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Morpho-Physiological and Transcriptional Regulation of Root System under Saline Conditions in *Nymphaea* Plants

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Abstract: Water lilies (*Nymphaea* L.) are ancient angiosperms that can be cultivated in both fresh and brackish water. Water lily plants have adapted morphologically and physiologically to the aqueous environment. Nonetheless, little is known about the regulatory mechanisms that enable water lily to acclimate to saline conditions, restricting its production and distribution. To illustrate the role of roots in water lily salinity tolerance, we investigated the adaptive regulation of the water lily root system under high salinity. Aspects of its root architecture, including root length, surface area, volume, and tip number, were significantly reduced by salt stress. Transcriptome sequencing showed that 120 genes were upregulated and 1214 genes were downregulated under salt stress. The differentially expressed genes were mainly enriched in oxidoreductase activity, structural molecule activity, and transmembrane transporter activity. Most ion transporter genes were downregulated, suggesting that water lily may partially close ion channels and/or transporters to avoid excessive ion accumulation or ion imbalance under long-term salt stress. Genes related to NO_3^- transport were both up- and downregulated, whereas genes related to ammonium transport were uniformly downregulated, suggesting that transcriptional changes may play a role in balancing nitrogen metabolism under long-term saline conditions. The roots showed relatively high concentrations of Na^+ and had the ability to hyper-accumulate Na^+ under salt stress. These findings provide insight into the regulatory mechanisms that enable water lily roots to tolerate salinity and lay a foundation for the breeding of salt-tolerant cultivars.

Keywords: water lily; root; salt stress; regulatory mechanism

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1. Introduction

Salt stress is a major abiotic stress that significantly restricts agricultural production worldwide [1,2]. Currently, about 1.125 billion hectares of agricultural land are affected by salt stress, and predictions suggest that 50% of cultivated land will be affected by increasing salinization by the middle of the 21st century [3]. Terrestrial plants have evolved a variety of strategies to cope with salt stress, including salt secretion, dilution, deposition, and exclusion. Terrestrial plants may also enhance their salt resistance through osmotic regulation, maintenance of ion balance, and enhancement of antioxidant capacity [4].

Roots have multiple important functions in plants, including the absorption and transport of water and inorganic salts from the soil and the synthesis of various organic compounds. Roots can sense environmental changes and coordinate with aerial plant parts to maintain normal growth and development in adverse environments. *AtHKT1* is a Na^+ transporter first identified in *Arabidopsis thaliana*; it enhances the salt tolerance of *Arabidopsis thaliana* by controlling the unloading of Na^+ from xylem vessels to xylem parenchyma cells and then promoting the transport of Na^+ to the roots through phloem recycling [5].

OsHAK5 improves the salt resistance of *Oryza sativa* by promoting root absorption of K^+ and mediating the transport of K^+ to the shoot. *OsHAK5* also promotes rice absorption of K^+ under low K^+ conditions [6]. Under saline-alkali stress, root nitrogen metabolism was improved by increasing glutamine synthase activity, thereby enhancing salt tolerance in wheat [7]. Over the course of long-term evolution, terrestrial plant roots have thus acquired specific regulatory mechanisms for coping with salt stress.

Aquatic plants have adapted morphologically and physiologically to the aqueous environment [8]. Their morphological traits include increases in air-filled tissues, thickening of epidermal cell walls, and reductions in root biomass [9,10]. Their leaves or petioles have evolved to absorb nutrients or ions directly from water, reducing their dependence on root uptake [11]. Most ion transport systems in the plasma membranes of aquatic plants have functional affinities with those of terrestrial plants. In the seagrass *Zostera marina*, a high-affinity Na^+ co-transport system was first shown to mediate the uptake of NO_3^-, and this Na^+/NO_3^--transporter is unique to aquatic plants [12,13]. The plasma membrane Na^+/H^+ cotransporter SOS1 was first identified in *Arabidopsis thaliana*, and the SOS3-SOS2-SOS1 signaling pathway plays a central role in the process of Na^+ exclusion [14]. However, in the seagrass *Cymodocea nodosa*, CnSOS1A was not activated by the SOS2/SOS3 system, which may be missing in aquatic plants [15]. Although studies of ion transport mechanisms in some submerged plants have been reported, details of their ion uptake and transport under high salinity remain to be clarified.

