SPSS software version 25.0. The statistical significance between different treatments was determined according to Tukey-Kramer test. P-values were used to estimate significance ($P < 0.05$), and different letters indicated significant differences among the various treatments.

Results

Growth, photosynthetic, and physiological responses to drought and salt stresses

To investigate the effects of prolonged drought and salt stress on the growth of *Populus*, this study measured and analyzed various growth, photosynthetic, and physiological parameters under different durations of stress. The results indicated that the leaves exhibited obvious wilting under 10 days of drought stress, which immediately recovered to their original state upon rewatering (Fig. 1a). There was no significant difference in seedling height between the stressed groups and the control group after 5 days of stress. After 10 days of drought stress (DS), the height was significantly lower than that of the control group (CK), with the difference becoming more significant after 10 days of stress (Fig. 1b). After 3 and 5 days of DS, there were minor changes in net photosynthetic rate (Pn), transpiration rate (Tr), and stomatal conductance (Gs) of the leaves. However, a significant reduction was observed after 10 days of DS. After rehydration, Pn, Tr, and Gs all gradually increase. The intercellular CO₂ concentration (Ci) in the leaves showed a slight decrease in response to the stress (Fig. 2a-d). In contrast, salt stress significantly decreased Pn, Tr, and Gs of the leaves after 3 days, but these parameters increased after 10 days of stress, while following rehydration, Pn, Tr, and Gs exhibited a slight decline. However, the intercellular concentration of carbon dioxide showed a slight decrease (Fig. 2a-d). This study simultaneously measured the physiological parameters of leaves under different stress treatments and rehydration conditions. The results showed that after 5 days of drought stress, the SOD activity in the leaves significantly increased, but decreased after 10 days of stress. Drought stress significantly increased the POD activity and proline content in the leaves, which also significantly increased after rehydration. Drought stress significantly increased the MDA content, but this content decreased after rehydration (Fig. 2e-h). On the other hand, the SOD activity in the leaves significantly increased after 5 days of salt stress, remaining at a high level even 1 day after rehydration (DS11), but decreasing after 3 days of rehydration (DS13). Following 3 days of salt stress, the POD activity in the leaves increased significantly, but decreased after 5 and 10 days of stress; a similar trend was observed for POD activity after rehydration as for SOD activity. Similarly, salt stress significantly increased the MDA and Pro content in the leaves, with these levels decreasing after rehydration (Fig. 2i-l).

Illumina sequencing and de novo assembly

To comprehensively explore the underlying mechanisms of *Populus* in response to salt and drought stress, transcriptome sequencing data from 33 samples, including continuous salt and drought stress for 0 d (CK), 3 d, 5 d, 10 d, and 11 d (1 d after rehydration), 13 d (3 d after rehydration). The 33 samples that were treated with drought and salt gave raw reads that ranged from 41.19 million to 85.18 million, with an average number of reads per sample of 54.40 million. The average size of the clean data for each sample was 7.97 Gb. The Q20 and Q30 ranged from 98.25% to 98.90% and from 93.21% to 95.88%, respectively. The percentage of GC ranged from 43.43% to 44.75%. (Table S2). About 83.65%~ 87.06% of the sequencing data for each sample were mapped to the *P. trichocarpa* genome (v4.1), indicating that the quality of transcriptome data was relatively high for bioinformatics analysis. Drought stress identified 33262 genes, salt stress identified 33103 genes, a total of 33685 genes were identified, of which 1714 were novel. Principal component analysis (PCA) was carried out among different treatments, for drought stresses, PCA explained 30.30% and 20.30% of the variances for PCA1 and PCA2, respectively (Fig. 3a), PC1 and PC2 explained 31.39% and 16.43% of the variance for salt stresses (Fig. 3b), respectively, indicating significant experimental repeatability and differentiation.

Analysis of differentially expressed genes (DEGs)

To primarily explore the molecular mechanisms underlying continuous drought and salt tolerance, differentially expressed genes at different time points were analyzed. From the perspective of drought stress analysis, compared to the control (CK), there were 2195, 1703, 3748, 4122, and 5659 DEGs after 3 d, 5 d, 10 d, 11 d, and 13 d of stress, respectively (Fig. 4a). Among these DEGs, there were 215 common differential genes across different treatments. 455 DEGs were commonly regulated during the drought stress period (DS3, DS5, and DS10). After rehydration, there were 1135 commonly regulated DEGs (DS11 vs DS10 and DS13 vs DS10) (Fig. S1a). Similarly, there were 3614, 2524, 5764, 2741, and 6776 DEGs after 3d, 5d, 10d, 11d, and 13d of salt stress, respectively (Fig. 4b). Among these DEGs, there were 456 common differential genes across different treatments. 2436, 1567, and 2848 genes were upregulated, and 1178, 957, and 2916 genes were downregulated at SS3, SS5, and SS10, respectively, compared with CK. There were 907 DEGs commonly regulated during the salt stress period (SS3, SS5, and SS10). After rehydration, there were 998 commonly regulated DEGs (SS11 vs SS10 and SS13 vs SS10) (Fig. S1b). Based on the same stress treatment time, this study found that under the stress conditions of 3, 5, 10, 11, and 13 d (compared to CK), the common DEGs were 1459, 1013, 1456, 1092, and 3160, respectively. Among these, the most abundant DEGs that were commonly regulated were found after 13 days of drought stress and salt stress.

This study also analyzed the number of relative DEGs under continuous drought and salt stress (Fig. 4d). The number of significantly differentially expressed genes was the smallest in DS5 vs DS3, the number of up-regulated and down-regulated genes was 239 and 402. Similarly, SS5 vs SS3 also had fewer differential genes, with 505 up-regulated and 999 down-regulated, respectively. Significantly differentially expressed genes were most abundant in DS10 vs DS13, and SS10 vs SS5, and SS10 vs SS11. Based on

the same stress treatment duration, this study revealed that after 3, 5, 10, 11, and 13 days of stress, there were 934, 652, 3400, 2435, and 2257 upregulated DEGs between the two stress conditions, and the downregulated DEGs were 414, 510, 3661, 1909, and 2365, respectively. Among these, 115 common DEGs were identified (Fig. 4c).

Functional annotation and GO classification

In this study, a total of 18860 DEGs were identified, with 12992 identified under drought stress and rehydration, and 15016 identified under salt stress and rehydration. To further elucidate the response mechanisms of poplar to drought and salt stress, the potential biological functions of the DEGs were analyzed using the GO database (Table S3). The GO enrichment analysis revealed that 46 and 44 GO terms were identified in drought stress and salt stress, respectively, including 22 terms of biological process (BP), 2 terms of cellular component (CC), and 22 terms of molecular function (MF) in drought stress. There were 22 terms of BP, 2 terms of CC, and 20 terms of MF in salt stress, respectively. For both stresses, most DEGs were enriched in cellular process and metabolic process in the BP category, cellular anatomical entity and protein-containing complex in the CC category, and binding and catalytic activity in the MF category. The difference between the GO enrichment results for the two types of stress was that under drought stress, there were two enriched terms, namely molecular sequestering activity and RNA folding chaperone.

