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Physiological and transcriptional reprogramming for salinity tolerance of endangered mangrove associate *Hernandia nymphaeifolia*

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Abstract

Background *Hernandia nymphaeifolia* is a typical mangrove associate with high ecological, ornamental, and medicinal values, but in China, it has become endangered in recent years, and an urgent protection is needed. Salinity is a key factor for growth and survival of mangrove seedlings, and thus a comprehensive understanding of salt tolerance in mangroves is important for their conservation and afforestation. However, little is known regarding salt-responsive mechanisms in *H. nymphaeifolia*.

Results In this study, we posed gradient salt treatments on *H. nymphaeifolia* seedlings and investigated their physiological and transcriptional reprogramming in response to salinity stress. The results revealed that hyper-salinity stress adversely impacted on leaf growth, cell integrity and photosynthetic performance of *H. nymphaeifolia* seedlings than those growing in fresh water or low salt conditions, mirroring its moderate salinity tolerance as a mangrove associate. Genes involved in osmotic sensing and regulation, reactive oxygen species (ROS) scavenging and ion homeostasis were differentially expressed to alleviate the destructive effects. Furthermore, our results identified some kinase-encoding genes as hub genes in co-expression networks, which may play a key role in regulating the synergistic expression of salt-responsive genes upon stress conditions.

Conclusion This research enriches our knowledge of the molecular mechanisms underlying the salinity tolerance of mangrove associates, which can theoretically assist the conservation and restoration of *H. nymphaeifolia*. Our findings also provide valuable genetic resources for future potential bioengineering applications in the fields of agriculture and forestry.

Keywords Ion homeostasis, Mangrove associate, Osmoregulation, Protein kinases, Salinity tolerance

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Introduction

Hernandia nymphaeifolia, also known as a "lantern tree", is a mangrove associate species of the family Hernandiaceae. *H. nymphaeifolia* widely grows in tropical coastal swamps and littoral forests along seashores without inundation of the periodic tides. *H. nymphaeifolia* has high ecological, ornamental, and medicinal uses [1]. For instance, *H. nymphaeifolia* is important for maintaining the coastal habitats [2], and it is also one of the excellent trees for landscaping due to its peculiar fruit morphology and shield-shaped leaves [3]. The wood of *H. nymphaeifolia* is light and perishable, thus can be used for fish net floats, canoe accessories and furnitures [4]. Moreover, its leaves can be used to relieve headaches, and its lignans have an effect on Ca^{2+} signaling in human neutrophils [5]. The natural distribution of *H. nymphaeifolia* in China is narrowly restricted to Hainan and Taiwan. Due to pressure from environmental changes and human activities, *H. nymphaeifolia* populations in China have suffered further shrinkage in recent years [6]. A field survey in 2022 recorded only 1,336 wild *H. nymphaeifolia* individuals in Hainan Island [7]. Now *H. nymphaeifolia* is listed on the List of National Key Protected Wild Plants in China [6], and an urgent protection is needed.

Salinity has been reported to be a key factor that largely affects growth and survival of mangrove seedlings [8, 9]. Excessive salinity would alter plant membrane permeability, disrupt ionic homeostasis, lead to physiological and metabolic disorders, and even cause plant cell death [10]. Thus, a comprehensive understanding of salt tolerance in mangroves is important for their conservation and afforestation. Compared to terrestrial plants, mangroves have evolved several morphological and physiological features to adapt to saline environments, such as salt glands, leaf succulence, salt sequestration in leaf vacuoles and ultrafiltration mechanisms for salt exclusion [1, 11]. However, different mangrove species have various adaptations to salinity [12]. Unlike true mangroves, which achieve the maximum growth in saline environments, mangrove associates are generally facultative halophytes and grow better with fresh water [11, 13]. For instance, high salinity was found to cause obvious damage to anatomical structure and photosynthesis of *Myoporum bontioides* leaves, and the activities of antioxidant system were enhanced accordingly, especially at the early stage, to combat the adverse impacts [14]. However, most current researches on *H. nymphaeifolia* mainly focuses on its chemical properties [15–17]. Little attention has been paid to the influence of salt stress on *H. nymphaeifolia* and its salt tolerance capacity.

In this study, we characterized physiological and transcriptional changes in leaves of *H. nymphaeifolia* seedlings upon salinity stress at different concentrations and durations. With these data, we aimed to (a) assess hyper-salinity-induced damage to *H. nymphaeifolia* and its corresponding physiological responses to salt stress, and (b) explore the key genes and pathways associated with the salt-responsive physiological reprogramming. These findings would unravel the salinity tolerance mechanism of *H. nymphaeifolia* at both physiological and genetic levels, and provide a basis for the conservation and restoration of *H. nymphaeifolia*.

Methods and materials

Plant materials and salt stress treatments

Seeds of *H. nymphaeifolia* were collected from the open field in Qionghai, Hainan, China (110°36′28.87″E, 19°12′11.29″N), and planted in the Agricultural Science Garden of Hainan University, Haikou, Hainan, China (110°19′24.88″E, 20°3′33.35″N). The average temperature during the cultivation was 26℃, with a maximum temperature of 31℃ and a minimum temperature of 17 ℃. The 2-year-old seedlings with good and similar growth status (~30 cm in height and with 6–9 leaves) were selected and transplanted individually in pots of 20 cm × 15 cm × 15 cm (upper caliber × lower caliber × height). Each pot was placed on a plastic tray. These seedlings were then assigned into five groups, where one group was treated with fresh water as control (CK), while the other four groups were treated with saline water with different NaCl concentrations (150, 300, 500 and 700 mM), to mimic various potential salinity conditions that *H. nymphaeifolia* may encounter in intertidal habitats [1, 13]. Each treatment consisted of three individuals as biological replicates. To mimic the semi-diurnal tide under natural condition, the seedlings were submerged to the height of the branches for 3 h every 12 h. During the experiment, water and fertilizer management, as well as pest and disease control, were carried out to guarantee the healthy growth, and pot position was adjusted randomly every week to ensure same light condition for each individual. For each seedling, the second and third to last pairs of leaves were collected after treatment for 7 d and 14 d, respectively, for downstream experiments [13, 18].