Water lilies are perennial aquatic herbaceous plants with floating leaves that belong to the *Nymphaeaceae* family of basal angiosperms [16]. Water lilies are widely used in landscaping for their colorful flowers, long bloom time, and strong adaptability to various stresses [16,17]. However, the use of water lilies is mainly restricted to fresh water, and their production in saline-alkali areas remains limited. Nonetheless, water lily plants have been reported to survive in 1.3% saline water in the coastal salt marshes of eastern England [18]. To investigate the role of roots in water lily salinity tolerance, we characterized their morpho-physiological and transcriptional responses to high salinity. The results provide insight into the regulatory mechanisms that enable water lily to survive under saline conditions and lay a foundation for the breeding of salt-tolerant cultivars.

2. Materials and Methods

2.1. Plant Materials and Salt Treatment

Nymphaea 'Colorado' plants were grown in the greenhouse of the Sun Yat-Sen Memorial Botanical Garden in Nanjing, China. The potted plants with 5–6 floating leaves were used as experimental materials. For NaCl treatment, plants were submerged in NaCl solutions with concentrations of 0, 50, 100, 150, and 200 mM.

2.2. Plant Growth and Root Measurement

After 3 weeks, the plants were photographed to document their morphological characteristics. Their roots were harvested and scanned on a ScanMaker i800 plus (MICROTEK) scanner. Total root length, total root surface area, total root volume, and number of root tips were determined using an LA-S plant root system analyzer. Root fresh weights were measured with an electronic balance (Sartorius BSA223S Sartorius Stedim Biotech Co., Beijing, China); the roots were placed in an oven at 105 °C for 30 min and then dried to constant weight at 72 °C for dry weight measurement. Data were analyzed by SPSS v.22 (IBM Corporation, Armonk, NY, USA).

2.3. Transcriptome Analysis of Roots

For the salt stress treatment (SR), plants were cultivated in 150 mM NaCl as described above for 3 weeks, and plants grown in fresh water were used as controls (CR). There were three biological replicates of each treatment. After sampling, the root tissues were frozen in liquid nitrogen and stored at −80 °C. RNA was extracted using the RNAprep Pure Plant Kit (QIAGEN, Mannheim, Germany), and cDNA was synthesized using random hexamer

primers and M-MuLV Reverse Transcriptase. RNA sequencing (RNA-seq) was performed on the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA), generating 150 bp paired-end reads. Clean data were obtained by removing reads containing adapter, reads containing N base and low quality reads from raw data. Then, the Trinity software (v2.6.6, Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA) was used to assemble the clean reads. Gene function was annotated based on Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KO and GO. The DESeq2 R package was used to identify differentially expressed genes (DEGs) between the NaCl treatments (based on padj < 0.05 and |log2FoldChange| > 1). GOseq (1.10.0, The Walterand Eliza Hall Institute of Medical Research, Melbourne, Australia) and KOBAS (v2.0.12, Peking University, Beijing, China) software were used for GO function enrichment analysis and KEGG pathway enrichment analysis of differential gene sets. FPKM (expected number of Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) was calculated to quantify the differentially expressed unigenes.

2.4. ICP-MS Analysis of Na^+ and K^+ Contents

Water lily roots were sampled at 0, 3, 12, 21, and 30 days and then dried to constant weight for ICP-MS analysis. There were three biological replicates for each treatment and sampling date. Samples were digested with a mixture of nitric acid and H_2O_2 and examined for Na^+ and K^+ content using an inductively coupled plasma mass spectrometer (ICP-MS; Agilent Technologies Co., Ltd., Beijing, China). SPSS v.22 was used for statistical analysis.

2.5. Quantitative Real-Time PCR

To validate RNA-seq data, ion transport related genes Cluster-3509.5272, Cluster-3509.89148, Cluster-3509.73386, Cluster-3509.19641, Cluster-3509.68592 and Cluster-3509.57359 were used as candidate genes. RNA was isolated with FastPure Universal plant Total RNA Isolation Kit (Vazyme, Nanjing, China). After reverse transcription, the gene expressions were analyzed by BIO-RAD CFX-Opus 96 (Bio-Rad, Berkeley, CA, USA). Actin was used for normalization.

