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Comparative Transcriptome Analysis Reveals the Molecular Mechanism of Salt Combined with Flooding Tolerance in Hybrid Willow (*Salix matsudana* × *alba*)

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Abstract: Plants in coastal areas often face the combined stress of salt and flooding, which can cause severe damage. The impact of multiple stresses on plant growth and survival is greater than that of individual stresses. However, the molecular responses of hybrid willow (*Salix matsudana* × *alba*) to the combination of salt and flooding have not been well understood. In this study, we conducted a comparative transcriptome analysis to investigate the molecular mechanism underlying the tolerance of hybrid willow to salt-flooding. Seedings were, respectively, treated with 200 mM NaCl and flooded with water or 200 mM NaCl solution for 3 d, 10 d, and 17 d. We identified 1842, 3350, and 2259 differentially expressed genes (DEGs) regulated by hybrid willow under single salt stress, single flooding stress, and combined salt and flooding stress, respectively. Many DEGs detected in single salt- and flooding-treated plants were expressed differentially after the combined salt and flooding. Most of the shared transcripts exhibited similar fold changes in common molecular responses such as detoxification of reactive oxygen species (ROS) and signaling pathways related to calcium, phytohormones, and protein kinases, which were also observed in plants exposed to each stress individually. Additionally, a large number of specific DEGs were identified under salt-flooding stress, primarily associated with cell wall remodeling, osmotic adjustments, stress signaling, primary metabolism, and ROS scavenging. KEGG (Kyoto Encyclopedia of Genes and Genomes) annotation indicated that hybrid willow leaves responded to salt-flooding stress mainly through phytohormone signaling and MAPK signaling pathways. Overall, this study provides new insights into the molecular mechanisms underlying the response of *Salix* species to multiple stresses and identifies potential candidate genes for enhancing the performance of hybrid willows.

Keywords: hybrid willow; salt stress; flooding stress; transcriptome profiles; differential gene expression

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1. Introduction

Coastal areas in Eastern China are facing an increasing threat of salinity and flooding due to both human-induced hydrological changes and natural processes. Furthermore, according to global climate change scenarios, larger coastal zones may experience a combination of salinity, flooding, and sea level rise [1]. The impact of salinity on plants can be observed in two main aspects: firstly, excessive salinity hinders water absorption by the root system, leading to physiological drought; secondly, the accumulation of salinity results in salt ion toxicity [2]. The secondary effects of salinity stress are more intricate, encompassing damage to cell components, oxidative stress, and metabolic disorders [3]. Flooding

stress, being one of the most significant abiotic stresses, affects various stages of terrestrial plant lifecycles, such as germination, seedling establishment, vegetative growth, and reproductive growth [4]. Flooding significantly restricts air exchange between plants and the atmosphere, thereby inhibiting aerobic respiration and reducing energy metabolism [5].

The interaction between salt and flooding can cause more damage than either salt or flooding alone [6]. While the effects of salt and flooding stress individually have been extensively studied using various methods, there have been limited studies on the combined effects of these two abiotic stresses. Both field and laboratory experiments have been conducted to assess the impact of combined stress on plant growth, antioxidant capacity, chloroplast ATP synthesis, fermentation enzyme activity, leaf photosynthesis, and aerenchyma formation [7–9]. However, the molecular regulatory networks involved are not well understood.

RNA sequencing has been utilized to investigate the molecular mechanisms underlying salt and flooding tolerance in trees. When exposed to salt stress, trees experience a rapid accumulation of Na^+ ions, which triggers the production of downstream signaling molecules that serve as regulators in the signaling pathway [10]. The Ca^{2+} signaling pathway is one of the most extensively studied pathways: excessive Na^+ induces the opening of intracellular Ca^{2+} channels, resulting in an increase in Ca^{2+} levels. This increase serves as an early signal for the plant's response to salt stress [11]. MYB, NAC, bHLH, and DREB are crucial transcription factor genes (TFs) involved in the removal of ROS [12], and they play essential roles in the salt stress response of trees [13]. A study employing virus-induced gene silencing (VIGS) to silence *SmERF B1-2* in *Salix matsudana* demonstrated a significant reduction in salt tolerance [14]. Another study focusing on the DREB transcription factor (TF) family in *Salix matsudana* revealed that *SmDREB A1-4* enhances plant tolerance to salinity by regulating ion homeostasis, reducing the Na^+/K^+ ratio, and improving proline biosynthesis [15]. These studies offer novel insights into the cultivation of salt-tolerant plants and shed light on the adaptive mechanisms of trees in salt environments.

Previous transcriptome studies have demonstrated the crucial role of key pathways in trees' response to flooding stress [16,17]. These pathways include photosystem, redox reactions, and alternative mobilization of energy reserves [16]. Furthermore, additional studies have partially revealed the mechanism of hypoxia-induced transcriptional and translational regulation in plants [18]. A study of *Morus alba* showed that genes associated with cytokinin, ethylene, and MAPK signaling were significantly upregulated under flooding treatment [19]. Another study focused on *Salix matsudana*, which overexpressed *SmTTF30* and enhanced its tolerance to flooding stress by increasing peroxidase activity and reducing malondialdehyde content [20]. These findings provide valuable insights into the mechanisms underlying submergence tolerance in trees.

Hybrid willow, a cross between *Salix matsudana* and *Salix alba*, is extensively cultivated in the eastern coastal areas of China and New Zealand and is characterized by rapid regeneration and growth. This species exhibits a remarkable tolerance to adverse environmental conditions such as flooding, drought, alkaline soil, high salinity, and high temperatures. Additionally, its strong wind-breaking ability makes it an ideal choice for coastal regions in China. The planting of hybrid willow in these areas not only aids in soil restoration but also helps in reducing salinity [1]. Compared to other *Salix* species, *Salix matsudana* × *alba* shows greater tolerance to salt and flooding. However, it is important to note that if salinity levels and flooding duration surpass a certain threshold, the survival and growth rates of hybrid willow can be significantly reduced.

To investigate the mechanisms of salt, flooding, and salt-flooding tolerance in hybrid willow, we conducted a comparative analysis of gene expression in seedlings exposed to normal, salt, flooding, and salt-flooding stress conditions. While the salt and flooding stress responses of *Salix* species are well documented [21–23], the combined effect of both stresses is not well understood. By unraveling the molecular mechanisms underlying the response of hybrid willow to multiple stresses, our study may contribute to the genetic improvement of coastal plants, the enhancement of saline soil, and the mitigation of coastal

area expansion. Additionally, our findings provide novel insights into the systemic response of plants to salt-flooding stress.

2. Materials and Methods

2.1. Plant Growth and Salt Stress Treatment

Hybrid willow plants were obtained from the Nanjing Forestry University, Jiangsu Province, China, in March 2022. This experiment was performed in the greenhouse at Southern Seed Testing Center of State Forestry Administration, Nanjing, China. Planting was conducted with clay containers (18 cm × 18 cm) containing sterilized vermiculite and irrigated every 2 days. The culture conditions were a 16 h light/8 h dark cycle and 1000 $\mu molm^{-2}s^{-1}$ canopy glare at 20–25 °C. After 60 days of growth, plants with uniform size were selected for the experiment.

To study the effects of salt and flooding stress combined, hybrid willow plants were flooded with 200 mM NaCl solution. Meanwhile, two groups of seedlings were either treated with 200 mM NaCl or flooded with water to compare the effect of single stress. For flooding stress, the containers were flooded with water to ensure that the roots of hybrid willow were submerged. Control groups were only irrigated with water to maintain 80~85% field capacity. According to our previous study, 3 days, 10 days, and 17 days may be the key time points for the salt-flooding stress response of hybrid willow. So, after a 3-, 10- and 17-day treatment, fresh leaf tissues of those seedings were obtained from control groups and treated plants (salt, flooding, and salt-flooding combined) and sent for transcriptome analysis. Three biological replicates (10 seedlings for each replicate) were performed for each treatment.

2.2. RNA Extraction, Library Preparation and Sequencing

Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and genomic DNA was removed using DNase I (TaKara) according to the manufacturer's instructions. RNA degradation and contamination were monitored on a 1% agarose gel. The quality, integrity, and purity of the total RNA were determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and quantified using the ND-2000 (NanoDrop Technologies, Wilmington, DE, USA). RNA purification, reverse transcription, library construction, and sequencing were performed by Shanghai Majorbio Biopharmaceutical Biotechnology Co., Ltd. (Shanghai, China) according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

The RNA-seq transcriptome library for *Salix matsudana* × *alba* was generated using the Illumina® Stranded mRNA Prep, Ligation Kit from Illumina (San Diego, CA, USA). A total of 1 μg of RNA was utilized in the library construction process. Initially, messenger RNA was isolated through polyA selection using oligo(dT) beads. Subsequently, the isolated mRNA was fragmented using a fragmentation buffer. After fragmentation, double-stranded cDNA synthesis was performed employing the SuperScript double-stranded cDNA synthesis kit (Invitrogen, San Diego, CA, USA) with random hexamer primers from Illumina. Following cDNA synthesis, the resulting cDNA underwent end-repair, phosphorylation, and the addition of an "A" base, as outlined by Illumina's library construction protocol. To obtain cDNA target fragments of approximately 300 bp, size selection was conducted on a 2% low-range ultra agarose gel. The size-selected fragments were then amplified via PCR using Phusion DNA polymerase (NEB) for a total of 15 PCR cycles. Once quantified using the Qubit 4.0, the paired-end RNA-seq sequencing library was subjected to sequencing on the NovaSeq Xplus platform, generating reads with 2 × 150 bp read length.

