

Transcriptomic Profiling Reveals the Contribution of Nitric Oxide to Maintaining Photosynthesis and Antioxidant Ability in *Hylotelephium erythrostictum* Leaves under Salt Stress

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Abstract

Hylotelephium erythrostictum is a plant species with high garden value and notable salt tolerance, yet the salt tolerance mechanism is poorly understood. In this study, we treated seedlings with 200 mM NaCl and performed transcriptome sequencing after 0, 5, and 10 d. A total of 123,008 unigenes were identified, of which 79,478 were functionally annotated. The majority of the differently expressed genes (DEGs) associated with photosynthesis and photosynthesis-antenna protein metabolic pathways were downregulated. In contrast, genes related to superoxide dismutase (SOD) and catalase (CAT) were significantly upregulated, and genes related to nitric oxide (NO) synthase also exhibited increased expression levels. As NO synthase-related genes in the transcriptome participate in the *H. erythrostictum* salt stress response, we treated the seedlings under salt stress with exogenous NO and discovered that the contents of peroxides, such as malondialdehyde (MDA), decreased, while soluble sugars and other osmotic substances increased. The chlorophyll fluorescence parameters, such as the maximum photochemical efficiency of PS II (Fv/Fm), actual quantum yield Y(II), and the photochemical quenching coefficient (qP) increased, whereas fluorescence (Fo) and the non-photochemical quenching coefficient (NPQ) decreased. Furthermore, the expression levels of *SOD*, *CAT*, *Cab*, and *psaA* were significantly upregulated. These results suggest that NO enhances the photosynthetic and antioxidant capacity of *H. erythrostictum* and improves salt tolerance.

Introduction

Plant growth and development are affected by a range of biotic and abiotic factors. Among these, salinity has emerged as a major challenge due to its negative effects on normal garden plant growth, agronomic field use, and global crop production (Singh et al. 2016; Mbarki et al. 2018; Sharif et al. 2019). Global statistics indicate that nearly 9.5438×10^8 hectares of cultivated land are under saline-alkali stress. A total area of about 1×10^8 hectares in China is affected by saline soil (Liu et al. 2021). As this situation is worsening, it is necessary to investigate plant adaptation to salt stress. Research has identified two ways in which salt toxicity impairs plant growth. First, high soil salinity causes drought. Drought reduces osmotic potential of the plant rhizosphere soil solution, with reduced water absorption and increased water loss from leaves (Van Zelm et al. 2020). Osmotic stress, ionic toxicity, and mineral imbalance occur due to reduced water and nutrient uptake by cells. However, plants engage in self-regulatory processes to alleviate the damage from salt stress, which triggers physiological and biochemical changes, such as a decrease in stomatal conductance, slowed photosynthesis, reduced reactive oxygen species (ROS) scavenging ability, and nutritional imbalance (Munns et al. 2008; Sirault et al. 2009). In conclusion, understanding the adaptation of plants to salt stress is crucial to mitigate the negative effects of salinity on growth, and crop production, and ensures global food security. Therefore it is essential to investigate the physiological and biochemical mechanisms to improve salt tolerance in plants.

Leaves are vital to plant growth and development, as they facilitate photosynthesis by receiving light. When plants are exposed to salt stress, the leaves undergo physiological and biochemical changes that

cause loss of green and mottling, which negatively affect photosynthesis. Hence, leaves act as a bridge in the response to salt stress. Salt stress leads to a Na^+ and Cl^- imbalance, which damages photosystem II (PS II) in the chloroplast, resulting in an increased proportion of absorbed light energy being dissipated as heat and fluorescence (Marriboina et al. 2022). Na^+ slows the photosynthetic rate by disrupting proton dynamics, chloroplast function, and carbon fixation enzyme activity (Van Zelm et al. 2020). Although the ROS produced by chloroplasts regulate energy metabolism and enhance the adaptability of plants to the environment (Foyer et al. 2022), reduced photosynthetic rates increase the production of ROS (Fichman et al. 2019). A high ROS concentration damages membrane lipids, resulting in increased permeability, peroxidation of membranes, leakage of intracellular electrolytes, and increased tissue leaching fluid conductivity (Ahmad 2010; Hayat S et al. 2012; AbdElgawad et al. 2016). ROS also act as signal transduction molecules to convey stress information to the nucleus and induce the stress response (Foyer et al. 2022).

Plants adapt to salt stress through a variety of mechanisms, including regulation by hormones, mineral homeostasis, salt compartmentalization, ion balance, osmotic regulation, formation of antioxidants, activation of enzymes, and production of nitric oxide (NO) (Fan et al. 2015; Chen et al. 2018; Yu et al. 2020; Wei et al. 2022; Yang et al. 2022). NO participates in the regulation of various physiological activities, such as root growth, stomatal opening, seed germination, flowering, and other development processes (Gayatri et al. 2017; Zhang et al. 2019; Ma et al. 2020; Abedi et al. 2021; Ciacka et al. 2022). As a stress signaling molecule, NO also plays a role in regulating protein activity and stability. Post-translational modifications, such as S-nitrosylation of cysteine and the tyrosine nitrosylation modification, enable NO to participate in various metabolic pathways (Abat et al. 2009; Radi et al. 2013; Sehrawat et al. 2014). NO also cooperates with ROS to induce hypersensitivity reactions, leading to local tissue cell death. Furthermore, NO maintains ROS homeostasis and improves stress tolerance through enzymatic oxidation and non-enzymatic antioxidant systems, such as activating the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX), also inducing non-enzymatic antioxidants, such as ascorbate (Wang et al. 2009; Wu et al. 2015; Tripathi et al. 2016; Lau et al. 2021). NO may play a role in regulating light energy allocation in wheat under drought stress (Shao et al. 2012), as well as in boosting the photosynthetic pigment content and photochemical efficiency of ryegrass (*Lolium perenne*) (Liu et al. 2010). Interestingly, applying exogenous NO under saline and non-saline conditions significantly affects several photosynthetic parameters, such as chlorophyll (Chl) *a*, Chl *b*, total Chl, and the rate of photosynthesis, stomatal conductance, transpiration, and subcellular CO_2 concentration (Tahjib-Ul-Arif et al. 2022). Thus, NO may be involved in the signal transduction process for the salt stress response.

Hylotelephium erythrostictum is a succulent plant that grows annually or perennially. This species is part of the group of facultative crassulacean acid metabolism (CAM) plants, which means its leaf stomata close during the day to conserve water while opening at night to assimilate CO_2 . Due to the ability to resist stress and high ornamental value, this plant is commonly found in landscaping, particularly in coastal areas. Despite the many studies on physiological changes in *H. erythrostictum* under stressful

conditions, few have examined the salt-tolerance genes. Transcriptome sequencing is a powerful tool to provide insight into functional gene structure and metabolism, identify candidate genes related to plant shape, and clarify specific biological processes. Here, we performed transcriptome analysis to identify and annotate the salt-tolerance genes of *H. erythrostictum* under NaCl treatment. We also used quantitative polymerase chain reaction (PCR) analysis to verify the differentially expressed genes (DEGs) related to the NO response pathway. Overall, our results provide a theoretical basis for further investigating the role of NO in the *H. erythrostictum* salt stress response. This study will lead to a better understanding of how this plant responds to stressful conditions and potentially develop ways to enhance its resilience by identifying salt-tolerance genes.