Physiological index measurement

Several physiological indices were measured for the seedlings under CK and salt treatment for 7 d and 14 d, respectively, to assess the impacts of salt stress on the physiological and biochemical processes of *H. nymphaeifolia*. For each individual, ~0.15 g fresh leaves were used for quantifying relative electrical conductivity (REC), which characterizes leakage and damage of plant cell membranes [19]. Collected samples were rinsed with deionized water and immersed in a centrifuge tube containing 20 mL of deionized water. The solution was shaken on a shaker at room temperature for 24 h, and the initial conductivity value C1 was determined by a

REX DDSJ-308 A Conductivity Meter (INESA Scientific Instrument Co., Ltd., Shanghai, China). The centrifuge tube was autoclaved at 120 ℃ for 20 min, and the conductivity value C2 was measured after it was cooled down. The REC was computed to the formula: REC (%) = C1/C2 × 100.

We further measured the relative water content (RWC) in leaves, which reflects the degree of osmotic adjustment of plants under salt stress. Approximately 0.15 g fresh leaves were taken and weighed as fresh weight (FW). They were soaked in water at room temperature for 24 h, then wiped dry and weighed as full weight (TW). After that, the leaves were dried at 65 ℃ until constant weight and weighed as dry weight (DW). The RWC of the leaves was calculated according to the formula: RWC (%) = (FW-DW)/(TW-DW) × 100.

Catalase (CAT) activity was determined by the ammonium molybdate-chromogenic method using the Catalase (CAT) Activity Assay Kit (Solarbio Life Science, Beijing, China), and peroxidase (POD) activity was measured following the guaiacol colorimetric method using the Peroxidase (POD) Activity Assay Kit (Solarbio Life Science, Beijing, China). Soluble sugars were determined using the anthrone colorimetry method with the Plant Soluble Sugar Content Assay Kit (Solarbio Life Science, Beijing, China), and proline content was quantified by the ninhydrin method using the Proline (Pro) Content Assay Kit (Solarbio Life Science, Beijing China). All these experiments were conducted following the manufacturer's instructions for the kits.

RNA sequencing and transcriptome analysis

Total RNA was extracted from the leaves of the seedlings at CK and 300 mM treatment for 7 d and 14 d, respectively, using the R6827-00 Plant RNA Kit (Omega Bio-tek, Inc., GA, USA). RNA purity and integrity were measured using the kaiaoK5500®Spectrophotometer (Kaiao Technology, Beijing, China) and the Bioanalyzer 2100 System (Agilent Technologies, CA, USA), respectively. The mRNA was further purified using Oligo(dT) attached magnetic beads, and sequenced using DNBSEQ-T7 platform (MGI Tech Co., Ltd., Guangdong, China). Data quality of the raw sequencing reads was assessed using FastQC v. 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For each sample, low-quality bases with a quality score < 20 were first trimmed at the end of each read, and the reads with > 15 ambiguous N bases, error rate > 10% or length < 50 bp were then filtered out. High-quality reads of all the samples were merged together and *de novo* assembled into a reference transcriptome using Trinity v. 2.15.0 [20]. CD-HIT [21] was employed to reduce redundancy of the assembled sequences. Gene expression in each sample was estimated using salmon software [22], with a correction of fragment-level GC biases. Compared to CK, differentially expressed genes (DEGs) were identified in each treatment scenario (7 and 14 d) using DESeq2 package [23]. The DEGs with $\log_2$-scaled fold change between treatment and CK > 1 and adjusted p-value < 0.05 were considered to be upregulated, while those with $\log_2$-scaled fold change between treatment and CK < -1 and adjusted p-value < 0.05 were downregulated.

Functional annotation was performed for the reference transcriptome using eggNOG-mapper v2 [24] based on predicted protein sequences, and an annotation database was constructed using the makeOrgPackage function of AnnotationForge package [25]. Gene Ontology (GO) enrichment analysis was then carried out for both up- and downregulated DEGs, respectively, using the enrichGO function of clusterProfiler package [26], with a p-value cutoff of 0.05.

DEGs between treatments for 7 d and 14 d were identified using DESeq2 package [23] following the same pipeline mentioned above. These DEGs were grouped by hierarchical clustering based on their expression trends across different time points. GO enrichment analysis was performed for each cluster of genes to assess their functional importance.

Weighted gene co-expression network analysis

Gene co-expression networks were constructed by the Weighted correlation network analysis (WGCNA) method via the corresponding R package [27]. Normalized expression counts were used for the analysis, and the low-expressed genes (average expression across samples < = 100) were filtered out. The analysis was conducted with the soft threshold of power set to 9, minModuleSize set to 5 and MergeCutHeight set to 0.25. The modules significantly correlated with salinity treatments (Student asymptotic p-value < 0.05) were retained for futher analysis. The networks of interested modules were visualized in Cytoscape [28].

Real-time quantitative PCR validation

Four upregulated DEGs were selected for real-time quantitative PCR (RT-qPCR) validation to verify the reliability of the gene expression levels inferred by RNA-seq. Primers were designed using Primer3 v. 4.1.0 and the sequences are listed in Table S1. Gene *U6* was employed as the internal reference because of its high expression stability in plants [29]. Experiments were carried out using the PerfectStart Green qPCR SuperMix kit (Transgen, Beijing, China) following the manufacturer's instructions. The $2^{-\Delta\Delta Ct}$ method was applied to infer the fold change in gene expression between each treatment group and CK. Consistency between the results of RT-qPCR and RNA-seq was quantified by Pearson correlation coefficient (PPC).