2.3. Gene Annotation, Differential Expression Analysis and Functional Enrichment

The raw paired-end reads were trimmed and quality controlled by fastp (https://github.com/OpenGene/fastp, accessed on 1 March 2023) with default parameters [24]. The clean data from the samples were then used for de novo assembly with Trinity (http://trinityrnaseq.sourceforge.net/, accessed on 1 March 2023) [25]. The assembled transcripts

were assessed and optimized using BUSCO (Benchmarking Universal Single-Copy Orthologs, http://busco.ezlab.org, accessed on 1 March 2023), TransRate (http://hibberdlab.com/transrate/, accessed on 1 March 2023) [26] and CD-HIT (http://weizhongli-lab.org/cd-hit/, accessed on 1 March 2023) [27]. BLASTX was employed to search all assembled transcripts in the NCBI protein non-redundant (NR, http://ftp.ncbi.nlm.nih.gov/blast/db/, accessed on 1 March 2023), Swiss-Prot (http://web.expasy.org/docs/swiss-prot_guideline.html, accessed on 1 March 2023), Pfam (http://pfam.xfam.org/, accessed on 1 March 2023), Clusters of Orthologous Groups of proteins (COG, http://www.ncbi.nlm.nih.gov/COG/, accessed on 1 March 2023), GO (http://www.geneontology.org, accessed on 1 March 2023) and KEGG (http://www.genome.jp/kegg/, accessed on 1 March 2023) databases to identify specific transcripts and search for the most similar proteins for annotations, with a typical cut-off E-value set below 1.0×10^{-5}.

To identify two groups of differentially expressed genes (DEGs), the TPM method (transcripts per million reads) was used to calculate the expression level of each gene. RSEM (http://deweylab.biostat.wisc.edu/rsem/, accessed on 1 March 2023) [28] was used to quantify gene abundances. Differential expression analysis was then performed using DESeq2 [29]. DEGs with $|\log2\ (\text{foldchange})| \geq 1$ and P-adjust ≤ 0.05 were considered to be significantly different expressed genes. In addition, functional enrichment analysis including GO (Gene Ontology, http://www.geneontology.org, accessed on 1 March 2023) and KEGG (http://www.genome.jp/kegg/, accessed on 1 March 2023) was performed to identify which DEGs were significantly enriched. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (https://github.com/tanghaibao/Goatools, accessed on 1 March 2023) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do, accessed on 1 March 2023) [30]. Statistical analyses were performed with GraphPad Prism 9 and Microsoft Excel 2021.

2.4. Quantitative Real-Time PCR (qRT-PCR) Validation

The qRT-PCR experiments were conducted in triplicates using a LightCycler Roche 480 instrument (Roche Applied Science, Mannheim, Germany). The primers for qPCR were designed using Primer Premier 6.0 (United States) and a 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) was employed to detect the double-stranded DNA synthesis. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

3. Results

3.1. Transcriptome Sequencing of Hybrid Willow under Salt, Flooding, and Salt-Flooding Stress

To investigate the mechanism of salt-flooding tolerance in hybrid willow, we collected leaf samples at different stages and sequenced them. After removing low-quality data, we obtained approximately 204.43 Gb of sequence data. These samples had >97% Q20 quality scores and approximately 44.5% GC content (Table 1). The percentage of clean reads that mapped to the reference transcriptome ranged from 77.33% to 82.16% (Table S1). To reduce redundancy in gene expression analysis, we retained the longest transcript (defined as unigene). In total, 163,243 unigenes were achieved with 1214 bp N50 length (Table 2). We also employed BUSCO to assess the completeness and quality of the assembly. The results showed that 71% of the unigenes were complete, including 68.1% single-copy and 2.9% duplicate transcripts, indicating high completeness of the assembled no-reference transcripts (Table S2). Among all the unigenes assembled, approximately 52% were between 200 and 500 bp, while approximately 27% were between 501 and 1500 bp. Unigenes ranging from 1501 to 3000 bp accounted for about 8% of the total, and about 4% exceeded 3001 bp (Figure 1).

Table 1. Summary of transcriptome sequencing data.

Sample	Total Raw Reads	Total Clean Reads	Total Clean Bases (GB)	Clean Reads Q20 (%)	Clean Reads Q30 (%)	Clean Reads Ratio (%)	GC Content (%)
CK_1	45,451,206	44,702,578	65.30	97.66	93.14	98.35	43.98
CK_2	44,426,128	43,848,962	63.78	97.89	93.72	98.7	44.98
CK_3	45,520,846	44,651,966	65.19	97.59	92.96	98.09	44.07
ST_3_1	44,216,724	43,664,338	64.37	97.62	93.01	98.75	44.51
ST_3_2	41,298,648	40,814,132	60.08	97.72	93.24	98.83	44.56
ST_3_3	45,022,232	44,478,730	65.57	97.63	93	98.79	44.44
ST_10_1	48,453,000	47,930,538	70.69	97.81	93.46	98.92	44.58
ST_10_2	43,512,372	43,153,996	63.89	97.68	93.09	99.18	44.45
ST_10_3	67,611,152	66,799,650	98.79	97.75	93.33	98.8	44.37
ST_17_1	51,127,904	50,643,462	74.61	97.85	93.53	99.05	44.82
ST_17_2	47,035,390	46,602,844	68.55	97.76	93.31	99.08	44.74
ST_17_3	41,987,562	41,580,708	61.23	97.8	93.42	99.03	44.81
WT_3_1	42,934,906	42,464,178	62.68	97.6	92.91	98.9	44.6
WT_3_2	44,325,100	43,796,830	64.50	97.71	93.23	98.81	44.44
WT_3_3	47,550,540	47,055,452	69.41	97.66	93.07	98.96	44.62
WT_10_1	46,635,646	46,043,560	67.73	97.59	92.95	98.73	44.27
WT_10_2	55,711,672	54,957,240	80.67	97.67	93.18	98.65	44.33
WT_10_3	48,916,490	48,299,826	71.28	97.69	93.21	98.74	44.27
WT_17_1	49,116,992	48,671,458	71.32	97.7	93.2	99.09	44.68
WT_17_2	44,362,276	43,857,480	64.34	97.67	93.14	98.86	44.53
WT_17_3	42,417,550	42,023,118	62.10	97.69	93.15	99.07	44.64
SWT_3_1	44,207,598	43,578,662	64.18	97.78	93.41	98.58	45.06
SWT_3_2	48,022,738	47,128,898	69.38	97.83	93.56	98.14	45.11
SWT_3_3	45,328,260	44,584,744	65.23	97.76	93.38	98.36	44.53
SWT_10_1	46,336,220	45,770,356	67.38	97.67	93.11	98.78	44.22
SWT_10_2	45,867,922	45,482,290	67.09	97.86	93.55	99.16	44.21
SWT_10_3	44,405,302	43,861,652	64.36	97.7	93.22	98.78	44.19
SWT_17_1	50,519,722	50,137,692	73.86	97.91	93.68	99.24	45.28
SWT_17_2	45,379,484	44,987,660	66.77	97.65	92.99	99.14	44.35
SWT_17_3	47,787,554	47,232,156	69.93	97.66	93.08	98.84	44.42

Note: CK: untreated; ST_3: treated with 200 mM NaCl for 3 days; ST_10: treated with 200 mM NaCl for 10 days; ST_17: treated with 200 mM NaCl for 17 days; WT_3: flooding treated for 3 days; WT_10: flooding treated for 10 days; WT_17: flooding treated for 17 days; SWT_3: flooded with 200 mM NaCl solution for 3 days; SWT_10: flooded with 200 mM NaCl solution for 10 days; SWT_17: flooded with 200 mM NaCl solution for 17 days.

Table 2. Summary of transcriptome assembly quality.

