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ORIGINAL ARTICLE

Analysis on the salt tolerance of *Nitraria sibirica* Pall. based on Pacbio full-length transcriptome sequencing

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Abstract

Key message ***Nitraria sibirica*** **Pall. regulates its tolerance to salt stress mainly by adjusting ion balance, modifying cell wall structure, and activating signal transduction pathways.**

Abstract *N. sibirica*, as a typical halophyte, can not only effectively restore saline-alkali land, but also has high economic value. However, studies on its salt tolerance at combining molecular and physiological levels were limited. In this study, the salt tolerance of *N. sibirica* was analyzed based on Pacbio full-length transcriptome sequencing, and the salt tolerance in the physiological level was verified by key genes. The results showed that 89,017 full-length transcripts were obtained, of which 84,632 sequences were annotated. A total of 86,482 coding sequences (CDS) were predicted and 6561 differentially expressed genes (DEGs) were identified. DEGs were significantly enriched in "sodium ion homeostasis", "response to osmotic stress", "reactive oxygen species metabolic process", "defense response by cell wall thickening", "signal transduction", etc. The expression levels for most of these DEGs increased under salt stress. A total of 69 key genes were screened based on weighted gene co-expression network analysis (WGCNA), of which 33 were first reported on salt tolerance. Moreover, *NsRabE1c* gene with the highest expression level was selected to verify its salt tolerance. Over-expression of *NsRabE1c* gene enhanced the germination potential and root length of transgenic *Arabidopsis thaliana* plants without salt treatment as compared to those of Col-0 and *AtRabE1c* mutant. The expression levels of *NsRabE1c* decreased in the growth stagnation phase, while significantly increased in the growth recovery phase under salt stress. We predicted that *NsRabE1c* gene help *N. sibirica* resist salt stress through the regulation of plant growth. The results of this study deepen the understanding of salinity resistance in *N. sibirica*.

Keywords *Nitraria sibirica* Pall. · Salt stress · Pacbio sequencing · Illumina sequencing · Full-length transcriptome · *NsRabE1c*

Introduction

Soil salinity seriously restricts agricultural production and ecological stability (Ahanger et al. 2020; Ahmad et al. 2016; Zhang et al. 2016b). The area of saline-alkali land in the world increases year by year. The current area has reached 9.54×10^8 hm^2, accounting for more than 6.5% of the total land area (Wang et al. 2021; Yang and Guo 2018b). In China, the total area of saline-alkali soil exceeds 9.91×10^7 hm^2, accounting for about 10% of the land area. Soil salinity has become one of the most serious abiotic stresses in nature (Zhao et al. 2021a). The expanding salinity area reduces the cultivated land area, affects food production and food security, and seriously hinders the sustainable development of economy and ecology (Flowers and Colmer 2008; Munns and Tester 2008).

Previous studies indicate that the effect of salt stress on plant growth and development mainly has two aspects. First, the water absorption capacity of plants under salt stress decreases due to osmotic stress and plants are in a state of water deficit, resulting in stomatal closure and slow growth. Second, with the increase of salt concentration, when it exceeds the load that the plant can bear, the ions in

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the plant body are out of balance (Faizan et al. 2021; Kaya et al. 2020). Na^+ takes the place of K^+, and the activities of many enzymes controlled by K^+ decrease (Xu et al. 2021; Zhao et al. 2020).

The main mechanisms of salt tolerance are to minimize the absorption of salt by roots, to accelerate salt efflux, and to distribute salt at the tissue and cellular levels (Munns 2005). The salt overly sensitive (SOS) pathway is the most important discovery to study how plants regulate ion balance, which mainly mediates the efflux of Na^+ from the cytoplasm. Vacuolar Na^+/H^+ antiporter mediates the partition of Na^+ (Zhao et al. 2020). The Casparian strip in the endodermis of vascular plant roots prevents the influx of salts into the stele through the apoplast under salt stress (Karahara et al. 2004). In addition, plants also alleviate salt stress by means of osmotic regulatory substances, antioxidant mechanisms, thickening cell wall structure, and signal transduction (Zhao et al. 2020). However, there are some differences in salt tolerance mechanism among different halophytes (Jin et al. 2016; Li 2008; Li et al. 2020; Lv et al. 2017; Tiika et al. 2021; Wei et al. 2022; Yu et al. 2022).

The remediation of saline soil by halophytes is a good choice to solve the problem of land salinization. *Nitraria sibirica* Pall., a dicotyledonous shrub belonging to *Nitraria*, is a typical halophyte. It mainly grows in the environment of salinity and drought, exhibits strong salt tolerance and environmental adaptability (Tang et al. 2021). Moreover, the fruits and leaves of *N. sibirica* have high nutritional, medicinal, and feeding values. Therefore, *N. sibirica* is an ideal plant to restore saline-alkali soil and to study the salt-resistant mechanism of halophytes.

The researches on *N. sibirica* under salt stress mainly focus on physiological ion balance (Tang et al. 2018), photosynthetic characteristics (Wang et al. 2019), reactive oxygen species metabolism (Zhao et al. 2021b), and seedling growth and development (Liu et al. 2021b). In the aspect of genetic engineering, only vacuolar Na^+/H^+ antiporter (NHX1), plasma membrane Na^+/H^+ antiporter (SOS1), and high affinity K^+ transporter (HKT1), which regulate ion balance, were cloned and studied (Geng et al. 2018; Li 2016; Wang et al. 2016). Furthermore, the transcriptomes of *N. sibirica* were investigated under 100 and 400 mM NaCl treatments (Li et al. 2017a, 2021). However, whether the molecular information of osmotic regulation, scavenging reactive oxygen species, cell wall structure, and signal transduction plays a role in the salt tolerance process of *N. Sibirica* has not been reported yet.

Pacbio sequencing and Illumina sequencing are effective means to analyze the salt tolerance of plants. Although Illumina sequencing technology has been employed in the study on the salt tolerance of *N. sibirica* (Li et al. 2017a), its shortcomings of short transcript splicing and incomplete transcript structure limit the research on molecular mechanisms (Sun et al. 2020). Pacbio sequencing technology has the advantages of longer reading length, more uniform coverage, and building a complete transcriptome, which can effectively avoid the problems of Illumina sequencing (Dong et al. 2015). However, Pacbio sequencing also has the defect of high error rate of single read length. The high accuracy of Illumina sequencing can make up for this defect (Xu et al. 2021). Sequencing analysis combining these two methods is a useful tool to obtain qualitative and quantitative transcriptome results, and it has been reported in many plants (Dong et al. 2015; Sun et al. 2020, 2021). However, the research on the salinity resistance of *N. sibirica* by Pacbio sequencing and Illumina sequencing has not been reported yet.

In this study, the full-length transcriptome analysis of *N. sibirica* under NaCl stress was conducted using Pacbio sequencing for the first time. Based on the next generation sequencing (NGS) data obtained by Illumina sequencing platform, the expression levels of genes involved in salt tolerance were compared and analyzed. The results revealed the changes of transcripts in *N. sibirica* at 24 h after the ending of NaCl stress at the molecular level. *NsRabE1c* with the top 10% connectivity and the highest expression level of unreported genes was selected for transgenic experiment, and salt stress verification test was carried out together with Col-0 and *AtRabE1c* mutants. These results will provide new data for further study on the mechanism of *N. sibirica* coping with salt stress.

Materials and methods

Cultivation and treatment of plant materials

Annual seedlings of *N. sibirica* were used as plant material in the present study. The seeds of *N. sibirica* were provided by Baicheng Academy of Forestry, Jilin Province. Seeds were soaked in water of 55 °C for 72 h and then sown in plastic hole plates in a greenhouse of Northeast Agricultural University. The culture substrate was raw soil/peat soil/river sand = 1/1/1 (v/v/v). The hole plates were exposed to 14 h of light at 26 °C and 10 h of darkness at 22 °C for 20 days with 50% Hoagland's nutrient solution. Then, when the seedlings grew 5–6 true leaves and spread completely, they were transplanted in 10 cm × 10 cm pots for treatment. Three plants were planted in each pot. The treatment were: (1) CK, control group, Hoagland nutrient solution; (2) Na^+, Hoagland nutrient solution + 300 mmol·L^{-1} NaCl. Three biological replicates were set for each treatment. Every five pots are treated as a group. There were 15 plants in each replicate.

To prevent the effect of salt shock, the plants in Na^+ treatment group were first watered with Hoagland + 50 mmol·L^{-1} NaCl as the initial concentration, then increasing 50 mmol·L^{-1} each day until the concentration reached

300 mmol·L^{-1}. The seedlings were continuously treated for 3 days, and irrigated with 300 mmol·L^{-1} NaCl once a day. The watering amount was 2 times of sand water-holding capacity. The plants in CK group were watered with Hoagland nutrient solution of the same volume. After stopping the salt treatment for 24 h (Ni et al. 2021), the leaves were collected from 15 plants for each replicate, well mixed, and then stored in −80 °C refrigerator immediately. Distilled water and deionized water were used to wash the leaves before collection.

RNA preparation for RNA-Seq

Total RNA was extracted with Trizol (Beijing, Dining). RNA degradation and contamination were monitored on 1% agarose gels. RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

NGS library preparation and sequencing

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using TruSeq RNA Library Preparation Kit (Illumina, USA) and index codes were added to attribute sequences to each sample. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First-strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3′ ends of DNA fragments, Illumina Adaptor was ligated to prepare for hybridization. To select cDNA fragments of preferentially 150–200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA) (Ni et al. 2021). Then, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers, and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. A total of 6 NGS libraries were established.

Data splicing, assembling, clustering, and sequencing

The original data (raw reads) obtained by sequencing were processed by the FastQC, the reads with low quality (Q20 ≤ 80%), joint contamination and high unknown base N content (Ns ≥ 5%) were filtered out, and the clean reads were obtained by de novo assembly with Trinity. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

Iso-Seq library preparation and sequencing

The Iso-Seq library was prepared according to the Isoform Sequencing protocol (Iso-Seq) using the Clontech SMARTer PCR cDNA Synthesis Kit and the BluePippin Size Selection System protocol as described by Pacific Biosciences (PN 100-092-800-03).

Acquisition of full-length transcript

Sequence data were processed using the SMRTlink 5.0 software. Circular consensus sequence (CCS) was generated from subread BAM files, parameters: min_length 50, max_drop_fraction 0.8, no_polish TRUE, min_zscore -9999, min_passes 1, min_predicted_accuracy 0.8, max_length 11,000. BAM files were output, which were then classified into full-length and non-full-length reads using pbclassify.py script, ignore polyA false, and minSeq Length 200. Non-full-length and full-length fasta files produced were then fed into the cluster step, which conducted isoform-level clustering (ICE), followed by final Arrow polishing, hq_quiver_min_accuracy 0.99, bin_by_primer false, bin_size_kb 1, qv_trim_5p 100, and qv_trim_3p 30.

