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The molecular regulatory mechanism of reed canary grass under salt, waterlogging, and combined stress was analyzed by transcriptomic analysis

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Abstract

Background Reed canary grass has been identified as a suitable species for restoring plateau wetlands and understanding plant adaptation mechanisms in wetland environments. In this study, we subjected a reed canary grass cultivar 'Chuanxi' to waterlogging, salt, and combined stresses to investigate its phenotypic characteristics, physiological indices, and transcriptome changes under these conditions.

Results The results revealed that the growth rate was slower under salt stress than under waterlogging stress. The chlorophyll content and energy capture efficiency of the PS II reaction center decreased with prolonged exposure to each stress. Conversely, while the activities of enzymes associated with respiratory metabolism, as well as MDA, PRO, Na^+, and K^+-ATPase, increased. The formation of distinct aerenchyma was observed under waterlogging stress and combined stress. Transcriptome sequencing analysis identified 5,379, 4,169, and 14,993 DEGs under CK vs. W, CK vs. S, and CK vs. SW conditions, respectively. The WRKY was found to be the most abundant under waterlogging stress, whereas the MYB predominated under salt stress and combined stress. Glutathione metabolic pathways and Plant hormone signal transduction have also been found to play important roles in stress.

Conclusion By integrating phenotypic, physiological, anatomical, and transcriptomic, this research provides valuable insights into how reed canary grass responds to salt, waterlogging, and combined stresses. These findings may inform the ecological application of reed canary grass in high-altitude wetlands and for breeding purposes.

Keywords Reed canary grass, Combined stress, Anatomical structure, Transcriptomics

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Introduction

Plants face a myriad of environmental stresses, including waterlogging, salinity, and extreme temperature (cold and heat) [1]. In agricultural production, plants often encounter multiple stresses simultaneously, which can result in more severe damage than individual stresses [2]. Therefore, understanding how plants respond and adapt to combined stresses to enhance agricultural productivity is crucial [3]. Wetlands store 40% of the global carbon pool and can regulate the water cycle and purify water quality [4]. Since the 18th century, with global warming and human irrationality in developing and using, wetlands have been shrinking in area, with 35% of the world's wetland area lost and more than a quarter of wetland species threatened with extinction [5, 6]. The Zoige Alpine wetland represents a significant ecological barrier area in the climate change zone of the eastern edge of the Tibetan Plateau, as well as an important water conservation area in the upper reaches of the Yangtze and Yellow Rivers, and a nationally defined key ecological function area [7]. However, it has been facing degradation problems, mainly manifested in the lowering of the groundwater table, significant reduction of the surface water area, the tendency of the wet environment to become semiarid, and the decline of species diversity [8]. To solve the degradation problem of the Zoige Alpine wetland, many attempts have been made by previous researchers, but due to the alpine seasonal waterlogging and high soil salinity characteristics of wetlands in the western Sichuan Plateau, which resulted in the poor adaptability of local grass species including *Elymus sibiricus* and *E. nutans* to wetlands, the management effect is not obvious [9], so the selection of appropriate native plants is the key to the conservation and restoration of wetlands in Southeastern Tibetan Plateau [10].

Reed canary grass (*Phalaris arundinacea*), a high-yielding cool-season forage grass, is commonly found along rivers and lakes in China, Europe, and North America [11]. It has shown potential for remediation of heavy metal pollution, wastewater purification, and soil erosion control. It is often used for the ecological restoration of major lakes in China, such as Poyang Lake and Dongting Lake [12]. Among the several registered varieties of reed canary grass in China, the cultivar 'Chuanxi' is highly valued because it is bred through the domestication and selection of wild germplasm from the western plateau region of Sichuan Province. It exhibits better waterlogging tolerance and salt tolerance compared to other varieties. Hence, it can be an important grass species for the restoration of the Zoige Alpine wetland.

However, the combined stress of salt and waterlogging poses a significant challenge in the ecological restoration of wetlands in the northwest Sichuan plateau. While previous studies have explored the molecular mechanisms of waterlogging in reed canary grass, they have not addressed the combined stress [13, 14]. To fill this research gap, we conducted a long-term study using 'Chuanxi' reed canary grass as the research material and subjected it to three abiotic stresses: waterlogging, salt, and combined stress. Our aim was to understand the differential genes and crucial pathways involved in the response of reed canary grass to combined stress. These findings can enhance our understanding of the adaptive mechanism of 'Chuanxi' reed canary grass to combined stress and provide valuable genetic information for its further utilization.

Materials and methods

Plant material and experiment treatment

The study used 'Chuanxi' reed canary grass, which was provided by the Hanchang Research Base of the Sichuan Academy of Grassland Science in Sichuan Province, China (30.45°N, 103.72°E). The plant was cultivated in the greenhouse of Sichuan Agricultural University, Sichuan Province, China (30.42°N, 103.51°E). Treated tillers were grown in sand and transplanted with Hoagland nutrient solution. When the plant reached the tillering stage and showed healthy growth, it was subjected to four treatments:

(1) Control (CK): grown under normal conditions.
(2) Waterlogging (W): the depth of waterlogging was the height of the whole plant height.
(3) Salt (S): plants were watered with NaCl treatment solution at a concentration of 300 mmo1·L^{-1}
(4) Salt-waterlogging (SW): waterlogging of the entire plant with a NaCl treatment solution at a concentration of 300 mmo1·L^{-1}.

Each treatment was replicated three times and lasted for 16 days.

Morphological observation and physiological indices

To assess the impact of abiotic stress, various measurements were taken, including growth rate, PS II Maximum photochemical efficiency, chlorophyll content, and electrical conductivity (EL). These measurements were conducted on days 0, 4, 8, 12, and 16 of each stress, with each stress being replicated three times. Harvested samples were promptly frozen in liquid nitrogen and stored at -80 °C for subsequent RNA sequencing and analysis of physiological indices. These indices included proline (Pro), glucose-6-phosphate dehydrogenase (G-6-P-H), malondialdehyde (MDA), total antioxidant capacity (TAC), Na^+, K^+-ATPase activities, ethanol dehydrogenase (ADH), and lactate dehydrogenase (LDH) activities in plant leaves. The data collected were expressed as mean and standard deviation and analyzed using

ANOVA. To identify significant differences between treatments, post hoc comparisons were performed using Duncan's test ($P<0.05$) with SPSS 20 software.

Observations on the anatomical structure of nutrient organs

To study the anatomical structure of reed canary grass, mature and healthy roots and leaves were carefully selected on day 8 based on the physiological index data. After sampling, these roots and leaves were precision-cut into approximately 1 cm segments using a sharp razor blade. The segments were immediately immersed in FAA fixative, which is a mixture of formalin, glacial acetic acid, ethanol, and distilled water in a ratio of 10:5:50:35, for 48 h to ensure optimal fixation. Following fixation, a series of procedures were carried out, including rinsing, initial staining, dehydration, wax immersion, sectioning, dewaxing, staining, and further dehydration. The data for each slice is counted by the case viewer software (Sysmex Europe, German) [15].