Statistical analyses

For each physiological index assay, the measurements were compared across different 7 d and 14 d treatments, respectively, by the least significant difference (LSD) test in IBM SPSS Statistics (IBM Corp., NY, USA). The differences at p-value < 0.05 were considered statistically significant and were indicated by different letters (lowercase for the experiments for 7 d, and uppercase for the experiments for 14 d) in the relevant figures. In addition, the differences in physiological indices between salinity treatments for 7 d and 14 d were assessed using two-tailed t-test.

Results

Effects of salinity changes on physiological processes of *H. nymphaeifolia* seedlings

When exposed to salinity stress, the growth status of *H. nymphaeifolia* seedlings got worse along with rising NaCl concentration (Fig. 1A and B). Plant growth conditions under the stress of low NaCl concentration were similar to the CK individuals. The leaves of the plants exposed to 500 and 700 mM were observed to turn yellow after 7 d (Fig. 1A). The adverse impact became further intensified when the stress extended to 14 d (Fig. 1B).

Accordingly, after 7 d of salinity stress, REC of leaves did not show obvious changes at most salt concentrations. It was substantially increased upon the treatment of 700 mM (Fig. 1C). In contrast, water content was slightly decreased in leaves with increased NaCl concentrations (Fig. 1D), indicating a modest impact of salinity stress on *H. nymphaeifolia* seedlings, except for that of extremely high concentration. Accordingly, CAT activity and proline content were significantly enhanced under 500 and 700 mM NaCl treatments (Fig. 1E and G). POD activity displayed a significant stepwise increase when the NaCl concentration increased from 0 to 500 mM, but showed a decrease at the concentration of 700 mM (Fig. 1F). In contrast to proline, the content of soluble sugar showed an overall downtrend with the increase of salinity (Fig. 1H).

With prolonging of treatment time, REC of *H. nymphaeifolia* leaves was significantly increased in most treatment groups (150, 300 and 700 mM) than in 7 d, and leaf RWC was lower than those for 7d (Fig. 1C and D), which was consistent with the increased degree of damage to leaves. To combat the adverse impacts, CAT activity and soluble sugar content were substantially increased in the seedlings upon 14 d of salt stress at 150 and 300 mM compared to 7 d (Fig. 1E and H). At 700 mM, POD was more activated in 14 d than in 7 d (Fig. 1F). The contents of proline after 14 d of stress were increased upon high salinity concentrations, but the levels were lower than those for 7 d (Fig. 1G).

Transcriptional reprogramming in response to salinity stress

We performed a comparative transcriptome analysis to investigate the molecular mechanisms underlying the response to salt stress at 300 mM NaCl in *H. nymphaeifolia*. A total of 21,67-21.70 million raw reads were obtained for the nine RNA samples. Q20 (base calling accuracy > = 99%) and Q30 (base calling accuracy > = 99.9%) rates of the clean reads ranged from 97.99 – 99.23% and 93.08 – 96.53%, respectively (Fig. 2A), which suggested the high quality of our sequencing data. A total of 416,949 unique genes were assembled as the reference transcriptome of *H. nymphaeifolia*. Euclidean distance was computed among samples based on their gene expression profiles, and the result revealed that the samples of each treatment group were more similar than those from different groups, indicative of the high consistency among biological replicates (Fig. 2B).

Compared to CK, 12,651 and 7,066 DEGs were detected upon 7 d and 14 d treatment of 300 mM NaCl, respectively (Fig. 2C). The large difference in DEG numbers between the two treatments suggested that distinct transcriptional responses were activated in *H. nymphaeifoli* seedlings along different durations of salt stress. Functional enrichment analysis revealed that the upregulated DEGs under 7 d NaCl treatment were overrepresented in the GO terms of response to hyperosmotic salinity stress, anion transmembrane transport and homeostasis, ethylene response, leaf senescence, wax metabolism, lateral root development, nitrate starvation response, auxin transport and auxin-activated signaling pathway (Fig. 2D). In contrast, the expression of the genes related to nonphotochemical quenching, photosystem II (PSII) repair and magnesium ion transport were substantially suppressed, indicating a negative impact of salt stress on photosynthesis. Comparatively, the genes upregulated upon 14 d treatment were primarily involved in responses to salinity, reactive oxygen species (ROS) and alcohol, inorganic anion transport, wax metabolism, abscisic acid (ABA)-activated signaling pathway, water transport and cellular pH regulation (Fig. 2E). Downregulated DEGs were highly represented in photosynthesis, brassinosteroid (BR) biosynthesis and ethylene metabolic process (Fig. 2E).

Quantitative real-time PCR validation

The qRT-PCR results showed a high consistency of the fold changes in gene expression with those of the RNA-seq, where the PPC was 0.76 and the p-value was 0.029 (Fig. 3). It suggested that the gene expressions inferred from the RNA-seq are reliable.