Error correction using Illumina reads

Additional nucleotide errors in consensus reads were corrected using the Illumina NGS data with the software LoRDEC.

ORF prediction and gene annotation

The obtained high-quality full-length transcripts were used for ORF prediction. Based on ORF prediction, the predicted CDS and protein sequences were obtained. To predict the longest and best transcripts, the longest ORFs were extracted, and protein libraries, peptides, or protein domains were searched with BlastP and Pfam.

The predicted high-quality protein information was annotated in six databases (GeneOntology (GO), EggNOG (cut-off Evalue ≤ 1e-3), Kyoto Encyclopedia of Genes and Genomes (KEGG, cut-off Evalue ≤ 1e-5), NCBI non-redundant Protein (NR, cut-off Evalue ≤ 1e-5), SwissProt (cut-off Evalue ≤ 1e-5) and TrEMBL). The NR, eggNOG, KEGG, and SwissProt annotations of the transcripts were obtained

by Blastx and Diamond software. GO annotation and classification were performed in the Blast2GO program based on the NR annotation results.

The annotated genes were classified by eggNOG function, enriched by KEGG and analyzed by GO distribution.

Quantification of the gene expression levels, identification, and function analysis of DEGs

Quantification of the gene expression level for each sample was performed by RSEM (Sun et al. 2020). The clean data generated by Illumina sequencing were mapped to SMRT sequencing data, and the read count of each gene was obtained from the mapping results. Based on the gene data with average counts > 5, the 2FC genes (|log2FC| ≥ 1) with FDR < 0.05 was used as the differentially expressed genes (DEGs). To further filter out more significant response genes under salt stress, the 16FC DEGs (|log2FC| ≥ 4) were chosen for analysis. 2FC indicates that the difference in gene expression level is more than 2 folds, and 16FC indicates that the difference in gene expression level is more than 16 folds.

Differential expression analysis was performed using the DeSeq2 to identify DEGs between the Na^+ treatment and CK. For function annotation, the GOseq R package was used to perform GO enrichment analysis on DEGs. The KEGG enrichment analysis of DEGs was carried out by KOBAS software.

WGCNA and gene network visualization

In order to further screen the key regulatory genes related to salt tolerance, weighted gene co-expression network analysis (WGCNA) was performed on all transcripts with more than 2 times differential expression (Ni et al. 2021; Sun et al. 2021). The genes that cannot be detected or expressed relatively low (TPM < 10) were discarded, the adjacency degree between the remaining differential genes and the similarity between genes were calculated according to the adjacency degree. The dissimilarity coefficient among genes was deduced, and the systematic clustering tree among genes was obtained.

The gene expression profile of each module was determined by the gene phylogenetic tree to test the modules related to salt tolerance. In co-expression network, the edge weight (ranging from 0 to 1) of any two genes connected was determined based on their topology overlap measure. Key genes were mined from modules related to salt-tolerant genes using Cytoscape according to the connectivity among genes.

After that, the modules with strong positive correlation with salt tolerance were obtained. GO enrichment analysis and KEGG enrichment analysis were carried out.

Generation of *NsRabE1C* overexpression *Arabidopsis thaliana* plants

The CDS sequence of *NsRabE1c* was linked into PCAMBIA1300 vector by homologous cloning method. The primers (RABE1c-ZTF, RABE1c-ZTR) used to amplify *NsRABE1c* were listed in Table S1. The plasmids were transferred into Agrobacterium GV3101 by heat shock method. Then, the Agrobacterium with PCAMBIA1300-*NsRabE1c* was transformed into *A. thaliana*. The T1 generation seeds were screened with 30 $mg{\cdot}L^{-1}$ hygromycin and confirmed by RT-PCR with primers (RabE1c-ZTF, RabE1c-ZTR). After three generations of screening and culture, homozygous overexpression *A. thaliana* was obtained. The homozygous overexpression *A. thaliana* plants were used for salt tolerance phenotype verification.

AtRabE1c mutant verification

The mutant seeds of *AtRabE1c* were purchased from Arabidopsis Mutant Service Center. In order to ensure the accuracy of homozygous mutants, three primers methods (rabe1c-LP, rabe1c-RP and rabe1c-LB) were used to identify mutant seedlings. Primers designed with T-DNA Primer Design (http://signal.salk.edu/tdnaprimers.2.html) website were shown in Table S1. The homozygous mutants *A. thaliana* plants were used for salt tolerance phenotype verification.

qRT-PCR validation of DEGs from the RNA-seq

In order to prove the accuracy of transcriptome data, 12 genes with the top 10% connectivity in the salt tolerance module were selected to verify their expression levels by qPCR. Quantitative RT-PCR primers (Table S1) were designed using Primer Premier 5.0 software. cDNA was reverse transcribed from the RNA with HiScript III RT SuperMix for qPCR (Vazyme) according to the manufacturer's instructions. ChamQ Universal SYBR qPCR Master Mix (Vazyme) was used for qPCR. The reaction conditions were as follows: 95 °C for 30 s, 40 cycles (95 °C for 5 s, 60 °C for 30 s). The *N. sibirica NsActin* gene was used as an internal control. Three different biological replicates were used to calculate the relative expression of gene by the $2^{-\Delta\Delta Ct}$ method (Sun et al. 2021).

In addition, the expression levels of *NsRabE1c* in *N. sibirica* treated with 300 $mmol{\cdot}L^{-1}$ NaCl at different periods (0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h) were analyzed in the same way.

Statistical analysis

The variance of the data was analyzed using the SPSS version 19.0 software (SPSS, Chicago, IL, USA), and the significance threshold was set at $P < 0.05$. The results were expressed as the mean ± standard deviation (SD) of at least three independent replicate experiments.

Results

Identification of full-length transcripts

In order to obtain the full-length transcriptome of *N. sibirica*, the leaves were sequenced using Iso-Seq and NGS techniques. The original data obtained on the PacBio Sequel platform and Illumina platform are about 323 GB and 97.5 GB, respectively. After filtering out incomplete CGs, 1,030,629 full-length non-chimeric reads (FLNC) were obtained (Table 1). After removing redundancy and clustering, 89,017 full-length transcripts (average length 2721.43 bp, N50 3009 bp, Q30 values 98%) were obtained, which were used as high-quality reference transcripts of *N. sibirica* (Table 1). Illumina platform obtained 651 million high-quality reads with Q30 higher than 98%. A total of 86,482 CDS sequences were predicted by TransDecoder software, among which 4000 genes without CDS may be small RNA, tRNA and transposition sequence (Table 1).

Gene annotation

In order to obtain more comprehensive gene annotation, 84,632 (95.07%) transcripts were annotated by GO, KEGG, NR, eggNOG, SwissProt and TrEMBL databases (Fig. 1a). Among them, 41,724 transcripts can be simultaneously annotated in 6 databases (Fig. 1a). 84,632 transcripts were annotated in at least one database (Fig. 1a), of which 84,478 (99.82%) transcripts were annotated in NR, 70,960 (83.85%) in Swiss-prot, and 84,467 (99.81%) in TrEMBL (Fig. 1b).

In addition, the eggNOG annotation demonstrated that 74,474 (88.00%) transcripts were assigned to 25 functional clusters, and "Signal transduction mechanisms" (6,181 transcripts) was the largest category, followed by "Posttranslational modification, protein turnover, chaperones" (6,026 transcripts) and "Intracellular trafficking, secretion, and vesicular transport" (3,877 transcripts) (Fig. 1b and c).

A total of 77,557 (91.64%) transcripts were annotated by the GO database (Fig. 1b). GO enrichment analysis on all DEGs was divided into three categories: biological processes, molecular function and cellular components. These transcripts were primarily enriched in "cellular process", "metabolic process", "biological regulation", "response to stimulus", "cell", "cell part", "organelle", "membrane", "binding", "catalytic activity", "transporter activity", "transcription factor activity, protein binding" and "signal transducer activity" (Fig. 1d).

To explore the main biological processes in *N. sibirica*, 45,886 (54.22%) transcripts were mapped to the KEGG database, where "carbohydrate metabolism" (4,719 transcripts), "translation" (3,695 transcripts) and "folding, sorting and degradation" (3,325 transcripts) were the most abundant subcategories, respectively (Fig. 1b and e).

Analysis on DEGs

To explore the variation in gene abundance and expression profiles under salt stress, clean reads from RNA-Seq were aligned to reference transcripts. Under the treatment of 300 mmol·L^{-1} NaCl, there were 6561 differentially expressed transcripts ($|\log2FC| \geq 1$, FDR < 0.05), of which the up-regulated and down-regulated genes were 2429 and 4132, respectively (Fig. 2a). In order to further filter out more significant response genes under salt stress, the 16FC DEGs ($|\log2FC| \geq 4$) were chosen for analysis. The up-regulated and down-regulated genes were 664 and 92, respectively (Fig. 2b).

GO enrichment analysis on 16FC DEGs showed that 176 and 224 DEGs were identified and respectively matched to "response to stress" and " response to stimulus" (Fig. 3a and c). A number of metabolism-related terms have been enriched, indicating that the synthesis of secondary metabolites will help to resist salt stress (Fig. 3a and c).