RNA extraction, cDNA preparation and sequencing

Mature leaves were sampled under stress on 8th, followed by rapid freezing in liquid nitrogen to preserve their RNA content. Total RNA was extracted from the frozen leaf tissues using TRIzol reagent, following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The quality and concentration of the extracted total RNA were determined using the NanoDrop ND1000® spectrophotometer (NanoDrop Technologies, United States). To ensure the integrity of the RNA samples, gel electrophoresis was performed as an additional examination. From these 12 samples, we screened for high-quality RNAs. The mRNAs featuring polyA tails were then enriched using Oligo dT-coated magnetic beads. These RNAs were fragmented using an interrupting buffer, followed using random N6 primers for reverse transcription to synthesize cDNA duplexes into double-stranded DNA. Library construction and sequencing processes were subsequently carried out using the Illumina HiSeq Xten platform at Wuhan Frasergen Biologicals Co Ltd (Wuhan, China).

Sequencing data filtering and assembly

The raw sequencing data were subjected to a series of filtering steps using SOAPnuke v2.1.0 software. The resulting clean reads were then used for transcript assembly using Trinity software. To obtain nonredundant collection of transcript sequences, these clean reads were aligned against NR, Swiss-Prot, GO and KEGG databases via BLAST2GO (https://www.blast2go.com/). To gain further insight, GO enrichment analyses were performed using the Wallenius noncentral hypergeometric distributions. These analyses were performed using the GOseq R package, allowing for a comprehensive understanding of gene ontology associations. In addition, KEGG pathway (http://www.genome.jp/kegg/) enrichment was statistically analyzed, providing valuable insights into the biological pathways associated with the transcriptomic data [16].

Identification of differentially expressed genes (DEGs)

To identify DEGs between the two samples, the expression level of each transcript was calculated using the transcripts per million reads (TPM) method. DEGs analysis was performed using the DESeq2 package in R, a statistical package. Significant DEGs were classified based on parameters such as at least a twofold difference in transcript abundance ($|\log2FC| \geq 1$), fold change (FC), and false discovery rate (FDR) <0.05. These rigorous analytical procedures facilitated the identification of DEGs and provided insights into the molecular mechanisms underlying the observed differences.

Weighted gene co-expression network analysis (WGCNA)

The R package was used to analyze gene expression networks using WGCNA. The clusters analyzed by WGCNA are called modules, and each module contains at least 80 genes. Models with unclassified expression patterns were classified as grey modules [17]. Phenotype-module correlation analyses were then performed on all modules using 8th physiological metrics, and the modules most closely related to the physiological metrics were analyzed to identify the set of genes associated with that physiological metric.

Validation of DEGs by real-time quantitative PCR (RT-qPCR)

To ensure the accuracy and reliability of the transcriptome data, we randomly selected nine highly expressed DEGs associated with abiotic stresses from RNA-seq (Table S1). Gene primer design was performed using Primer5 software, and primer synthesis was carried out by You kang Biotechnology (Chengdu, China, Table S2). RNA was reverse transcribed into cDNA using the Evo M-MLV Plus cDNA Synthesis Kit from Accurate Biology (Changsha, China). The cDNA samples were then subjected to qPCR analysis using the Maxima SYBR Green qPCR Master Mix (Accurate, Changsha, China). The reaction conditions included an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 57.5 °C for 30 s, and extension at 72 °C for 15 s. Three biological replicates and three technical replicates were tested for each sample. Eukaryotic translation initiation factor 4E (*ETF*) was used as an endogenous reference gene, and transcript expression was normalized using the $2^{-\Delta\Delta Ct}$ method. The data were analyzed and plotted using Graph Prism 8 [18].

Results

Phenotypic changes in reed canary grass under abiotic stress

The phenotypes of tested plants showed different changes under different stresses. Under salt stress, the plants began to exhibit wilting symptoms on the 8th day, ultimately leading to their demise by the 16th day (Fig. 1). Under waterlogging stress, plants showed maximum growth rate on the 4th day, followed by a gradual decrease after the 8th day when the leaves were accompanied by a slight loss of greenness (Figs. 1 and 2A). Under combined stress, the treated plants gradually wilted and died. The growth rate was higher than that of salt stress, although lower than that of waterlogging stress (Figs. 1 and 2A).

Physiological indicators of reed canary grass under abiotic stress

Under abiotic stress, the chlorophyll content and PS II Maximum photochemical efficiency decreased gradually with time (P <0.05, Fig. 2B). The chlorophyll content was found to be significantly lower on day 16 than on day 0 (P<0.05, Fig. 2C). EL, PRO, and MDA content increased gradually under abiotic stress, and the values of these metrics were significantly higher on day 16 than on day 0 (P<0.05, Fig. 2D and E, and 2F). The trends of three enzyme indices related to plant respiratory metabolism, G-6-P-H, ADH, and LDH, followed the same trend. The activities of these enzymes increased gradually with the duration of stress and were significantly higher (P<0.05, Fig. 2G, H, I and J)) on the 16th day compared to other time points. Additionally, TAC was greater under stress than control, and the value on day 8 was significantly higher than other time points (P<0.05, Fig. 2K).

Anatomical dissection on reed canary grass under combined stress

The leaf blade of reed canary grass consists of the epidermis, phloem, and stomata. Under salt stress, the epidermal cells of the leaf blade are severely damaged, and their shape is altered. The leaf air cavities were more pronounced in the waterlogged and combined stresses. In this study, air cavities were clearly observed under salt stress, however, air cavities were not evident under waterlogging and combined stress due to stomatal closure (Fig. 3).

The roots of reed canary grass mainly consist of the epidermis, aerenchyma, and vascular column. Under waterlogging and combined stress, the roots formed more pronounced aerenchyma. Comparison of aerenchyma area, it was observed that the waterlogged stress exhibited the largest area of aerenchyma, measuring at 120,467 μm². This was followed by the combined stress, which measured 30,387 μm². In contrast, there was no aerenchyma observed in both the salt stress and control treatments (Fig. 3). Additionally, we observed two adjacent xylem secondary vessels in the control and salt stress, while there was only one xylem secondary vessel in the waterlogging and combined stress (Fig. 3).