3. Results

3.1. Morphological Characterization of Water Lily Plants

High salt concentrations significantly inhibited leaf and root growth of the water lily plants. As NaCl concentration increased, biomass decreased dramatically, and most leaves decayed in the 200 mM NaCl treatment (Figure 1a,b). There was a slight positive effect of low NaCl concentration on biomass: compared with control plants, water lilies in the 50 mM salt treatment had 11.19% higher fresh weight (FW) and 23.49% higher dry weight (DW). Nonetheless, NaCl concentrations greater than 100 mM significantly reduced root biomass. In the 200 mM salt treatment, fresh weight and dry weight decreased significantly by 43.72% and 53.22%, respectively (Figure 1c). With increasing salt concentration, total root length, surface area, volume, and tip number decreased significantly (Figure 1d–g). Compared with control plants, total root length was decreased by 56.20%, total root surface area by 65.82%, total root volume by 63.74%, and root tip number by 39.61% in the 200 mM NaCl treatment. These results demonstrate that NaCl concentrations of $\leq$50 mM had no inhibitory effect on water lily biomass and root system structure, but NaCl concentrations $\geq$150 mM significantly inhibited root system growth.

3.2. Analysis of RNA-Seq Data

Water lily plants treated with or without 150 mM NaCl were used to investigate changes in root gene expression. Approximately 5.7–6.7 Gb of clean bases were obtained from each of three replicate samples per NaCl level. The error rate was controlled at 0.03%; Q20 was approximately 97%, Q30 approximately 93%, and GC content approximately 47% (Table 1). We identified 1334 DEGs between the two NaCl treatments: 120 genes upregulated and 1214 downregulated under 150 mM NaCl (Figure 2).

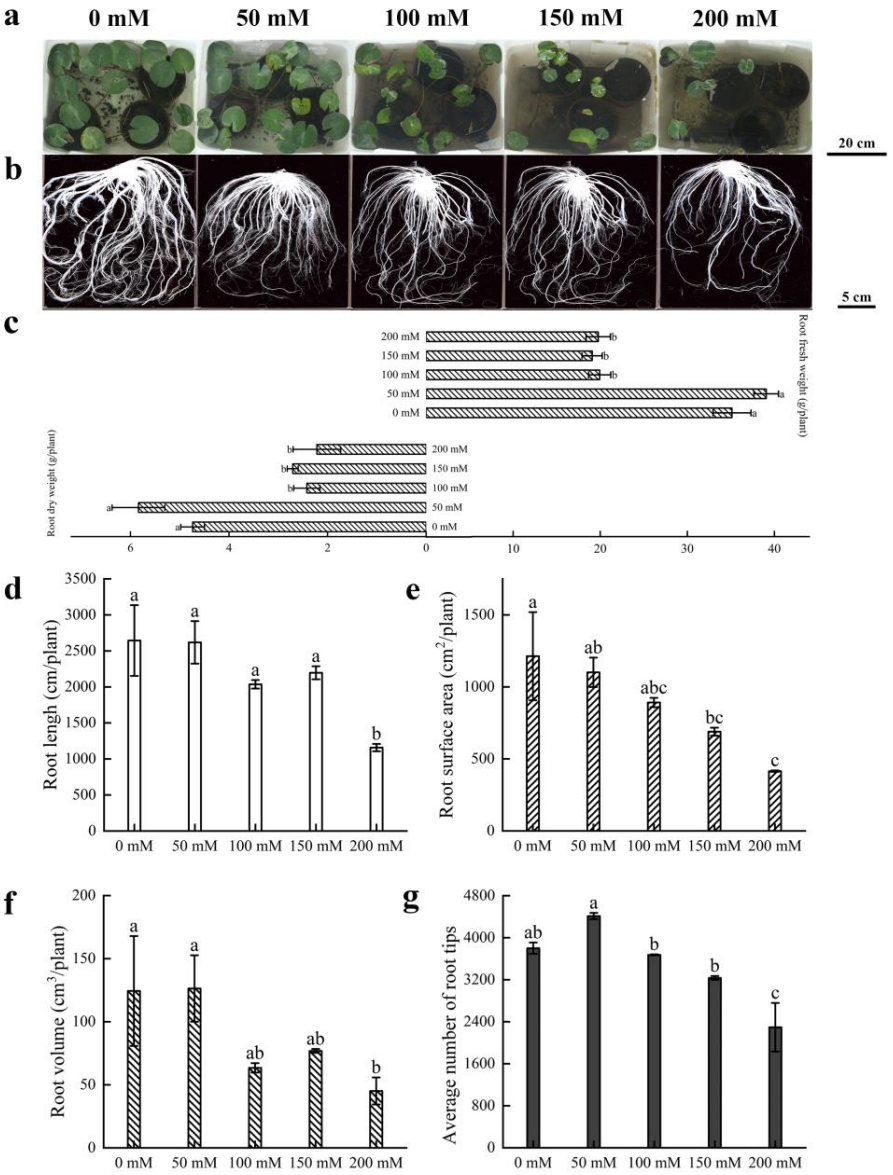

Figure 1. Growth status of water lilies in control and NaCl treatments. (**a**) Growth status of water lilies. (**b**) Scanned images of water lily roots from different NaCl concentrations. (**c**) Root fresh and dry weights. (**d**–**g**) Root length (**d**), root surface area (**e**), root volume (**f**), and root tip number (**g**) of water lily roots from different NaCl concentrations. Data are mean (±SD), $n = 3$. Different letters above bars indicate significant differences ($p < 0.05$, Duncan's test).