To further reveal the functional differences between these two parts of DEGs, the KEGG metabolic pathway enrichment analysis was conducted. Pathways such as "Carbon metabolism", "Phosphatidylinositol signaling system", "Protein processing in endoplasmic reticulum" and "Ubiquitin

Table 1 Summary of PacBio and Illumina transcripts

PacBio sequel platform						NGS platform		
Subreads base (G)	Complete Non-chimera reading (FLNC)	Full-length transcripts	Average length of subreads	N50	Q30	Subreads base (G)	High-quality reads	Q30
323 GB	1,030,629	89,017	2721.43	3009	98%	97.5 GB	651 million	98%

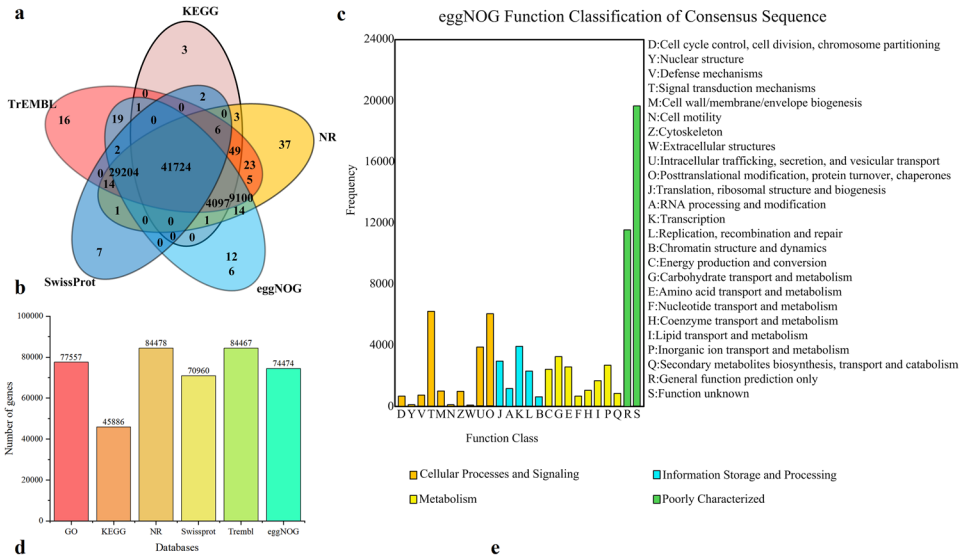

Fig. 1 Annotation of *N. sibirica* transcripts. **a** Gene function annotations in 5 databases (Nr, eggNOG, KEGG, SwissProt and TrEMBL). **b** The number of genes annotated in 6 databases. **c** Annotation of the eggNOG function of the *N. sibirica* transcripts. **d** Annotation of the GO function of the *N. sibirica* transcripts. **e** Annotation of the KEGG function of the *N. sibirica* transcripts

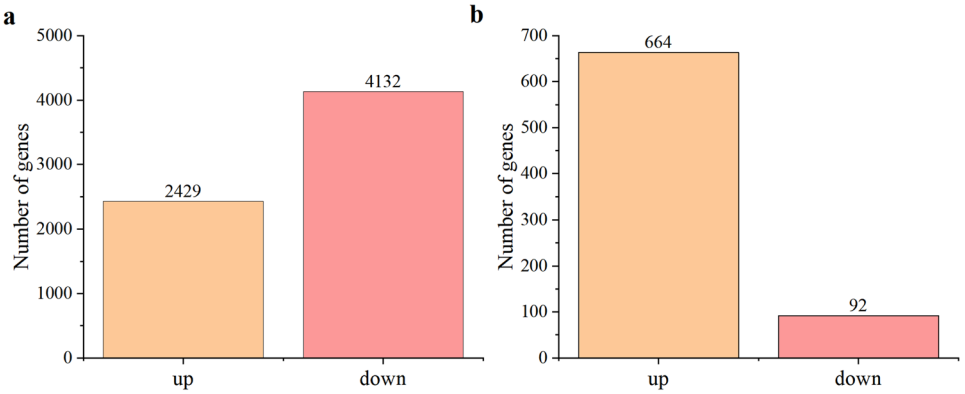

Fig. 2 The numbers of differentially expressed genes (DEGs). **a** 2FC. **b** 16FC. 2FC indicates that the difference in gene expression is more than 2 folds, and 16FC indicates that the difference in gene expression is more than 16 folds

mediated proteolysis", etc., were significantly enriched, indicating an extraordinary effect in response to salt stress (Fig. 3b and d).

Acquisition of key genes

In order to further screen the key regulatory genes related to salt tolerance, WGCNA was performed on all transcripts with more than 2 times of differential expression (Fig. 4a). Genes are classified into 24 different hierarchical clustering modules. The different modules were represented by

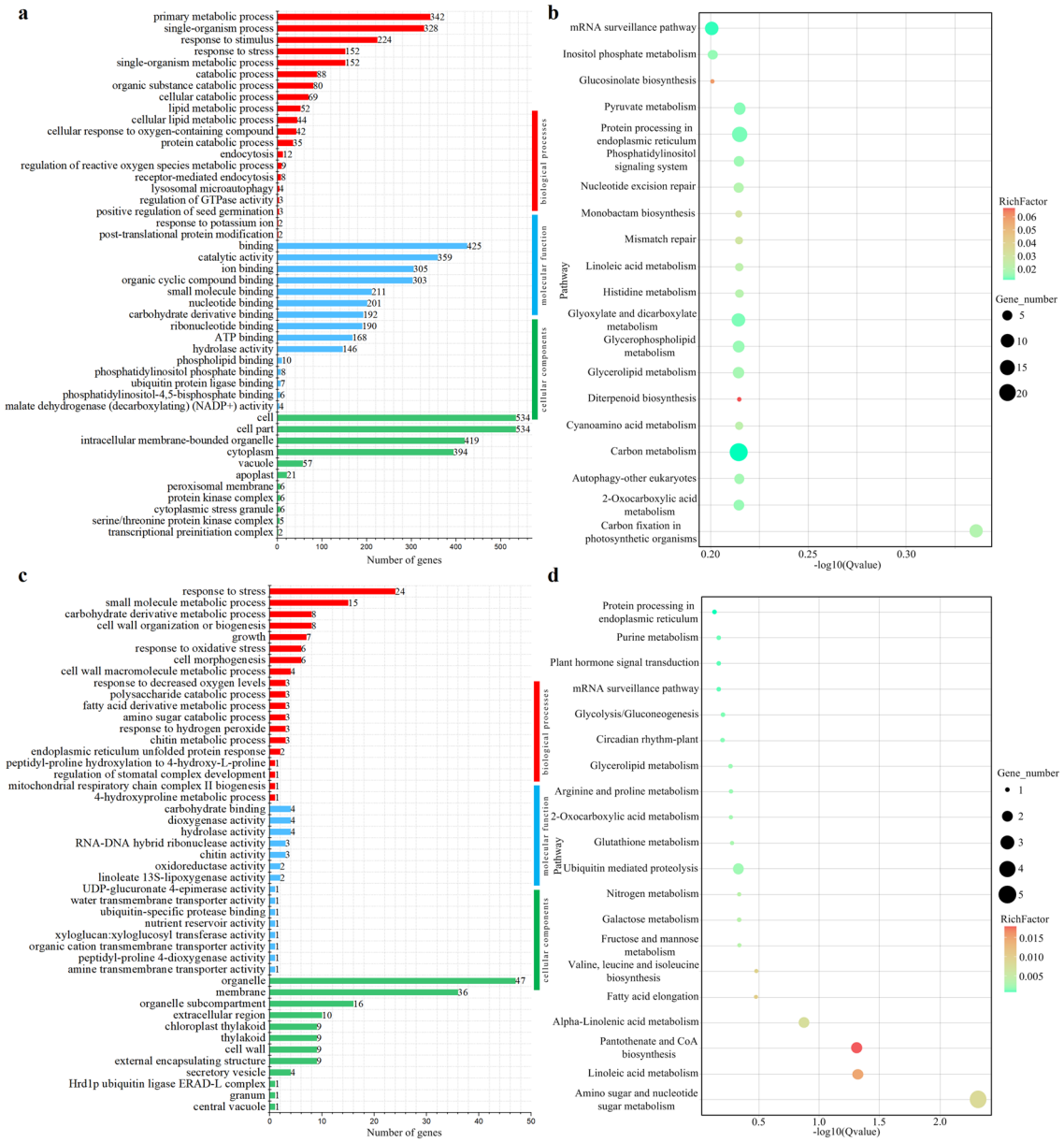

Fig. 3 GO and KEGG analysis of 16FC DEGs in *N. sibirica* under salt stress. **a** GO analysis of 16FC up-regulated DEGs. **b** KEGG analysis of 16FC up-regulated DEGs. **c** GO analysis of 16FC down-regulated DEGs. **d** KEGG analysis of 16FC down-regulated DEGs

different colors (Fig. 4b). Based on the analysis of gene expression profiles of each module, it was found that the correlation coefficients of green, lightcyan and magenta modules related to salt stress were the highest (Fig. 4b).

These three modules contained 420, 82 and 252 genes, respectively (Fig. 4c). Through GO enrichment analysis, it was found that many single genes in the green module were mainly enriched in ion balance, signal transduction, active oxygen scavenging and cell wall structure (Fig. 5a). The terms related to cell wall structure and composition and osmotic regulation were significantly enriched in the lightcyan module (Fig. 5c). In the magenta module, many single genes were enriched in terms related to hormones, osmotic regulation and signaling (Fig. 5e). The above results showed that these aspects play an important role in the regulation of salt stress in *N. sibirica.* In addition, there are some terms in all three modules, such as "response to salt", "response to salt stress", and "hyperosmotic salinity response", indicating that *N. sibirica* possesses the ability to deal with salt stress (Fig. 5a, c and e).

KEGG enrichment analysis showed that "Plant hormone signal transduction", "MAPK signaling pathway-plant", "Phosphatidylinositol signaling system" were significantly enriched in these three modules (Fig. 5b, d and f). It