Transcriptome sequencing data filtering and assembly

The sequencing data was processed and assembled to obtain high quality transcripts (Table S3). Then, the transcripts were functionally annotated, and it was found that some transcripts had a high degree of similarity to sequences in known databases, while some did not have matching sequences. We counted the annotation results of commonly used GO databases, and the top three GO terms for all three comparison groups were: cellular processes, cellular anatomical entities, and catalytic activity

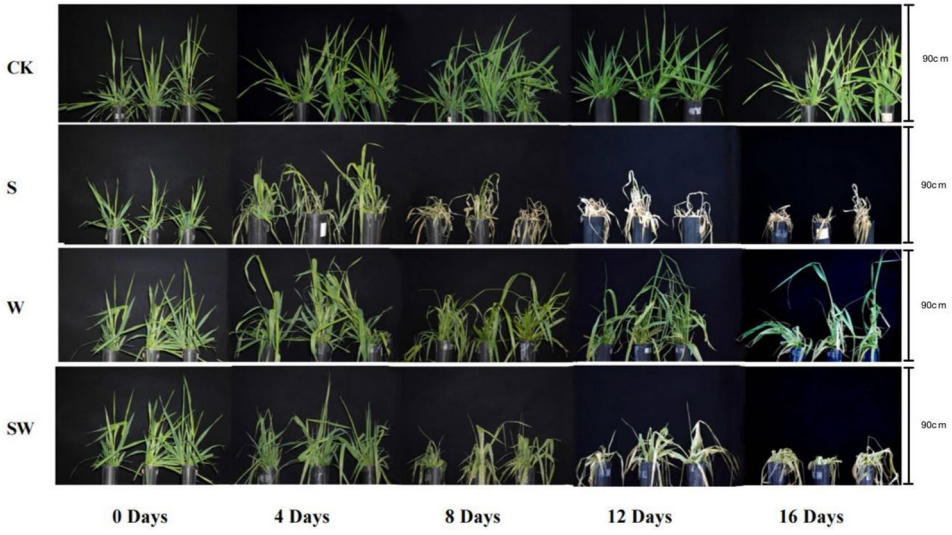

Fig. 1 Growth and development of reed canary grass under waterlogging, salt, and combined stresses

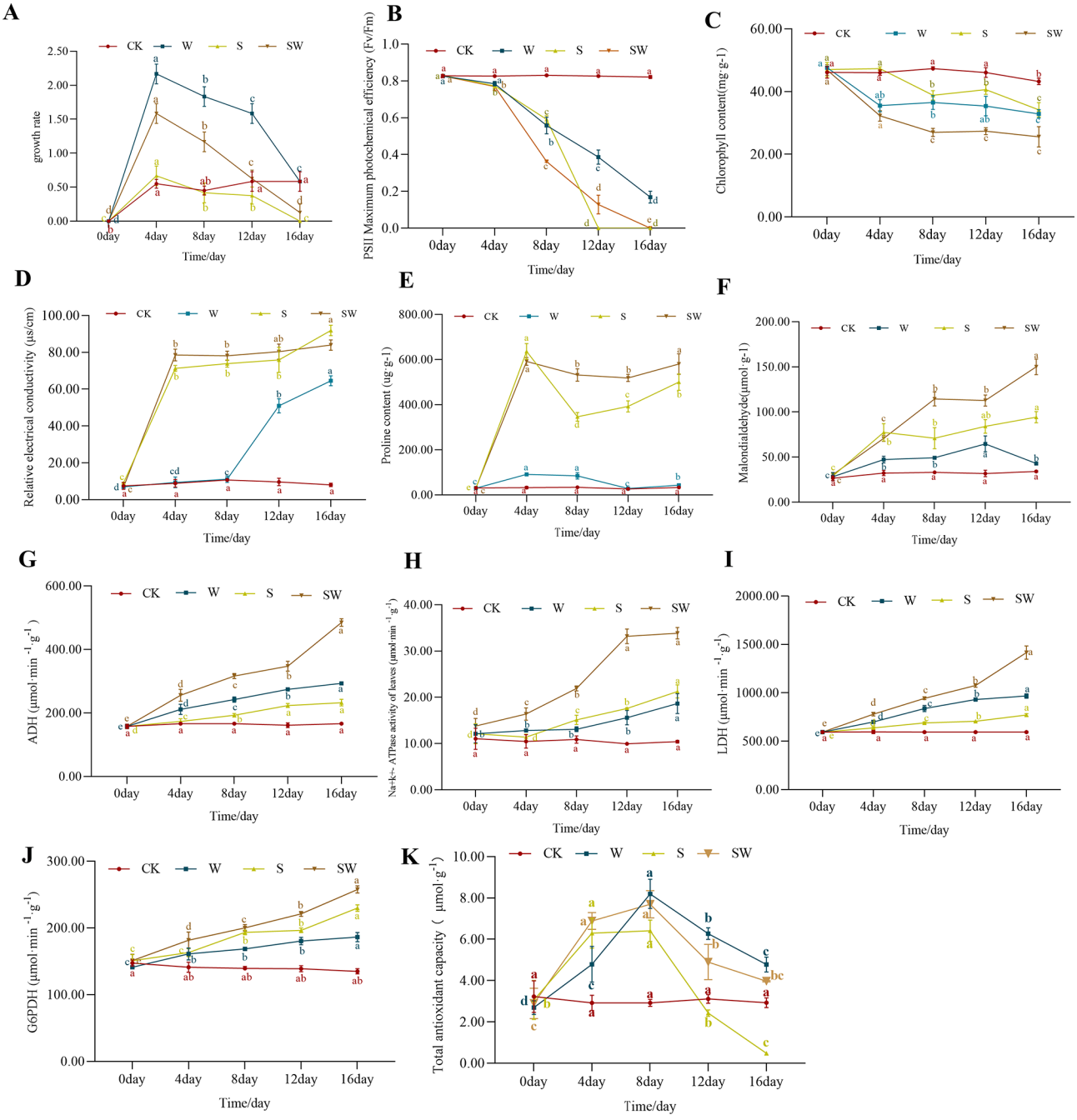

Fig. 2 Effects of waterlogging, salt, and combined stress on physiological indices of reed canary grass at each stress time. **A**–**D** refer to growth rate, PS II maximum photochemical efficiency, and relative conductivity, respectively; **E** and **F** refer to proline and malondialdehyde, respectively; and **G**–**K** refer to enzyme dehydrogenase (ADH), Na^+, and K^+-ATPase activities, ethanol and lactate dehydrogenase (LDH) activities, glucose-6-phosphate dehydrogenase (G-6-P-H), and total antioxidant capacity (TAC).)Notes: Different colored lower-case characters in the graphs represent the significance of differences in indicators for the same treatment at different time points, $P < 0.05$, the same applies hereinafter

(Figure S1A, B & C). In the KEGG pathway analysis, in the three comparative groups of CK vs. S, CK vs. W and CK vs. SW, the pathways with high number of annotated genes were mainly in the areas of carbohydrate metabolism, energy metabolism and amino acid metabolism. metabolism, in which we found that among the top-ranked pathways, CK vs. SW had the highest number of genes in the response pathway (Figure S1 D, E & F).