Table 1. Sequencing statistics of Nymphaea 'Colorado' under control conditions (CR) and 150 mM NaCl treatment (SR).

Sample Name	Raw Reads	Clean Reads	Clean Bases	Error Rate (%)	Q20 (%)	Q30 (%)	GC Content (%)
CR1	22,947,598	22,451,618	6.7G	0.03	97.58	93.25	48.55
CR2	22,741,980	22,228,034	6.7G	0.03	97.72	93.55	47.91
CR3	22,409,365	21,936,861	6.6G	0.03	97.63	93.25	48.17
SR1	23,281,962	21,788,747	6.5G	0.03	97.86	93.75	46.52
SR2	22,502,300	22,344,183	6.7G	0.03	97.89	93.92	47.56
SR3	20,460,810	19,022,924	5.7G	0.03	97.99	94.03	43.76

Q20 (%), Q30 (%): Percentage of bases with Phred quality scores ≥20 and ≥30, respectively. Q20 and Q30 correspond to incorrect base call probabilities of 0.01 and 0.001, respectively.

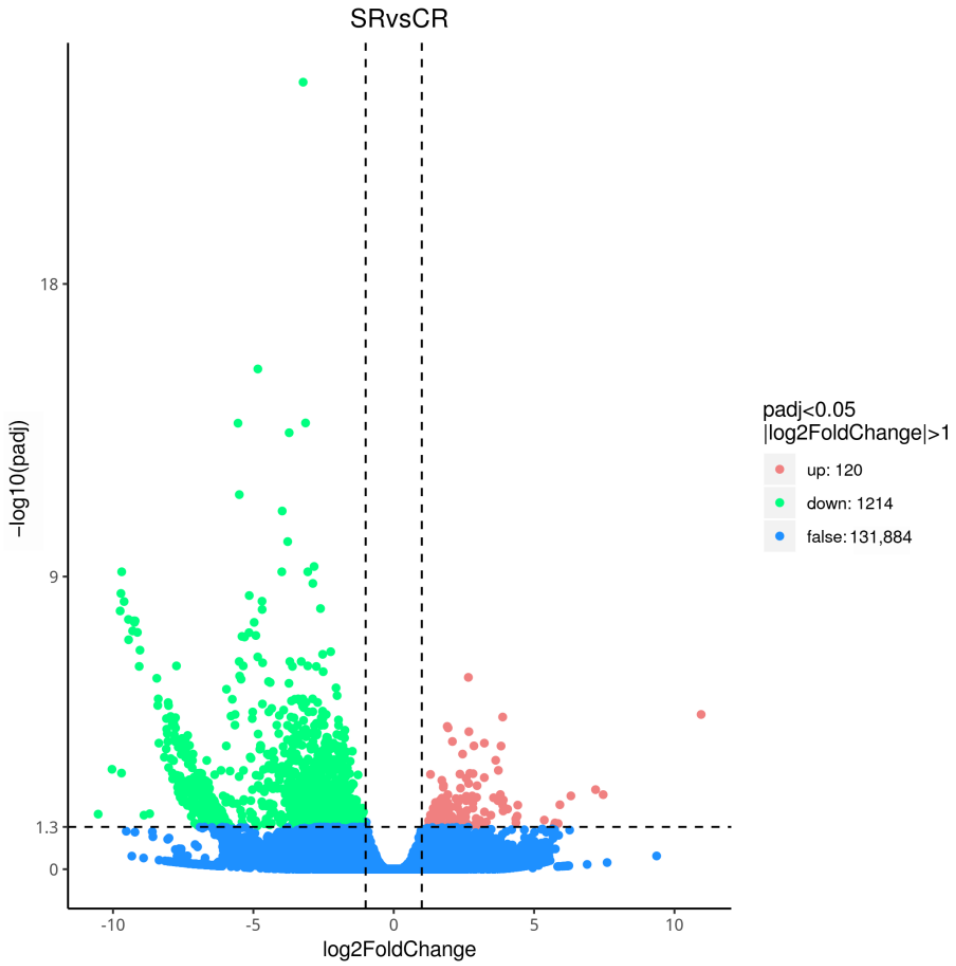

Figure 2. Volcano plot showing differentially expressed genes in water lily roots under control vs. 150 mM NaCl conditions.