Materials and methods

Plant materials

We cultured seedlings in 1/4 Hoagland nutrient solution in an artificial climate chamber with a 14-hour light and 10-hour dark cycle. The temperature and humidity were regulated at 25/18°C during the day/night and 60%, respectively. The culture solution was replaced every 3 days. Once the seedlings grew approximately 10 cotyledons, those with a consistent growth status and that were free of pests and diseases were selected for the stress treatment.

NaCl treatment for RNA sequencing

Randomly selected seedlings with 10 cotyledons were exposed to the 200 mM NaCl treatment, which was prepared by dissolving NaCl in 1/4 Hoagland nutrient solution. The treatment concentration was maintained by replacing the solution every 3 days. Leaves treated with 200 mM NaCl were collected at 0 (T0 control), 5 (T5), and 10 (T10) d, treated with liquid nitrogen, and stored at – 80°C for the RNA sequencing and gene expression analyses. Each treatment was performed in triplicate.

RNA extraction and purification

Total RNA was isolated separately from each sample using an RNAPrep Pure Plant Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. The purified RNA was quantified using a NanoDrop 2000 spectrophotometer, and RNA integrity was evaluated using the Agilent 2100 Bioanalyzer.

Library preparation and Illumina sequencing

Ori-Gen Science and Technology Co., Ltd. (Beijing, China) prepared the library and sequencing for transcriptome analysis. Two RNA samples were taken and 10 µg of total poly(A) mRNA was extracted from each sample using Sera-mag Magnetic Oligo (dT) Beads (ThermoFisher, Waltham, MA, USA). The mRNA was broken into short fragments, adapters were added, and PCR amplification was carried out. The fragments were treated with fragmentation buffer to interrupt the mRNA to create short fragments, and a random hexamer primer was used to synthesize the first-strand cDNA. The second-strand cDNA was synthesized using 20 µL of second-strand buffer (Invitrogen, Carlsbad, CA, USA), along with 10 mM

dNTP Mix, 5 U/uL RNaseH, and 10 U/uL DNA polymerase I. The short fragments were purified with the Qiaquick PCR Purification Kit, resolved with EB buffer for end reparation, and augmented with poly(A) before connecting them with sequencing adapters. Suitable fragments were selected for PCR amplification as templates. The Illumina HiSeq™ 2500 platform was used to sequence the paired-end (PE) RNA-seq libraries according to the manufacturer's protocols. Base calling and data analysis were carried out using the Illumina instrument software. Ori-Gen Science and Technology Co., Ltd. stored one copy of the RNA samples at – 80°C for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments. A 1–2 µg portion of total RNA was used as the template in the qRT-PCR reactions with reverse transcriptase, following the manufacturer's instructions.

RNA-seq quality pre-processing and analysis

The RNA-seq results that met the Q20 standard were used, indicating acceptable sequencing quality. Raw reads generated from sequencing machines often contain dirty reads, involving adapters, as well as unknown or low-quality bases. As such, these data would compromise any bioinformatics analysis. Thus, dirty raw reads, such as reads with adapters, unknown nucleotides > 5%, and low-quality reads were eliminated, after which a *de novo* assembly of the transcriptome was conducted using the Trinity short reads assembling program. Trinity software connected the contigs and yielded sequences, known as unigenes. Subsequently, the unigenes were blasted using BLASTX and annotated against the SwissPort, COG, non-redundant (Nr), KEGG, and GO protein databases using a cut-off E-value of 0.00001. The Gene Ontology (GO) database is comprised of molecular functions, cellular components, and biological processes. The GO annotations for the unigenes were obtained using the Blast2GO program and Nr annotation. After the GO annotation was obtained, WEGO software facilitated the GO functional classification, offering an understanding of the distribution of gene functions of all unigenes. Furthermore, the KEGG database allowed for the analysis of gene products and related gene functions during metabolic and cellular processes. The pathway annotations were obtained for the unigenes by performing KEGG pathways annotation using Blastall software against the KEGG database.

The findings of this study were determined through the differential expression analysis of fold changes in expression levels (with a p-value of < 0.05), carried out in Cufflinks (version 2.0.2). The DEGs were subjected to COG analysis and clustered based on Fisher's test and the chi-square test.

qRT-PCR

Total RNA samples used for transcriptome sequencing were also used for qRT-PCR. The reactions were performed on a Roche LightCycler® 96 Real-Time PCR instrument according to the manufacturer's instructions using a FastQuant RT Kit (with gDNase) (Tiangen Biotech). Primers were designed using Primer-Blast to amplify the 80–200 bp products for each gene of interest. Gene names, annotations, and the primers used for the qRT-PCR analysis are listed in Table S1. Each sample was analyzed at least three times to ensure accuracy.

Sodium nitroprusside (SNP) treatment

To investigate the effect of NO on the response of *H. erythrostictum* leaves to NaCl, we utilized 50 μ M SNP as an exogenous NO donor. SNP was added to a 200 mM NaCl solution to achieve the desired treatment concentrations. Each treatment was replicated three times.

Physiological indices

The leaves from the treatment and control groups were washed with deionized water and blotted dry with absorbent paper. Subsequently, relative electrical conductivity (REC) was measured using a relative conductivity meter. MDA and soluble sugar contents were assessed using the MDA Assay Kit and Plant Soluble Sugar Content Assay Kit (Solarbio Science & Technology Co. Ltd, Beijing, China) respectively, as per the manufacturer's instructions.

Chlorophyll fluorescence parameters

The study utilized a PAM-2500 portable modulated chlorophyll fluorometer from WALZ (Effeltrich, Germany) for measurements. Mature leaves were carefully selected to avoid veins and were dark-adapted for 20 min using leaf clips. The fluorescence parameters, including F_o , F_v/F_m , $Y(II)$, qP , and NPQ were measured.

Results

Transcriptome analysis of *H. erythrostictum* leaves under salt stress

High-throughput transcriptome sequencing and de novo assembly

We collected 81.523 Gb of raw data from nine samples, which were carefully filtered to remove low-quality reads containing adapters, unknown bases, or other deficiencies. Through a stringent quality check and data cleaning, we obtained a clean read count of 571.719 Mb (Table S2). The contigs were assembled into 200,924 scaffolds using paired-end joining and gap-filling techniques. *De novo* assembly of these scaffolds resulted in 124,008 unigenes (Table S3).

Gene annotation and functional classification

We analyzed the transcriptome library and evaluated its completeness and annotation quality. All unigenes were aligned to the protein databases using BLAST, and an E-value threshold of 10^{-5} was set. Our analysis showed that 64.1% of the 12,408 annotated unigenes had significant BLAST matches in the Nr database, while 39.0% had matches in the Swiss-Prot database. Additionally, 55,665 (44.9%) and 63,457 (51.2%) of the unigenes had significant matches in the Pfam and GO databases, respectively, and 26,712 (21.5%) were similar to proteins in the KEGG database (Table S4).

To ensure the completeness of our transcriptome library, we searched for genes with KOG classifications and assigned 61,508 unigenes (Fig. S1b). Among the 25 KOG categories, the largest group represented 'general functional prediction' with 7,604 unigenes (18.88%), followed by 'post-translational modification,

protein turnover, chaperones' (4,540, 11.27%), 'translation, ribosomal structure and biogenesis' (3,663, 9.10%), and 'signal transduction mechanisms' (3,009, 7.47%).