To gain further insights into the time-dependent biological processes, GO enrichment analysis was performed on the differentially expressed genes at each time point. The gene annotations were ranked in descending order based on the number of associated genes, and the top 10 significantly enriched GO terms were selected for each of the three categories: BP, CC, and MF (Fig. 5). Comparative analysis of the GO enrichment results for drought stress (DS) and salt stress (SS) revealed that both conditions share common response mechanisms at these three levels, while also exhibiting significant differences. In the BP category (Fig. 5a), both stress conditions were associated with the GO terms GO:0009698 (phenylpropanoid metabolic process), GO:0006720 (isoprenoid metabolic process), GO:0044550 (secondary metabolite biosynthetic process), and GO:0008299 (isoprenoid biosynthetic process). Additionally, GO:0006721 (terpenoid metabolic process) was presented in DS3, DS5, SS3, and SS5. Furthermore, GO:0042546 (cell wall biogenesis) and GO:0071669 (plant-type cell wall organization or biogenesis) were commonly detected in DS5, DS10, and SS3. However, with prolonged exposure to stress, drought stress gradually enriched GO:0042546 and GO:0071669, while salt stress demonstrated an opposite trend, instead enriching GO:0009753 (response to jasmonic acid) over time. Notably, a significant difference in enrichment results was observed between drought stress and salt stress after 10 days of exposure. In the CC category (Fig. 5b), GO:0031225 (anchored component of membrane), GO:0009505 (plant-type cell wall), GO:0031226 (intrinsic component of plasma membrane), and GO:0099503 (secretory vesicle) were consistently identified in both stress conditions, with anchored component of membrane (GO:0031225) notably showing significant enrichment across all time points for DS and SS. Additionally, GO:0031977 (thylakoid lumen), GO:0009543 (chloroplast thylakoid lumen), and GO:0031978 (plastid thylakoid lumen) were enriched in DS3, DS5, and SS5. However, as stress

exposure continued, drought stress exhibited a gradual decrease in the enrichment of GO:0009521 (photosystem), while salt stress displayed an increasing enrichment of this term. Moreover, GO:0009505 was present in all drought stress conditions and only in SS3, whereas GO:0009705 (plant-type vacuole membrane) exhibited the opposite pattern, being exclusive to DS10 and all salt stress conditions, with GO:0005774 (vacuolar membrane) being unique to salt stress. In the MF category (Fig. 5c), GO:0038023 (signaling receptor activity), GO:0052689 (carboxylic ester hydrolase activity), GO:0015291 (secondary active transmembrane transporter activity), and GO:0046527 (glucosyltransferase activity) were enriched in both DS and SS. GO:0016835 (carbon-oxygen lyase activity) and GO:0004857 (enzyme inhibitor activity) showed enrichment in DS3, DS5, and SS3. Over extended periods of stress exposure, GO:0015267 (channel activity) gradually became enriched in both stress conditions, while GO:0008289 (lipid binding) was enriched across all salt stress conditions and specifically in DS3. Furthermore, GO:0035251 (UDP-glucosyltransferase activity) was exclusively enriched in drought stress (DS5 and DS10), while GO:0015399 (primary active transmembrane transporter activity) was exclusive to salt stress.

Under rehydration conditions, the enrichment patterns of GO:0044264 (cellular polysaccharide metabolic process) and GO:0042546 (cell wall biogenesis) were consistent, both being enriched in DS11 and SS13. Moreover, GO:0012501 (programmed cell death), GO:0044550 (secondary metabolite biosynthetic process), and GO:0009698 (phenylpropanoid metabolic process) were enriched in DS13, SS11, and SS13, while GO:0071669 (plant-type cell wall organization or biogenesis) and GO:0016052 (carbohydrate catabolic process) were enriched in DS11, DS13, and SS13. In the CC category, GO:0009505 (plant-type cell wall), GO:0031225 (anchored component of membrane), GO:0031226 (intrinsic component of plasma membrane), and GO:0046658 (anchored component of plasma membrane) were the terms with the highest abundance across all rehydration conditions. Regardless of DS or SS treatment, the enrichment of GO:0005887 (integral component of plasma membrane) and GO:0031012 (extracellular matrix) was mainly observed one day after rehydration, while GO:0022626 (cytosolic ribosome) and GO:0005874 (microtubule) were enriched three days after rehydration. About the MF category, there was a major enrichment in the following three functions: GO:0038023 (signaling receptor activity), GO:0046527 (glucosyltransferase activity), GO:0052689 (carboxylic ester hydrolase activity), and GO:0004888 (transmembrane signaling receptor activity). GO:0042803 (protein homodimerization activity) and GO:0005507 (copper ion binding) were enriched three days after rehydration (DS13 and SS13). GO:0008194 (UDP-glycosyltransferase activity), GO:0015267 (channel activity), and GO:0022803 (passive transmembrane transporter activity) were collectively enriched in DS11 and SS13, while GO:0015399 (primary active transmembrane transporter activity) was exclusively enriched in SS13.

In conclusion, compared to the control, differentially expressed genes at different time points under drought stress and salt stress were predominantly enriched in GO:0009698, GO:0044550, GO:0031225, GO:0009505, and GO:0031226. GO:0035251 was exclusively enriched under drought stress, while GO:0005774 and GO:0015399 were specifically enriched under salt stress.

KEGG pathway enrichment analysis

To identify core pathways involved in stress responses, we performed KEGG pathway annotation on 12992 DEGs under drought stress and rehydration, as well as 15016 DEGs under salt stress and rehydration. The results indicated that both groups were mainly concentrated in cellular processes, environmental information processing, genetic information processing, metabolism, and organismal systems. There were 24 and 13 KEGG pathways significantly enriched in response to drought and salt stress, respectively (Table S4). Among the commonly enriched pathways were ko04626 (plant-pathogen interaction), ko04075 (plant hormone signal transduction), ko04016 (MAPK signaling pathway-plant), ko00052 (galactose metabolism), ko00196 (photosynthesis-antenna proteins), ko00950 (isoquinoline alkaloid biosynthesis), ko02010 (ABC transporters), ko00053 (ascorbate and aldarate metabolism), and ko00051 (fructose and mannose metabolism).

To elucidate the temporal specificity of pathways in plants responding to drought and salt stress, we conducted KEGG enrichment analysis of DEGs at each stress time point separately, observing the temporal dynamics of the pathways, and analyzing the significantly enriched pathways ($P < 0.5$). Significant differences in metabolic pathway characteristics were observed across different treatments (Table S4). DS5 vs CK exhibited the most pronounced differences, encompassing 27 significant enriched pathways, while DS13 vs CK was only associated with 9 enriched pathways. Notably, the pathways ko01100 (metabolic pathways), ko02010, and ko04626 displayed significant enrichment as core regulatory pathways across 8 treatments. Additionally, ko00904 (diterpenoid biosynthesis), ko00905 (brassinosteroid biosynthesis), and ko00950 were consistently enriched in 7 treatments, suggesting a close association with plant stress responses and rehydration recovery mechanisms. Further analysis revealed that ko00073 (cutin, suberine, and wax biosynthesis), ko00910 (nitrogen metabolism), ko01110 (biosynthesis of secondary metabolites), ko04016, and ko04075 were commonly present in 6 treatments. Interestingly, ko00910 was significantly enriched during the 3, 5, and 10-day phases of drought and salt stress treatments, though no activation was detected during rehydration. Similar characteristics were observed in ko00350 (tyrosine metabolism), ko00380 (tryptophan metabolism), ko00592 (alpha-linolenic acid metabolism), and ko00945 (stilbenoid, diarylheptanoid and gingerol biosynthesis). Meanwhile, ko00053 exhibited a biphasic response, being significantly enriched in the DS3, DS5, SS3 vs CK, and DS13, SS13 vs CK treatment groups. ko00500 (starch and sucrose metabolism), ko00940 (phenylpropanoid biosynthesis), and ko00941 (flavonoid biosynthesis) were continuously activated throughout the drought-rehydration process, with ko00500 specifically enriched in SS13 vs CK and ko00940 in SS3 vs CK. The activation of ko00941 and ko00906 (biosynthesis of various plant secondary metabolites) was strictly limited to drought stress and rehydration processes. Furthermore, a treatment-specific pathway was identified; for instance, ko00942 (anthocyanin biosynthesis) was significantly enriched only in the DS3 vs CK, while ko00999 (biosynthesis of various plant secondary metabolites) was detected as a specific response pathway in DS5 vs CK. Moreover, 16 common enriched pathways were identified in both DS3 vs CK and SS3 vs CK, whereas 13, 7, 7, and 5 shared enriched pathways were detected at 5, 10, 11, and 13 days of drought and salt stress, respectively. This study also performed KEGG enrichment analysis of DEGs between different treatments

at the same time points. The results indicated that the pathways ko00052, ko01100, ko04016, and ko04075 were commonly enriched at 3, 5, 10, and 11 days of stress.