Type	Total Number	Largest Length (bp)	Smallest Length (bp)	Average Length (bp)	N50 Length (bp)	Fragment Mapped Percent (%)	GC Percent (%)	BUSCO Score
Unigene	163,243	36,559	201	809.39	1214	50.871	40.51	C: 71% (S: 68.1%; D: 2.9%)
Transcript	336,728	36,559	201	947.02	1479	73.365	40.62	C: 89.1% (S: 25.1%; D: 64%)

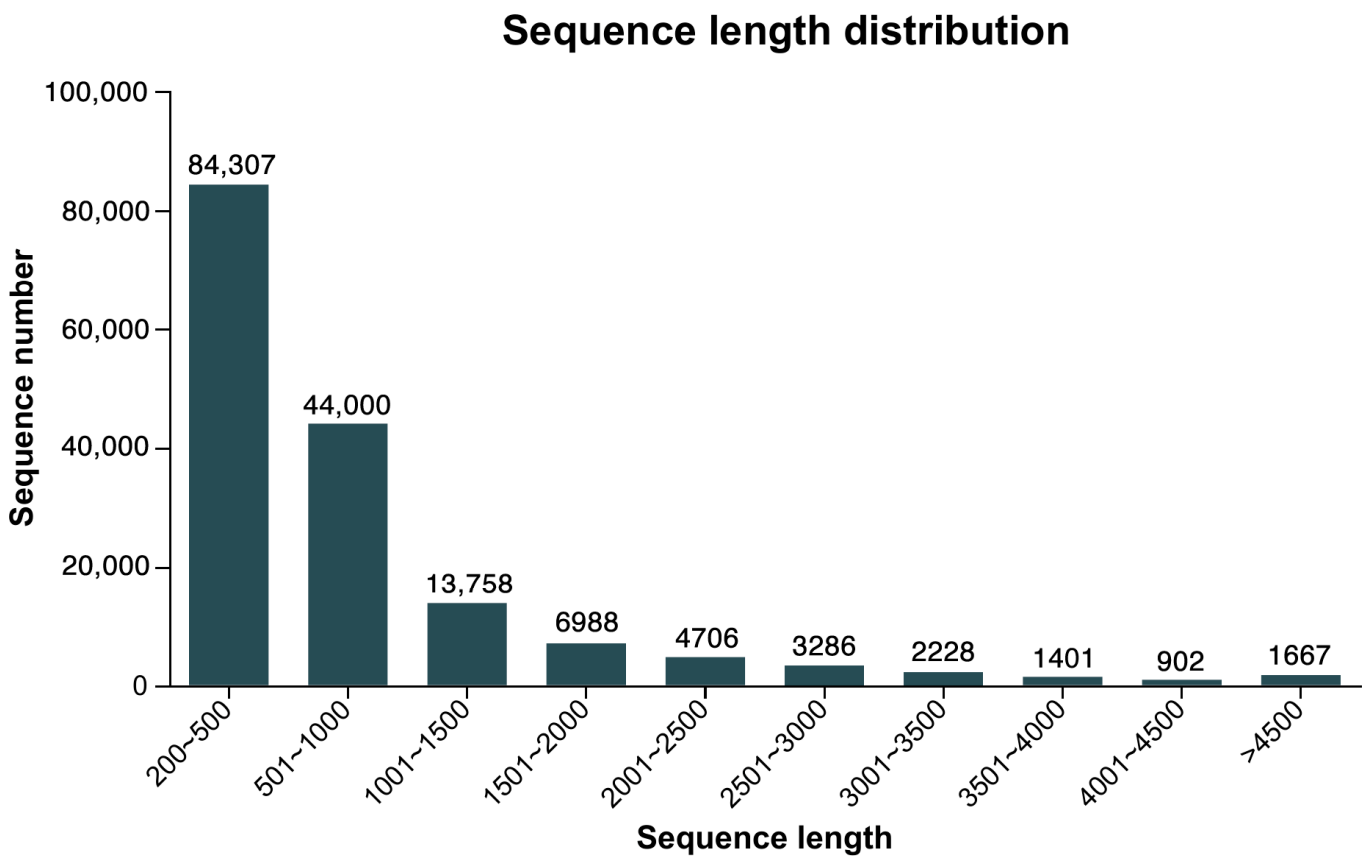

Figure 1. Summary of sequence length distribution.

The TPM data were used for principal component analysis to examine the relationship between transcriptomes of the different samples. A two-dimensional plot revealed that leaf samples from the same treatment (three biological replicates) clustered together, while the groups appeared scattered under different conditions (Figures S1–S3). Furthermore, the expression patterns of unigenes in hybrid willow samples from different groups exhibited some degree of variation (Figure S4).

3.2. Functional Annotation of Hybrid Willow Transcriptome

A total of 163,124 unigenes were annotated in various databases including GO (55,678), KEGG (44,746), eggNOG (90,623), NR (101,829), Swiss-Prot (66,359), and Pfam (43,236). The study found that the overall annotation success rate was 66.54%. Table 3 provides the success rate of gene annotation, which ranged from 26.5% (Pfam database) to 62.42% (NR database). However, due to the lack of high-quality hybrid willow genomic data, the remaining unigenes could not be annotated.

Table 3. Success rate of gene annotation.

	Unigene Number	Percentage (%)
GO	55,678	52.52
KEGG	44,746	27.43
eggNOG	90,623	55.55
NR	101,829	62.42
Swiss-Prot	66,359	40.68
Pfam	43,236	26.5
Total_anno	108,550	66.54
Total	163,124	100

In this study, a total of 55,678 unigenes were clustered into 52 GO terms (Figure 2, Table S3), which can be classified into three categories: molecular function, cellular component, and biological process. In the molecular function category, the most abundant term was binding (47,694), followed by catalytic activity (41,903) and transporter activity (4936). The cellular component category mainly included the cell part (39,993), membrane part (32,429), and organelle (22,944). Moreover, genes related to the cellular process (40,977), metabolic process (38,044), and biological regulation (13,230) were highly represented in the biological process category. Furthermore, through comparison and analysis using the KEGG database (Figure 3), a total of 44,746 unigenes could be classified into five major metabolic pathways: metabolism (9945), genetic information processing (7838), environmental information processing (2021), cellular processes (2086), and organismal systems (956). These pathways were further subdivided into 20 sub-metabolic pathways. The majority of sub-metabolic pathways were related to carbohydrate metabolism (3416), translation (3249), folding, sorting, and degradation (2917), transport and catabolism (2086), among others. The results of functional annotation were utilized for further analysis.

3.3. Differential Gene Expression Analysis of Hybrid Willow under Salt, Flooding, and Salt-Flooding Stress

To investigate gene expression in hybrid willow under salt stress, we compared the changes in gene expression between the control group (CK) and the treatment groups (ST_3, ST_10, and ST_17). Most genes showed high expression levels (Table S4). We screened for DEGs using a significance level of $p < 0.05$ and a fold change of more than double, and calculated gene expression using TPM values. Figure 4 illustrates the identification of DEGs in the hybrid willow samples at each sampling time. The largest difference in DEGs was observed between ST_10 and CK, with 6651 upregulated and 8535 downregulated genes, while the smallest differences were found between ST_3 and CK, with 2277 upregulated and 2298 downregulated genes. Moreover, we detected 949 upregulated and 860 downregulated genes that were consistently present in all treatment groups.

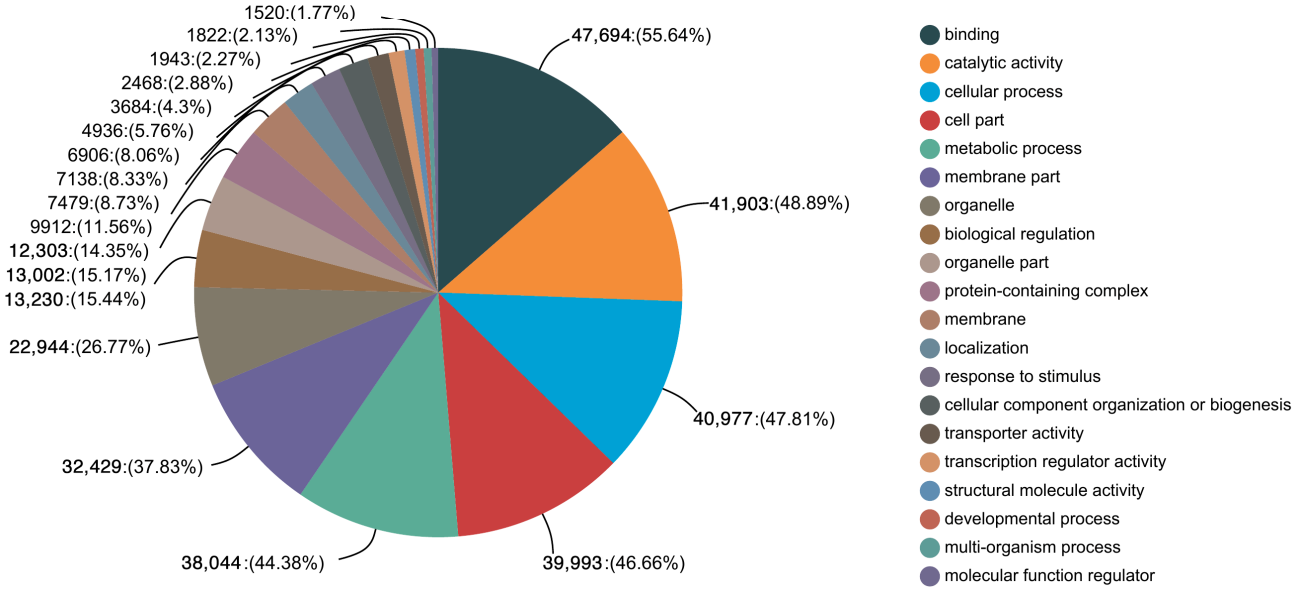

Figure 2. GO classification of unigenes.