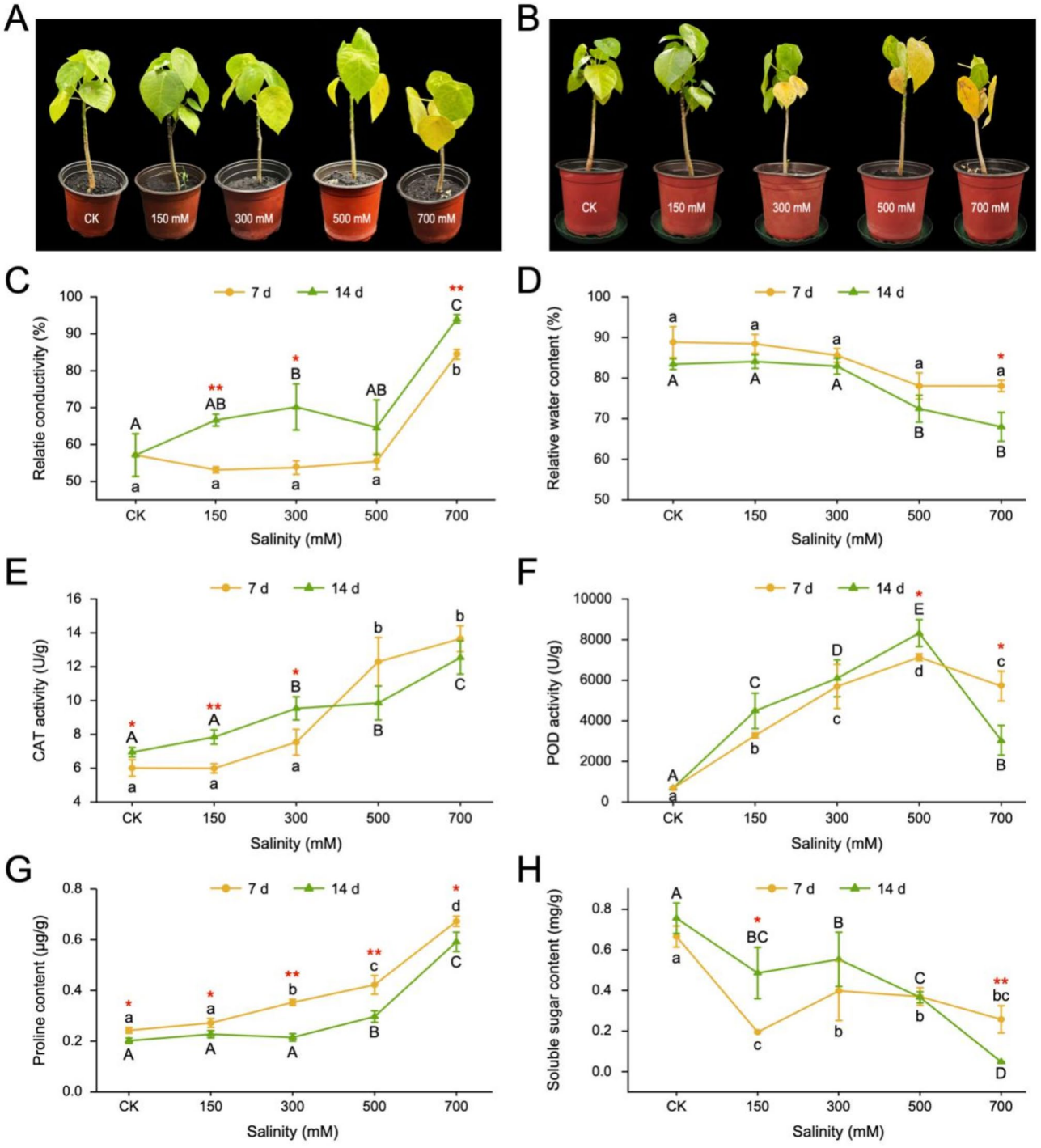

Fig. 1 Growth status and physiological changes in the leaves of *H. nymphaeifolia* seedlings in response to different salinity treatments. A-B: Growth status of *H. nymphaeifolia* seedlings under salt stress at different concentrations (0 (CK), 150, 300, 500 and 700 mM) for 7 d (**A**) and 14 d (**B**). C-H: Changes in relative conductivity (**C**), relative water content (**D**), catalase (CAT) activity (**E**), peroxidase (POD) activity (**F**), proline content (**G**) and soluble sugar content (**H**), respectively, across different treatment time and salinity concentrations. Different lowercase and uppercase letters represent significant differences at the *p*-value < 0.05 level of the LSD test among the 7 d and 14 d treatments, respectively, of different salinity concentrations. * and ** indicate significant differences between 7 d and 14 d treatments at the *p*-value < 0.05 and 0.01 level, respectively, of the two-tailed t-test

A

Treatment	Replicates	No. of raw reads	No. of clean reads	Q20 rate (%) R1/R2	Q30 rate (%) R1/R2
CK	1	21,694,759	21,690,050	99.21/98.14	96.45/93.49
	2	21,688,167	21,682,957	99.17/98.10	96.26/93.29
	3	21,683,343	21,678,209	99.17/98.02	96.23/93.09
7 d	1	21,682,394	21,677,470	99.16/98.24	96.28/93.94
	2	21,696,446	21,691,399	99.23/98.16	96.53/93.60
	3	21,691,445	21,686,575	99.22/98.12	96.51/93.51
14 d	1	21,666,797	21,662,020	99.19/97.99	96.35/93.08
	2	21,677,244	21,672,362	99.21/98.08	96.47/93.40
	3	21,698,178	21,693,486	99.19/98.19	96.35/93.70

No., Number.