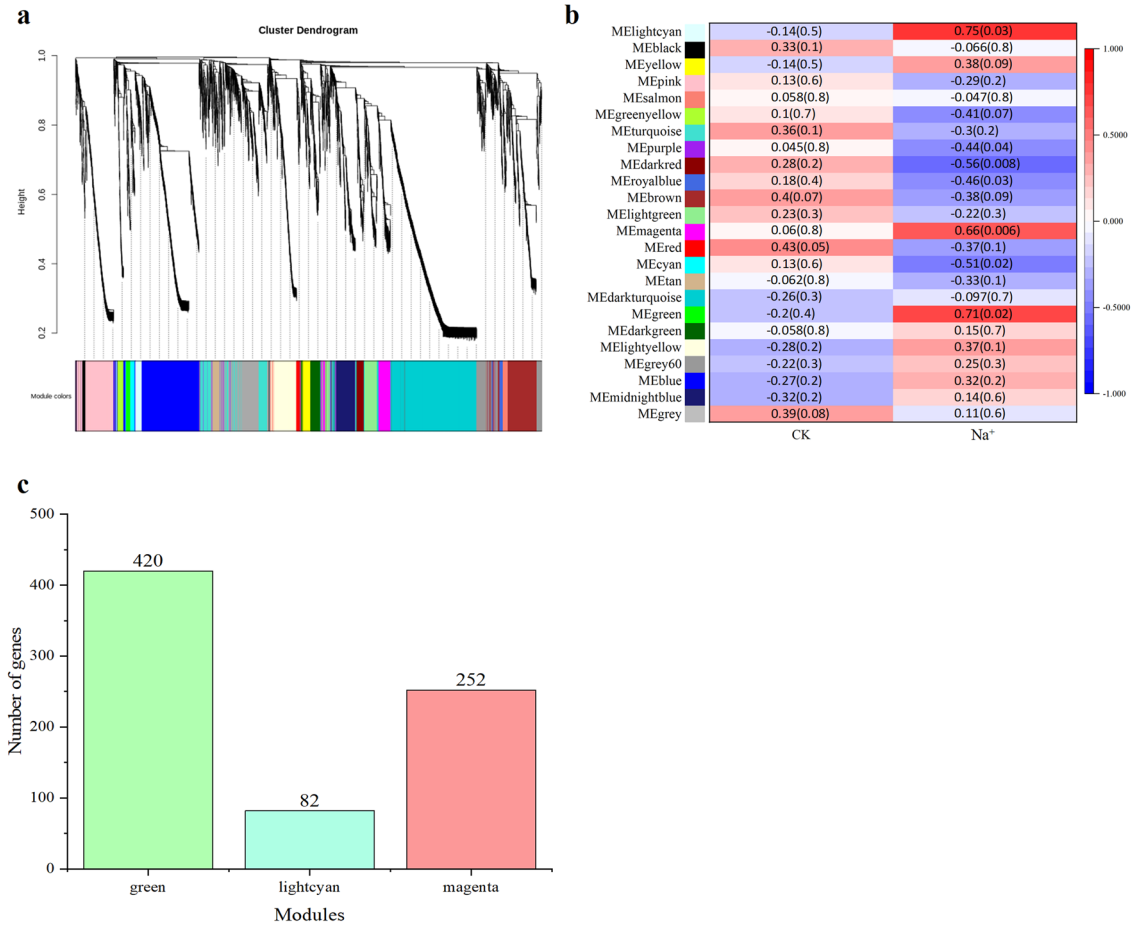

Fig. 4 Identification of co-expression network modules in *N. sibirica*. **a** Gene dendrogram obtained by hierarchical clustering with the module color. **b** Relationships of modules and samples from different treatments. Each row in the table corresponds to a module, and each column corresponds to a sample. **c** The number of genes contained in the three modules related to salt stress

indicated that *N. sibirica* can transmit salt stress signals to the regulation process of plants through these three kinds of signal transduction, so as to deal with salt stress injury. In addition, "Arginine and proline metabolism", "Protein processing in endoplasmic reticulum" and "Endocytosis" were also significantly enriched (Fig. 5b, d and f).

The connectivity of genes in these 3 modules was analyzed by the Cytoscape software. The genes with top 10% connectivity are listed in Tables 2, 3, and 4, respectively. It indicated that these genes play an important role in the salt stress resistance of *N. sibirica*.

DEGs involved in the salt tolerance of *N. sibirica*

Identification of DEGs related to Na^+ transport

Under salt stress, 9 transporters regulating Na^+ influx were identified as non-selective cation channel (*NSCC*s) genes. These genes included 5 cyclic nucleotide gated channels (*CNGC*s) and 4 glutamate receptors (*GLR*s) (Fig. 6). In addition, salt stress induced differential expression levels of *SOS1*, *NHX2* and *NHX6* (Fig. 6). Furthermore, 8 high affinity potassium transporter (*HAK*s) and 1 potassium channel (*AKT*s) were identified as DEGs (Fig. 6), which maintained the balance between Na^+ and K^+ under salt stress.

Identification of DEGs related to osmotic regulation

Five DEGs were identified to be associated with osmosensors, which included 1 hyperosmolality-gated Ca^{2+}

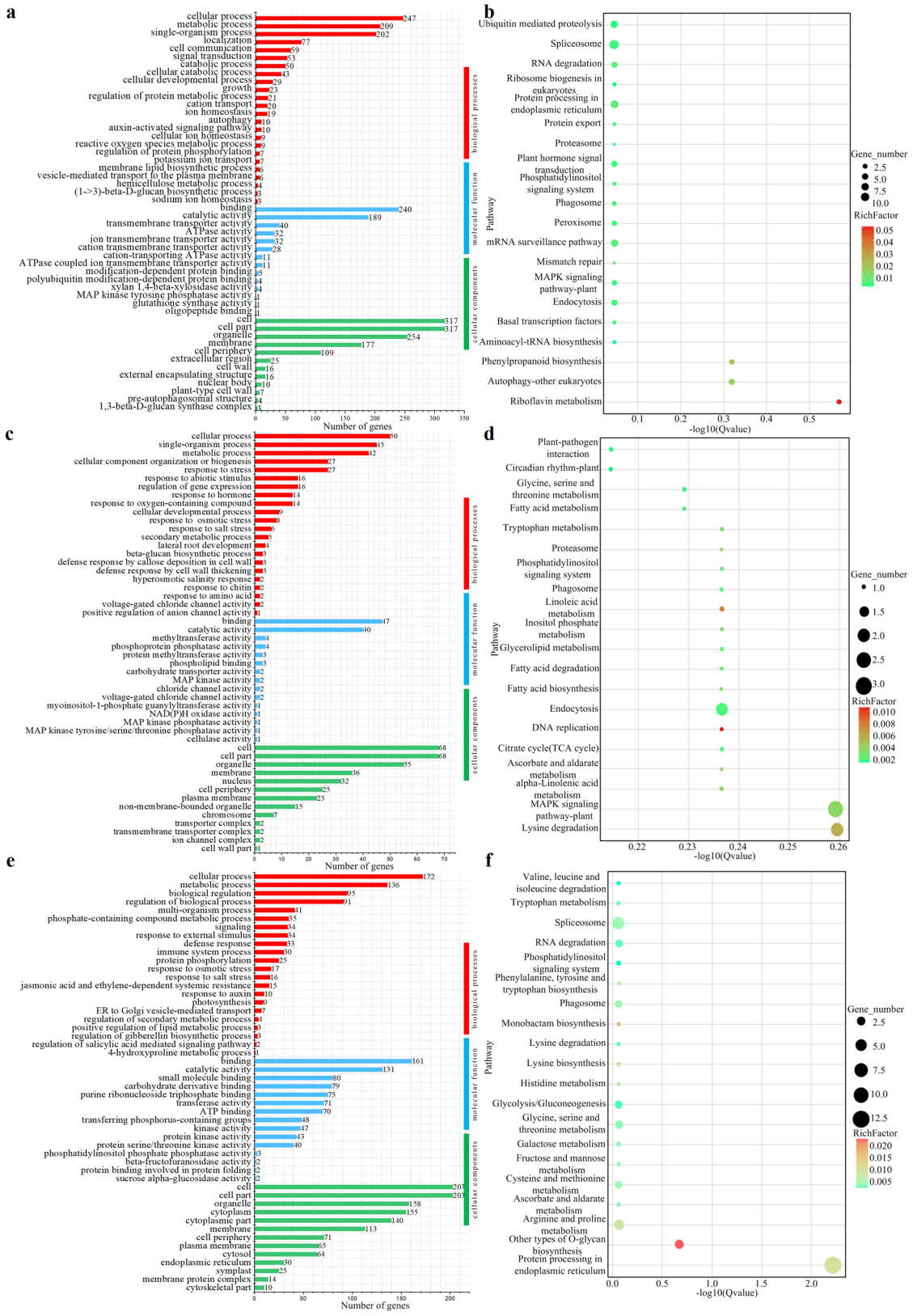

Fig. 5 GO and KEGG analysis of modules related to salt stress in *N. sibirica*. **a** GO analysis of green module. **b** KEGG analysis of green module. **c** GO analysis of lightcyan module. **d** KEGG analysis of lightcyan module. **e** GO analysis of magenta module. **f** KEGG analysis of magenta module (colour figure online)

Table 2 Information of the genes with the top 10% of connectivity in green modules

Gene ID	Protein name	Gene name	Average relative expression		Description
			NaCl treatment	CK	
transcript_20611	Cadmium/zinc-transporting ATPase HMA2	*HMA2*	45.37	1.53	Unreported
transcript_22002	Receptor-like protein EIX2	*EIX2*	13.41	2.5	Unreported
transcript_25556	Phosphatidylinositol 4-kinase alpha 1	*PI4KA1*	3.53	0	Unreported
transcript_27571	Phosphoenolpyruvate carboxylase 2	*PPC2*	3.67	0.8	
transcript_30685	Potassium transporter 7	*HAK7*	5.74	3.10	
transcript_30703	Protein MEI2-like 2	*ML2*	10.02	2.1	Unreported
transcript_33089	Plasma membrane ATPase 4	*PMA4*	10.23	3.7	
transcript_33864	Nudix hydrolase 3	*NUDT3*	4.77	0.21	Unreported
transcript_3893	Proteasome activator subunit 4	*PA200*	3.42	0.74	Unreported
transcript_42472	Calcium-transporting ATPase 4, endoplasmic reticulum-type	*ECA4*	5.61	1.0	
transcript_43048	Phospholipase D alpha 1	*PLD1*	28.71	8.37	
transcript_46594	Cell division cycle protein 48 homolog	*CDC48*	4.6	1.8	
transcript_48491	Protein OBERON 4	*OBE4*	24.33	4.82	
transcript_51239	Alpha-L-arabinofuranosidase 1	*ASD1*	6.98	2.46	Unreported
transcript_59460	ATP-dependent Clp protease ATP-binding subunit ClpA homolog CD4B, chloroplastic	*CD4B*	41.60	10.74	Unreported
transcript_60351	3-ketoacyl-CoA synthase 20	*KCS20*	42.47	5.85	
transcript_61807	SURP and G-patch domain-containing protein 1-like protein	*At3g52120*	5.41	1.6	Unreported
transcript_65449	ATPase 11, plasma membrane-type	*AHA11*	7.52	0.8	
transcript_66601	Protein CHUP1, chloroplastic	*CHUP1*	15.83	3.89	Unreported
transcript_6960	Clustered mitochondria protein	*CLU/FMT*	6.06	1.49	Unreported
transcript_70176	NADP-dependent malic enzyme		11.98	1.39	Unreported
transcript_71010	Ferredoxin–nitrite reductase, chloroplastic	*NIR1*	8.5	0.13	
transcript_74799	Triose phosphate/phosphate translocator, chloroplastic	*CTPT*	19.23	3.67	Unreported
transcript_75893	Protein PIN-LIKES 6	*PILS6*	24.57	12.9	Unreported
transcript_78193	Heat shock 70 kDa protein 4	*HSP70-4*	20.81	5.6	
transcript_78942	Light-inducible protein CPRF2	*CPRF2*	12.1	6.06	
transcript_80854	Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplastic	*RCA1*	24.32	7.4	
transcript_81299	Protein PAM71-homolog, chloroplastic	*PAM71-HL*	25.08	10.74	Unreported
transcript_83484	Protein TWIN LOV 1	*TLP1*	50.36	10.2	
transcript_84351	Eukaryotic translation initiation factor 5	*EIF5*	100.13	30.84	
transcript_85617	Cytochrome P450 98A2	*CYP98A2*	30.65	8.63	Unreported
transcript_85688	Ras-related protein	*RABE1c*	66.25	5.55	Unreported
transcript_86881	Leucoanthocyanidin dioxygenase	*ANS*	138.57	10.2	
transcript_86950	Serine/arginine-rich splicing factor RS41	*RS41*	97.68	20.85	
transcript_87330	Cathepsin B-like protease 2	*CATHB2*	61.86	10.85	
transcript_87852	3-isopropylmalate dehydratase large subunit, chloroplastic	*IIL1*	46.26	3.37	Unreported
transcript_88255	Eukaryotic translation initiation factor 5A	*eIF-5A*	136.13	30.85	
transcript_88301	40S ribosomal protein S8	*RPS8*	20.89	7.35	Unreported
transcript_9428	Pre-mRNA-processing-splicing factor 8A	*PRP8A*	2.82	0.07	Unreported
transcript_86816	Actin-7	*ACT7*	115.29	100.94	