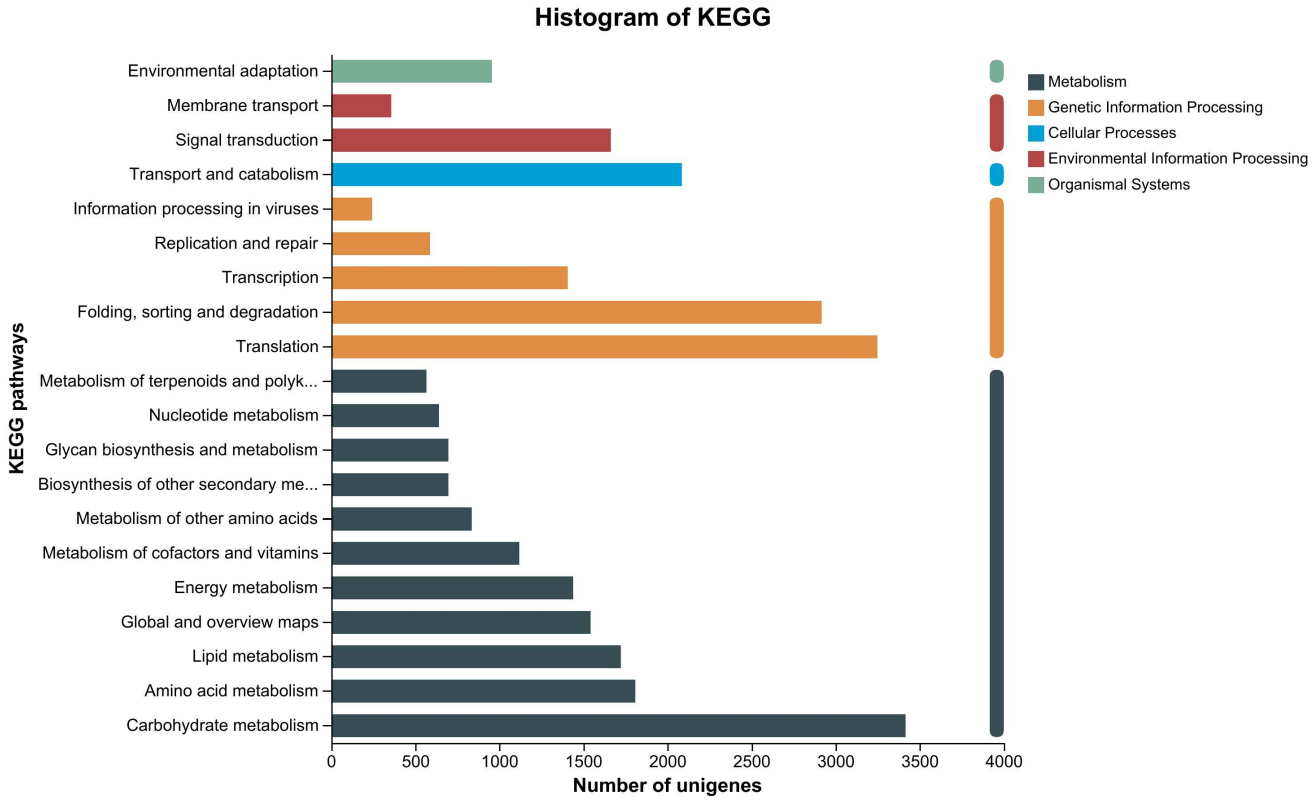

Figure 3. Histogram of KOG classification of unigenes.

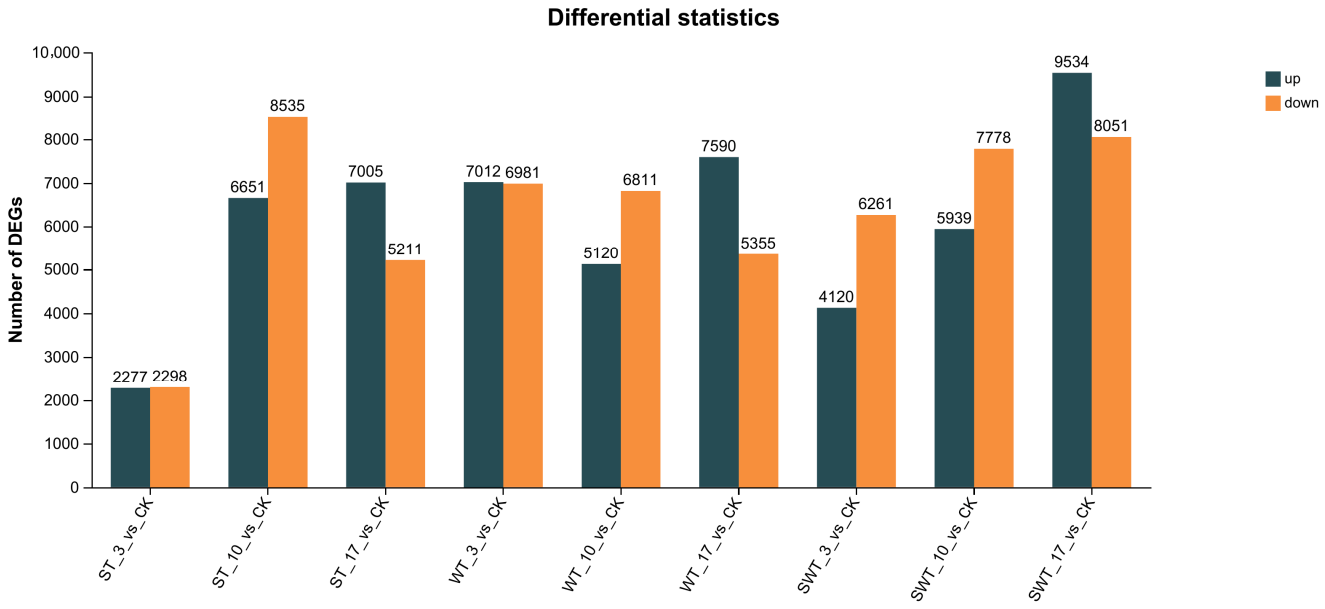

Figure 4. Summary of the number of upregulated and downregulated DEGs.

To investigate the transcriptional responses to flooding stress, we conducted an experiment using hybrid willow seedlings that were planted in a submerged condition. A total of 24,526 DEGs were identified. We then compared the DEGs between the flooding stressed and control samples to determine changes in gene expression. Among these DEGs, 8833 were upregulated and 8794 were downregulated. Additionally, we detected a number of unique DEGs. For instance, when comparing WT_3 vs. CK, WT_10 vs. CK,

and WT_17 vs. CK, we found 7012, 5120, and 7590 upregulated DEGs, and 6981, 6811, and 5355 downregulated DEGs, respectively.

To reveal the effects of salt combined with flooding stress on hybrid willow, we compared the salt-flooding stress samples with the control group. Specifically, we analyzed the differences between SWT_3 and CK, SWT_10 and CK, and SWT_17 and CK, which resulted in 10,381, 13,717, and 17,585 DEGs, respectively. Interestingly, we observed that the number of DEGs in hybrid willow increased continuously with treatment time when exposed to salt-flooding stress (Figure 4). This suggests that the response mechanism of hybrid willow to salt-flooding stress is complex, and the transcriptome level is influenced by the duration and intensity of salt, flooding, and salt-flooding treatments.

3.4. Functional Classification of DEGs

To elucidate the functional characteristics of the DEGs, we conducted GO analysis to understand the mechanisms underlying salt, flooding, and salt-flooding tolerance in hybrid willow. Figure 5 shows that twenty GO terms were identified in hybrid willow. The DEGs from the salt stress treatment vs. control group, flooding stress treatment vs. control group, and salt-flooding stress treatment vs. control group were enriched in similar functional categories, as shown in Tables S5–S7. In the cellular component category, the majority of the DEGs were associated with the cell part, membrane part, and organelle. In terms of biological processes, most of the DEGs were involved in cellular processes, metabolic processes, and biological regulation. In the molecular function category, the DEGs were primarily associated with binding and catalytic activity.