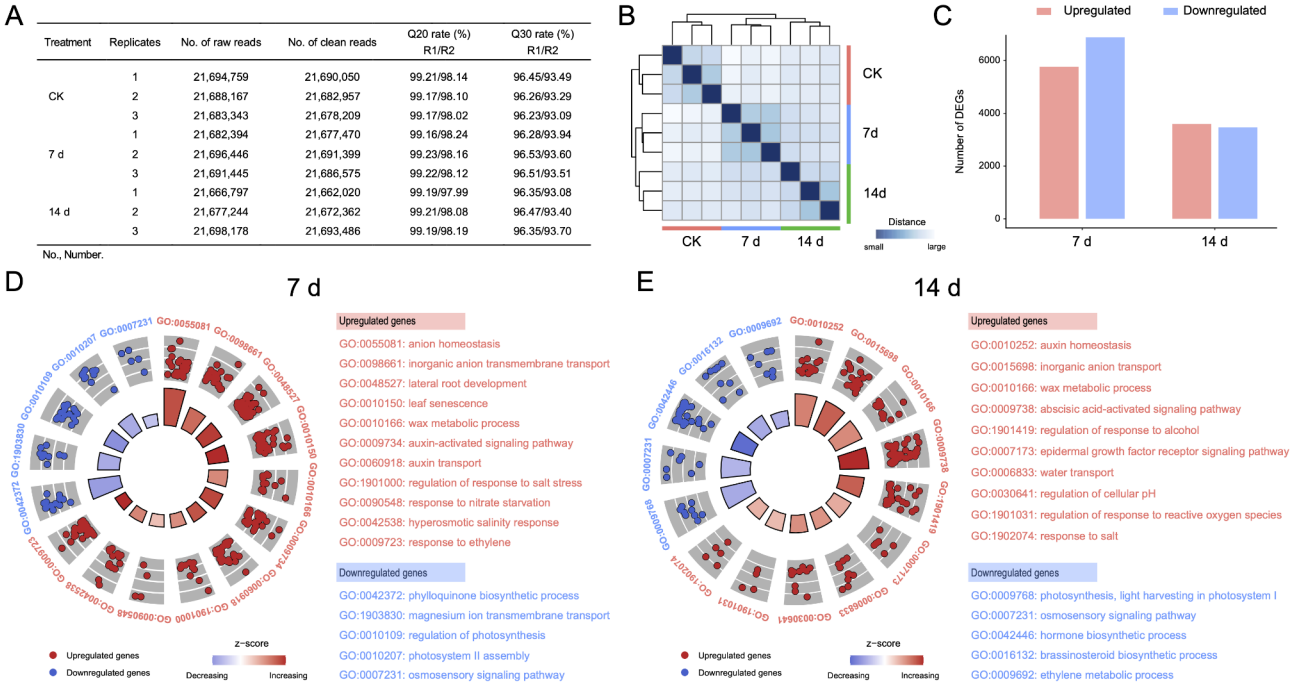

Fig. 2 RNA-seq analysis revealing transcriptional reprogramming in *H. nymphaeifolia* seedlings in response to 300 mM salinity treatments. **A**: Summary statistics of data quality of the RNA-seq data. **B**: Distance matrix illustrating transcriptomic similarities among different samples. Smaller distance is shown in darker color. **C**: Numbers of upregulated and downregulated genes under 7 d and 14 d treatment, respectively, compared to CK condition. **D-E**: Circular plot showing the representative Gene Ontology (GO) terms enriched for upregulated (red) and downregulated genes (blue) under 7 d (**D**) and 14 d salinity treatment (**E**). In each panel, GO term IDs and names are listed at right

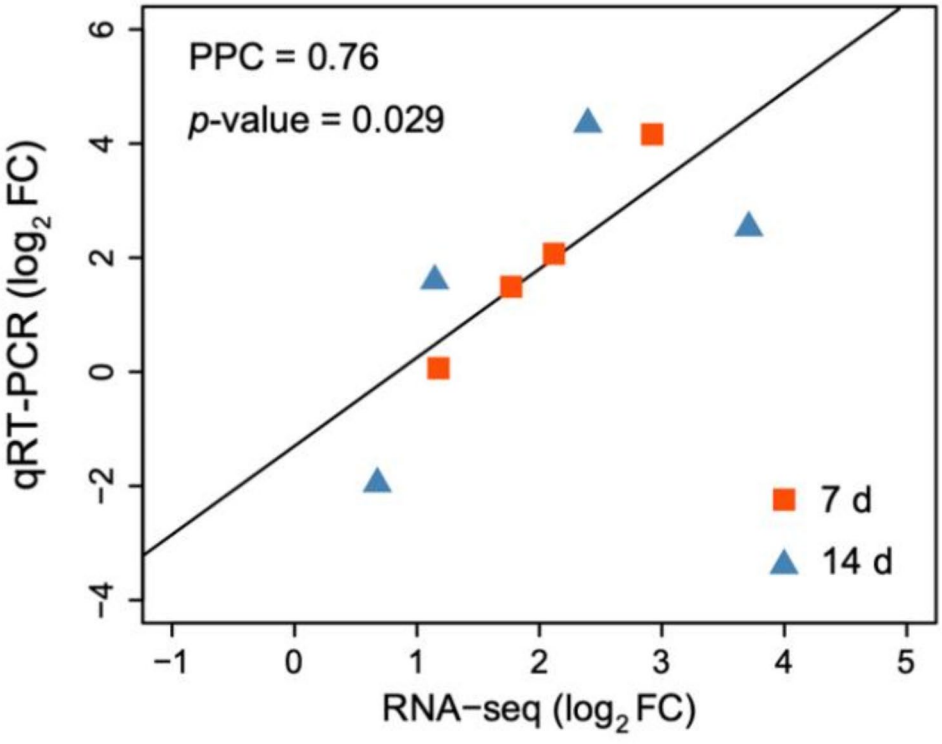

Fig. 3 Scatter plot illustrating the consistency between the $\log_2$-scaled fold change (FC) of four examined genes upon 7 d (red rectangles) and 14 d salinity treatment (blue triangles) inferred by RNA-seq (x axis) and qRT-PCR (y axis). The Pearson correlation coefficient (PCC) and *p*-value are listed at the top left of the plot

Gene expression dynamics across different treatment durations

We further investigated the temporal dynamics of gene expression in *H. nymphaeifolia* under salt stress. With the extension of NaCl treatment from 7 d to 14 d, 3,193 and 1,949 genes were further upregulated and downregulated, respectively. Notably, expression of the genes associated with anion homeostasis, extracellular stimulus response, sulfur assimilation and transport, proline biosynthesis and calcium ion transport were enhanced from CK to 7 d, but apparently decreased with the lapse of treatment time to 14 d (Fig. 4A). The genes participating in phytohormone regulation, such as BR metabolism and karrikin responses, were downregulated continuously as stress time was extended. In contrast, some DEGs were found to be specifically highly expressed upon 14 d salinity stress. Functional enrichment analysis of these genes revealed an overrepresentation of cell wall organization, auxin metabolism regulation and gibberellin response (Fig. 4B). The biological processes including photosynthesis and energy quenching, abscisic acid (ABA) homeostasis and responses to iron and zinc ion starvation were suppressed by 7 d treatment, but got recovered with the prolonging of time. These results indicated that *H. nymphaeifolia* might reallocate energy and resources for growth optimization, enabling better adaptation to relative long-term stress.