DEGs under abiotic stress

Comparing the DEGs under different treatment groups (CK vs. W, CK vs. S, and CK vs. SW), we found that the

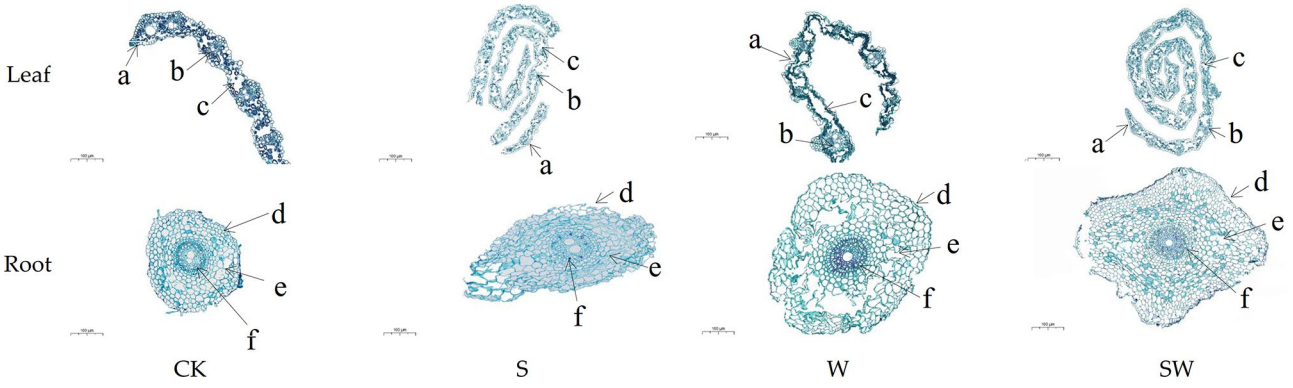

Fig. 3 Cross-sectional structure of leaf/root slices of reed canary grass under waterlogging, salt and combined stress. a represents the leaf epidermis, b represents the vascular bundle, c represents the air cavity, d represents the root epidermis, e represents the root aerenchyma, f represents the root column

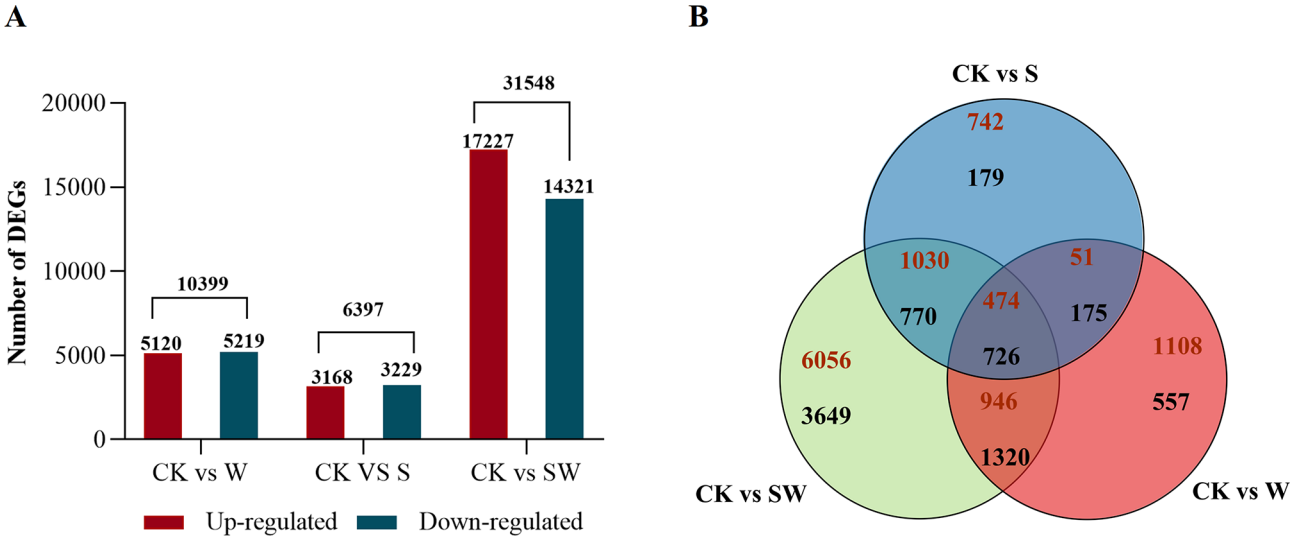

Fig. 4 DEGs statistics of reed canary grass under waterlogging, salt, and combined stress versus control. **A** indicates histogram of the number of up- and down-regulated DEGs in each comparison group; **B** represents a Venn diagram of the number of DEGs in each comparison group. in which red numbers represent up-regulation and black numbers represent down-regulation

highest number of DEGs, 14,993, was found in CK vs. SW (Fig. 4A).

Specifically, in the CK vs. W, 2,579 genes were up regulated, and 2,800 genes were down regulated; in the CK vs. S, 2,297 genes were up regulated, and 1,872 genes were down regulated; in the CK vs. SW, 8,506 genes were up regulated, and 6,487 genes were down-regulated (Fig. 4A). Further analysis of the DEGs in these three comparison groups identified a total of 1,200 DEGs, with 474 genes up-regulated and 726 genes down-regulated (Fig. 4B). Taken together, these findings suggest that, compared with those under single stresses, gene expression in reed canary grass under combined stress is significantly influenced by these two stresses, resulting in more complex and noticeable changes.

Analysis of differential transcription factors (TFs)

To examine how reed canary grass responds to different types of stress, we analyzed the top 10 TFs families in three comparison groups. The findings showed that under combined stress, reed canary grass produced 936 TFs, while under salt stress and waterlogging stress it produced 380 and 248 TFs, respectively. Additionally, the research revealed that the MYB had the highest number of differential TFs in the CK vs. SW and CK vs. S, while the WRKY had the highest number under waterlogging stress (Fig. 5A and B, and 5C). These results suggest that the number of differential TFs in response to combined stresses is more significant than those in response to individual stresses. Furthermore, the CK vs. SW had the highest number of TFs families, specifically 52 families. Among these families, 33 were shared with the CK vs. S and 38 were shared with the CK vs. W (Fig. 5D).