Differentially expressed transcription factors during drought and salt stresses

In this study, a total of 2754 transcription factors associated with drought stress were identified, including 188 MYBs, 157 AP2/ERFs, 154 bHLHs, 139 NACs, 118 C₂H₂-type transcription factors, and 98 WRKYs (Fig. S2a). In addition, a total of 2732 salt stress-related transcription factors were identified, including 187 MYBs, 155 AP2/ERFs, 152 bHLHs, 138 NACs, 117 C₂H₂-type transcription factors, and 98 WRKY genes (Fig. S2b). In this study, differential gene analysis was conducted on members of the bHLH, ERF, MYB, and NAC gene families. Under drought stress, a total of 51 bHLH, 71 ERF, 75 MYB, and 63 NAC genes exhibited differential expression at least at one time point. Clustering analysis of their expression patterns was performed, and the heatmap results revealed that genes within these four families could be classified into 4, 4, 4, and 5 clusters, respectively (Fig. 6). Under salt stress, 54 bHLH, 72 ERF, 69 MYB, and 64 NAC genes were identified, and similar clustering analysis showed that these gene families could be divided into 5, 4, 3, and 5 clusters, respectively (Fig. S3). Interestingly, during the early stage of drought stress, most genes exhibited low expression levels. However, they showed significant upregulation after 10 and 11 days of drought stress (DS10 and DS11), particularly among ERF and NAC genes. Conversely, under salt stress, most genes displayed high expression levels during the initial phase of stress, with significant upregulation observed at 3- and 5-day post-treatment, followed by a marked decline in expression at 10 days.

Co-expression networks in response to drought and salt stresses

WGCNA was used to construct a gene co-expression network to identify the key molecular mechanism and identify novel functional genes involved in the response to drought and salt stress at different time points. In this study, all detected genes were subjected to filtering, and only genes with an average FPKM value greater than 1 across samples were used for WGCNA analysis. After screening the raw data, 22645 genes and 22573 genes for drought stress and salt stress were retained to conduct WGCNA, and 18 and 25 distinct co-expression modules were obtained, respectively (Fig. 7). Under drought stress, module-trait correlation analysis identified five key modules exhibiting significant and specific associations with distinct time points. Specifically, the midnightblue, grey60, red, turquoise, and brown modules showed significant positive correlations with drought stress exposure at 3, 5, 10, 11, and 13 days, respectively. DS3, DS5, DS10, DS11, and DS13 contained 132, 73, 1564, 4394, and 2999 genes, respectively. Similarly, under salt stress, the red, lightgreen, darkred, pink, and yellow modules demonstrated significant positive correlations with salt stress exposure at 3, 5, 10, 11, and 13 days, respectively. SS3, SS5, SS10, SS11, and SS13 contained 1279, 146, 71, 547, and 2109 genes, respectively.

Subsequently, GO enrichment analyses were performed on the genes within the ten significant modules associated with drought and salt stress. The GO enrichment analysis revealed that 102, 98, 227, 422, and 524 significant GO terms were identified in DS3, DS5, DS10, DS11, and DS13, respectively (Fig. 8, Table S5), and 266, 115, 96, 137, and 261 significant GO terms were identified in SS3, SS5, SS10, SS11, and SS13, respectively (Fig. S4, Table S5). At the early drought stress response phase (DS3), GO enrichment revealed significant activation of jasmonate-mediated defense (GO:2000022, GO:0009753, GO:0009867, GO:0071395, and GO:0031347), ion homeostasis (GO:0006816, GO:0015250, GO:0022836, GO:0005261, and GO:0005216), and secondary metabolism (GO:0009808, GO:0009809, GO:0009698, and GO:0009699). At the mid-term drought stress adaptation phase (DS5), the most genes showed significant GO enrichment in protein homeostasis (GO:0006457, GO:0071824, GO:0034620, and GO:0042026) and heat shock protein binding (GO:0031072, GO:0051879, and GO:0030544). At the long-term drought stress tolerance phase (DS10), genes were enriched in hypoxia response (GO:0001666 and GO:0071456), ROS detoxification (GO:0036293, GO:0070482, GO:0036294, and GO:0071453), ethylene (GO:0009873, GO:0071369, and GO:0009723) and salicylic acid pathways (GO:0009751 and GO:0009696). At the rewatering recovery phase, the genes were initially enriched in protein catabolic process (GO:0043632, GO:0019941, GO:0006511, GO:0043161, and GO:0010498) and autophagic mechanism (GO:0006914, GO:0061919, GO:0061136, and GO:1905037). After rehydration for 3 days, the genes were enriched in ribosome biogenesis (GO:0022613, GO:0042254, GO:0034660, GO:0005840, GO:0044391, and GO:0022626) and mitochondrial translation (GO:0005740, GO:0031966, GO:0005743, GO:0007005, GO:0070585, GO:0072655, and GO:0098798). However, for salt stress, most of the genes were mainly enriched in carbohydrate biosynthetic process (GO:0009698, GO:0034637, GO:0016051, GO:0045492, GO:0009699, GO:0009808, and GO:0009809), stress signaling and defense (GO:0009867, GO:2000022, GO:0009788, and GO:0009611, GO:0031347), and ion homeostasis (GO:0055062 and GO:0055081) at SS3. At SS5, the genes were enriched in light system regulation (GO:0010218, GO:0071482, GO:0009644, and GO:0007602), oxidative stress (GO:0000302, GO:0036294, GO:0071453, GO:0070482, and GO:0036293), and G-protein signaling (GO:0007188, GO:0062197, and GO:0007186). At SS10, the genes were enriched in meristem development (GO:0048508, GO:0010072, GO:0048507, GO:0090421, and GO:0010014), ion homeostasis (GO:0055081 and GO:0000041), and hormonal crosstalk (GO:0071215, GO:0009738, and GO:0009735). After rehydration, the genes were enriched in phosphatase activity (GO:0080163, GO:0043666, GO:0010921, and GO:0004864) and germination and development (GO:0010029, GO:1900140, GO:0009911, and GO:0051094) at SS11. After rehydration for 3 days, the genes were enriched in cell wall remodeling (GO:0042546, GO:0045488, GO:0044036, GO:0010393, GO:0010383, and GO:0044038), ribosome biogenesis (GO:0042255, GO:0042254, GO:0005840, and GO:0044391), and energy production (GO:0042775, GO:0006839, GO:0005743, GO:0042775, GO:0042773, and GO:0006757).

Additionally, the top 20 genes with the highest connectivity degree within each of the ten modules were identified as hub genes (Fig. 9, Fig. S5). Among them, there were 3 (Tify), 2 (1 HD-Zip and 1 HSF), 4 (1 NAC, 2 ERF, and 1 LOB), and 2 (1 HD-Zip and 1 NAC) transcription factors were identified in DS3, DS5, DS10, DS11, and there were 2 (1 NAC and 1 MADS-MICK), 6 (2 HSF, 3 ERF and 1 WRKY), and 1 (WRKY)

transcription factors were identified in SS3, SS5, and SS10, respectively. None of the hub genes in DS13, SS11, and SS13 were transcription factors. Finally, the expression patterns of the identified hub TFs were graphically presented in the heatmap (Fig. S6).

To validate the reliability of transcriptome analysis data, 10 common DEGs related to drought stress and salt stress were verified using qRT-PCR. The results of qRT-PCR were generally consistent with the RNA-seq, which confirmed the authenticity of the DEGs in this study (Fig. S7).

Discussion

Plants were frequently exposed to various abiotic and biotic stresses, which might occur simultaneously or sequentially (Li et al. 2024a). Among these, drought and salt stress, despite their distinct origins, both could induce cellular dehydration, osmotic imbalance, and associated oxidative stress, involving complex molecular regulatory mechanisms (Zhang et al. 2017). In this study, the physiological and molecular responses of *Populus* to drought and salt stresses were investigated, aiming to elucidate the molecular regulatory networks underlying their stress tolerance.