3.3. Pathway Enrichment Analyses

To analyze the molecular basis of root system changes under high salinity, we performed gene ontology (GO) enrichment analysis of the DEGs. Sixteen GO terms were significantly enriched in the root DEGs (Figure 3a). The top three enriched molecular function GO terms were 'oxidoreductase activity' (130 DEGs), 'structural molecule activity' (88 DEGs), and 'transmembrane transporter activity' (87 DEGs). The biological process GO terms 'carbohydrate metabolic process' (86 DEGs) and 'transmembrane transport' (77 DEGs) were also significantly enriched. Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-Based Annotation System (KOBAS), we identified six significantly enriched KEGG pathways (Figure 3b), including 'ribosome', 'flavonoid biosynthesis', and 'phenylpropanoid biosynthesis'.

3.4. Analysis of Genes Related to Ion Channels and Transporters

We next focused in detail on the expression of 17 ion transporter genes identified as DEGs in water lily roots under high salinity (Table 2). One aluminum-activated malate transporter gene was upregulated, whereas genes encoding one Na^+ transporter, four K^+ transport-related transporters, two boron transporters, one anion transporter, two cation transport-related transporters, and six ABC transporters were downregulated. Roots may, therefore, have downregulated the expression of specific transporters to avoid ionic toxicity caused by excessive ion accumulation.