We also used GO to classify the functions of the predicted unigenes. The GO analysis revealed that 63,457 sequences were categorized into 56 functional groups (Fig. 1). The unigenes were assigned to the cellular component, molecular function, and biological process categories. The cellular component category had the highest representation, followed by the biological process and molecular function categories. Cellular processes (39,809, 62.73%) and metabolic processes (34,690, 54.67%) represented the majority of biological processes. Cell (44,534, 70.18%), cell parts (44,402, 69.97%), and organelles (34,998, 55.15%) were the most prominently represented in the cellular component category. Binding (36,919, 58.18%) and catalytic activity (30,781, 48.51%) represented the majority in the molecular function category, while 6.63% (4,210) were involved in transporter activity. These findings suggest a high level of conservation in cellular and molecular processes across different species (Fig. 1).

Our study assigned 26,712 unigenes to various pathways, classified as Organismal Systems, Metabolism, Human Diseases, and Genetic Information Processing, Environmental Information Processing, and Cellular Processes. As shown in Fig. 2, more than 51.00% of the unigenes were involved in Metabolism, followed by Genetic Information Processing. This annotation was an essential resource for exploring the processes, functions, and pathways associated with salt tolerance mechanisms.

Analysis of differentially expressed genes

Notable distinctions in gene expression were detected among the three treatment groups. Specifically, the expression of 27,975 DEGs changed significantly between the 5-d and 10-d treatment groups, compared to the 0-d group. The changes included 13,501 upregulated unigenes and 14,474 downregulated unigenes. Furthermore, 311 unigenes were upregulated and 415 were downregulated simultaneously in the three group comparisons (Fig. 3a, b). These DEGs were enriched in various GO terms and KEGG pathways. This result indicates that a myriad of transcriptional regulatory events occurs during salt stress (Fig. S2).

The results of the GO and KEGG analysis suggested that the response of plants to salt stress is a complex process involving multiple genes and regulated by various biological metabolic processes. During salt stress, many genes related to photosynthetic pathways, the ROS system, and NO synthase were identified (Table S5). Some genes involved in photosynthesis were upregulated under salt stress, such as deoxychlorophyll-*a* oxygenase, phosphoenolpyruvate carboxylase, and NADP-malic enzyme, while others such as the chlorophyll *a/b* binding protein, PS II protein D1, ferredoxin-NADP reductase, and the ATP synthase CF1 α chain were downregulated. The majority of the genes encoding antioxidant enzymes were upregulated in response to salt stress, but genes associated with ascorbate oxidase were downregulated. Additionally, all NO-related genes were upregulated (Table S5). To validate the transcriptome data, several genes were selected for qRT-PCR analysis, which revealed expression patterns consistent with the TPM values (Fig. 4).

Effect of NO on the plasma membranes of seedlings under salt stress

The REC of *H. erythrostictum* leaves increased with longer NaCl treatment times. Specifically, the REC was 1.16 and 1.17 times higher than the control on days 5 and 10 of salt stress, respectively (Fig. 5a). Interestingly, adding SNP significantly lowered the REC of the seedlings, particularly on day 10 (reduction of nearly 10.00%) when compared to the NaCl treatment alone ($P < 0.05$). Moreover, the MDA contents in the group that was treated with 200 mM NaCl were 4.78 and 4.65 times higher than the control on days 5 and 10, respectively (Fig. 5b). However, there was a considerable decrease of 52.4% and 60.8% in the SNP-added group on days 5 and 10, respectively, when compared with the NaCl treatment group (Fig. 5b). This finding suggests that NO minimizes damage to the plasma membranes brought about by salt stress.

Effect of NO on the osmotic adjusting substances of seedlings under salt stress

The soluble sugar content in leaves increased under the NaCl treatment compared to the control group. Specifically, the NaCl treatment group had soluble sugar contents that were 1.96 and 2.65 times higher than those in the control group at different treatment times (Fig. 6). The NaCl + SNP group also showed an increase in soluble sugar content compared to the control group, but the value was lower than the NaCl treatment by approximately 14.01%.

Effect of NO on the chlorophyll fluorescence parameters of seedlings under salt stress

The photosynthetic findings showed that the F_o and NPQ of leaves increased as salt stress time was extended under NaCl treatment. These values were 1.24 and 2.35 times higher than the control on day 10 of the NaCl treatment, respectively (Fig. 7). The F_v/F_m value decreased by 4.40%, the $Y(II)$ decreased by 20.30%, and qP decreased by 11.00%. In contrast, F_v/F_m , $Y(II)$ and qP increased by 1.03, 1.05, and 1.04 times, respectively, in the NaCl + SNP group, compared to the single NaCl treatment group on day 10 after treatment ($P < 0.05$). However, F_o and NPQ were only 0.83 and 0.69 times that of the single NaCl treatment (Fig. 7).

The expression levels of the *SOD*, *CAT*, *Cab*, and *psaA* genes were detected by qRT-PCR. The results showed that salt stress upregulated *SOD* and *CAT* (Fig. 8a, b), but downregulated *Cab* and *psaA* expression (Fig. 8c, d). After adding SNP, all four genes were upregulated, indicating that NO enhanced the antioxidant and photosynthetic systems under salt stress in *H. erythrostictum*.

Discussion

Salt stress is a challenging environment that limits plant growth and development. *H. erythrostictum* is a salt-tolerant plant that can withstand 300 mM NaCl, and has significant potential in landscaping.

Although many studies have explored the physiological indicators and traits of *H. erythrostictum* under salt stress, the salt tolerance mechanism remains unknown. RNA-seq is the method of choice to study gene expression, and is crucial for analyzing unknown genes and novel transcript isoforms (Hrdlickova et al. 2017). In our study, the transcriptome analysis of leaves treated with 200 mM NaCl revealed that the response to salt stress involved multiple genes and metabolic processes. Most of the DEGs enriched in photosynthesis and photosynthesis-antenna protein metabolic pathways were downregulated after the salt treatment. Examples of downregulated genes include PS II protein D1, ferredoxin-NADP reductase, chlorophyll *a/b* binding protein, ATP synthase CF1 α chain, and PS I P700 apoprotein A. This finding indicates that salt stress has a significant effect on the structure of the photosynthetic electron transport chain and photosynthesis, resulting in a decrease in the photosynthetic rate. On the other hand, the dehydroascorbate reductase and glutaredoxin genes, particularly the glutathione S-transferase (GST) gene, were upregulated in *H. erythrostictum*. Furthermore, the expression levels of genes associated with glutathione peroxidase, SOD, and CAT were also significantly upregulated, indicating that *H. erythrostictum* has significant antioxidant capacity. NO is a stress-signaling molecule that directly or indirectly regulates the expression of redox-related genes and participates in the regulation of salt stress. The transcriptome analysis revealed that the expression of genes related to the NO synthetase pathway increased. NO enhances APX activity through S-nitrosylation, regulates the AsA-GSH cycle, reduces toxic effects, such as H₂O₂, and enhances antioxidant capacity (Begara-Morales et al. 2014). Furthermore, NO affects the photosynthetic electron transport chain and structure, effectively protecting the light reaction center and ensuring the normal transmission of electrons (Astier et al. 2018; Solymosi et al. 2021).