Divergent physiological strategies underpin drought and salt stress adaptation

In this study, poplar trees exhibited distinctly different physiological responses under long-term drought and salt stress. Although both stresses ultimately inhibited growth (as reflected in reduced height), drought led to delayed photosynthetic inhibition, primarily manifested as a slight decrease in C_i at 10 days (DS10), which was attributed to non-stomatal limitation. Previous studies had indicated that stomatal limitation was the main factor reducing photosynthetic rate under mild drought, whereas non-stomatal factors were the main reason for the decline of the photosynthetic rate under severe drought conditions, which was similar to the results of this study (Flexas et al. 2004). In contrast, salt stress caused growth inhibition mainly through rapid stomatal closure at SS3 (stomatal limitation), leading to significant decreases in $P_n/Tr/G_s$ at 3 days. Salt stress triggers rapid stomatal closure (within minutes) to reduce water loss and maintain plant growth, albeit at the cost of lowered photosynthesis (Sirault et al. 2009). Notably, proline contents in leaves continuously accumulated over time under drought stress, indicating osmotic adjustment as an adaptive response to water deficit. Meanwhile, MDA content increased significantly, reflecting progressive membrane damage. POD activity also markedly increased, contributing to scavenging excess H_2O_2 (Per et al. 2017). One day after rewatering (DS11), proline and MDA levels remained elevated with no signs of recovery, and POD activity persisted at high levels, suggesting ongoing reactive oxygen species (ROS) generation and active scavenging mechanisms (Liang et al. 2023). Under salt stress, proline content and SOD activity peaked at SS11 but declined sharply three days after rewatering (SS13), with MDA content returning to near-control levels. This indicated that physiological recovery from salt stress occurred more rapidly than from drought stress, likely due to more efficient ion homeostasis regulation. Interestingly, POD activity increased significantly

at SS3 but showed an abnormal decrease at SS5, coinciding with elevated SOD levels, suggesting a transient imbalance in the antioxidant enzyme network during early stages of salt stress.

Temporal regulation of gene expression during stress response and recovery

This study utilized transcriptomic sequencing to systematically analyze temporal responses of a novel hybrid poplar genotype under drought and salt stress. A total of 33 transcriptome libraries were constructed, identifying 33,685 genes, among which 18,860 were differentially expressed (DEGs) in at least one time point under either stress. Notably, more DEGs were identified under salt stress than drought at comparable time points, likely due to ion homeostasis disruption caused by salt (Chen et al. 2021). During rewatering, the number of DEGs under salt stress was lower than under drought stress, suggesting that rehydration rapidly mitigated ionic toxicity. In contrast, drought appeared to cause more severe physical damage, requiring extensive transcriptional reprogramming for recovery—evidenced by enrichment of terms such as GO:0042546 and GO:0071669 at DS5 and DS10. Three days after rewatering, the number of DEGs peaked under both stress conditions, likely reflecting the transition from stress alleviation to active repair, metabolic reactivation, and growth recovery (Wang et al. 2022a). These processes involve highly coordinated biological responses, requiring substantial differential gene regulation.

At the gene expression level, drought and salt stress shared common enriched pathways such as phenylpropanoid biosynthesis (GO:0009698), terpenoid metabolism (GO:0006720), lignin biosynthesis (GO:0044550), and other metabolic processes related to structural reinforcement and stress adaptation—consistent with reported drought-induced lignin accumulation (Xie et al. 2024; Li et al. 2022). Molecular functions, including anchored component of membrane (GO:0031225), chloroplast envelope (GO:0009505), intrinsic component of plasma membrane (GO:0031226), and clathrin-coated endocytic vesicle (GO:0099503), were also significantly enriched, indicating their roles in stress regulation. However, distinct regulatory strategies emerged in the stress response. Drought stress rapidly activated terpenoid metabolism (GO:0006720) and cell wall biogenesis (GO:0042546), while salt stress immediately activated jasmonate signaling (GO:0009753) and ion transporter activity (GO:0015399). As stresses progressed, these differences became more pronounced: drought enhanced cell wall biosynthesis (GO:0042546/GO:0071669) and UDP-glucosyltransferase activity (GO:0035251), promoting lignin deposition and osmolyte synthesis for water retention (Yan et al. 2021). Salt stress, on the other hand, immediately activated vacuolar ion transport (GO:0005774/GO:0015399) and jasmonic acid response pathways (GO:0009753), highlighting a reliance on ion compartmentalization and hormone-mediated signaling (Yu et al. 2020). KEGG analysis further supported these findings: salt stress specifically activated flavonoid biosynthesis (ko00941) for ROS scavenging, while drought preferentially enriched cuticle and suberin biosynthesis (ko00073) to reduce water loss (Ayaz et al. 2021).

During rewatering, functions related to cell wall remodeling (GO:0042546/GO:0071669) and carbohydrate catabolism (GO:0016052) remained active, indicating ongoing physical repair. Notably,

there was significant enrichment of programmed cell death (GO:0012501) in the late rewatering stage (13 days post-treatment), suggesting that stresses induced substantial accumulation of ROS within plants, thereby triggering programmed cell death processes. Subsequently, plants facilitated tissue regeneration by eliminating irreversibly damaged cells (Zhang et al. 2023c). Furthermore, ascorbate metabolism (ko00053) remained active during this phase, implying continued oxidative damage repair. In contrast, nitrogen metabolism pathways (ko00910) were active during the stress phase but downregulated during rehydration, indicating a shift in nitrogen allocation toward regeneration (Li et al. 2024b). Differential recovery mechanisms were also observed: drought exclusively activated autophagy (GO:0006914) for damage clearance, while salt stress induced phosphatases (GO:0080163) to remodel energy metabolism and ion homeostasis.

The dynamic response mode of transcription factors

Transcription factors played crucial roles in both drought and salt stress responses. In this study, we analyzed the dynamic expression of four key TF families: bHLH, ERF, MYB, and NAC. Under drought stress, most ERF and NAC genes were lowly expressed initially but significantly upregulated after 10–11 days (DS10 and DS11), consistent with previous studies in *Populus alba* × *Populus glandulosa* where *PagERF162B* and *PagERF28A* were strongly induced (Zeng et al. 2023). In contrast, bHLH and MYB genes showed variable expression patterns. Under salt stress, most TFs were highly expressed early (SS3 and SS5) but declined by SS10, similar to the observation in *Populus tomentosa* (Han et al. 2024). After rewatering, their expression gradually increased. Numerous studies had also characterized the response patterns of the bHLH and MYB gene families in poplar under drought and salt stress conditions, elucidating their roles in stress adaptation and regulatory networks (Zhang et al. 2024; Yang et al. 2021b). These findings revealed distinct dynamic regulation patterns among different transcription factor families in response to drought and salt stress, providing valuable insights into the complex mechanisms underlying plant stress responses.

Using WGCNA, we identified modules associated with each stress period and their hub genes, which included 19 transcription factors: 5 ERFs, 3 NACs, 3 Tifys, 2 WRKYs, 2 HD-Zips, 2 HSFs, 1 LOB, and 1 MADS-box gene. Among these, *Potri.006G226800* (*PtrHsfA2*) was a hub gene in both DS5 and SS5. Previous studies indicated that *PtrHsfA2* co-expression networks were enriched in temperature stimulus response, ROS detoxification, and abiotic stress response (Zhao et al. 2023). *PsnHSF14* from *P. simonii* × *P. nigra* had also been implicated in salt tolerance (Wang et al. 2021b). *Potri.018G038100* (*ERF194*) was significantly upregulated at DS10. Previous studies had demonstrated that *ERF194* could modulate drought tolerance via ABA signaling pathways and ROS scavenging (Wang et al. 2022b).

Conclusion

In conclusion, this study demonstrated that *Populus* employed divergent molecular and physiological strategies to mitigate drought and salt stress. While both stresses triggered shared responses related to osmotic adjustment and oxidative defense, drought tolerance primarily relied on enhanced cell wall biogenesis and late-phase transcriptional reprogramming, whereas salt adaptation depended on early

ion transport and hormonal signaling. This finding could provide valuable insights into the mechanisms of abiotic stress adaptation in woody plants and offer a theoretical foundation for the development of stress-resilient forestry varieties.

Declarations

Author Contributions

Heng Zhang: Writing – original draft, Methodology, Funding acquisition, Investigation, Formal analysis, Data curation. Meng Wang: Methodology, Investigation, Formal analysis. Xizhuo Xing: Data curation, Conceptualization. Dong Zeng: Supervision, Software. Xuanchen Liu: Supervision. Zhanqi Ren: Supervision. Shuo Yu: Validation, Conceptualization. Hongfei Liu: Writing – review & editing. Songjia Yu: Writing – review & editing. Chenguang Zhou: Methodology, Investigation, Formal analysis, Data curation, Conceptualization. Guanzheng Qu: Writing – review & editing, Funding acquisition, Conceptualization.

Acknowledgments

This work was supported by Biological Breeding-National Science and Technology Major Project (2023ZD0405601) and “the Fundamental Research Funds for the Central Universities, (No. 2572022AW02)”.