permeable channel 1 (*OSCA1*), 1 histidine kinase 1 (*AHK1*) and 3 aquaporins (*SIP1-1*, *TIP2-1* and *TIP1-3*) (Fig. 7). Eleven MAPKs were identified as DEGs, which regulated osmotic stress (Fig. 7). Five abscisic acid receptors (*PYLs*) were identified from the annotation data, among which 1 *PYL* was identified as DEG in response to salt stress (Fig. 7). In addition, Ornithine aminotransferase, Osmotin-like protein OSM34, Phosphoinositide phosphatase SAC6, CBL-interacting serine/threonine-protein kinase 9, serine/threonine-protein kinase SIS8 and Phosphatidylinositol

Table 3 Information of the genes with the top 10% of connectivity in lightcyan modules

Gene ID	Protein name	Gene name	Average relative expression		Description
			NaCl treatment	CK	
transcript_29609	Lipoxygenase 2, chloroplastic	*LOX2*	1.94	0.7	
transcript_31210	Receptor-like protein kinase THESEUS 1	*THE1*	5.843	2.01	
transcript_34108	RNA polymerase II C-terminal domain phosphatase-like 2	*CPL2*	4.06	0.8	
transcript_41271	Long chain acyl-CoA synthetase 8	*LACS8*	2.16	0.06	Unreported
transcript_45959	Probable beta-D-xylosidase 2	*BXL2*	0.58	5.21	Unreported
transcript_473	Callose synthase 12	*CALS12*	0.87	0	Unreported
transcript_61306	Myb family transcription factor PHL6	*PHL6*	2.78	1.2	
transcript_68267	Probable transcription factor At3g04930	*At3g04930*	2.85	1.5	

Table 4 Information of the genes with the top 10% of connectivity in magenta modules

Gene ID	Protein name	Gene name	Average relative expression		Description
			NaCl treatment	CK	
transcript_11764	Protein transport protein SEC31 homolog B	*SEC31B*	0.23	2.73	Unreported
transcript_17437	Leucine-rich repeat receptor protein kinase HPCA1	*HPCA1*	1.3	4.72	
transcript_18627	Cyclin-dependent kinase G-2	*CDKG-2*	3.95	8.28	
transcript_18768	Serine/threonine-protein phosphatase BSL1	*BSL1*	1.07	6.33	Unreported
transcript_20585	Probable leucine-rich repeat receptor-like serine/threonine-protein kinase At3g14840	*LRR-RLK*	0.04	1.54	
transcript_21102	Protein transport protein Sec24-like At3g07100	*At3g07100*	0.71	2.89	Unreported
transcript_24285	Copper-transporting ATPase PAA1, chloroplastic	*PAA1*	0	1.54	Unreported
transcript_27363	Probable serine/threonine-protein kinase SIS8	*SIS8*	2.67	9.06	
transcript_39797	G-type lectin S-receptor-like serine/threonine-protein kinase At4g27290	*At4g27290*	4.2	9.71	
transcript_43414	Ferric reduction oxidase 7, chloroplastic	*FRO7*	1.37	5.21	Unreported
transcript_48736	30-kDa cleavage and polyadenylation specificity factor 30	*CPSF30*	2.01	5.43	
transcript_52401	Beta-glucosidase-like SFR2, chloroplastic	*SFR2*	3.09	10.73	
transcript_52747	Phosphoinositide phos phatase SAC6	*SAC6*	2.51	10.32	
transcript_56691	RuBisCO large subunit-binding protein subunit alpha,chloroplastic		0.124	5.43	Unreported
transcript_58466	Probable polyol transporter 4	*PLT4*	1.341	10.76	
transcript_60952	ATP-dependent Clp protease ATP-binding subunit ClpA homolog CD4B, chloroplastic	*CD4B*	4.29	11.04	Unreported
transcript_64837	Chitinase domain-containing protein 1		0.742	3.86	Unreported
transcript_66736	Switch-associated protein 70		2.61	8.54	Unreported
transcript_68424	Transcription factor bHLH48	*BHLH48*	1.24	7.54	
transcript_77006	Methionine aminopeptidase 1B, chloroplastic	*MAP1B*	7.74	32.81	Unreported
transcript_77119	Serine acetyltransferase 4	*SAT4*	1.92	10.36	

3,4,5-trisphosphate 3-phosphatase participated in osmotic stress, and the expression levels of which changed (Fig. 7).

Identification of DEGs related to antioxidant capacity

A total of 8 antioxidant-related DEGs belonging to the catalase (*CAT*), peroxidase (*POD*), ascorbate peroxidase (*APX*), glutathione reductase (*GR*) and glutathione peroxidase (*GPX*) were identified, of which 3 genes were up-regulated (2 *CAT*s and 1 *POD*) (Fig. 8). Salt stress induced differential expressions of 4 non-enzymatic antioxidant genes, namely glutathione synthase (*GSH2*), Cytochrome P450 711A1 (*MAX1*), Protein ACTIVITY OF BC1 COMPLEX KINASE 1 (*ABC1K1/BDR1/PGR6*), and COP1-interacting protein 7 (*CIP7*) (Fig. 8). Moreover, Leucine-rich repetitive receptor

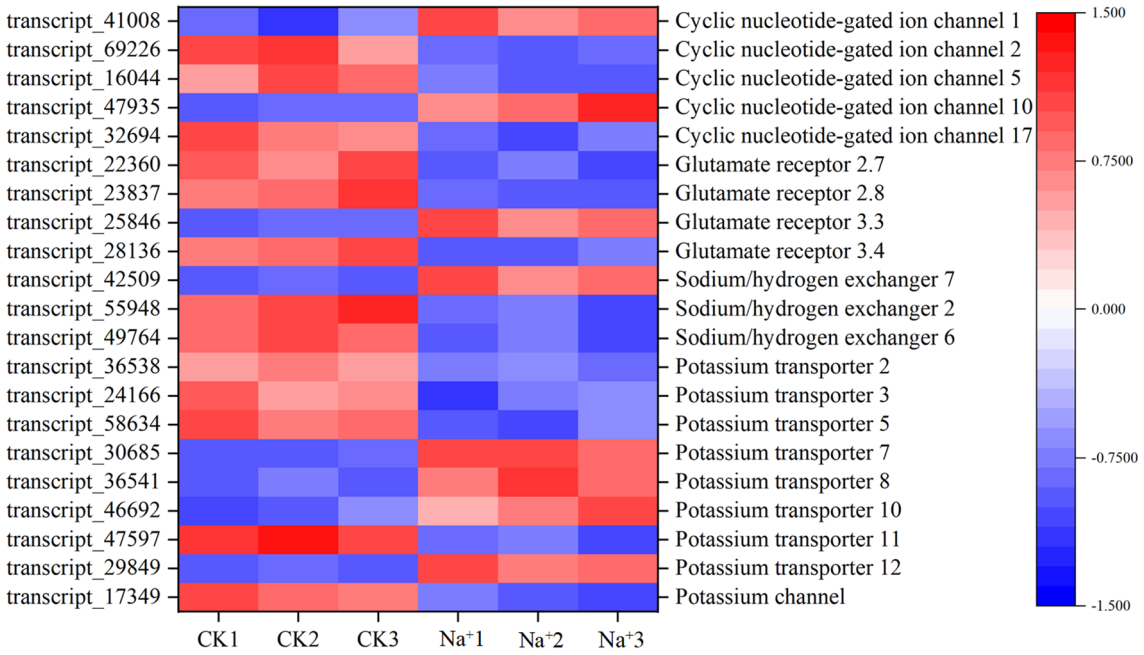

Fig. 6 DEGs related to Na^+ transport in *N. sibirica* under salt stress

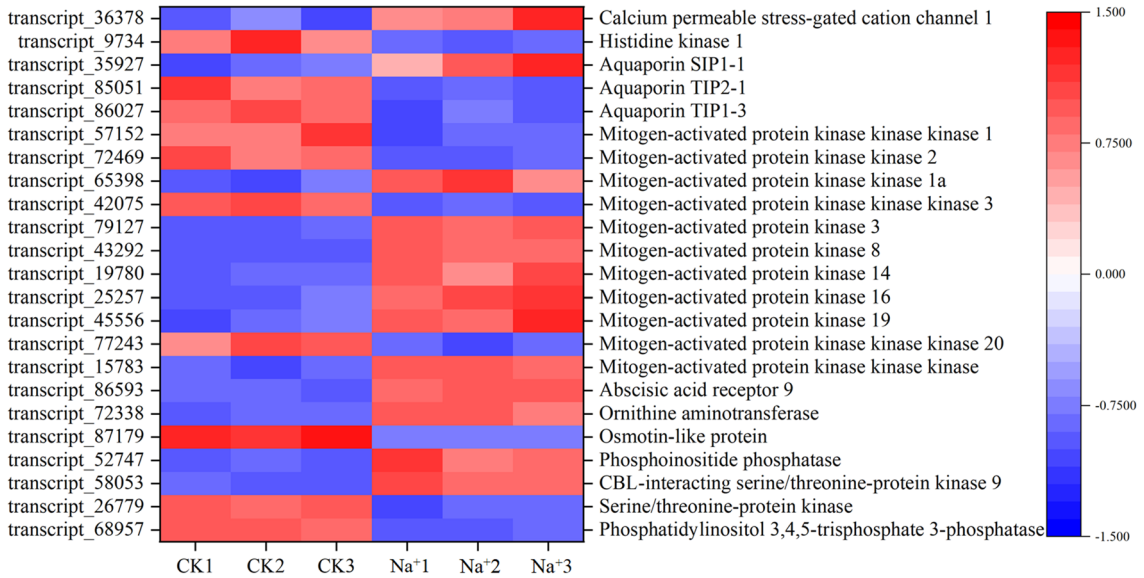

Fig. 7 DEGs related to osmotic regulation in *N. sibirica* under salt stress

kinase HPCA1 (*HPCA1*), Inactive poly [ADP-ribose] polymerase RCD1 (*RCD1*), Mitogen-activated protein kinase 8 (*MPK8*), NADPH oxidase RBOHA and Primary amine oxidase were identified as DEGs under salt stress (Fig. 8).