Co-expression networks associated with responses to salinity stress

WGCNA identified 14 gene modules that were significantly correlated with salinity stress (Fig. 5A). Of them, "darkolivergreen", "coral2" and "mediumpurple3" modules were positively correlated to 7 d salinity treatment, while four modules ("green", "darkred", "purple" and "turquoise") display negative relationships. GO enrichment analysis showed that the genes of the "darkolivergreen" module was highly represented in negative regulation of photomorphogenesis, vacuole organization and vacuolar transport, protein import into nucleus, autophagy, nucleotide-excision involved in interstrand cross-link repair and phospholipid catabolic process (Fig. 5B). In this module, some gene were of high connectivity (hub genes), including those encoding protein kinase, DnaJ protein, WAT1-related protein and hrq1 (Fig. 5C). These genes may play regulatory roles in modulating the expression of other genes. Three modules ("darkred", "bisque4" and "orangered4") were positively related to the salinity stress of 14 d, while four modules were negatively correlated (Fig. 5A). The genes in the "orangered4" module were significantly enriched for responses to cation stress and photooxidative stress, sodium ion import across plasma membrane, stomatal closure regulation, and positive regulation of kinase activity (Fig. 5D). The hub genes of this module mainly encode protein kinase, Beclin-1-like protein ATG6, Zinc finger A20 and AN1 domain-containing stress-associated protein (SAP) and ubiquitin-conjugating enzyme (Fig. 5E).

Discussion

Compared to true mangroves, mangrove associates capture the special ecological niche between mangrove swamps and terrestrial ecosystems and play an important role in function maintenance and artificial afforestation of coastal ecosystems [1, 14, 30]. However, few attentions have been paid for understanding the salt tolerance capacity, as well as the underlying mechanisms, of mangrove associates, which restricts their conservation and usage in afforestation. In the current study,

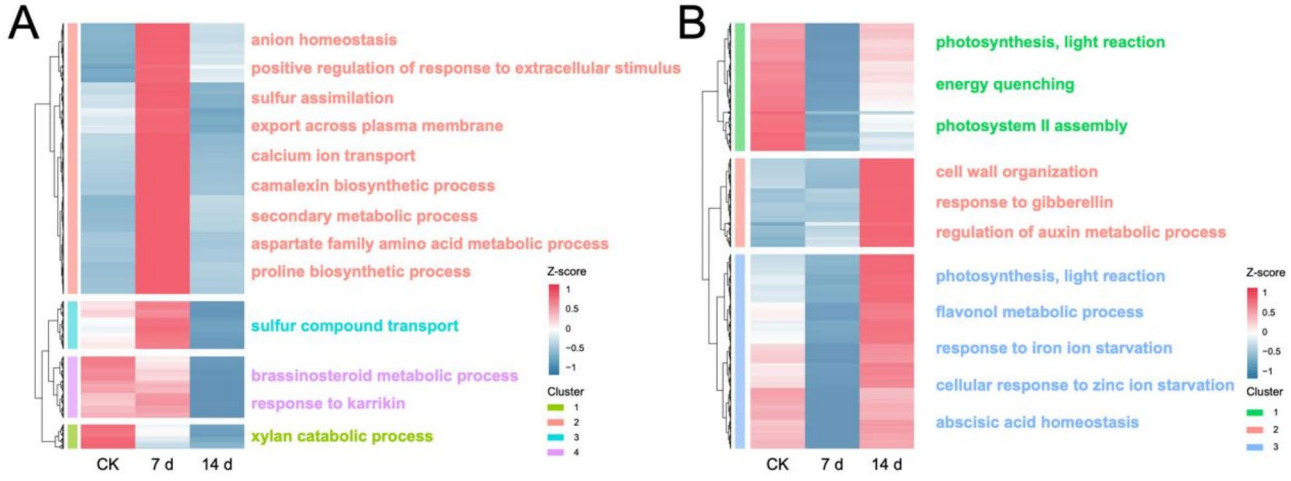

Fig. 4 Gene expression dynamics across different treatment durations. **A**: Heatmap illustrating expression trend of the genes upregulated upon 7 d treatment over 14 d treatment. **B**: Heatmap illustrating expression trend of the genes upregulated upon 14 d treatment over 7 d treatment. For both panels, genes are grouped by hierarchical clustering. Representative Gene Ontology (GO) terms enriched for each cluster of genes are listed at right