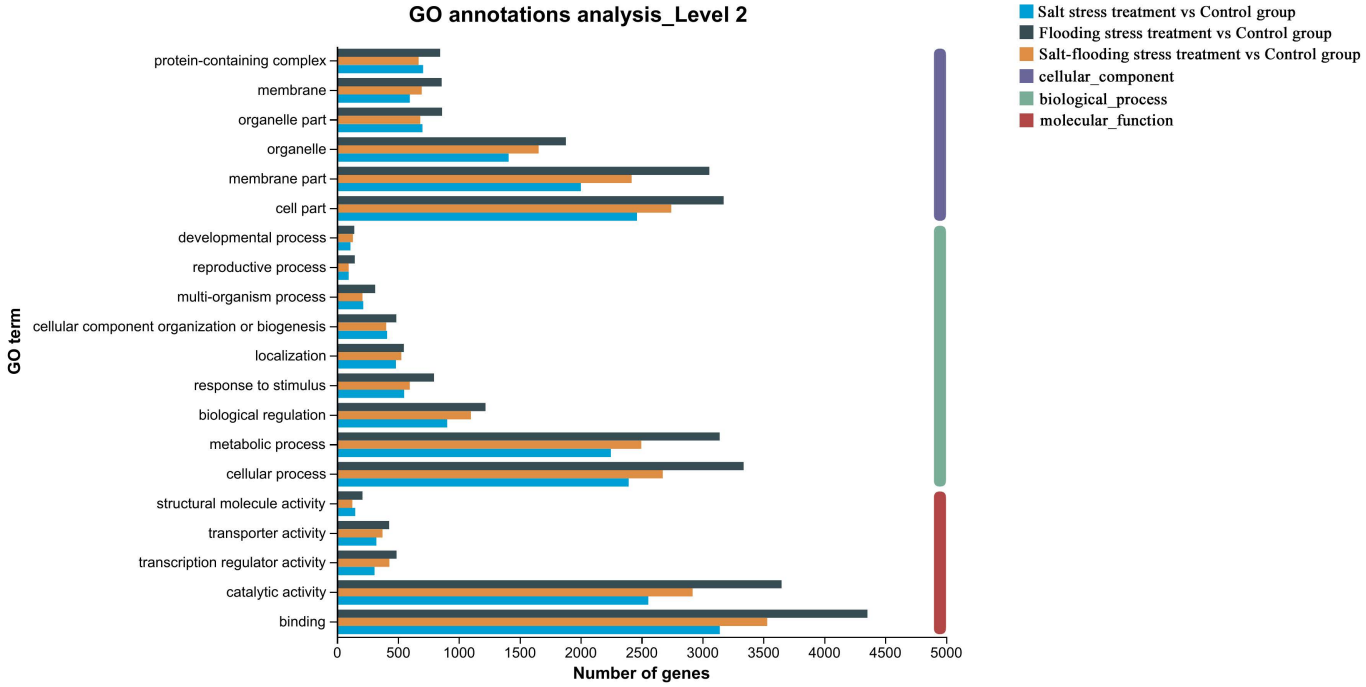

Figure 5. GO annotations analysis of DEGs in the leaves of hybrid willow.

The KEGG enrichment analysis of DEGs under salt, flooding, and salt-flooding stress was conducted, and the results are shown in Figure 6. For the salt stress and salt-flooding stress treatments, the DEGs were categorized into 127 pathways (Tables S8 and S9). In contrast, for the flooding stress treatment, 22,545 DEGs were categorized into 135 pathways (Table S10). Among these, three pathways were significantly overrepresented under salt stress treatment ($p \leq 0.01$), namely inositol phosphate metabolism, circadian rhythm, and glycerolipid metabolism (Table S8). Five pathways were significantly overrepresented under flooding stress, including plant hormone signal transduction, pathogen interaction, MAPK signaling pathway, α-Linolenic acid metabolism, and circadian rhythm (Table S10).

When the salt and flooding stresses were combined, a total of seven pathways were significantly overrepresented. Interestingly, unlike the single stress conditions, the first two pathways overrepresented under the combined stress were plant hormone signal transduction (Figure 7) and MAPK signaling pathway (Figure 8).

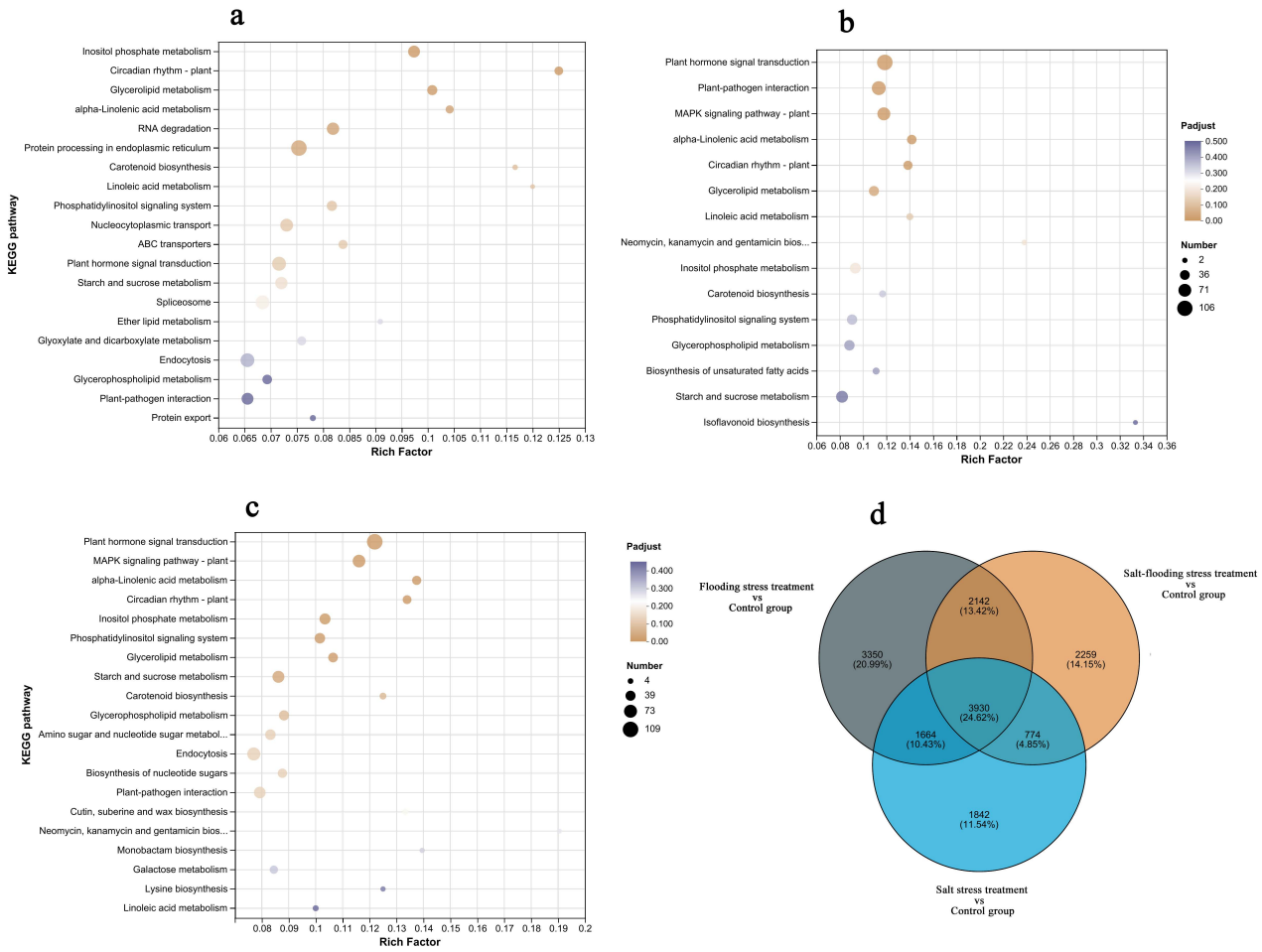

Figure 6. (**a**) KEGG enrichment of DEGs under salt stress, (**b**) KEGG enrichment of DEGs under flooding stress, (**c**) KEGG enrichment of DEGs under salt-flooding stress, (**d**) Venn diagram analysis of DEGs.

3.5. Identification of Key DEGs in Response to Salt, Flooding, and Salt-Flooding Stress

Under salt stress, 118 DEGs identified were associated with cell wall modification or synthesis. Most of them, such as the cellulose synthase-like, xyloglucan endotransglucosylase/hydrolase, mechanosensitive ion channel, and polygalacturonase genes were downregulated under salt stress. These genes were involved in cell expansion, loosening, and elongation. Some genes associated with ion transporting were identified under salt stress, including 15 potassium transporters, 11 phosphate transporters, 9 cyclic nucleotide-gated ion channels, 7 ligand-gated ion channels, 6 potassium channels, 6 magnesium transports, 2 sodium/calcium exchangers, 3 vacuolar cation/proton exchangers, 2 sodium/hydrogen exchangers, 2 chloride channels, 1 sodium/calcium exchanger, 1 magnesium/proton exchanger, and 1 boron transporter. In this study, a total of 64 DEGs were identified that related to hormone signal transduction, including 17 ethylene, 8 cytokinin, 7 auxin, 7 abscisic acid (ABA), 6 jasmonic acid (JA), 3 salicylic acid, and 3 brassinosteroid genes. Furthermore, a total of 22 antioxidant-related DEGs were identified which belong to the superoxide peroxidase, dismutase, and ascorbate peroxidase families.