Identification of DEGs related to cell wall

Thirty-five DEGs that are associated with cell wall sensing, synthesis or modification were identified (Fig. 9). Most of them [e.g., Receptor-like protein kinase FERONIA (*FER*), THESEUS1 (*THE1*), Cell wall-associated kinase 1 (*WAK1*), Cellulose synthase (*CesA*), cellulose synthase interactive 1 (*CSI1*), KORRIGAN1 (*KOR1*), Pectinase (*PME*), Pectin acetylesterase (*PAE*) and Callose synthase (*CALS*)] were up-regulated under salt stress (Fig. 9). *FER*, *THE1* and *WAK1* are cell wall receptors. Some genes, such as *CesA*, *CSI1*, *KOR1*, *PME*, *PAE* and *CALS* are related to cell wall synthesis. However,

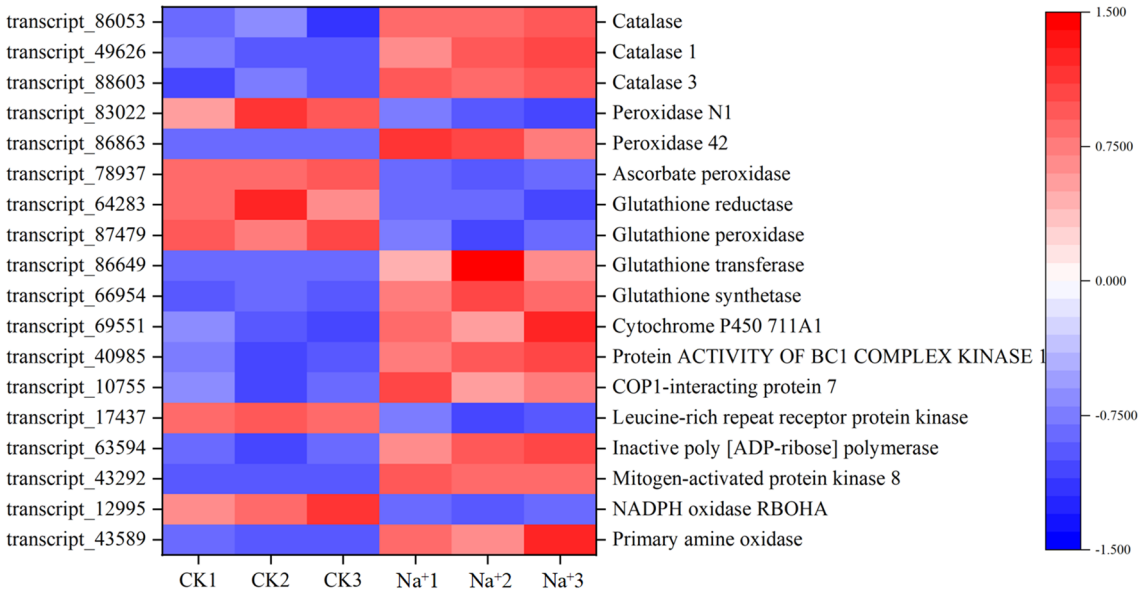

Fig. 8 DEGs related to antioxidant capacity in *N. sibirica* under salt stress

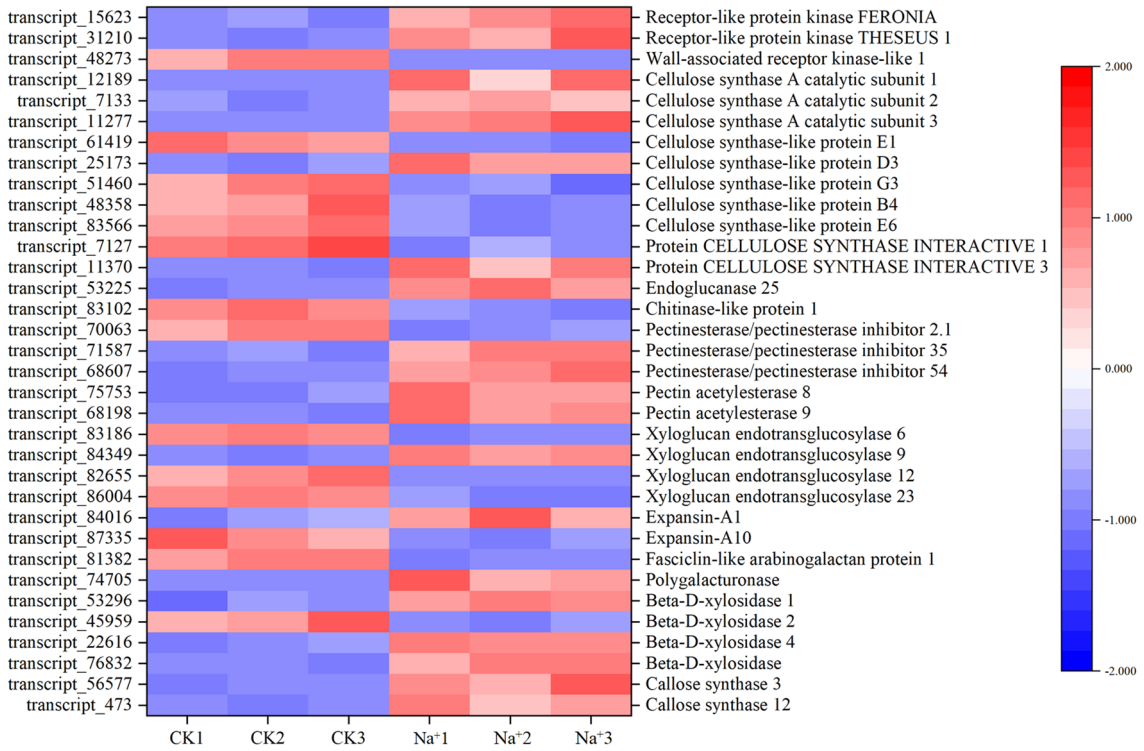

Fig. 9 DEGs related to cell wall in *N. sibirica* under salt stress

fasciclin-like arabinogalactan-protein (*FLA*), Xyloglucan endotransglucosylase (*XTH*) and expansin (*EXP*) related to cell wall modification were mostly down-regulated under salt stress (Fig. 9).

Identification of DEGs related to signal transduction

Several genes related to signal regulation were found in these DEGs. Eighteen DEGs involved in plant hormone

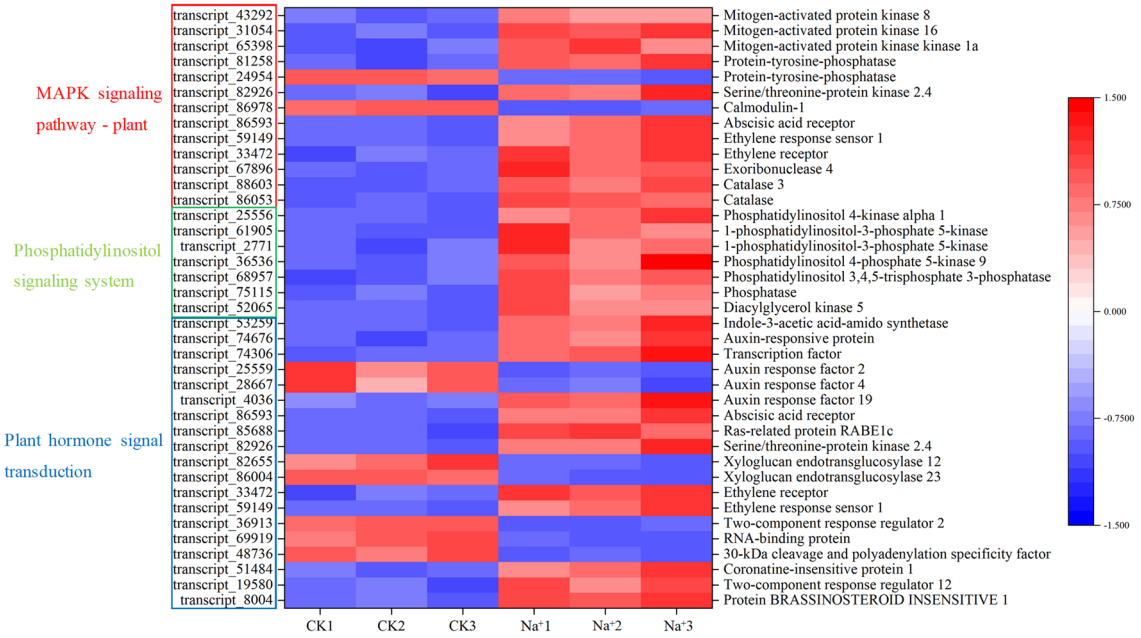

Fig. 10 DEGs related to signaling transduction in *N. sibirica* under salt stress

signal transduction pathways were screened out (Fig. 10). These DEGs included 6 auxin (IAA) signaling process genes (*GH3.6*, *IAA9*, *BHLH105*, *ARF2A*, *ARF4* and *ARF19*), 5 abscisic acid (ABA) signaling process genes (*PYL9*, *RabE1c*, *SNRK2.4*, *XTH12* and *XTH23*), 3 ethylene (ETH) signaling process genes (*ETR1*, *ERS1* and *ARR2*), 2 salicylic acid (SA) signaling process genes (*BRN1* and *CPSF30*), 1 jasmonic acid (JA) signaling process gene (*FBL2*), 1 cytokinin (CK) signaling process gene (*ARR12*) and 1 brassinosteroid (BR) signaling process gene (*BRI1*). A total of 7 transcripts encoded Phosphatidylinositol signaling system genes, including *PI4KA1*, *FAB1A*, *FAB1B*, *PIP5K9*, *PTEN2A*, *IMPL1* and *DGK5*, all of which were up-regulated under salt stress (Fig. 10). Moreover, a total of 14 transcripts encoded MAPK signaling pathway-plant genes, including 4 Mitogen-activated protein kinase, 2 Protein-tyrosine-phosphatase, 1 SNF1-related kinase 2.4, 1 Calmodulin, 1 Abscisic acid receptor, 2 Ethylene receptor, 1 Protein ETHYLENE INSENSITIVE 5 and 2 Catalase (Fig. 10).