Excess ROS production hinders the electron transport processes in the mitochondria and chloroplasts (Speijer et al. 2020), leading to an imbalance in ROS metabolism and oxidative stress. To mitigate the damage caused by oxidative stress, plants manufacture or accumulate osmoregulatory substances, such as soluble sugars, in response to oxidative stress signals (Ahmad et al. 2021). These substances alter the osmotic potential of the cell, leading to expansion and adjustments to the stomata and photosynthetic systems, ultimately promoting better growth. Studies have suggested that the secondary antioxidant NO directly eliminates stress-induced ROS or regulates the enzyme system responsible for scavenging ROS (Arora et al. 2016). Additionally, adding SNP enhances the expression of the *SOD*, *CAT*, and *APX* genes in chickpeas (*Cicer arietinum*) under salt stress, thus reducing electrolyte extravasation and H₂O₂ and MDA contents (Ahmad et al. 2016). Our study demonstrated that SNP significantly increased the expression of the *SOD* and *CAT* genes under salt stress, resulting in a reduced MDA content and preventing an excessive surge in soluble sugar levels, which would increase cell osmotic pressure. These findings highlight how NO modulates antioxidant enzyme genes to protect the antioxidant defense mechanism, prevent NaCl-induced membrane damage, and improve salt tolerance.

Fo and NPQ levels increased after exposure to salt stress, while Fv/Fm, Y (II), and qP levels decreased, indicating damage to the cell membranes, inhibited PS II reaction center activity, and disruption of the photosynthetic process. These findings are consistent with studies on *Hedera nepalensis* var. *sinensis*,

Populus euphratica, and *Solanum lycopersicum* under salt stress (Shin et al. 2020; Li et al. 2022; Tuerxun et al. 2022). However, applying exogenous NO effectively increased Fv/Fm, Y (II), and qP of leaves under salt stress, but reduced Fo and NPQ. This result indicates that NO maintains high photochemical activity and ensures the normal progress of photosynthesis under salt stress. Moreover, the expression of genes related to chlorophyll *a/b* binding proteins and PS I P700 apoproteins were upregulated after adding exogenous NO, indicating that NO maintained the photosynthetic pigments, effectively protected the photoreaction center, and ensured the normal transport of electrons. NO also improves the photosynthetic rate by synthesizing ATP and regulating two respiratory electron transport chains in mitochondria (Gupta et al. 2018). We speculate that NO response to salt stress in multiple ways. As a major player in the signaling network, NO also interacts with various hormone signaling pathways, such as gibberellic acid, abscisic acid, ethanol, salicylic acid, and auxins (Vidal et al. 2018; Prakash et al. 2021). In this study, genes related to nitric oxide synthase, abscisic acid, calmodulin, auxin, and gibberellin were significantly upregulated under salt stress, indicating that a signaling network system jointly participates in the salt tolerance mechanism of the plants under salt stress.

The *SOD*, *CAT*, *Cab*, and *psaA* genes are crucial in the salt stress response in plants. The *SOD* gene encodes SOD, which clears detrimental oxygen from the system. Overexpressing the *TaSOD2* gene in wheat results in a significant increase in SOD activity, enhancing the capacity for salt tolerance and producing antioxidant effects (Wang et al. 2016). *CAT* encodes a series of small proteins that catalyze the decomposition of H₂O₂ to maintain ROS equilibrium (Zhang et al. 2022). *Cab* expression preserves the thylakoid membranes, regulates the distribution of light energy, and improves adaptability to different conditions (Feng et al. 2014). The *psaA* gene encodes the P700 binding protein A1, which is a pigment involved in PS I and acts as the primary reaction center in photosynthesis. *H. erythrostictum* showed increased expression levels of the *SOD* and *CAT* genes but downregulated expression of the *Cab* and *psaA* genes under salt stress. However, after adding SNP, the expression levels of all of these genes increased, indicating that exogenous NO enhances antioxidant and photosynthetic capabilities under salt stress.

As a facultative CAM plant, *H. erythrostictum* presents C3 plant characteristics when grown under favorable conditions, but switches to the CAM pathway to use water more efficiently when faced with an extreme environment, such as saline-alkali, high temperature, or drought. Phosphoenolpyruvate carboxylase (PEPase) activity and malate content are essential indicators of the CAM pathway (Sunagawa et al. 2010; Winter et al. 2022). The process of transitioning from C3 to CAM mode in *H. erythrostictum* under drought stress significantly increases PEPase activity and malic acid content (Hu et al. 2015). The present study indicates that the PEPase gene and NADP-malic enzyme gene expression were upregulated in *H. erythrostictum* under salt stress, suggesting that the CAM pathway was activated as a mechanism to enhance salt tolerance. In conclusion, our findings suggest that *H. erythrostictum* has a sophisticated and intricate system to respond to salt stress, indicating that it is a good salt-tolerant ornamental plant.

Conclusion

We conducted transcriptome sequencing and gene annotation of *H. erythrostictum* seedlings under NaCl stress, and discovered many DEGs involved in the photosynthetic, ROS scavenging, and NO synthetic pathways. Based on the transcriptome data, the signaling molecule NO enhanced salt tolerance of *H. erythrostictum* by regulating its photosynthetic and antioxidant systems.

Declarations

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Availability of data and materials

Primers and other supplementary materials designed in this paper are included in the additional file.

Author contribution statement

All authors contributed directly to this article. WJX analyzed the data and wrote the manuscript, CZX performed experiments, LPS and HZH designed and directed the experiments, HZH reviewed and revised the manuscript.