Declaration of Competing Interest

The authors declared no conflicts of interest.

Data Availability

The data supporting the findings of this work are available from the supporting information or upon request.

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Figures

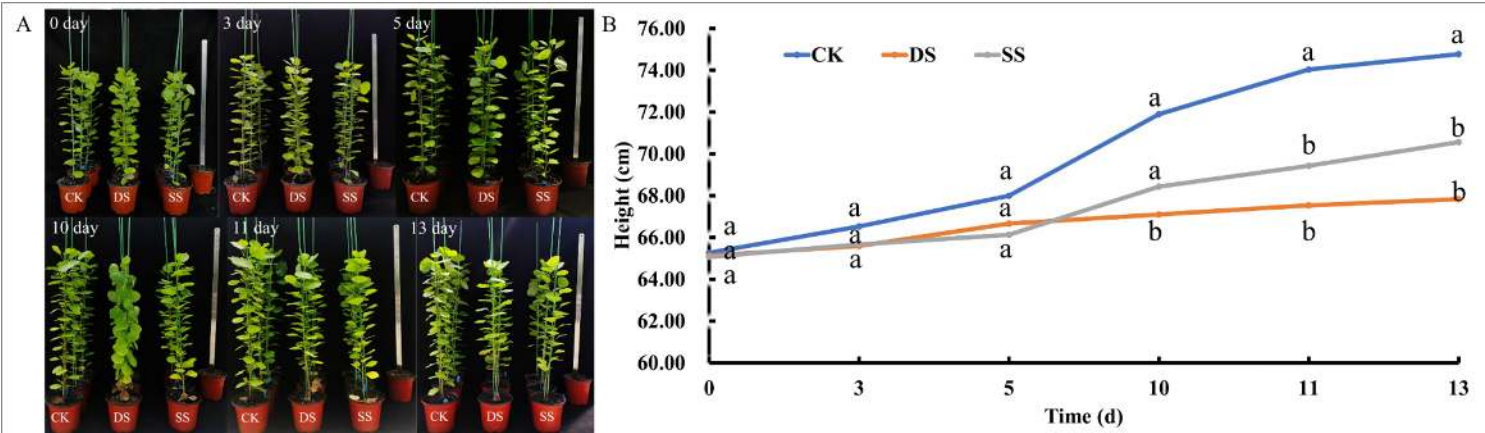


Figure 1

The growth phenotypic characteristics under drought and salt stress. (a) Growth phenotype (b) Height. Different letters above bars represent significant differences at $P < 0.05$.

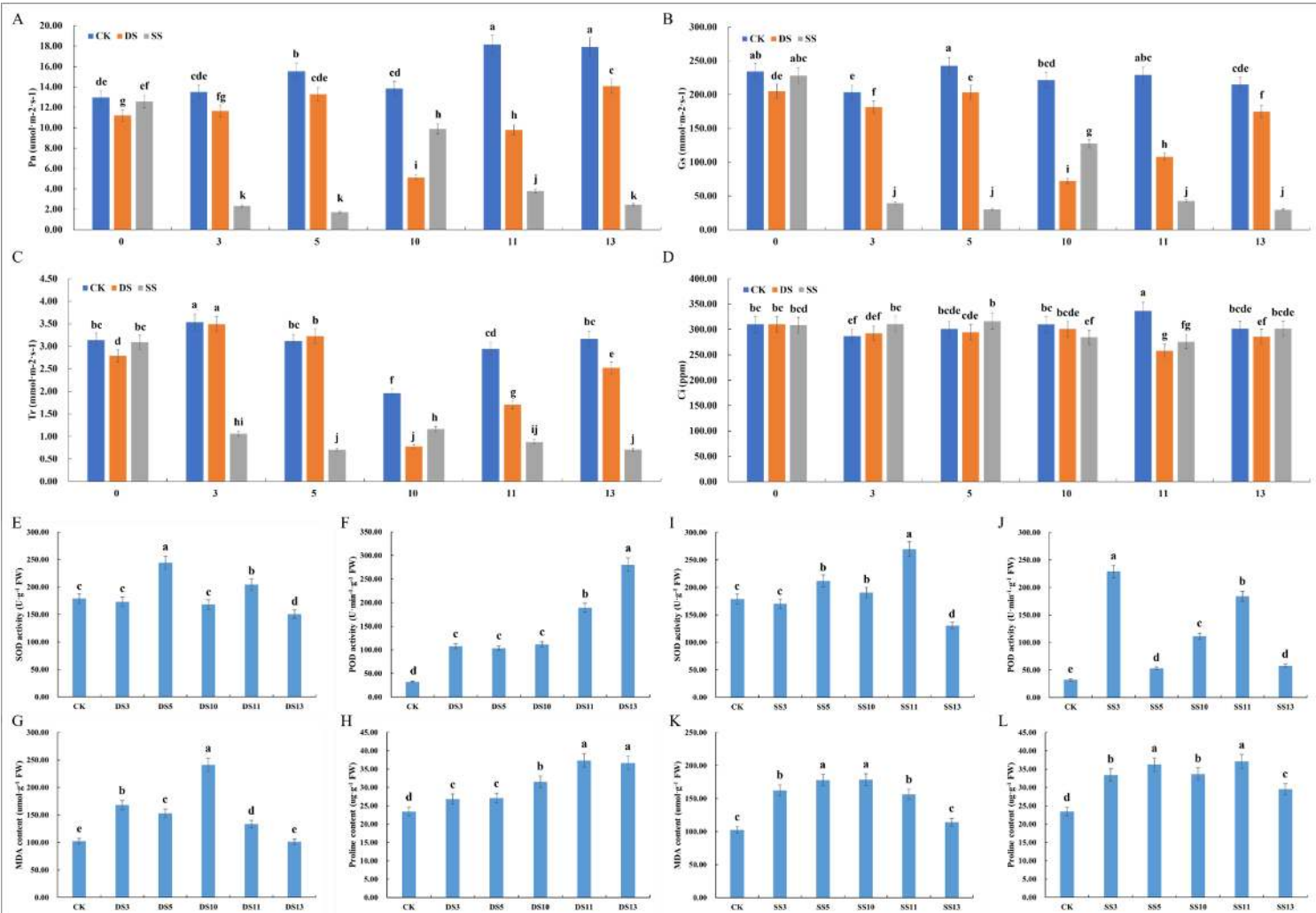


Figure 2

The photosynthetic and physiological parameters under drought and salt stress. (a~d) Photosynthetic indexes including net photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO₂ concentration (Ci). (e~h) Physiological parameters including superoxide dismutase activity (SOD), peroxidase activity (POD), malondialdehyde content (MDA), and proline content (Pro) under drought stress. (i~l) Physiological parameters under salt stress. Different letters above bars represent significant differences at $P < 0.05$.

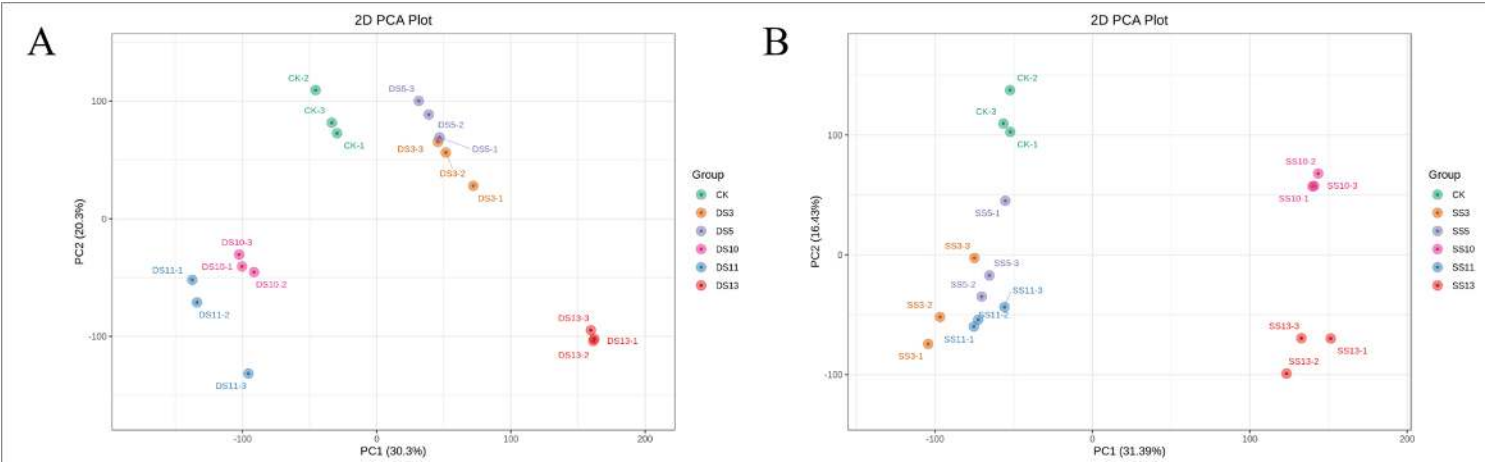


Figure 3

Principal component analysis (PCA) of the two stresses at various time points. (a) Drought stress (b) Salt stress.