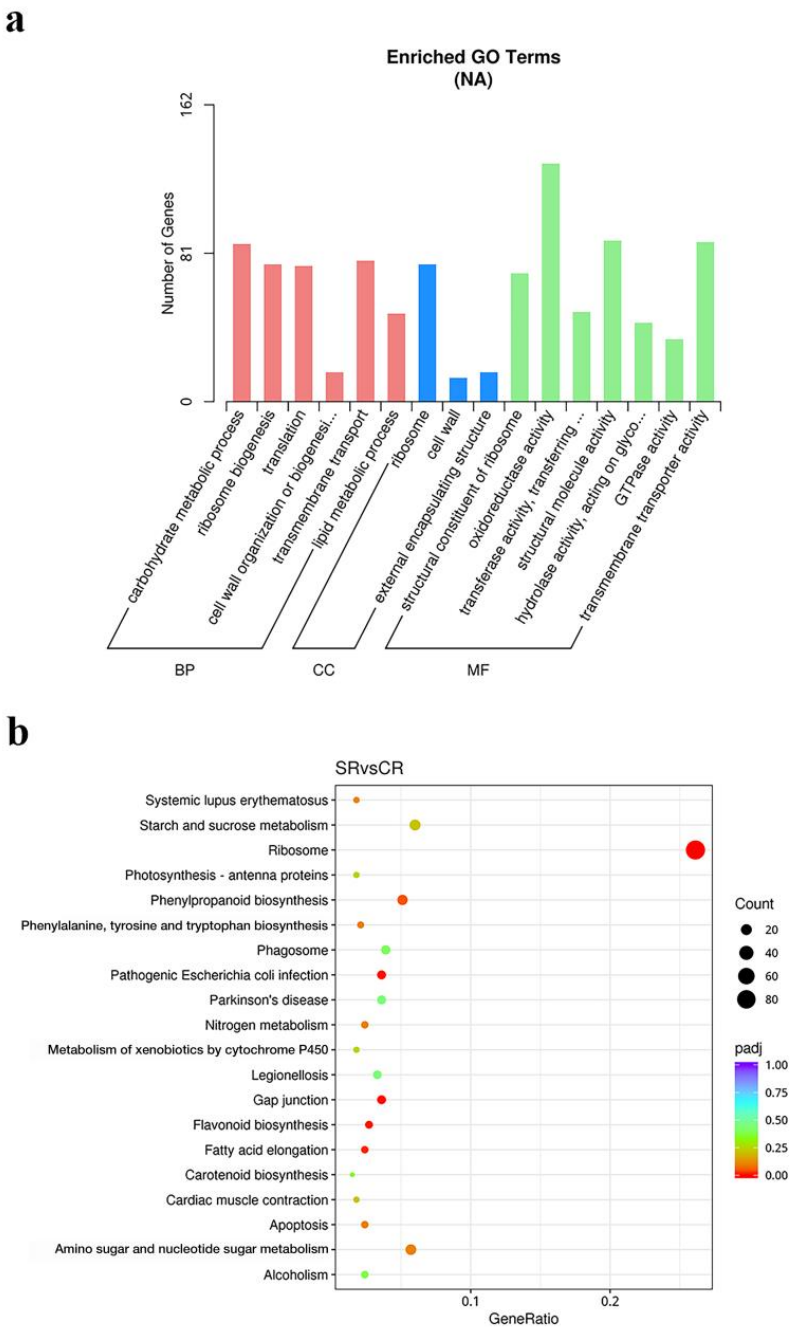

Figure 3. GO (**a**) and KEGG (**b**) pathway enrichment of differentially expressed genes in water lily roots from control vs. 150 mM NaCl conditions.

Because disruption of nitrogen metabolism is another potential mechanism of salt injury, we also examined the expression of 18 DEGs encoding transporters for various nitrogen forms (Table 2). Three transporter genes were upregulated, and 15 transporter genes were downregulated. Notably, NO_3^--related transporter genes, including an aluminum-activated malate transporter gene and an NRT1/PTR family gene, were upregulated. By contrast, ammonium transporter genes were all downregulated. These findings suggest that water lily plants may absorb NO_3^- efficiently under saline conditions. To validate RNA-seq data, expression patterns of candidate genes were further analyzed using RT-qPCR (Figure 4). Generally, the expression models of candidate genes were consistent with the transcriptome data.

Table 2. Differentially expressed genes related to the transport of ions in water lily roots under salt stress.

Gene ID	log_2FC	*p*-Value	Description
		Downregulated	
Cluster-3509.89148	−3.11	2.67×10^{-3}	sodium transporter HKT1
Cluster-3509.60818	−4.40	9.59×10^{-3}	potassium transporter 5
Cluster-3509.73386	−2.68	5.13×10^{-3}	potassium transporter 5-like
Cluster-3509.79079	−1.72	4.51×10^{-2}	potassium transporter 5-like
Cluster-3509.78332	−1.58	3.22×10^{-2}	K+ uptake permease 4
Cluster-3509.41717	−1.34	1.31×10^{-2}	boron transporter 2
Cluster-3509.64063	−1.47	2.36×10^{-4}	boron transporter 2
Cluster-3509.65281	−2.94	2.04×10^{-4}	anion transporter 3, chloroplastic
Cluster-3509.82188	−1.25	3.10×10^{-2}	organic cation/carnitine transporter 7-like
Cluster-3509.72310	−1.05	4.78×10^{-2}	cation-transporting P-type ATPase
Cluster-3509.19641	−2.02	7.55×10^{-3}	ABC transporter G family member 36
Cluster-3509.96046	−3.61	9.84×10^{-4}	ABC transporter G family member
Cluster-3509.61142	−1.33	3.79×10^{-2}	ABC transporter G family member 29
Cluster-3509.60854	−5.93	8.54×10^{-3}	ABC transporter G family member 21
Cluster-3509.98196	−2.31	1.13×10^{-2}	ABC transporter I family member 17-like
Cluster-3509.82719	−1.14	3.20×10^{-2}	ABC transporter I family member 6, chloroplastic
Cluster-3509.68592	−2.68	1.59×10^{-2}	ammonium transporter 1 member 1-like
Cluster-3509.60927	−2.84	2.33×10^{-2}	ammonium transporter 2-like
Cluster-3509.57872	−2.78	1.36×10^{-3}	ammonium transporter 1 member 1-like
Cluster-3509.78326	−4.61	4.17×10^{-3}	ammonium transporter 3 member 1-like
Cluster-3509.57359	−3.13	8.47×10^{-5}	high-affinity nitrate transporter 2.1
Cluster-3509.63374	−3.31	1.47×10^{-4}	high-affinity nitrate transporter-activating protein 2.1-like
Cluster-3509.66911	−3.23	6.24×10^{-25}	urea-proton symporter DUR3
Cluster-3509.51839	−2.80	6.07×10^{-3}	urea-proton symporter DUR3
Cluster-3509.81777	−1.57	3.48×10^{-2}	proton-dependent oligopeptide transporter family
Cluster-3509.18628	−6.08	5.55×10^{-3}	proton-dependent oligopeptide transporter family
Cluster-3509.40878	−1.70	7.53×10^{-3}	oligopeptide transporter 4
Cluster-3509.77846	−1.43	4.91×10^{-2}	peptide transporter
Cluster-3509.97247	−1.53	3.45×10^{-2}	amino acid transporter
Cluster-3509.81032	−1.55	6.73×10^{-3}	amino acid/polyamine transporter I
Cluster-3509.22635	−1.91	3.95×10^{-2}	cationic amino acid transporter 1
		Upregulated	
Cluster-3509.5272	3.56	6.35×10^{-3}	aluminum-activated malate transporter
Cluster-3509.5272	3.56	6.35×10^{-3}	aluminum-activated malate transporter
Cluster-3509.69281	2.29	7.37×10^{-3}	NRT1/PTR FAMILY 7.2
Cluster-3509.41673	1.51	2.56×10^{-2}	amino acid transporter AVT6A-like