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Figures

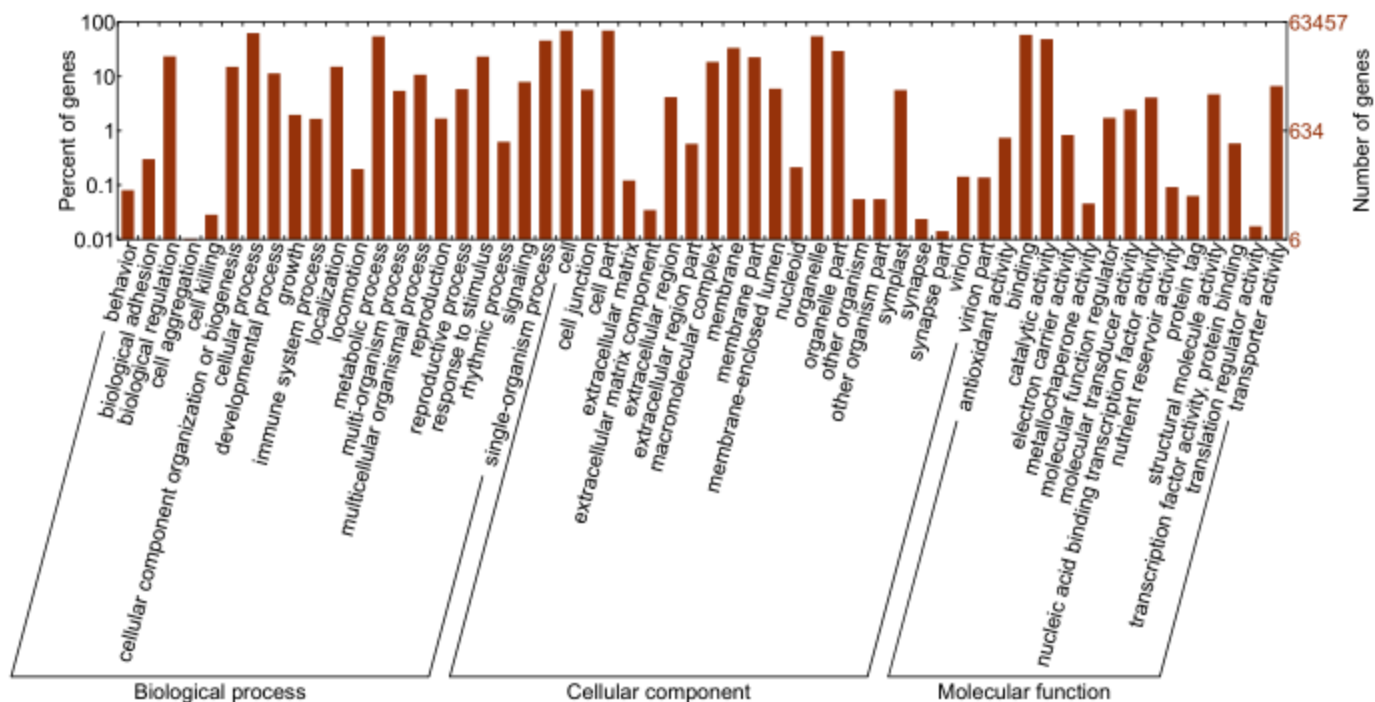


Figure 1

Histogram presentation of Gene Ontology (GO) classifications. Left y-axis indicates the percentage of unigenes in subcategories of each main category. Right y-axis indicates the number of unigenes in each subcategory.

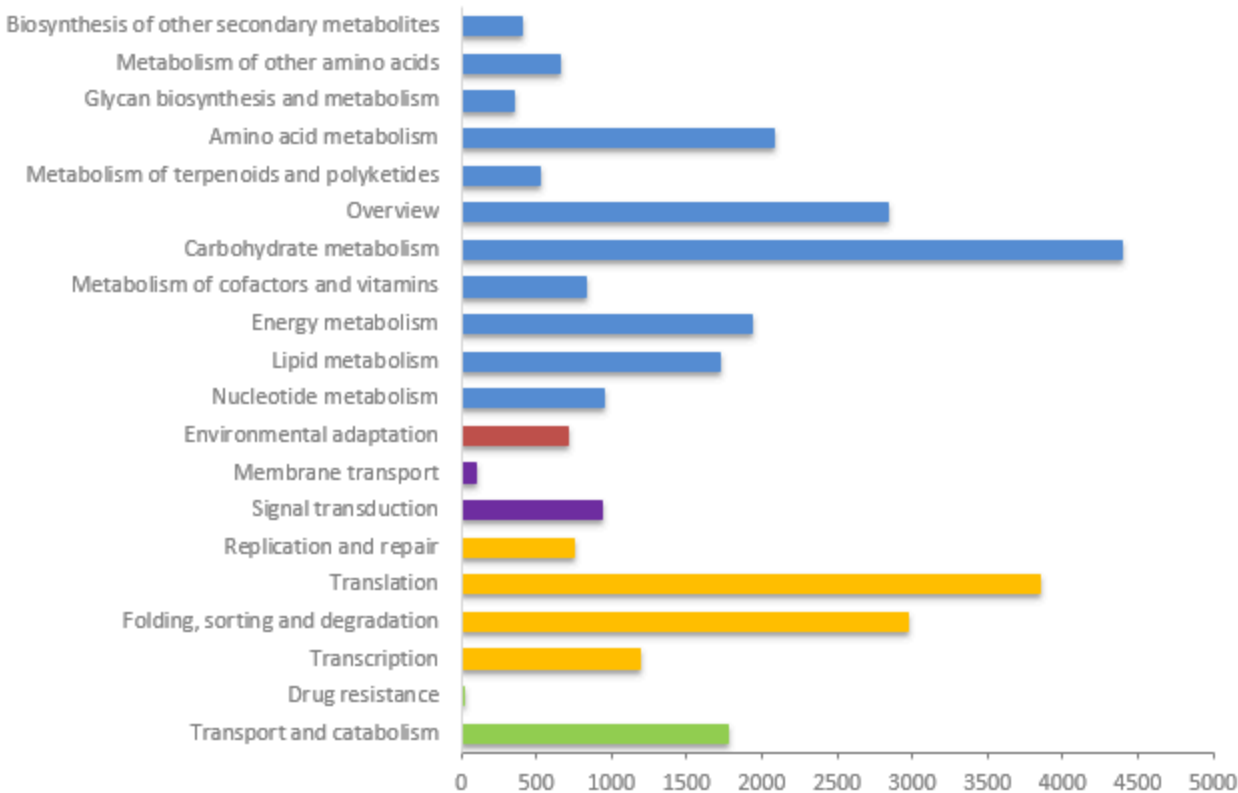


Figure 2

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KEGG classification of unigenes of *H. erythrostictum* leaves. In the figure, blue represents metabolism, red represents organismal systems, purple represents environmental information processing, yellow represents genetic information processing and green represents cellular processes.