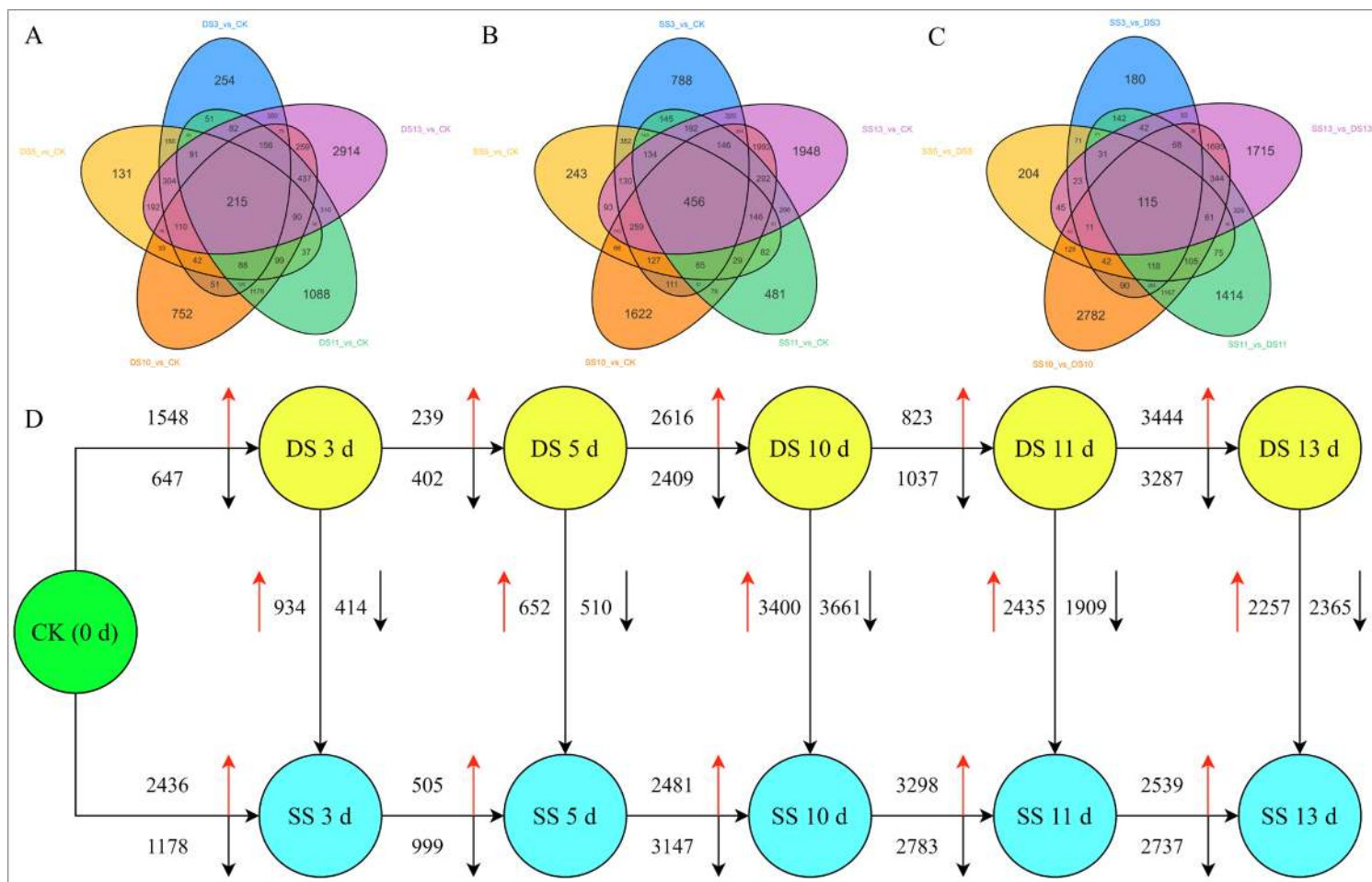


Figure 4

Venn diagrams and the number of DEGs were counted for the same period of both stresses at various time points. The numbers of upregulated and downregulated genes were marked in red and black, respectively. DEGs, differentially expressed genes. (a) Venn diagram among drought stresses (b) Venn diagram among salt stress (c) Venn diagram between the two stresses at the same time points (d) The number of relative DEGs under continuous drought and salt stress.

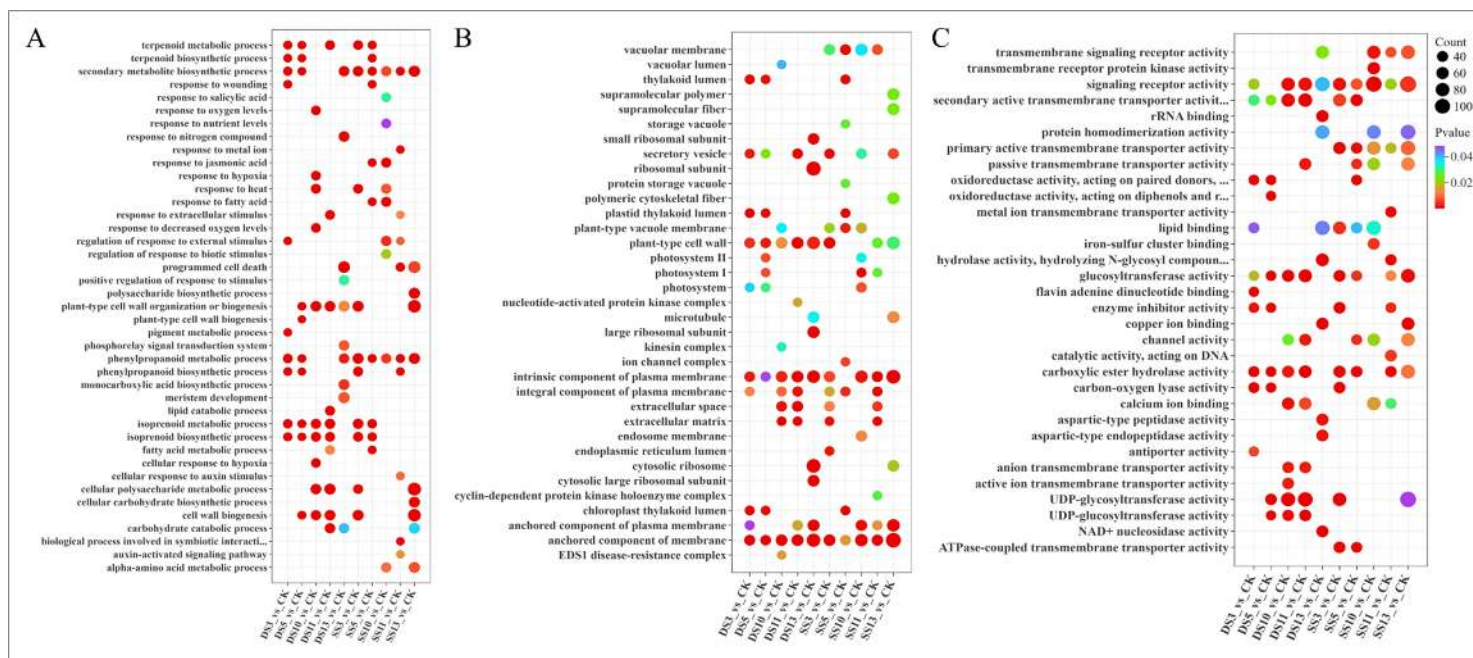


Figure 5

Top 10 significantly enriched GO terms among differentially expressed genes in drought and salt stress treatments compared to CK. (a) Biological process (b) Cellular component (c) Molecular function.