3.5. Analysis of Na^+ and K^+ in Water Lily Roots

When water lilies were grown in 150 mM NaCl, root Na^+ concentration first increased continuously over 12 days, then plateaued from 12 to 30 days. The Na^+ concentration of roots in the high-salinity treatment reached 43.71 g/kg DW, nearly twice that of control plants (Figure 5a). At the same time, root K^+ concentration increased slightly (and non-significantly) from 0 to 3 days, decreased significantly from 3 to 12 days, and remained relatively stable thereafter (Figure 5b). Water lily plants may have evolved a unique strategy for Na^+ and K^+ accumulation to adapt to long-term salt stress.

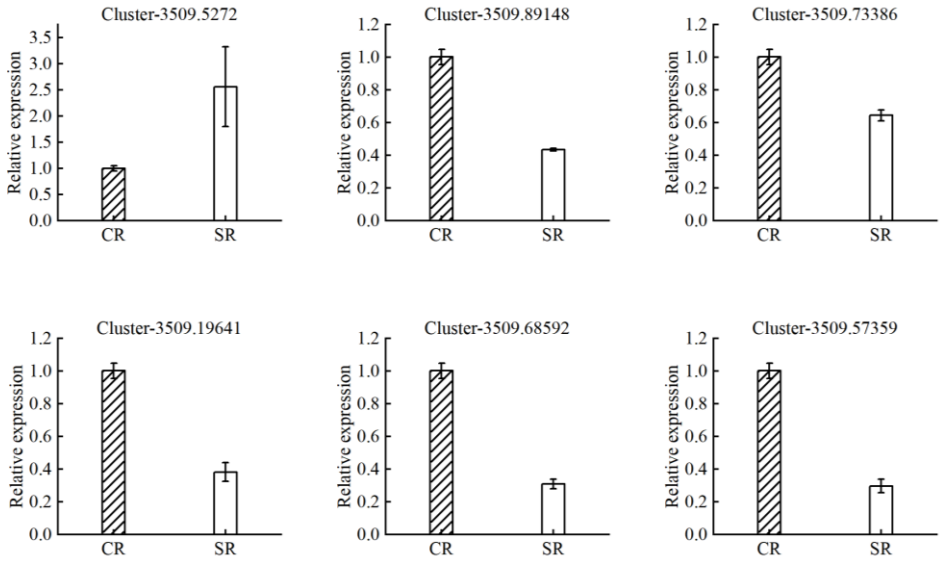

Figure 4. Analysis of gene expression in water lily roots under control conditions (CR) and 150 mM NaCl treatment (SR) using RT-qPCR. Actin was used for normalization.

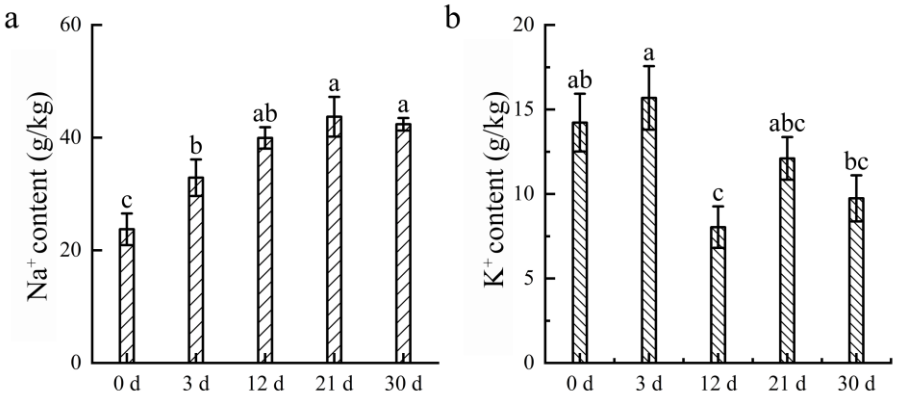

Figure 5. Na^+ and K^+ ion concentrations in water lily roots under high-salinity conditions. (**a**) Na^+ content. (**b**) K^+ content. Water lilies were cultivated in a solution containing 150 mM NaCl for 0, 3, 12, 21, and 30 days. Data are mean (±SD), $n = 3$. Different letters above bars indicate significant differences ($p < 0.05$, Duncan's test).