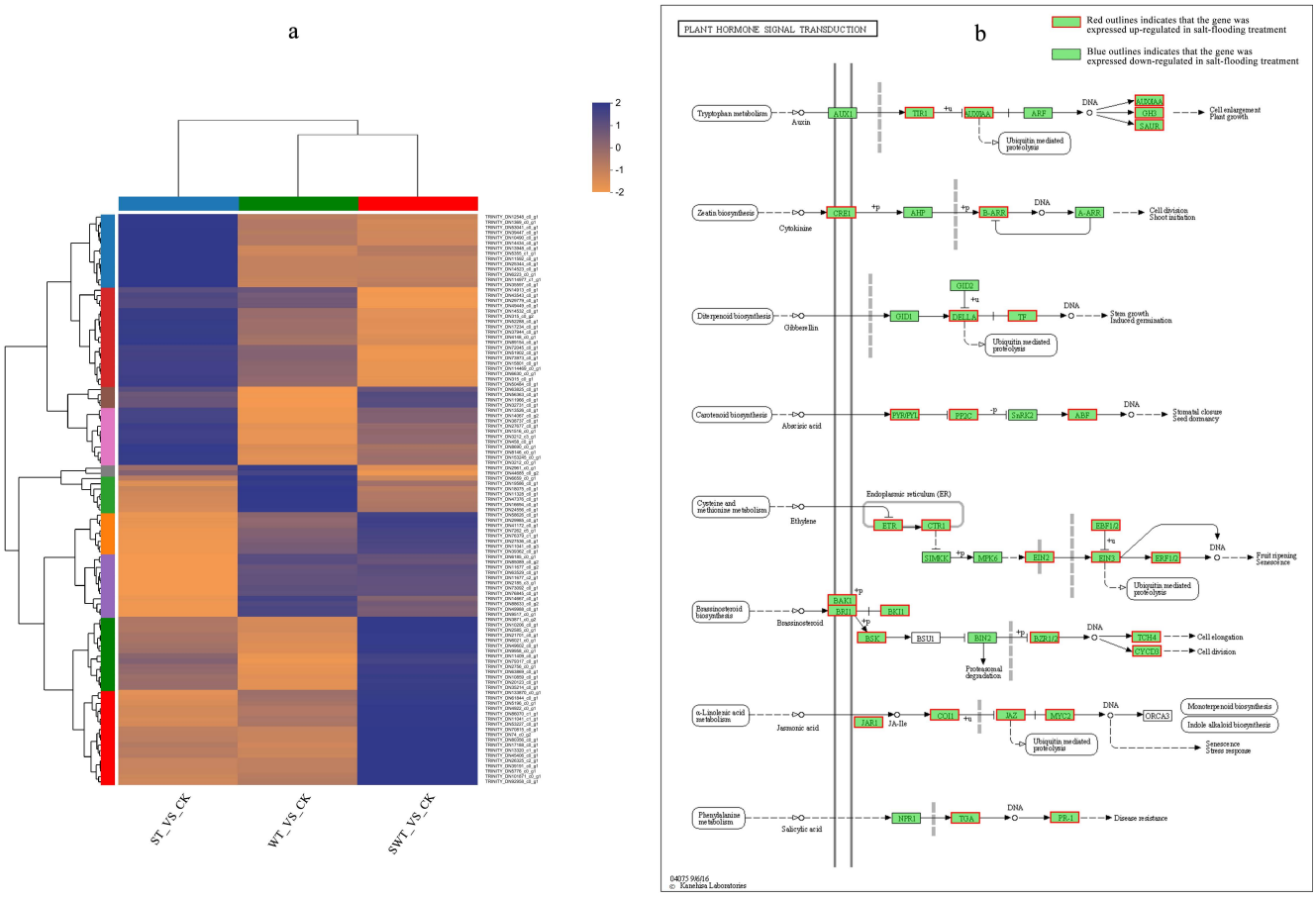

Figure 7. (**a**) Heatmaps of DEGs that were enriched in plant hormone signal transduction pathway in hybrid willow under salt, flooding, and salt-flooding stress; expression levels were quantified using TPM values, (**b**) plant hormone signal transduction pathway involved in the response to salt-flooding stress. The yellow color in the heatmap shows the downregulation of DEGs and the blue color shows the upregulation when the DEGs were compared among various combinations as mentioned at the bottom of each column. Green in pathway diagram represents annotated DEGs in salt-flooding treatment. Red outlines indicate that the gene was expressed upregulated and blue outlines indicate that the gene was expressed downregulated. ST: salt-treated group; WT: flooding-treated group; SWT: salt-flooding-treated group; Ck: control group.

Under flooding stress, a total of 162 DEGs were identified that related to cell wall modification or synthesis. Similar to salt stress, cellulose synthase-like, xyloglucan endotransglucosylase/hydrolase, mechanosensitive ion channel, and polygalacturonase genes were downregulated under flooding stress. Some genes encoding ion transport were identified as differentially expressed under flooding stress, including 23 phosphate transporters, 13 potassium transporters, 8 ligand-gated ion channels, 7 magnesium transports, 5 cyclic nucleotide-gated ion channels, 5 potassium channels, 5 chloride channels, 4 sodium/calcium exchangers, 3 vacuolar cation/proton exchangers, and 1 sodium/hydrogen exchanger. Compared to salt stress, there were more DEGs related to hormone signal transduction under flooding stress, with 106 in total; mainly including auxin, ethylene, ABA, cytokinin, and JA-related genes. Meanwhile, antioxidant-related DEGs were also more identified in flooding stress, almost twice as much as in salt stress. These DEGs belong to the superoxide peroxidase, dismutase, and ascorbate peroxidase families.

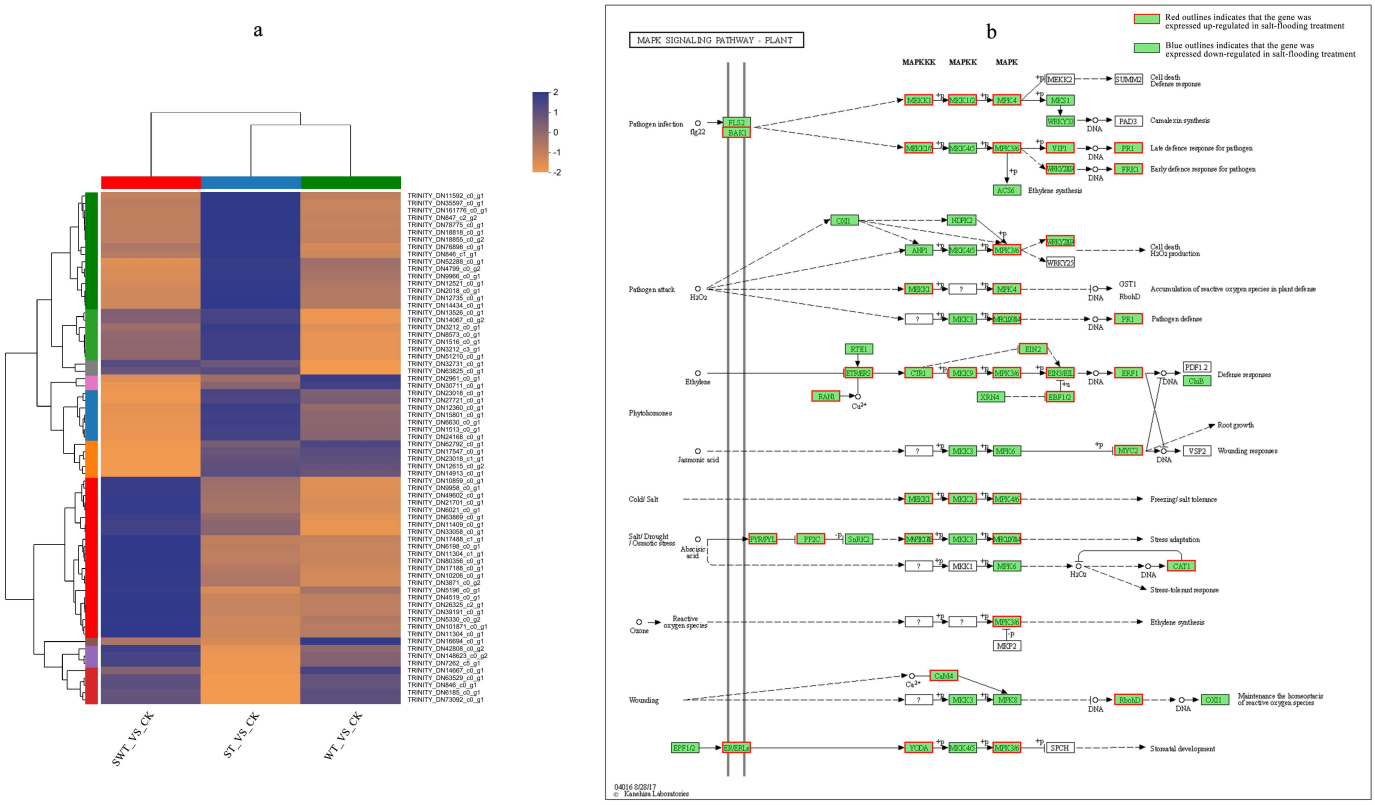

Figure 8. (**a**) Heatmaps of DEGs that were enriched in MAPK signaling pathway in hybrid willow under salt, flooding, and salt-flooding stress; expression levels were quantified using TPM values, (**b**) MAPK signaling pathway involved in the response to salt-flooding stress.