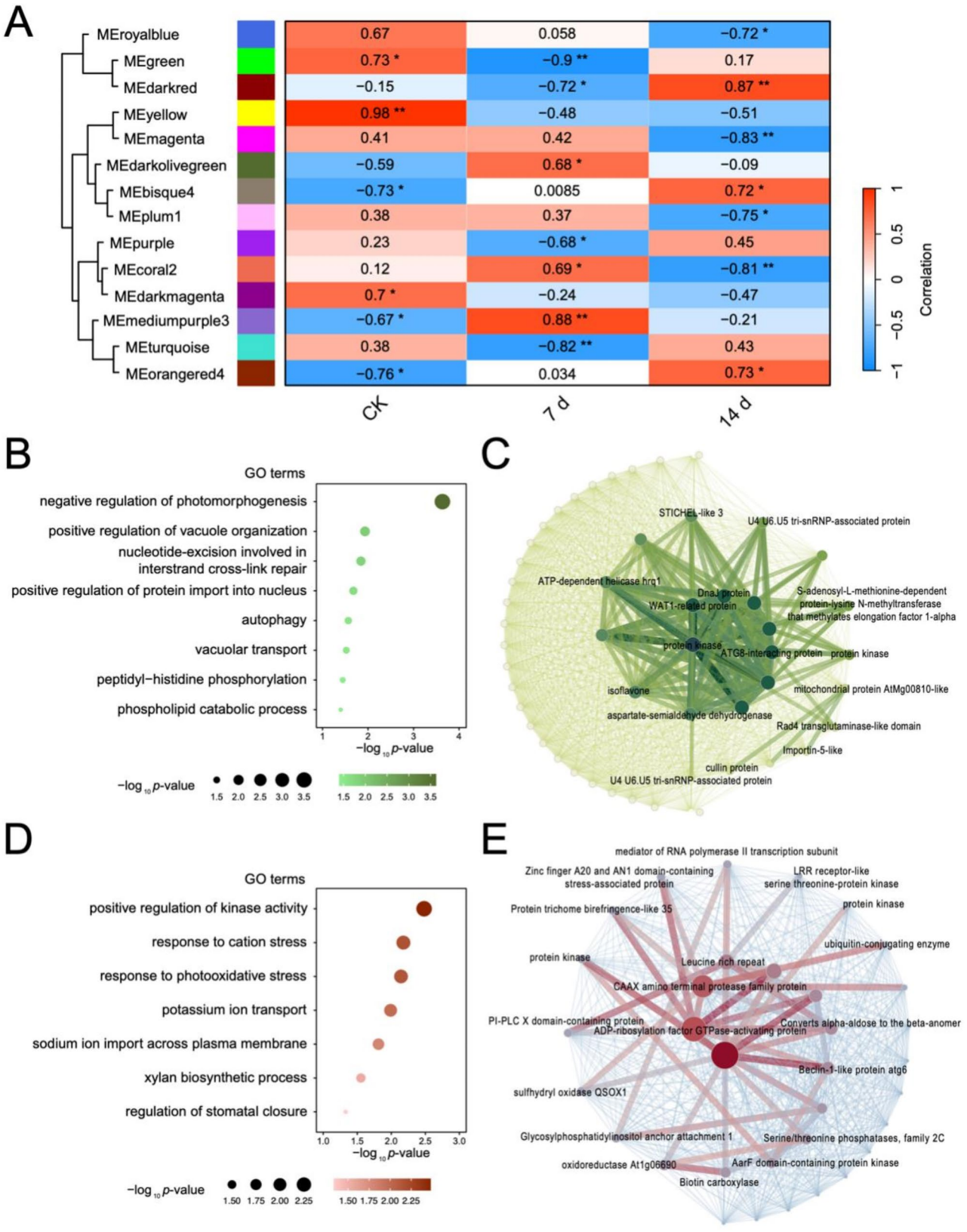

Fig. 5 (See legend on next page.)

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Fig. 5 Gene co-expression networks associated with salinity responses. **A**: Gene modules significantly correlated with the response to salinity stress. Red and blue colors note positive and negative correlation, respectively. * and ** represent *p*-value < 0.05 and 0.01, respectively. The relationships among modules are illustrated by a hierarchical tree at left. **B**: Representative Gene Ontology (GO) terms enriched for the genes of the "darkolivegreen" module, which is positively correlated with the response to 7 d salinity stress. **C**: Gene interaction network for the "darkolivegreen" module. Dot size and color corresponds to the number of connections the gene has. **D**: Representative GO terms enriched for the genes of the "orangered4" module, which is positively correlated with the response to 14 d salinity stress. **E**: Gene interaction network for the "orangered4" module. Dot size and color corresponds to the number of connections the gene has

a comprehensive investigation was performed on the impacts of salinity stress on the seedlings of a typical mangrove-associated plant *H. nymphaeifolia*. Our results revealed that the *H. nymphaeifolia* seedlings growing with fresh water or low salt stress showed similar and good conditions. However, the growth status got worse when exposed to salt stress at median and high concentration for 7 d (Fig. 1A and B), mirroring a moderate tolerance of *H. nymphaeifolia* to salinity stress. A similar phenomenon was also observed in other mangrove associates, for example, *Clerodendrum inerme* [31]. It is consistent with their nature as glycophytes with the capacity to adapt to low salinity environment [1, 14, 30]. Accordingly, when exposed to 700 mM salt stress for 7 d, the leakage of leaf plasma membrane was significantly increased, and the water content in leaves was substantially decreased (Fig. 1C and D). Compared to true mangrove plants, mangrove associates like *H. nymphaeifolia* in general have lower osmolality [12], and thus are prone to lose water under salt stress. As a response, the genes associated with osmosensory signaling pathway were differentially expressed in stressed individuals (Fig. 2D and E). For instance, the genes encoding Ca^{2+}-permeable mechanosensitive channels (mid1-complementing activity protein, MCA) were significantly downregulated. In soybean (*Glycine max*), the decreased mRNA expression of *MCA2* and *MCA3* were found to respond to high salt and dehydration stress, respectively [32]. Accordingly, Ca^{2+} transport was substantially enhanced in *H. nymphaeifolia* seedlings upon 7 d salt stress (Fig. 4A). The mRNA abundance of the gene encoding a histidine kinase (HK) was also significantly changed upon salt stress. In plants, HKs have multiple functions, including ethylene receptors, osmosensors, cytokinin receptors and intracellular sensors, and they act a regulatory role in stress responses [33]. To further combat the adverse impacts of cellular dehydration, biological processes related to proline biosynthesis and responses to extracellular stimulus were activated in the leaves of salt-stressed *H. nymphaeifolia* plants (Figs. 1G and 4A). Proline is a typical osmoregulator in halophytes, and the increased accumulation of free proline can promote cell osmotic pressure and reduce water loss [34]. In contrast, the concentration of soluble sugar was downregulated upon salt stress (Fig. 1H). It is different from the observation in *C. inerme* [31], indicating that a different osmoregulatory mechanism was employed by *H. nymphaeifolia*.