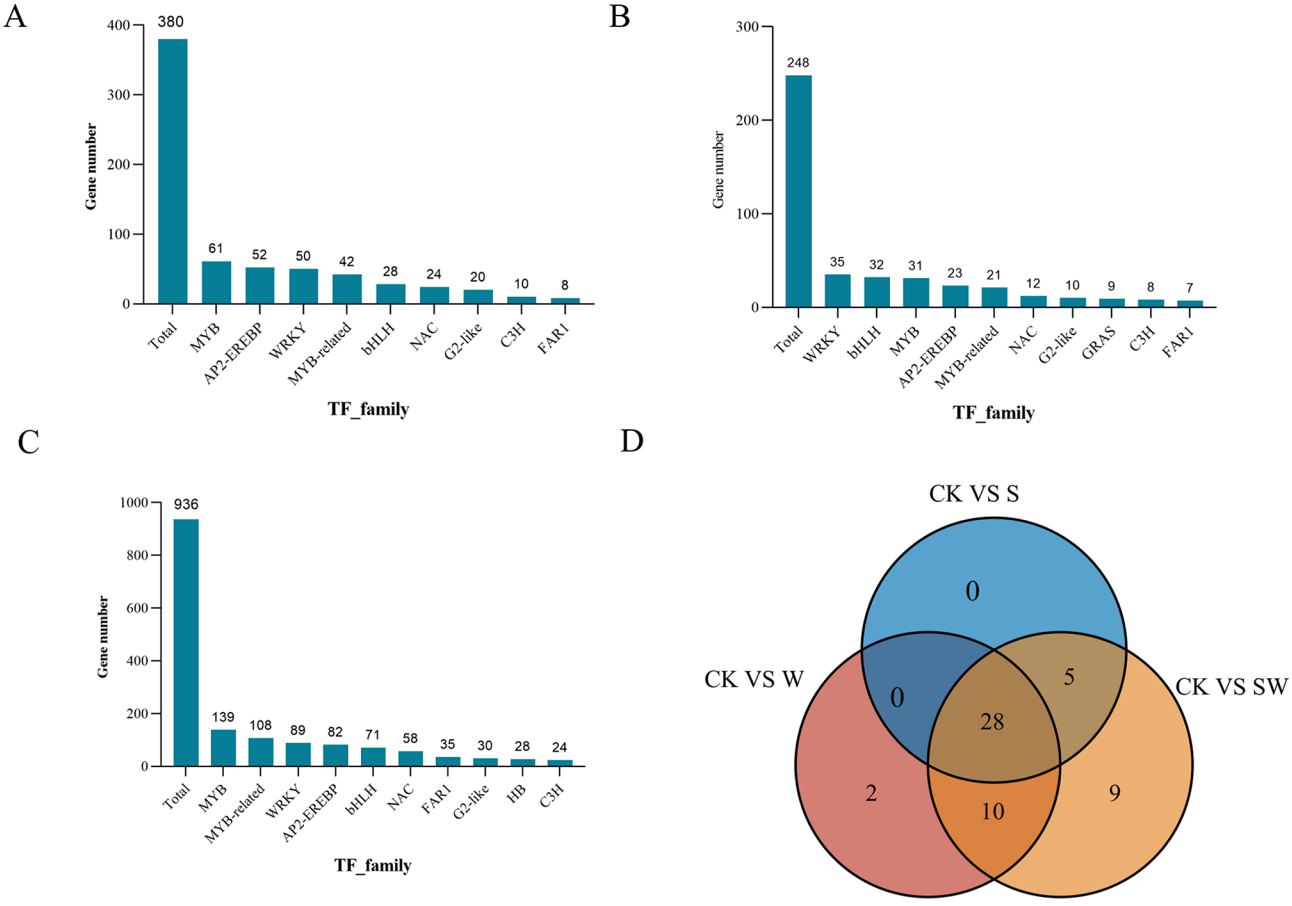

Fig. 5 TFs that differ for each comparison group can be observed. **A**, **B**, and **C** indicate the number of TFs present in the top 10 ranked transcriptional families, while **D** represents the Venn diagram of transcriptional families for the three comparison groups

Weighted gene co-expression network analysis

We conducted a WGCNA of reed canary grass in response to waterlogging and salt stress, using 250,336 detected genes. A total of 10 modules, represented by different colors, were identified (Fig. 6A and B). By examining the Pearson correlations between modules, traits, and samples, we found that the absolute value of the correlation coefficients of the four modules exceeded 0.8 (Fig. 6C). Specifically, the magenta module showed a significant negative correlation with ADH (correlation coefficient −0.93, $P<0.05$), the red module showed a significant positive correlation with Na^+/K^+ ATPase (correlation coefficient 0.86, $P<0.05$); the blue module showed a significant positive correlation with PRO (correlation coefficient 0.86, $P<0.05$), the yellow module showed a significant positive correlation with LDH (correlation coefficient 0.91, $P<0.05$) and a significant negative correlation with PRO (correlation coefficient −0.9, $P<0.05$).

We counted the number of genes in the four modules, of which the magenta, red, blue and yellow modules contained 213, 534, 1,349 and 932 genes, respectively(Fig. 6D). By annotating these genes with KEGG, we found that the genes in the magenta module might be related to ADH, and these genes were mainly involved in the pathways of peroxisome, pyruvate metabolism, and glyoxylate and dicarboxylic acid metabolism. In the red module, many genes were associated with pathways such as ascorbate and aldarate metabolism, and glyoxylate and dicarboxylic acid metabolism. In the blue module, genes are annotated to be involved in energy metabolism and signaling pathways such as glycolysis/glycolysis, oxidative phosphorylation and phosphatidylinositol signaling system. The genes in the yellow module are mainly involved in amino acid metabolism and energy metabolism pathways such as arginine and proline metabolism, oxidative phosphorylation (Table S5).

Glutathione metabolism pathway analysis

It is well known that glutathione metabolism, as an antioxidant defense system, is essential for protecting cells from abiotic stresses. In the above study WGCNA analysis, we identified a few genes associated with the glutathione metabolism pathway (Table S4, Figure S3). Among these genes, γ-glutamyl transferase, a regulatory gene responsible for converting L-γ-glutamyl to