The molecular mechanisms involved in salt-flooding tolerance in hybrid willow were more complicated. Many DEGs may be related to salt-flooding stress response, including 138 cell wall modification or synthesis, 170 ion transport, 109 hormone signal transduction, and 30 antioxidant-related genes (Table S4). Although the molecular mechanism of tolerance to combined stress and single stress in hybrid willow has some similarities, the number of DEGs identified in each group was different. Meanwhile, the expression levels and patterns of key DEGs in response to different stress vary greatly (Table S11).

3.6. Identification of TFs

To identify salt, flooding, and salt-flooding stress-related genes in hybrid willow leaves, BLASTX was used to search unigene sequences, and 1725 TFs were identified and divided into 33 families (Table S12). MYB_superfamily, AP2/ERF, C2C2, bHLH, NAC, GRAS, WRKY, and bZIP were involved in plant responses to abiotic stresses. For all comparison groups in this study, AP2/ERF, bHLH, and NAC were the first three upregulated TFs, and the first three downregulated differential TFs were MYB_superfamily, C2C2, and AP2/ERF. According to the hierarchical cluster analysis of TFs under salt, flooding, and salt-flooding stress (Figures S5–S7), the TFs can be divided into an upregulated group and a downregulated group. It suggests that different TF families are functionally biased in response to different treatments.

3.7. Verification of RNA-Seq Data by qRT-PCR

To validate the RNA-seq results and analyze the changing expression patterns of the DEGs, we selected nine candidate genes for qRT-PCR. These genes include *WRKY32*, *WRKY41*, *WRKY51*, calcium-binding protein (CML3), and serine/threonine-protein kinase (CTR1), among others (Table S13). All the selected genes were related to the salt and flooding stress. The expression patterns of nine selected unigenes under different salt-

flooding stress durations were measured by qRT-PCR and the results were found to be similar to those of RNA-seq. This indicates that the RNA-seq data presented in this study are reliable (Figure 9).

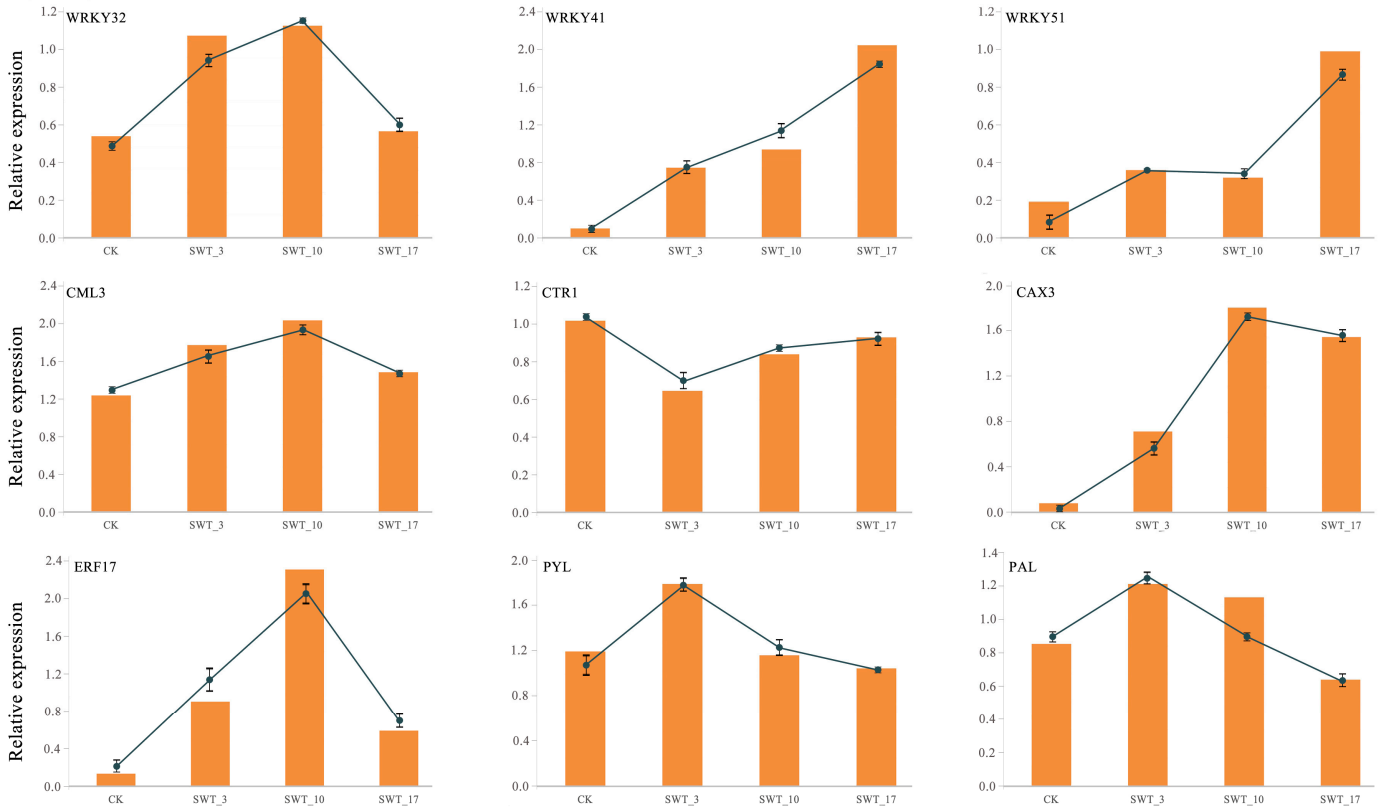

Figure 9. Expression patterns of 9 selected unigenes between the control group and salt-flooding stress group in hybrid willow. The relative expression levels of each DEG are indicated on the *y*-axis.

4. Discussion

Plants in coastal areas often face harsh environments such as salt and flooding, which can lead to cellular ionic toxicity, osmotic stress, and oxidative stress. These conditions can result in wilting, slow growth, or even death of most plants [31].

Over the course of long-term evolution, plants have developed complex regulatory networks to adapt to these challenging environments. Salt-flooding tolerant species, for example, can thrive in coastal areas and be used to protect saline wetlands, mitigate soil erosion, and aid in bioremediation. Hybrid willow, known for its high tolerance to biotic stresses, has been widely utilized for various purposes such as pulp production, timber, and afforestation. However, the research on the molecular mechanisms of hybrid willow in response to abiotic stresses is in its early stages. In this study, we investigated the tolerance mechanisms of hybrid willow to salt, flooding, and their combination by analyzing the transcriptome data of hybrid willow leaves under different treatment conditions.

4.1. Hybrid Willow Responds to Salt-Flooding Stress through Plant Hormone Signal Transduction

In this experiment, a total of 127 pathways were identified in the KEGG differential gene enrichment pathways analysis. The top pathways included plant hormone signal transduction, MAPK signaling pathway, and α-Linolenic acid metabolism, indicating that the salt-flooding stress affected a wide range of physiological processes.

Phytohormones play a crucial role in plant response to abiotic stresses. Plant growth regulators, such as ABA, ethylene, gibberellin, auxin, and cytokinin, are involved in signaling pathways that influence cellular responses to flooding stress. The analysis of hormonal pathways revealed significant changes in transcript levels related to ABA

signaling under flooding conditions. The expression of the main ABA receptors (PYL, PYR) was significantly altered. Additionally, changes in DEGs involved in the ethylene network were also observed in this study. Furthermore, the study revealed that several auxin-induced proteins IAAs were found to be overexpressed, indicating the potential involvement of auxin-mediated factors in the response of hybrid willow to flooding stress. The upregulation of MYC2, a key transcription factor (TF) for JA responses, was also observed in hybrid willow leaves subjected to salt-flooding stress. Additionally, the study showed that various signaling pathways mediated by cytokinins, gibberellins, ethylene, and salicylic acid were differentially regulated in hybrid willow (Figure 7).