Over-expression of *NsRabE1c* gene improves the sensitivity of *A. thaliana* to salt stress

A gene with the top 10% connectivity was selected for salt tolerance analysis. *NsRabE1c* has not been reported in salt stress yet. Among the genes with top 10% connectivity that have not been reported, the expression level of *NsRabE1c* under salt stress was the highest (Tables 2, 3 and 4). Therefore, *NsRabE1c* was selected for the analysis of salt tolerance in this study.

A. thaliana with over-expressed *NsRabE1c* was treated with salt stress along with *AtRabE1c* mutant and Col-0. The *AtRabE1c* mutant was identified as homozygous (Fig. S1). As shown in Fig. 11a, the germination rates of these three types of *A. thaliana* changed with time under different salt concentrations. When the salt concentration was 0, the germination rates of these three types all reached 100% with the increasing of time. However, there were significant differences in the germination potential, in the order of OE > Col-0 > *AtRabE1c*. The results demonstrated that the overexpression of *NsRabE1c* could accelerate plant germination. Under salt stress, the germination potentials of three types of *A. thaliana* were all limited. Comparing with the *AtRabE1c* and Col-0, the germination potential of OE was more limited. The germination potentials of these three types were *AtRabE1c* > Col-0 > OE when salt concentrations were 100 and 150 mmol·L^{-1}.

When the salt concentration was 150 mmol·L^{-1}, the germination rates of Col-0 and OE decreased, with that of OE decreased by 35%. In addition, the growth states of these plants were observed to the 10th day, and the survival rate and root length were recorded. It was found that the growth of *AtRabE1c* was better than that of Col-0, and Col-0 was better than OE with the increase of salt concentration (Fig. 11b). The survival rate was consistent with growth, and *AtRabE1c* had the highest survival rate, followed by Col-0 and OE (Fig. 11c). The changing trend of root length was similar to that of the germination rate with the increase of salt concentration. When salt concentration was 0, the root length of OE was significantly longer than those of Col-0

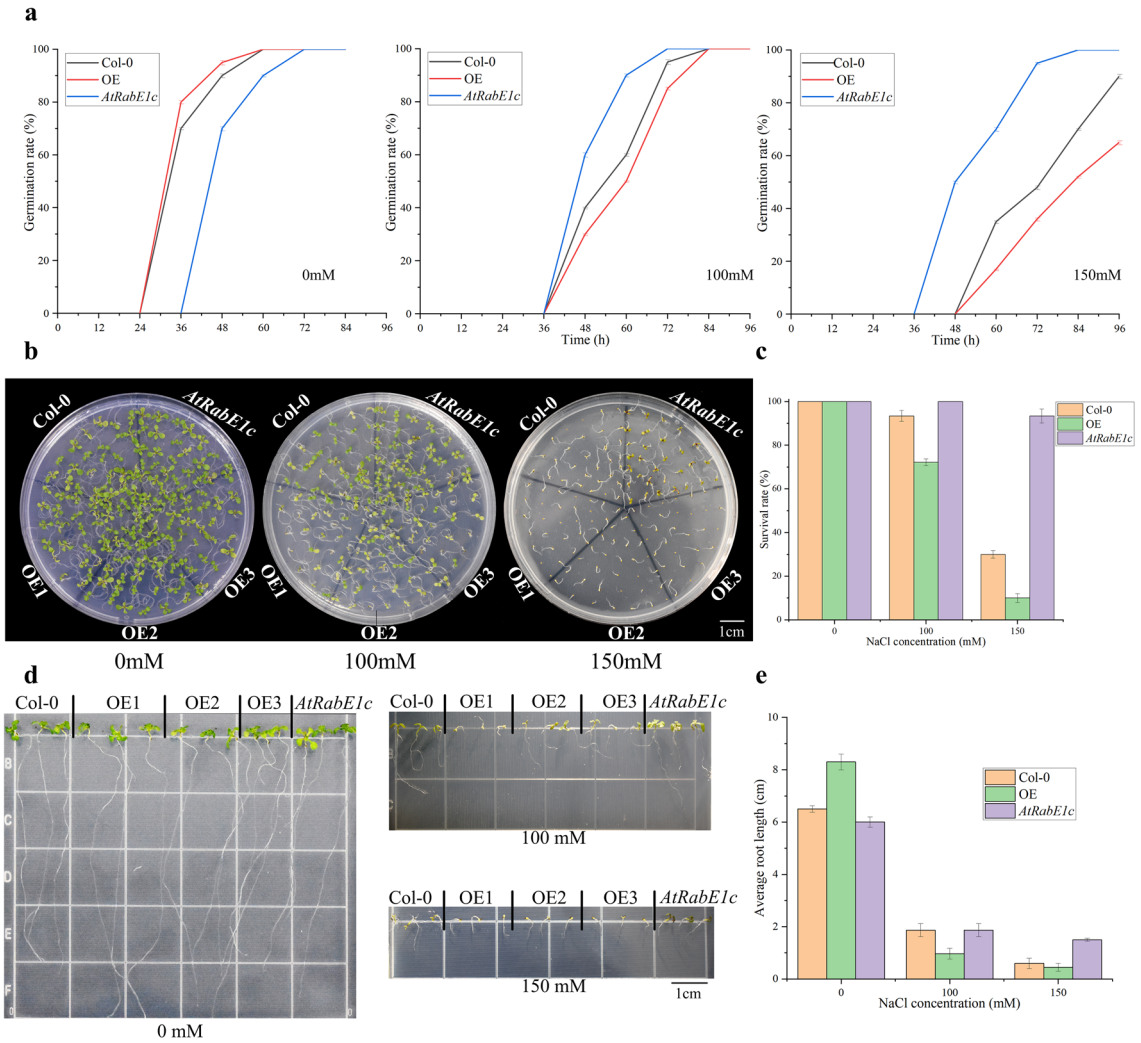

Fig. 11 Function verification of *NsRabE1c* in *A. thaliana*. **a** The germination rates of transgenic, Col-0 and *AtRabE1c* mutant plants. **b** The growth states of transgenic, Col-0 and mutant plants under 0, 100 and 150 NaCl stress at the 10th day. **c** The survival rates of transgenic, Col-0 and mutant plants. **d** The root growth states of transgenic, Col-0 and mutant plants under 0, 100 and 150 NaCl stress at the 10th day. **e** The root lengths of transgenic, Col-0 and mutant plants

and *AtRabE1c* (Fig. 11d and e). When salt concentrations were 100 and 150 mmol·L^{-1}, the root lengths of *AtRabE1c* and Col-0 were significantly longer than that of OE (Fig. 11d&e). Based on the above phenomenon, it seemed that *NsRabE1c* gene significantly responded to salt stress and negatively regulated plant salt tolerance.

qRT-PCR validation of DEGs from the RNA-seq analysis

To validate the reliability of transcriptome analysis data, 12 DEGs related to salt stress were verified using qRT-PCR. The results of RNA-seq and qRT-PCR revealed a high-rank consistency, indicating that the RNA-seq data are dependable and accurate (Fig. 12).

Discussion

The continuous expansion of saline-alkali soil area seriously affects agricultural development and ecological environment (Flowers and Colmer 2008; Munns and Tester 2008). Halophytes survive on and effectively restore saline-alkali soil, which have the potential to cover saline wasteland and reduce soil erosion (Flowers and Colmer 2008). *N. sibirica*, a typical perennial woody halophyte, can grow in saline soil because of its ability to isolate excessive Na^+ into vacuoles and stabilize the concentration of K^+ in cells (Tang et al. 2021). The physiological and molecular mechanisms involved in the salt tolerance of *N. sibirica* have been extensively investigated. At present, Illumina sequencing technology has been used to analyze the salt tolerance of *N. sibirica* treated with 100 and 400 mM NaCl (Li et al. 2017a,

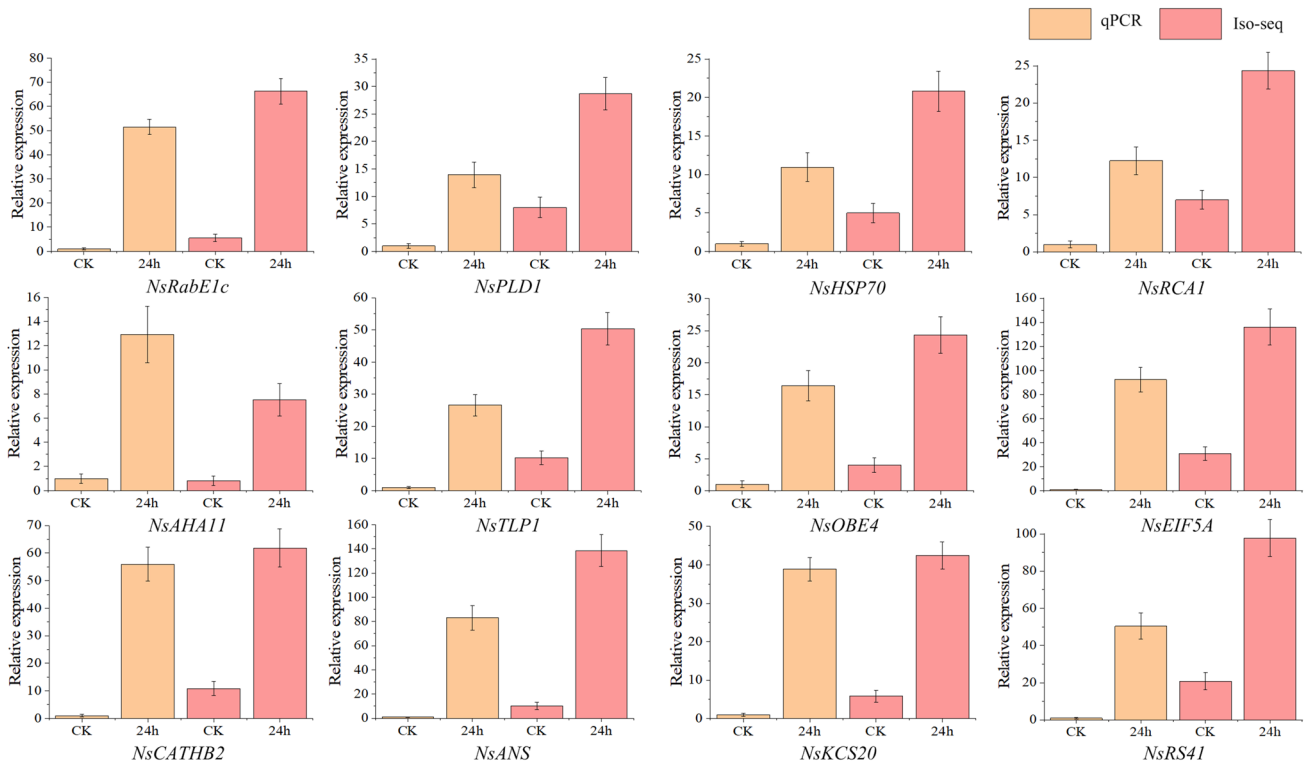

Fig. 12 qRT-PCR verification on the expression patterns of some unreported but highly differentially expressed genes in comparison to the RNA-seq data. The relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method, with the actin reference gene serving as a control