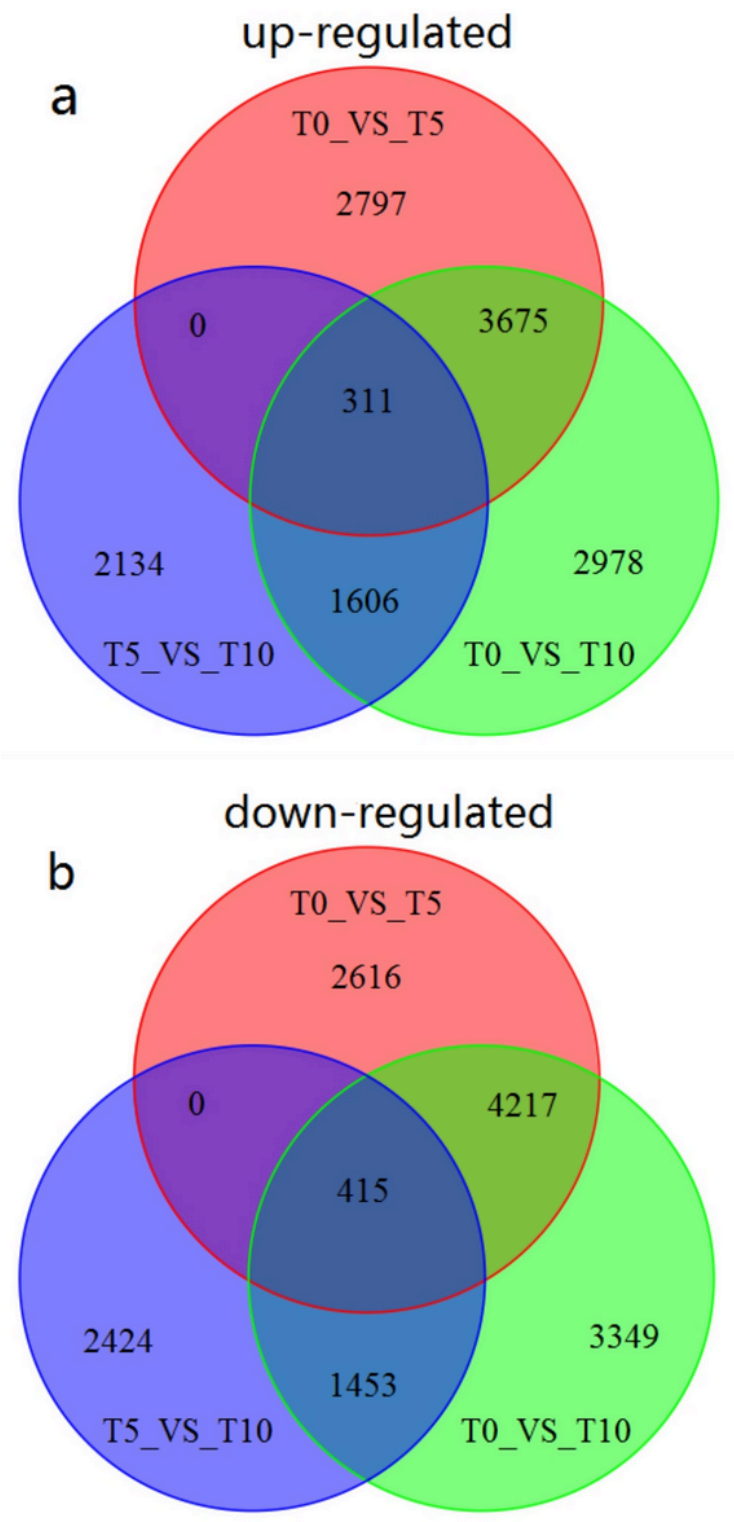


Figure 3

The number of differential expressed genes between samples. (a,b) Differential gene expression of the three samples. We performed a statistical analysis on the genes with an FPKM value > 0.3 to reduce

false positives and false negatives, we concluded that it was reliable when applied to genes with an FPKM value > 0.3 in at least one of these three treatment groups. We filtered the data using an FDR ≤ 0.001 and |log2 (fold change)| ≥ 1.

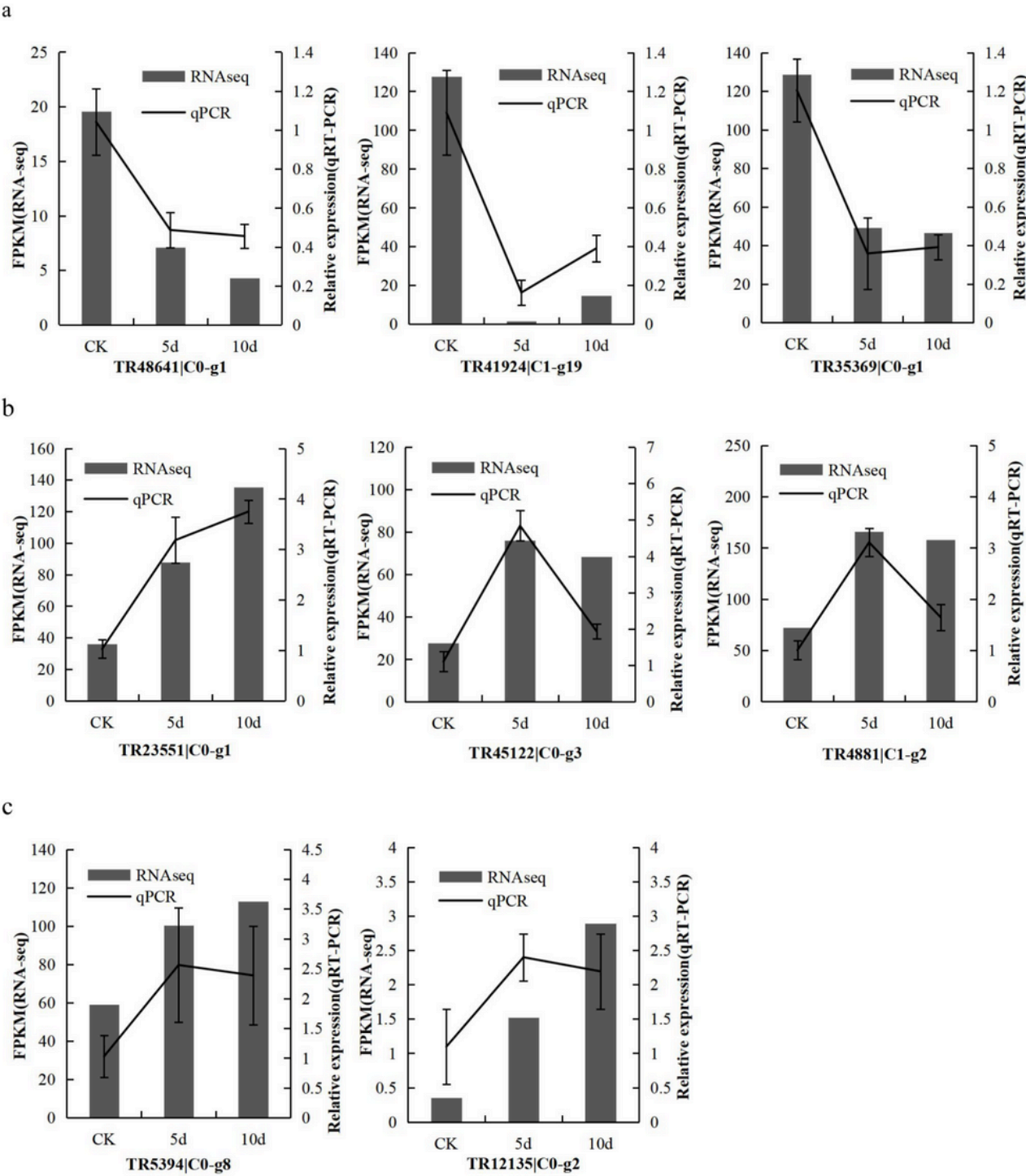


Figure 4

The relative expression levels of nine DEGs based on qRT-PCR. (a) Genes involved in the Photosynthetic. (b) Genes involved in the removal of ROS. (c) Genes involved in the NO synthase. The values represent

the means of three independent biological replications, and standard errors are shown. Statistical significance [least significant difference (LSD)] of the difference in gene relative expression levels is indicated by different small letters ($P < 0.05$).