4. Discussion

Terrestrial plants can deploy a number of strategies to adapt to salt stress, including morphological changes, physiological adaptations, and changes in gene expression at the transcriptional level. Under salt stress, plant roots may exhibit phenotypic plasticity through the regulation of root system architecture [19–21]. In Arabidopsis, lateral root initiation and development play an important role in this adaptive system; low and moderate salt stress had no remarkable effect on lateral root formation, but higher NaCl concentrations inhibited lateral root initiation and emergence [22]. When plants were exposed to salt stress, root epidermal cells declined substantially, resulting in significantly lower root hair numbers. These changes in root structure under salt stress helped to reduce the active sites on the root surface, thereby limiting excessive root absorption of salt ions and avoiding their negative effect on roots. In the present study, NaCl concentrations $\leq$50 mM had no inhibitory effect on root system growth, whereas NaCl concentrations $\geq$150 mM significantly inhibited root biomass and altered root architecture (Figure 1). Thus, water lily roots may have limited the influence of salt stress by reducing their root biomass and changing their root architecture.

Na^+ accumulation involves the activity of various ion channels and other transporters, and excessive Na^+ uptake causes specific damage to plants [23,24]. In this study, the Na^+ concentration in water lily roots was 23.73 g/kg DW under non-saline conditions, indicating that water lily can naturally and safely store abundant Na^+ in roots. In the 150 mM NaCl treatment, the Na^+ concentration increased continuously over the first 12 days, reaching a maximum of 43.71 g/kg DW, suggesting that water lily can hyper-accumulate additional Na^+ under high salinity. As the salinity treatment continued beyond 12 days, the Na^+ concentration remained at a plateau, suggesting that it had reached a saturated concentration (Figure 5). At the same time, Na^+ transport-related genes were downregulated in water lily roots, suggesting that the plant may limit the abundance of Na^+ channels or transporters to avoid excessive ion accumulation in the roots (Table 2).

Different forms of soil N, including NO_3^- and NH_4^+, can contribute to plant nutrition [25]. In general, salt stress has a negative effect on nutrient uptake and transport, leading to nutritional disorders in plants [26]. Changes in N metabolism have been reported in different plant species in response to salinity. In sorghum, NH_4^+-grown plants showed higher salinity tolerance than NO_3^--grown plants [27]. NH_4^+ was reported to be the preferred N form for *Trigonella foenum-graecum*, and the use of ammonium as a major N source improved its salinity adaptation [28]. In halophyte species, *Spartina alterniflora* Loisel also preferred NH_4^+ as an inorganic N source [29]. By contrast, NH_4^+ was reported to increase the sensitivity of *Pisum sativum* to salt stress [24]. The results of the present study showed that genes encoding ammonium transporters and high-affinity nitrate transporters were downregulated, whereas those encoding an aluminum-activated malate transporter and an NRT1/PTR family low-affinity nitrate transporter were upregulated (Table 2). These results suggest that NO_3^- may be a preferred inorganic N source during long-term adaptation to saline conditions.

5. Conclusions

In conclusion, water lily may adapt to saline conditions by flexible, plastic development of its root system, including reductions in biomass and changes in root architecture. Over the short term, roots had the ability to hyper-accumulate Na^+ under salt stress, and the maximum Na^+ concentration was greater than 40 g/kg DW. Over long-term NaCl treatment, water lily may limit Na^+ channels or transporters to avoid excessive ion accumulation in roots. Nitrogen metabolism, especially NO_3^- absorption, may also have a role under long-term saline conditions. However, the regulation mechanism underlying the absorption and transportation of important ions in water lily plants under salt stress still needs further investigation.

Author Contributions: Conceptualization, S.C., X.L. and D.Y.; methodology, S.C. and X.L.; validation, S.C., X.L. and D.Y.; formal analysis, J.C. and F.D.; resources, F.D. and Z.Z.; data curation, Y.C. and Q.H.; writing—original draft preparation, S.C. and F.D.; writing—review and editing, S.C. and X.L.; visualization, S.C. and X.G.; supervision, X.L. and D.Y.; funding acquisition, X.L. and D.Y. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0003793. https://db.cngb.org/search/?q=CNP0003793 (accessed on 2 April 2022).

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

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