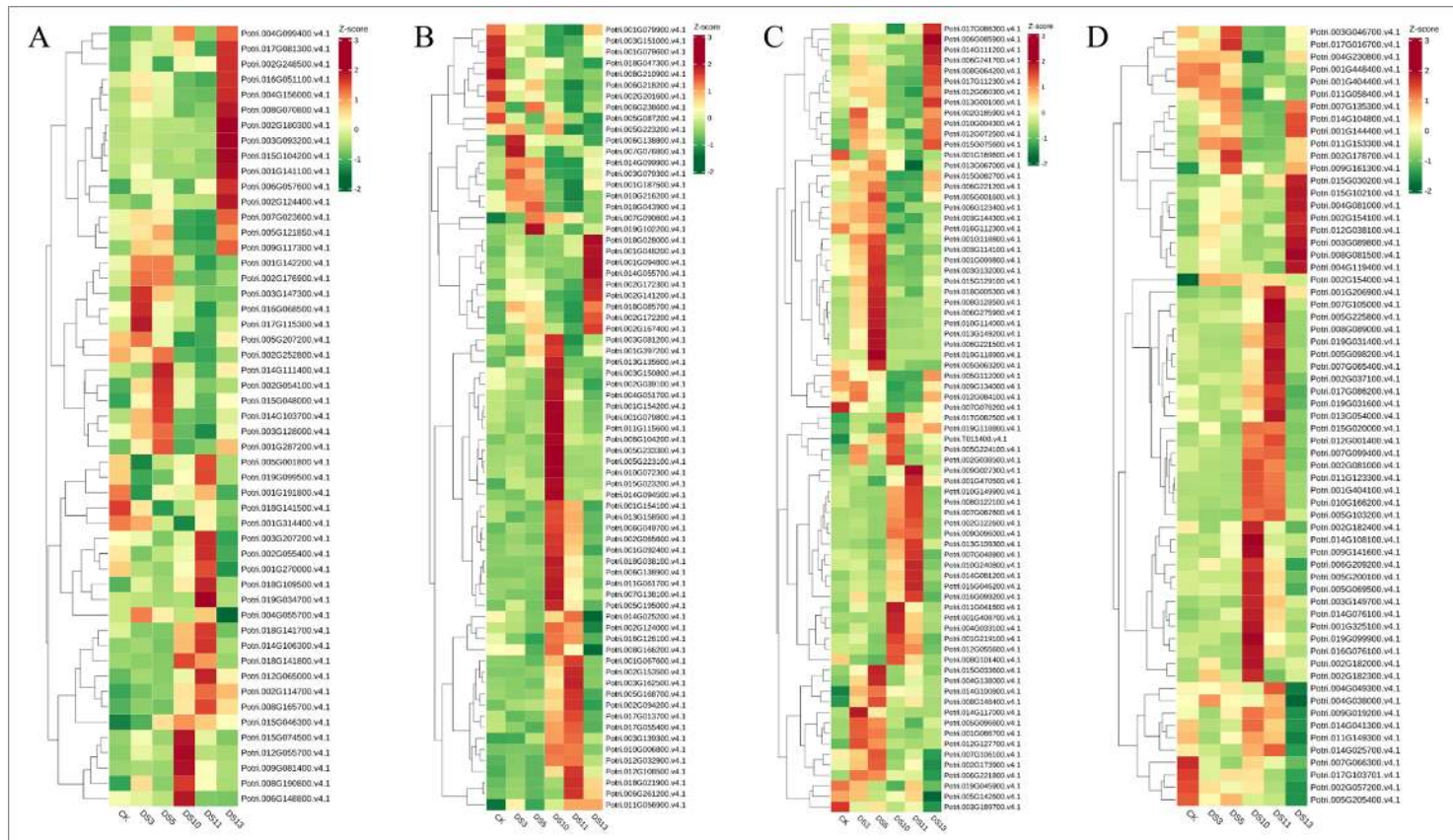


Figure 6

Heatmap of differentially expressed transcription factors across four families in drought stress. (a) bHLH gene family (b) ERF gene family (c) MYB gene family (d) NAC gene family

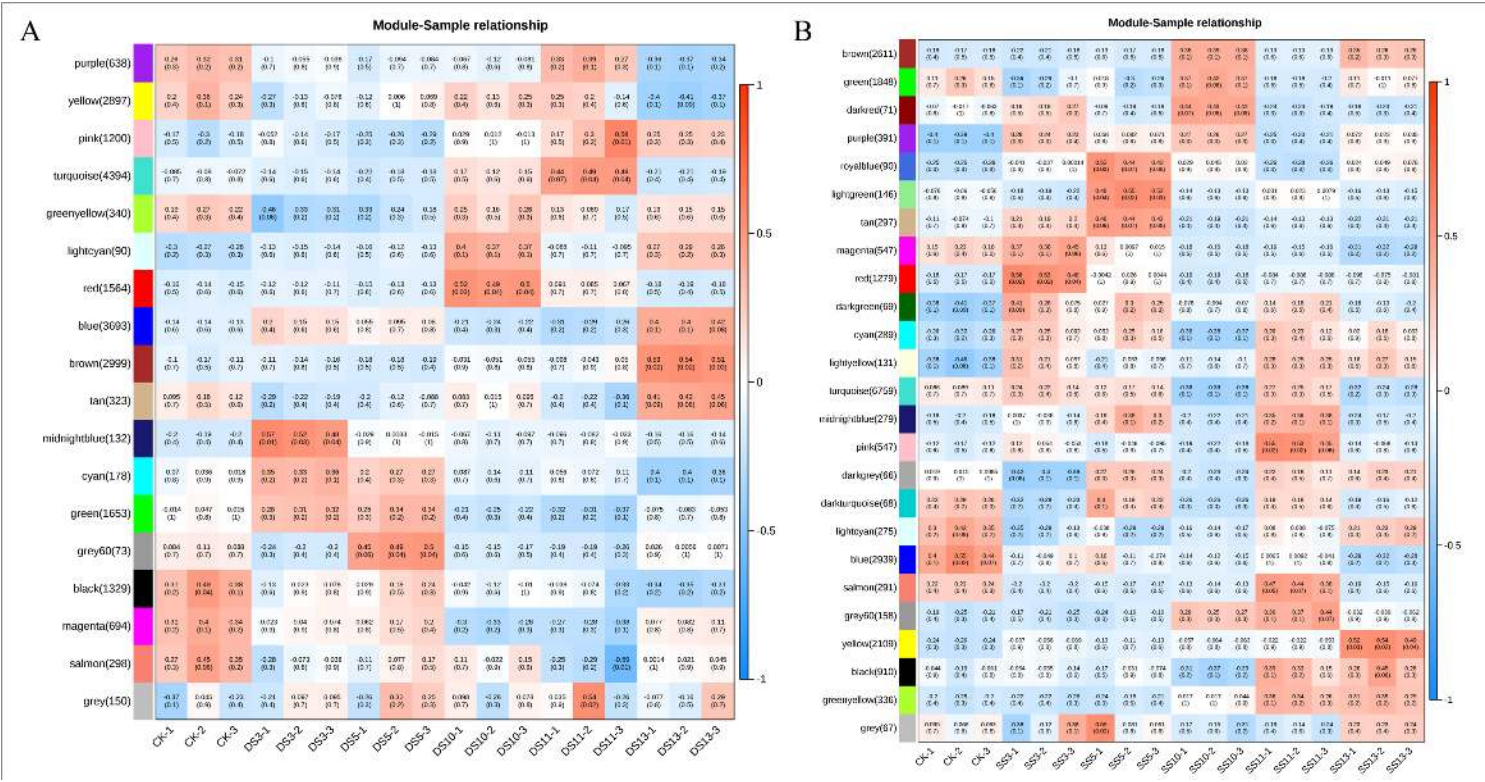


Figure 7

Correlation heatmaps of modules and samples from different treatments. Each row represented a module. Each column corresponded to a specific sample. The values in each cell represented the correlation coefficients (r) and p-values (in parentheses). The colors represented the correlation coefficient between the module and the sample; red indicated a positive correlation between the module and the sample, and blue indicated a negative correlation.