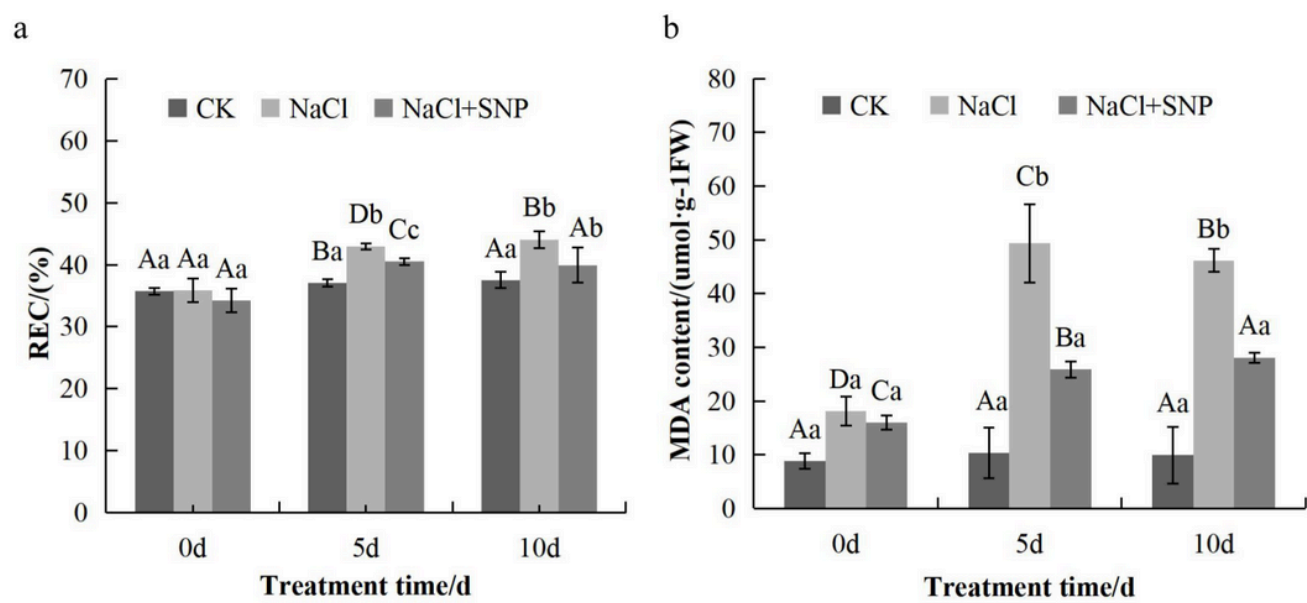


Figure 5

The effect of NO on plasma membrane of seedlings under salt stress. (a) Effect of NO on REC of seedlings under salt stress. (b) Effect of NO on MDA of seedlings under salt stress. CK represents the control group, NaCl represents the experimental group treated with 200 mM NaCl, NaCl+SNP represents the experimental group treated with 200 mM NaCl and 50 μM SNP. Different capital letters indicate significant difference among different treatments at the same time point ($P < 0.05$), different small letters indicates significant difference among different time points under the same treatment ($P < 0.05$).

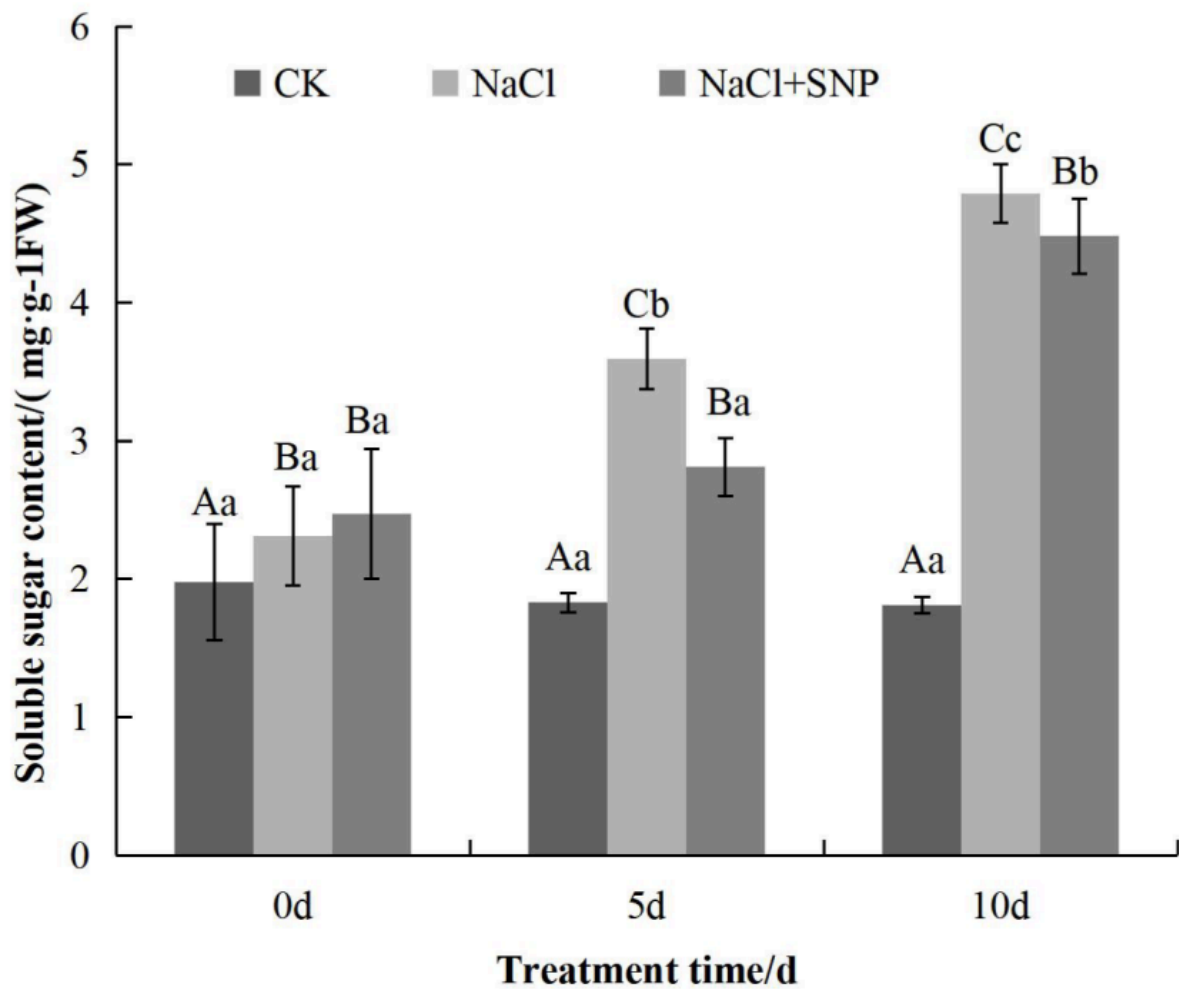


Figure 6

The effect of NO on soluble sugar content in *H. erythrostictum* leaves under salt stress. Different capital letters indicate significant difference among different treatments at the same time point ($P<0.05$), different small letters indicates significant difference among different time points under the same treatment ($P<0.05$).