4.2. MAPK Signaling Pathway Is a Key Pathway for Hybrid Willow to Respond to Salt-Flooding Stress

The activation of the MAPK signaling pathway primarily occurs in response to osmotic stress, with reactive oxygen species (ROS) believed to be the main inducers of this pathway [32]. This pathway consists of three components: MAP3K, MAP2K, and MAPK. MAP3K activates MAP2K by phosphorylating two serine or threonine residues, which in turn phosphorylates MAPK on threonine and tyrosine residues. Eventually, MAPK activates downstream genes [33]. The transcriptome profile of hybrid willow has revealed that genes involved in the MAPK signaling pathway play a role in responding to salt-flooding stress. When exposed to salt-flooding stress, the balance of ROS within hybrid willow cells is disrupted, leading to a rapid increase in ROS levels and subsequent activation of this pathway. Several studies have shown that RAF-like MAPKKKs are activated by hyperosmotic stress and participate in the activation of downstream SnRK2, both ABA-unresponsive and ABA-activated, thereby integrating the early osmolarity and ABA signaling cascades [34,35]. In our study, we observed that a differentially expressed gene, annotated as *MAP3K3*, was significantly upregulated in leaves subjected to salt, flooding, and salt-flooding treatment. We also found that WRKY, a key transcription factor (TF), is phosphorylated through the MAPK signaling pathway [36]. Furthermore, we identified three differentially expressed genes (DEGs) in leaf samples, all annotated as WRKY TFs, which were upregulated under salt-flooding stress. These findings suggest that these TFs may play a crucial role in the MAPK signaling pathway.

4.3. EDGs Related to Ion Transport in Hybrid Willow under Salt-Flooding Stress

Characterization of the transcriptional profile of hybrid willow revealed a potential relationship between ion transport genes and salt-flooding tolerance. When exposed to high salinity environments, roots absorb significant amounts of Na^+, which can be detrimental to plants due to disturbances in intracellular K^+/Na^+ balance and inhibition of enzyme activities. In salt-tolerant plants, cytoplasmic calcium disruptions activate the salt-sensitivity pathway [37], which in turn promotes the restoration of ion balance by activating NHX and facilitating vacuolar Na+ dissociation [38]. NHX assists in the segregation of Na^+ into vacuoles in leaf cells under salt stress. The accumulation of Na^+ in vacuoles acts as a regulator to reduce cell water potential, thereby facilitating water uptake. Thus, NHX may be involved in the transport of cytoplasmic Na^+ to vesicles and is upregulated under salt-flooding stress. Calcium, as a second messenger for abiotic stress responses, plays a crucial role in salt signaling mechanisms [39]. Changes in cellular calcium levels are regulated by a group of calcium ion-binding proteins, which are key players in response to environmental and developmental stimulation [40]. CBL and CML, two calcium ion binding proteins, were found to be overexpressed in hybrid willow samples. The expression pattern of CBL and CML in hybrid willow was consistent with a previous study on *Salix* [21], showing significant upregulation in the leaves.

4.4. Alterations of Antioxidants System in Hybrid Willow under Salt-Flooding Stress

Abiotic stresses such as drought, salt, high temperature, and flooding can result in the generation of reactive oxygen species (ROS), which can cause damage to membrane

systems and DNA, leading to cell death [41]. Flavonoids possess antioxidant effects due to their ability to chelate and scavenge metal ions, thereby preventing the generation of ROS [42]. Flavonoids are secondary metabolites of plant polyphenols and have a wide range of physiological functions. The flavonoid pathway is derived from the general phenylpropanoid pathway, and PAL serves as an important restriction enzyme that catalyzes the initial step in phenylpropanoid metabolism [43]. Salt–flooding stress can affect the accumulation or reduction of flavonoids in plants [44]. In this study, we found that phenylpropanoid biosynthesis and flavonoid biosynthesis pathways were significantly enriched, as shown in KEGG analysis (Table S9). Most genes involved in these pathways were upregulated during the first three days of treatment and then downregulated. Additionally, antioxidant enzymes such as superoxide peroxidase and dismutase play a role in scavenging ROS [45]. Our study also observed the upregulation of most antioxidant genes under salt-flooding treatment.

Furthermore, salt-flooding stress leads to the accumulation of dual-action ROS, acting as both signal molecules and toxic compounds [46]. Based on our previous research, we observed increased activities of antioxidant enzymes including superoxide peroxidase, dismutase, and ascorbate peroxidase after flooding treatment. Similarly, in the current research, we found significant expression of ROS scavenging genes encoding superoxide peroxidase, dismutase, and glutathione-S-transferase in hybrid willow leaves.

4.5. Differential Gene Expression under Combined Salt and Flooding Treatment

When comparing responses to single and combined stress, it was observed that many differentially expressed genes (DEGs) detected in plants treated with single salt and flooding also showed differential expression after the combined salt and flooding treatment. This suggests that there are common molecular responses, such as the detoxification of ROS and the activation of calcium-, phytohormone-, and protein kinase-signaling pathways [47], which have been observed in other studies on plant responses to multiple stress combinations. The fold changes between single and combined stress were similar for most shared transcripts. Additionally, when two individual stresses displayed different expression patterns, the final response was often determined by the more severe condition [47]. In this study, shared transcripts have shown opposite expression patterns between single and combined stress, suggesting the emergence of new regulators under combined stress.

When hybrid willow is under salt-flooding stress, lots of specific DEGs were identified. These genes are primarily involved in cell wall remodeling, osmotic adjustments, stress signaling, primary metabolism, and ROS scavenging. These genes were upregulated only if both stresses were involved at the same time, whereas not regulated in hybrid willow treated with a single stress. For example, the regulation of ROS production and detoxification in hybrid willow differs at the transcriptional level when exposed to salt stress alone compared to salt stress combined with flooding. Conversely, transcripts encoding cationic peroxidase are upregulated in both salt stress and flooding stress, while transcripts encoding the glutathione transferase gene family are specifically activated only by the combined salt and flooding stress. It is noteworthy that genes related to hormone signal transduction are regulated by the combined stress to a greater extent than by salt stress or flooding stress alone. Among these genes, two auxin-responsive genes were overexpressed and one transcript encoding auxin-induced protein is downregulated only under combined treatment.

The results obtained from this study demonstrate that predicting the responses of hybrid willow to combined stresses is challenging, and it cannot be directly inferred from its responses to individual stresses.

5. Conclusions

Despite the gradual utilization of the abiotic stress tolerance advantage of hybrid willow, the molecular mechanism behind this advantage remains unclear. In this study, we conducted the first transcriptome analysis to understand the mechanism of response to the

combined stress of salt-flooding in hybrid willow. Our findings reveal that the expression patterns of a complex set of genes, which respond to individual stresses, are affected by salt-flooding stress. Transcriptome profiling shows that flooding has a significant impact on altering gene expression profiles, while salt stress has a comparatively lesser effect. Moreover, when salt stress is combined with flooding treatment, the alteration of hybrid willow's transcriptome is more pronounced compared to either treatment alone. Various differentially expressed genes (DEGs) were identified in response to salt-flooding stress, primarily related to cell wall remodeling, osmotic adjustments, stress signaling, primary metabolism, and ROS scavenging. KEGG annotation revealed that hybrid willow leaves primarily responded to salt-flooding stress through phytohormone signaling and MAPK signaling pathways. The main objective of this study was to compare the transcriptome changes between salt-flooding stress and two single stresses in order to accurately identify specific changes. These changes could either be unique to a particular stress or shared between two stress responses. Although many genes exhibited similar trends in expression under both single and combined stresses, there were significant differences in their expression levels. This suggests the presence of common mechanisms underlying salt, flooding, and salt-flooding responses at the transcriptome level. These findings provide valuable insights and opportunities for enhancing salt-flooding tolerance in *Salix* and other plant species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14091858/s1, Figure S1. PCA analysis of unigenes under salt stress; Figure S2. PCA analysis of unigenes under flooding stress; Figure S3. PCA analysis of unigenes under salt-flooding stress; Figure S4. Unigene expression patterns of samples; Figure S5. Hierarchical cluster analysis of all differently expressed TFs under salt stress; Figure S6. Hierarchical cluster analysis of all differently expressed TFs under flooding stress; Figure S7. Hierarchical cluster analysis of all differently expressed TFs under salt-flooding stress; Table S1. Summary of hybrid willow transcriptome assemblies; Table S2. Summary of transcriptome assembly quality; Table S3. GO classification of unigenes; Table S4. DEGs altered by salt, flooding, and salt-flooding stress in hybrid willow; Table S5. GO functional classification of assembled unigenes under salt stress; Table S6. GO functional classification of assembled unigenes under flooding stress; Table S7. GO functional classification of assembled unigenes under salt-flooding stress; Table S8. KEGG metabolism pathway categories of assembled unigenes under salt stress; Table S9. KEGG metabolism pathway categories of assembled unigenes under salt-flooding stress; Table S10. KEGG metabolism pathway categories of assembled unigenes under flooding stress; Table S11. Expression levels of DEGs in response to salt, flooding, and salt-flooding stress; Table S12. Summary of Transcription factors identified in the DEGs; Table S13. Primer sequences.

Author Contributions: Writing—original draft preparation, Z.C.; conceptualization, H.G.; data curation, H.W.; investigation and sample collection, J.R. and Z.C.; supervision, X.X. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available because they are being used to apply for projects.

Conflicts of Interest: The authors declare no conflict of interest.

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