High salinity-induced osmotic impacts can also cause secondary damage to plant cells. ROS formation and accumulation is one of the main culprits [35, 36]. In *H. nymphaeifolia*, photosynthesis and photomorphogenesis were found to be suppressed by hyper-salinity stress (Figs. 2D and E and 5B), which was similar to the observations in other mangrove plants, such as *C. inerme* [31], *Kandelia candel* and *Bruguiera gymnorrhiza* [37]. The photoinhibition would restrict CO_2 fixation, further disrupt the balance between absorption and utilization of light energy and finally accelerate ROS production [38]. Moreover, ABA-mediated signaling pathway was also highly activated when the treatment was extended to 14 d (Fig. 2E). In plants, although ABA can improve leaf tolerance to water loss by shutting up stoma, stomatal closure would further reduce CO_2 fixation in chloroplast as a by-product and boost the formation of ROS, which intensifies lipid peroxidation and causes damage to the structure and integrity of cells and organelles [39]. To alleviate the adverse impacts of ROS accumulation, antioxidative enzymes were activated and DNA repair was also enhanced (Figs. 1C and D and 5B). Moreover, some of them were coordinately expressed with other genes in the co-expression network upon 7 d salinity stress (Fig. 5C). For example, the genes encoding DnaJ protein and ATP-dependent helicase Hrq1 were identified as hub genes by WGCNA. DnaJ, also known as heat shock protein 40 (Hsp40), is a type of chaperone proteins that play crucial roles in protein folding [40]. In tomato (*Solanum lycopersicum*), DnaJ proteins participate in removal of ROS and alleviation of stress-induced photoinhibition [41]. Hrq1 is a homolog to RecQ helicases, and it has been shown to participate in DNA repair in *Arabidopsis* plants [42, 43]. In addition, the boosting of ROS may also trigger plant leaf senescence. In *H. nymphaeifolia* seedlings, expression of the genes involved in leaf senescence and autophagy were found to be positively correlated with 7 d salt stress (Figs. 2D and 5B). Leaf senescence in plants primarily involves chloroplast breakdown, nutrient recycling and translocation, and programmed cell death, which can improve material utilization efficiency and environmental tolerance of plants [44]. The enhancement of these biological processes conferred the early responses of *H. nymphaeifolia* seedlings to salinity stress.

When the stress extended to 14 d, the relative electrolyte leakage and water loss were substantially increased in the leaf cells of the salt-stressed *H. nymphaeifolia*

seedlings, in particular under exposure to 700 mM NaCl (Fig. 1A and B), suggesting intensified damage of cell integrity. To alleviate the adverse impacts, genes associated with Na^+ and K^+ transport, cation stress response, photooxidative stress response, stomatal closure, and phytohormone metabolism and regulations were particularly upregulated in response to 14 d salt stress (Figs. 4B and 5D). Na^+ accumulation in plant cytosol may reduce the activity of K^+-dependent enzymes and disrupt normal nutrient uptake and metabolism [39]. For mangrove plants, excessive Na^+ in leaf cells can be sequestered in vacuoles to avoid their destructive effects [11]. In contrast to Na^+, enhancement of K^+ acquisition can improve ion homeostasis and antioxidant enzyme activities in plants, thus ameliorating salt stress-induced ROS accumulation and promoting their tolerance to salt stress [45–47]. Moreover, an increase in K^+ concentration can also help plants restore the activities of PSII and photosystem I (PSI) proteins, as well as photosynthetic electron flow and photochemical yield [48]. Thus, the synchronous enhancement of Na^+ and K^+ transport can assist *H. nymphaeifolia* seedlings in maintaining ion homeostasis, alleviating oxidative damage to cell components, and promoting photosynthetic performance under long-term stress. In addition, some stress-responsive kinases and regulatory proteins, such as serine/threonine phosphatases, zinc finger A20 and AN1 domain-containing SAP and sulfhydryl oxidase QSOX1, were supposed to play pivotal roles in modulating transcriptomic reprogramming in response to 14 d salt stress (Fig. 5E). Protein kinases play a crucial role in stress sensing and signaling transduction in plants [49]. In tomato, mitogen-activated protein kinase (MAPK) was shown to mediate regulation of the balance among phytohormones ethylene, ABA and jasmonic acid (JA), and activation of antioxidant enzymes during salt stress [50]. Thus, the upregulation of MAPK in *H. nymphaeifolia* may induce photooxidative stress response and stomatal closure (Fig. 5D and E), which further alleviate the water loss and destructive effects caused by salt stimulus. Zinc finger A20 and AN1 domain-containing SAPs are another type of participants in the responses of plants to multiple abiotic stresses [51]. The interaction between SAPs and receptor-like cytoplasmic kinase 253 (RLCK253) can activate the downstream signaling cascade and improve salt stress tolerance of *Arabidopsis* plants [52]. These key regulatory genes may serve as potential candidate targets for future genetic transformation and editing to confer *H. nymphaeifolia* with high resistance to salinity.

Conclusion

In this study, we elucidate the transcriptional alterations in *H. nymphaeifolia* that regulate its physiological and biochemical reprogramming in responses to hyper-salinity threats. The findings largely broaden our knowledge of the underlying mechanisms for *H. nymphaeifolia*'s tolerance to environmental changes, which provides theoretical guidance to the conservation and afforestation of endangered mangrove species. Moreover, our study identifies some pivotal regulators that modulate the synergistic expression of salt-responsive genes, which might be used for future bioengineering improvement of salt tolerance of mangrove associates and other glycophytic crops.

Supplementary Information

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Supplementary Table 1

Author contributions

Y. Y. and Y. Z. designed and supervised the study; Z. F., X. X., C. Z., Y. L., C. Z., M. E. T., and J. H. analyzed the data and visualized the results; Z. F., X. X., S. S., Y. Z., and Y.Y. wrote the initial manuscript; All other authors contributed critically to the writing of the final manuscript and gave approval for publication.

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Data availability

Sequence data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) database of the National Center for Biotechnology Information (NCBI) with accession code GSE277704.

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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