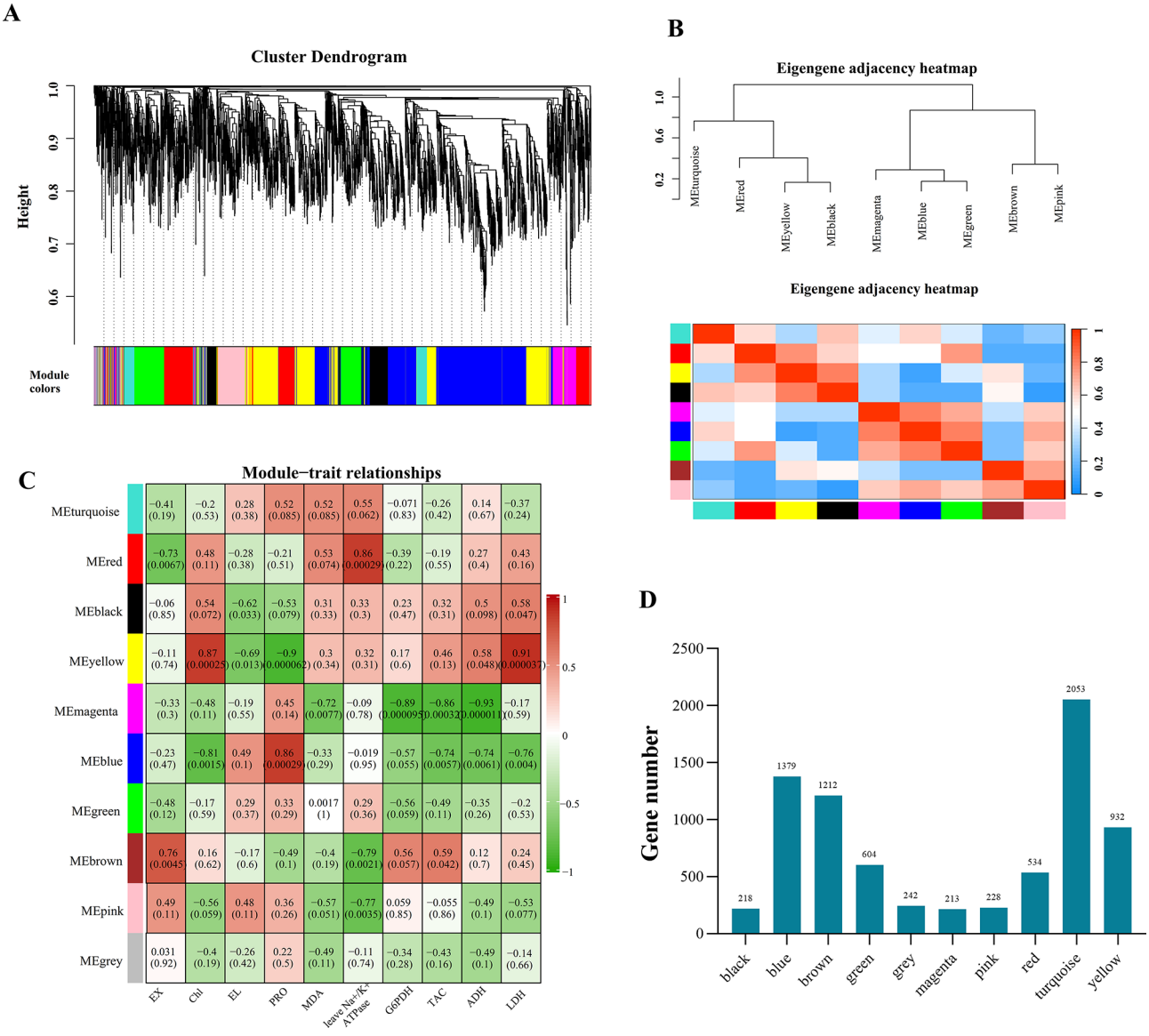

Fig. 6 Visualization of WGCNA. **A** Diagram of the results of WGCNA using 250,336 genes; **B** Module clustering dendrograms and heat maps; **C** Module-Trait Relationship Diagram; **D** Histogram of the number of genes contained in the top 10 TFs families

L-amino acids, showed up-regulation. Additionally, pyridine nucleotide disulfide oxidoreductase and thioredoxin reductase, essential enzymes for glutathione conversion, displayed up or down-regulation in the expression of six genes with thioredoxin reductase function and 15 genes with pyridine nucleotide disulfide oxidoreductase function, particularly under salt-waterlogging stress. Furthermore, three enzymes involved in the $NADP^+$/NADPH transition process, namely NADP-dependent isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate-1-dehydrogenase, were also associated with glutathione metabolism. Overall, our findings indicate that the glutathione pathway relies on multiple gene interactions to maintain intracellular redox homeostasis, antioxidant defense, detoxification, and immune regulation (Fig. 7).

Plant hormone signal transduction

Plant hormone signal transduction as a common response pathway to waterlogging stress has been studied in numerous studies [19]. In this study we analyzed the gene expression of key hormones in the plant hormone signal transduction. In the auxin pathway, abiotic stress led to both upregulation and downregulation of the growth hormone receptor transport inhibitory response protein 1 (TIR1) gene, along with upregulation of most growth AUX/IAA genes. Three out of the four growth auxin response factor (ARF) genes were upregulated, except under salt stress, where one gene was

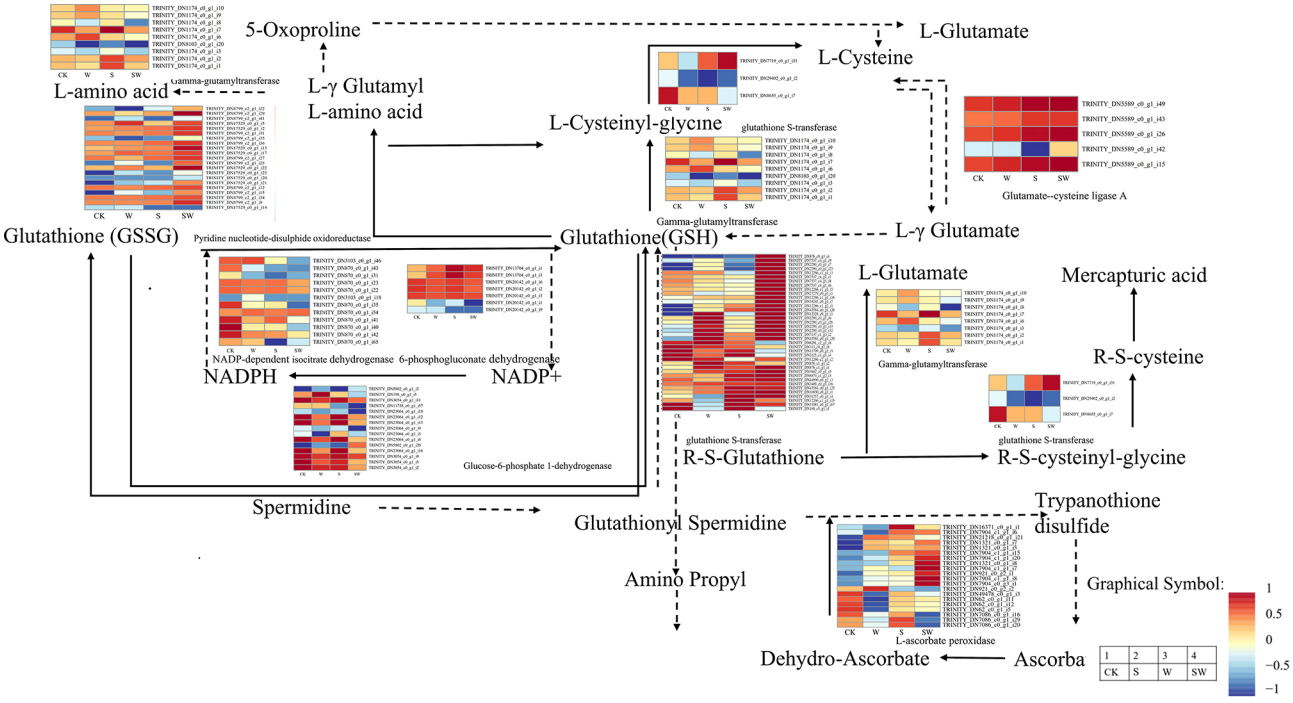

Fig. 7 Map of glutathione metabolic pathways under waterlogged, salt, and combined stresses

downregulated. Two Gretchen Hagen 3 (GH3) genes and one out of 29 SAUR genes were downregulated under different stresses. Among the genes in the gibberellin pathway, two GID1 genes were upregulated in response to different abiotic stresses, and the majority of the 26 IF genes were upregulated. However, four genes exhibited downregulated expression under waterlogging and combined stress but upregulated expression under salt stress. In the ethylene pathway, four ETR genes and two CTR1 genes were upregulated, with the latter being specific to salt treatment. Most of the genes in EIN2 and EIN3 were upregulated, while three EBF1/2 genes were upregulated under salt stress. Overall, the findings indicate that salt stress elicits different responses in plant hormone signal transduction compared to waterlogging and the combination of both stresses in salt, waterlogging, and combined treatments (Fig. 8).