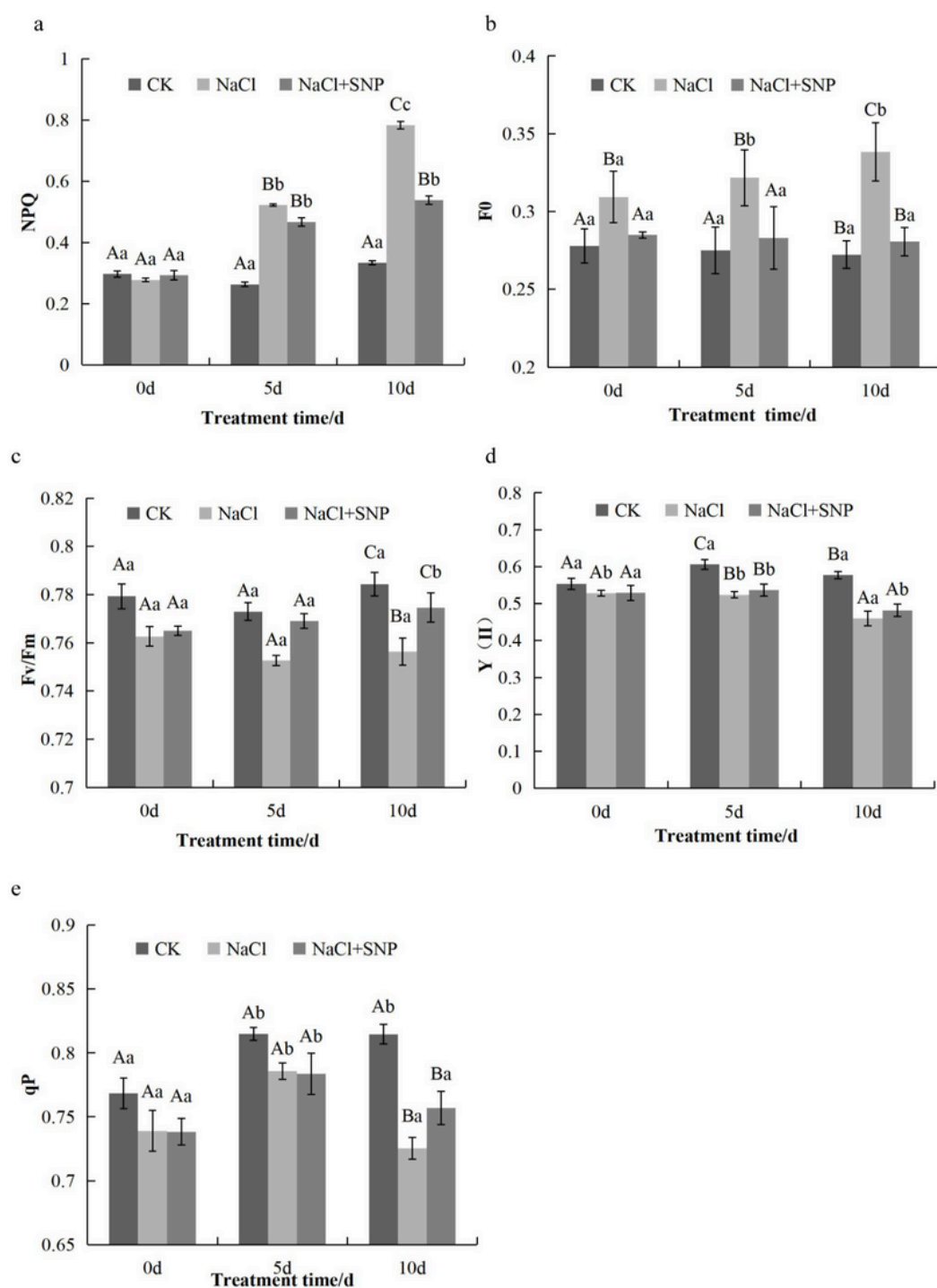


Figure 7

Effect of NO on Chlorophyll Fluorescence parameters of seedlings under salt stress. CK represents the control group, NaCl represents the experimental group treated with 200 mM NaCl, NaCl+SNP represents the experimental group treated with 200 mM NaCl and 50 μ M SNP. Different capital letters indicate significant difference among different treatments at the same time point ($P < 0.05$), different small letters indicates significant difference among different time points under the same treatment ($P < 0.05$).

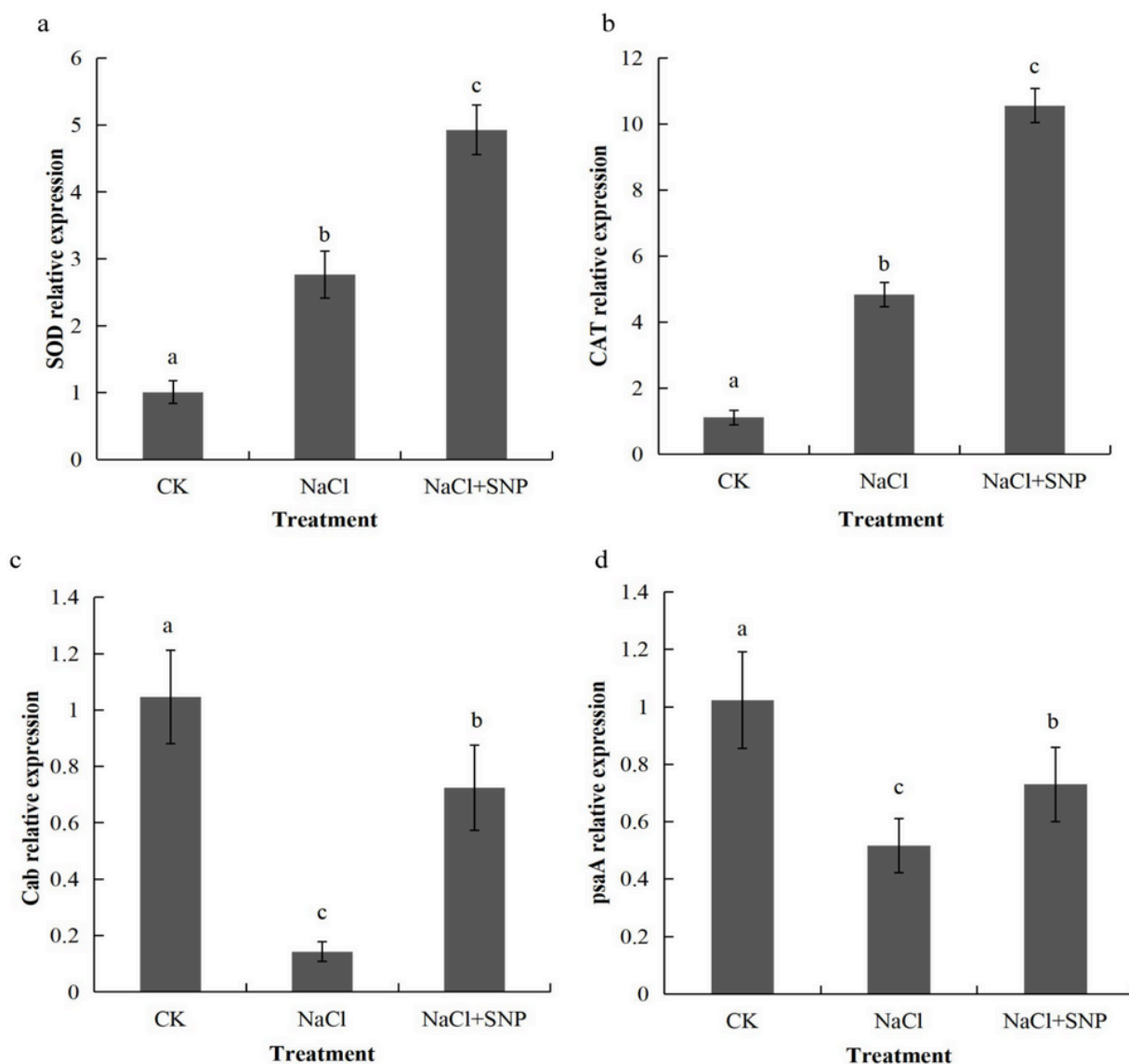


Figure 8

The relative expression of *SOD*, *CAT*, *Cab*, *psaA* in different treatment of *H. erythrostictum*. (a) The relative expression of *SOD* in different treatment of *H. erythrostictum*. (b) The relative expression of *CAT* in different treatment of *H. erythrostictum*. (c) The relative expression of *Cab* in different treatment of *H. erythrostictum*. (d) The relative expression of *psaA* in different treatment of *H. erythrostictum*.

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