(a) Drought stress (b) Salt stress.

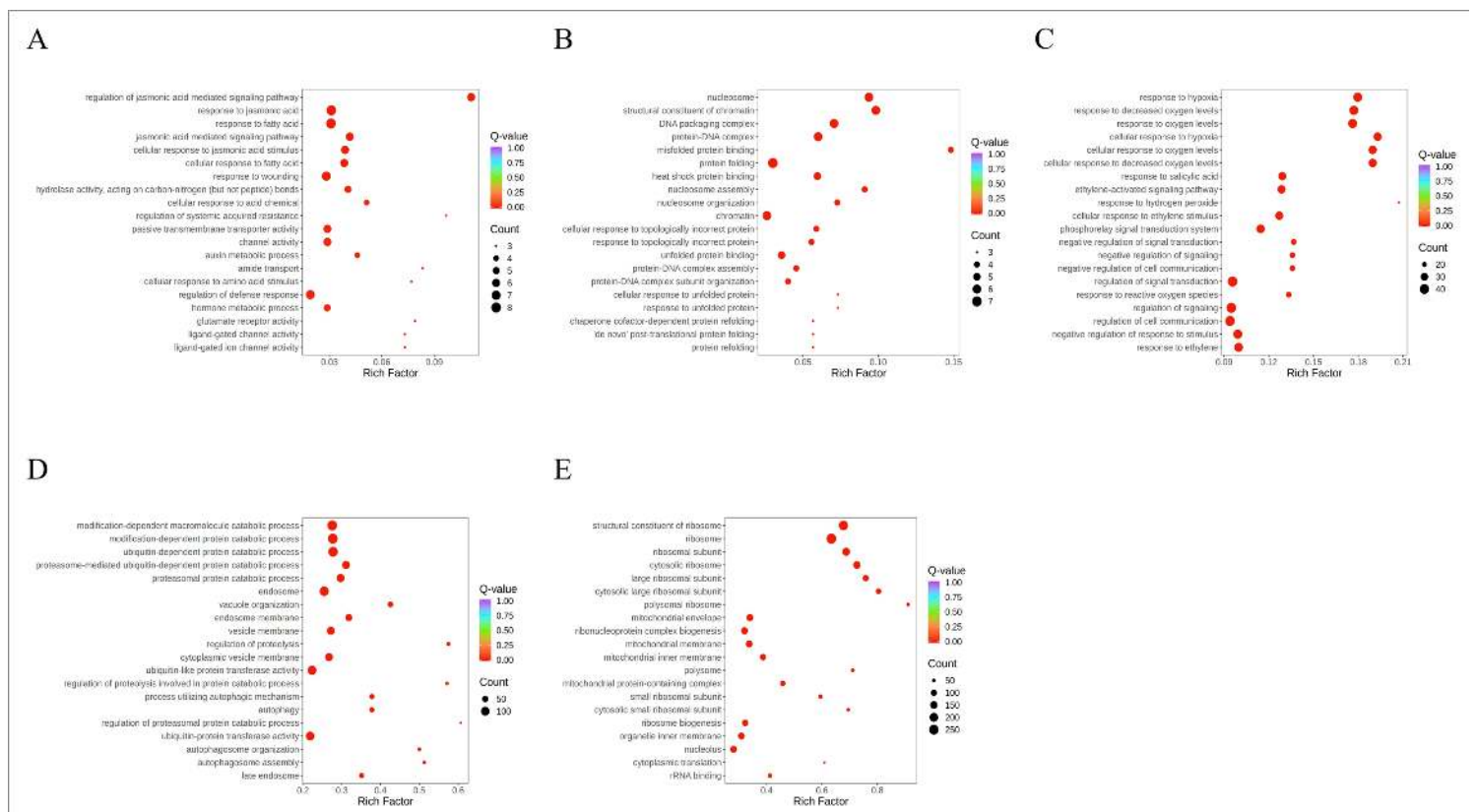


Figure 8

GO enrichment analyses on the genes within the five significant modules associated with drought stress. (a) Midnightblue module related to DS3 (b) Grey60 module related to DS5 (c) Red module related to DS10 (d) Turquoise module related to DS11 (e) Brown module related to DS13.

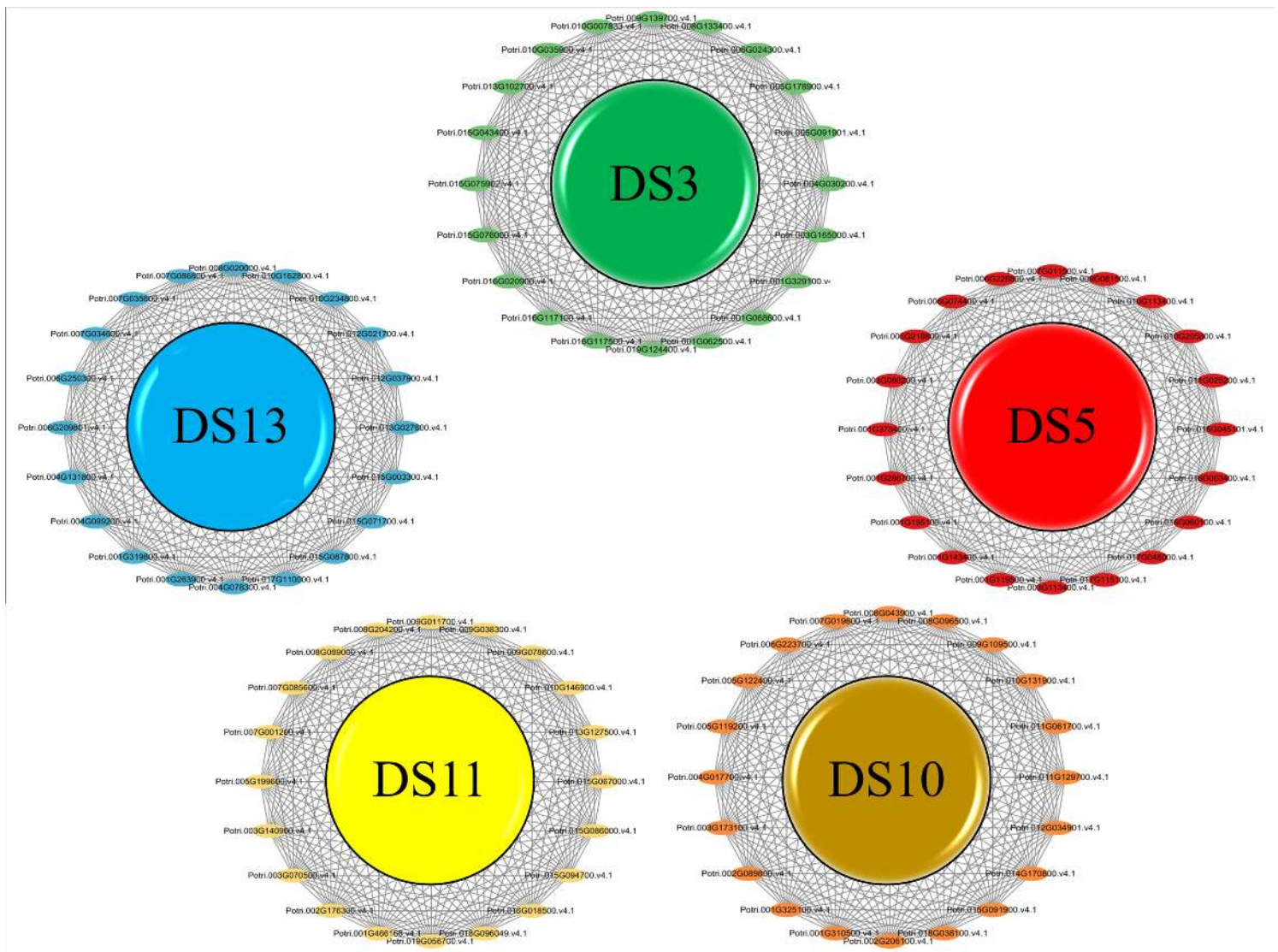


Figure 9

The top 20 genes with the highest connectivity degree within each module in drought stress.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial.zip](#)