Transcriptome validation

Nine genes were randomly selected for validation of transcriptome sequencing results. Based on the test results, the trend of the qRT-PCR data was basically consistent with the RNA-seq data, confirming the reliability of the RNA-seq analyses in this study (Figure S3).

Discussion

Seasonal waterlogging and high soil salinity are significant challenges for plateau wetland restoration. The adaptation mechanism of 'Chuanxi' reed canary grass, a key forage species for plateau wetland restoration, remains unclear. Therefore, this transcriptomics-based study revealed the mechanisms of differences in phenotypes, physiological indicators, and anatomical structures of reed canary grass under waterlogging stress, salt stress and combined stress.

Phenotypic and anatomical differences in reed canary grass under combined stress

Plant phenotypes reflect their morphological characteristics and are influenced by growth, development, and adaptation to the environment [20]. Different stresses induce plants to exhibit varied phenotypes and trigger the production of specific substances to counteract adverse effects. In our study, we observed that plants exposed to salt stress exhibited a lower growth rate compared to other stress conditions, with the growth rate gradually approaching zero starting from the 12th day of stress [21]. This phenomenon may be attributed to the disruption of plant cells under high salt conditions, resulting in significant inhibition of photosynthesis and other metabolic processes, as well as necrosis of leaf tissues [22]. This conclusion was validated in the context of cabbage (*Brassica rapa* L. ssp. *Pekinensis*) under salt stress [23]. In the study, it was observed that under waterlogging stress, the leaf blades of reed canary grass gradually lose their green color and eventually become transparent. This is attributed to prolonged wetness and lack of sunlight, which hinder the synthesis and accumulation of chlorophyll, greatly reducing the efficiency of photosynthesis [24]. Additionally, waterlogging damages the epidermal

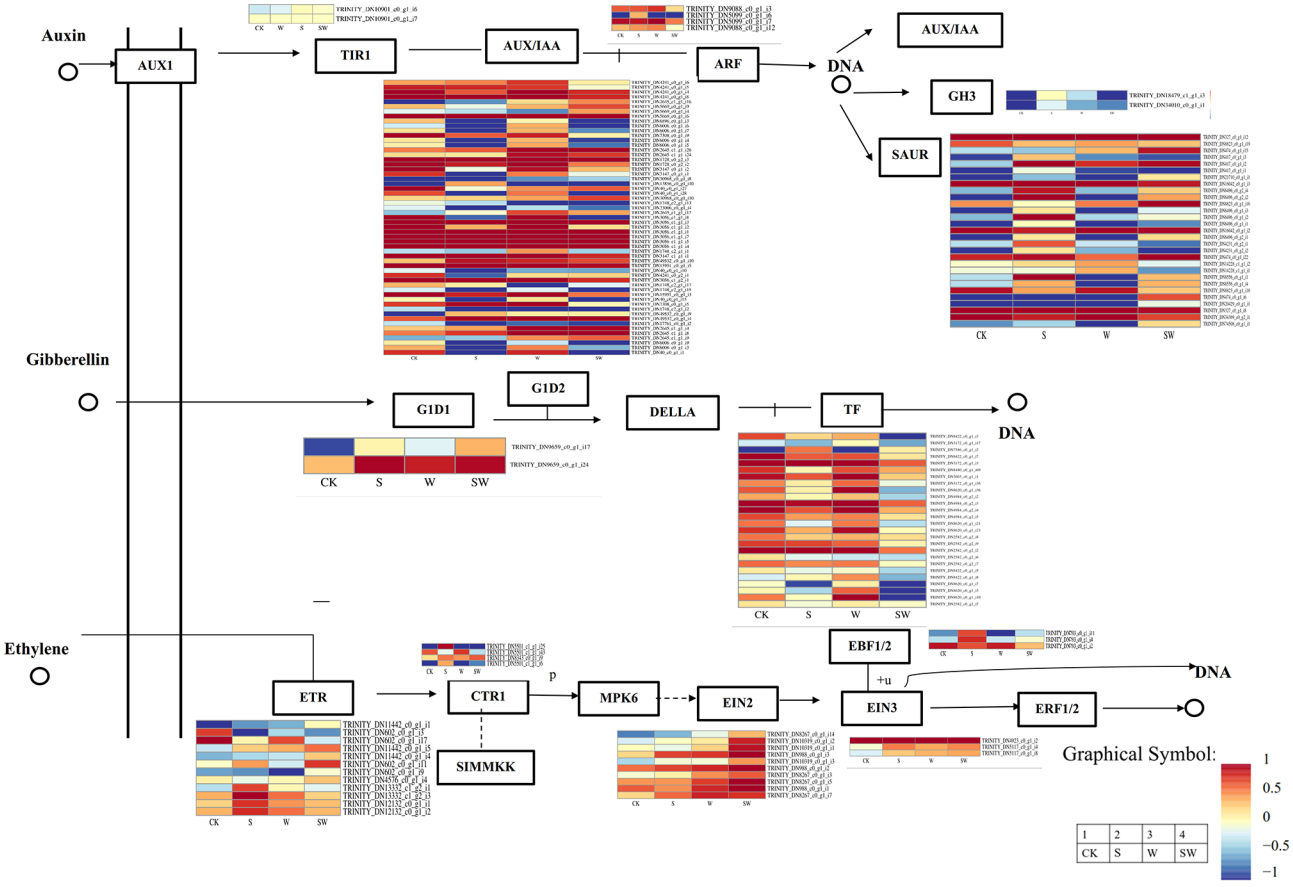

Fig. 8 Plant hormone signaling pathway under combined waterlogging, salt and combined stress

layer and stomatal structure of leaves, impeding carbon dioxide uptake and oxygen release, ultimately leading to leaf degradation and reduced transparency [25]. However, plants under waterlogging stress can enhance their survival by keeping the upper leaves above the water surface, enabling them to access sufficient oxygen, light, and carbon dioxide [26]. Consequently, at the onset of waterlogging stress in the study, the growth rate of reed canary grass was higher compared to other treatments. It is important to note that while combined stress negatively affects plant metabolism and physiology, the presence of oxygen in the water can mitigate some of these negative effects. Salt stress alone may produce saline toxicity, which can be attenuated in water environments.

Abiotic stresses can wreak havoc on plants, disrupting physiological and metabolic activities and potentially leading to their demise. Specifically, under salt stress, a substantial increase in Na^+ concentration can lead to cell swelling and altered osmotic pressure, which in turn causes cellular dehydration and a rise in electrical conductivity [27]. Moreover, both waterlogging and salt stress can trigger the accumulation of MDA, Pro, and TAC. These conditions also impact respiratory metabolic pathways, culminating in the oxidation of cellular lipids, proteins, and nucleic acids. This cascade of events compromises plant cell functionality, resulting in injury or cell death [28]. In our research, we found that EL, MDA, Na^+/K^+-ATPase, and proline levels were significantly elevated under salt stress and combined stress conditions compared to waterlogging stress alone. Notably, EL intensified as stress duration increased, indicating that salt stress inflicted more pronounced damage on the cell membrane and influenced the release of intracellular substances. Additionally, the activity of enzymes associated with respiratory metabolism experienced conspicuous changes, particularly those related to anaerobic respiration—ethanol dehydrogenase and lactate dehydrogenase—which saw an increase along with stress duration and were more pronounced under combined stress conditions. This suggests that plants adapt to adverse environments by modulating the activities of anaerobic respiratory enzymes as a strategy to sustain growth and development [29, 30].

Differences in TFs of reed canary grass under combined stress

TFs are proteins that bind to DNA and regulate gene transcription, influencing plant responses to stress [31].

This study showed that combined stress conditions resulted in the highest number and variety of transcription factors compared to single stress conditions. This is likely due to compound stress activating new pathways and responses that require new TFs, involving signaling, metabolic, and antioxidant systems to enhance plant adaptation [32]. We found that salt stress and combined stress conditions had the highest number of MYB, while waterlogging stress conditions had the highest number of WRKY [33]. MYB are known to be activated or up-regulated under salt stress conditions and can regulate genes involved in salt stress response WRKY regulate genes related to oxygen supply, respiration, and hormones in response to waterlogging stress [34]. WGCNA revealed that key modules contained transcription factors mainly from the ARF, bHLH, and C2C2-GATA. ARF activated under hypoxic conditions promotes lactic acid fermentation for energy production. C2C2-GATA regulates energy metabolism, redox homeostasis, sugar metabolism, and mitochondrial function under hypoxic condition [35]. In summary, these TFs families play vital roles in plant stress response by regulating stress-related genes and enhancing plant adaptation through gene expression regulation.

Reed canary grass metabolic pathway differences under combined stress

Plant adaptation to different stresses involves the activation of metabolic pathways and the regulation of gene expression [36]. Waterlogged salt complex stresses were found to have more severe and widespread negative effects on plants compared to single stresses. To adapt to these combined stresses, plants must regulate gene expression, accumulate specific substances such as glutathione (GSH/GSSG), and maintain ion homeostasis. Pyruvate metabolism, glutathione metabolism, and MAPK signaling pathways contribute to the adaptive capacity of plants [37].

Glutathione plays a key role in trapping reactive oxygen species (ROS), reducing oxidative damage, and maintaining intracellular redox balance during waterlogging stress [38]. Under salt stress, glutathione interacts with ion channels and transporter proteins to regulate ion homeostasis. Glutathione also influences the expression of genes related to antioxidant enzymes, indirectly affecting plant resistance to abiotic stress [39]. In this study, we observed significant up- and down-regulation of genes in the glutathione metabolic pathway, particularly those involved in the synthesis of pyridine nucleotide-disulfide oxidoreductase and thioredoxin reductase, which are responsive to combined stress. Further research into the interactions between glutathione metabolism and combined stress will deepen our understanding of plant response mechanisms and pave the way for practical applications in agriculture [40].

Plant hormones play a crucial role in sensing and responding to abiotic stresses by modulating gene expression, protein synthesis, and metabolite accumulation [41]. In this study, the negative regulator of auxin, Aux/IAA, was mostly up-regulated under waterlogging stress, inhibiting the activity of growth hormone response factor (ARF) and leading to a decline in growth hormone synthesis; Ethylene promotes plant growth, metabolic activity, and antioxidant capacity, thereby reducing the damage caused by abiotic stresses [42]. Under salt stress, ETR can maintain intra- and extracellular ion homeostasis by regulating the expression of ion channels, ion transport proteins, and antioxidant-related genes to mitigate the effects of salt stress on ion production [43]. Under waterlogging stress, ETR acts as a TF involved in oxygen supply, root respiration, and alcohol fermentation to help plants adapt [44]. In this study, ETR were up regulated under different stresses. Gibberellins stimulate root growth, enhancing water and nutrient uptake capacity. DELLA protein, a negative regulator of gibberellin signaling, was up-regulated under salt stress but down-regulated under combined stress, indicating a response to the stress [45]. Streamlining the interactions between hormones and abiotic stress responses will provide insights into plant molecular mechanisms and guide agricultural applications.

Conclusions

We used Illumina RNA-seq technology to study the response mechanisms of reed canary grass to three abiotic stresses: waterlogging, salt, and combined stress. By analyzing anatomical and physiological indicators, we observed significant changes in the morphological and physiological traits of reed canary grass on the 8th day of stress. Notably, aerated tissues appeared in leaves and roots. Transcriptome sequencing revealed a higher number of DEGs under combined stress, with the MYB family being the most abundant under salt and combined stress, and the WRKY family being the most abundant under waterlogging stress. Additionally, phytohormone signaling and glutathione metabolism pathways were found to play important roles in plant resilience to adversity. This study provides valuable insights into the complex adaptive mechanisms of reed in salt and waterlogging environments and lays a foundation for further exploration of relevant pathways and genes.

Abbreviations

NR	Non-Redundant
Swiss-Prot	Swiss-Protein Knowledgebase
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes
GSEA	Gene Set Enrichment Analysis

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

X.M., D.L., S.B., and J.Z.; methodology: X.J. (Xuejie jia), Y.X. (Yi Xiong) and Y.X. (Yanli Xiong); formal analysis: X.J. (Xuejie jia), M.Y. and X.J (Xiaofei Ji); writing—original draft preparation: X.J(Xuejie jia). and Y.X. (Yi Xiong); writing—review and editing: X.J. (Xuejie jia), Y.X. (Yi Xiong), X.M. and J.Z; visualization: X.L. and X.J(Xuejie jia); supervision, M.Y and X.M.; project administration: X.M. and J.Z.; funding acquisition: X.M. and J.Z. All authors have read and agreed to the published version of the manuscript.

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Data availability

The second-generation sequencing raw data have been deposited in the database of the National Gene Bank of China (https://db.cngb.org/) under the accession numbers CNP0004425.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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