2021). It was found that "cell wall", "metabolic process", "MAPK signal pathway", "carbon metabolism" and "amino acid metabolism" pathways were significantly enriched in GO and KEGG enrichment analysis. In the present study, in addition to the above pathways, "sodium ion homeostasis", "response to osmotic stress", "reactive oxygen species metabolic process", "defense response by cell wall thickening" and "Phosphatidylinositol signaling system" pathways were found to be significantly enriched, and the involved genes of the above pathways were analyzed. Moreover, Li et al (2021) found that *AMY2*, *BAM1*, *GPAT3*, *ASP1*, *CML38* and *RPL4* genes played important roles in the regulation of salt tolerance in *N. sibirica*. In this study, 69 salt tolerance-related genes were screened out by WGCNA, of which 33 have not been reported in salt tolerance in previous literature. The above 33 genes can be used as candidate genes for further researches on salt tolerance. Importantly, 89,017 full-length transcripts were obtained in this study, which provided favorable materials for the subsequent verification of gene function.

Expression of ion transport genes in *N. sibirica*

Because the concentration of Na^+ in salinized soil is higher than that in plants, an electrochemical gradient is formed, which leads to the passive entry of Na^+ into the cytoplasm (Xu et al. 2021). Na^+ enters cells through NSCCs, such as CNGCs, GLRs, etc. (Duszyn et al. 2019; Kronzucker and Britto 2011). In this study, 5 *CNGC*s DEGs were identified, 2 of which were up-regulated and 3 of which were down-regulated (Fig. 6). In addition, 4 *GLR*s DEGs were also identified, 1 of which was up-regulated and 3 of which were down-regulated (Fig. 6). When salt stress was applied longer than 9 h, the plant was at the growth recovery stage (van Zelm et al. 2020). Therefore, the down-regulation of *NSCC*s genes in this study proved that the inflow of Na^+ decreased, which led to the continuous recovery of plant growth.

Increasing the outflow of Na^+ and regulating the balance of Na^+/K^+ are also the main ways for plants to cope with salt stress (van Zelm et al. 2020). After being triggered by cytoplasmic Ca^{2+}, SOS pathway alleviates salt stress by exporting excess Na^+ (Kronzucker and Britto 2011). In this study, the expression of *SOS1/NHX7* was found to be stimulated by salt stress (Fig. 6), which promoted the excretion of Na^+.

N. sibirica isolates excess Na^+ into vacuole, so that reducing the content of Na^+ in cytoplasm (Wang et al. 2016). This process is controlled by Na^+/H^+ antiporters. In this study, *NHX1* did not respond as a DEG, and the expression of *NHX2* was inhibited (Fig. 6), indicating that the compartmentation of Na^+ may have completed at 24 h after the

ending of NaCl treatment. It has been reported that NHX2 could transport K^+ into vacuoles in *A. thaliana* (Bassil et al. 2011). A previous study showed that the root of *N. sibirica* had a better retention rate of K^+ (Tang et al. 2021). In this study, the decreased expression level of *NHX2* in leaves may restrict the transport of K^+ to vacuoles and store more K^+ in roots. The inhibition on the expression of *NHX2* gene was also found in tobacco under salt stress (Luo et al. 2019).

Many studies have shown that whether plants can survive in saline environments largely depends on their ability to maintain the K^+/Na^+ balance under salt stress (Sun et al. 2009; Tang et al. 2018). Therefore, plants have to modulate the Na^+/K^+ homeostasis through maintaining high K^+/Na^+ ratio under salt stress (Zhao et al. 2021a). HAKs and AKTs contribute to the absorption of K^+ and maintain the balance of Na^+/K^+ (Nieves-Cordones et al. 2014). In this study, 8 *HAK*s were identified as DEGs(Fig. 6). Several *HAK*s have been cloned in rice and *A. thaliana,* and demonstrated different functions such as regulating the absorption and transport of K^+, participating in osmotic stress regulation downstream of plant ABA and auxin signaling pathways, promoting root development and negatively regulating shoot growth related to swelling in plants (Li et al. 2017b). The expression levels of *HAK*s in *N. sibirica* under salt stress were up-regulated or down-regulated, which may exert the same functions as those in rice and *A. thaliana*.

Some studies have shown that aquaporins can also regulate the influx of Na^+ and the efflux of K^+ (Nongpiur et al. 2020). However, the overexpression of wheat aquaporin gene *TaNIP* in *A. thaliana* was found to decrease Na^+ concentration and increase K^+ concentration (Gao et al. 2010). The contradictory results showed that different subfamilies of aquaporins may have different functions, and the Na^+ transport ability of aquaporins needs to be further verified. In this study, it was found that the expression levels of three aquaporins varied under salt stress, one was up-regulated and the other two were down-regulated, indicating that three aquaporin genes also responded to salt stress in different ways (Fig. 7).

The above results indicated that *N. sibirica* reduces the harm of Na^+ by reducing the inflow of Na^+, increasing the outflow of Na^+, compartmentalizing the excess Na^+ into vacuoles and maintaining the ion balance of K^+/Na^+ during salt stress.

Expression of osmotic-related genes in *N. sibirica*

Under salt stress, ion imbalance and water deficiency in the plant cell cause osmotic stress (Zhao et al. 2021a). Activation of salt-mediated osmotic stress pathways induces the biosynthesis and accumulation of compatible osmolytes to reduce the cell osmotic potential as well as stabilize the structures of proteins and cells (Yang and Guo 2018b). The osmotic-related receptor proteins are the potential master switches for triggering and coordinating the stress response (Nongpiur et al. 2020). Some studies have shown that there may be more than one receptor of osmotic stress in plants (Nongpiur et al. 2020).

In *A. thaliana*, it has been found that OSCA1 is an osmosensor, forms hyperosmolality-gated calcium-permeable channels (Yuan et al. 2014). Osmotic receptor AHK1 is a positive regulator of drought and salt stress in *A. thaliana* (Tran et al. 2007). However, some studies have shown that *ahk1* mutants were not sensitive to salt stress, and the growth of *ahk1* mutants under 150 mM NaCl treatment was even better than that of WT (Kumar et al. 2013). These results indicated that AHK1 may possess other functions in addition to being an osmotic sensor, which needs to be further confirmed. Some studies have shown that aquaporins directly detected the water potential difference across the membrane to sense osmotic stress. However, more studies are needed to prove that aquaporins can be used as osmotic sensors (Hill and Shachar 2015). In this study, *CSC1*/*OSCA1*, *AHK1* and aquaporins were found to be differentially expressed and may also be identified as osmotic stress receptor genes. These results indicated that they are essential in regulating osmotic stress in *N. sibirica* (Hill and Shachar 2015; Nongpiur et al. 2020; Tran et al. 2007). In addition, it has been found that cell wall monitoring proteins can detect mechanical bending and the damage of cell wall caused by osmotic stress (Nongpiur et al. 2020). In this study, three kinds of cell wall receptors genes (*FER*, *THE1* and *WAK1*) were also found to detect cell wall damage caused by osmotic stress (Fig. 9).

Under salt stress, MAPK cascades are the connection between salt stress receptors and target genes (Lin et al. 2021). Studies have shown that MAPK cascades regulate osmotic stress caused by salt stress in an ABA-dependent manner. The functions of many genes constituting *MAPK* cascades have been verified (Lin et al. 2021). In this study, 11 *MAPK*s DEGs and 1 ABA receptor DEGs were identified, most of which were up-regulated (Fig. 7), indicating that these genes regulate osmotic stress in *N. sibirica*. This result is consistent with the studies of MAPK cascade genes involved in salt stress of *A. thaliana*, maize, rice and other plants (Kim et al. 2011; Kong et al. 2011; Ning et al. 2010; Teige et al. 2004; Wang et al. 2010).

Expression of antioxidant genes in *N. sibirica*

Plants have evolved a good antioxidant mechanism to cope with oxidative stress. This mechanism consists of enzyme and non-enzyme antioxidant components (Ahmad et al. 2018; Nadarajah 2020; Yang and Guo 2018a). In this study, we identified many enzymes that regulate oxidative stress, including 3 *CAT*s, 2 *POD*s, 1 *APX*, 1 *GR*, 1 *GPX* and 1 *GST*.

We also identified several genes related to non-enzymatic regulator synthesis, such as *GSH2*, *MAX1*, *ABC1K1/BDR1/PGR6* and *CIP7*. The expression levels of most of the above genes were up-regulated under salt stress (Fig. 8). These results suggested that *N. sibirica* maintains a low level of ROS in cells and alleviates oxidative stress by activating antioxidant defense system.

HPCA1 is a kind of H_2O_2 receptor, which can detect the increase of H_2O_2 under salt stress and trigger the influx of Ca^{2+} to achieve stomatal closure (Wu et al. 2020). RCD1 is an important transcription regulator of oxidative stress, and interacts with SOS1 to play a role in oxidative stress tolerance in *A. thaliana* (Katiyar-Agarwal et al. 2006). In addition, the MAPK cascade can regulate the gene expression of antioxidative response and increase antioxidative enzyme activities to detoxify ROS and sustain ROS homeostasis (Lin et al. 2021; Takahashi et al. 2011). For example, MPK8 can converge Ca^{2+} and MAP kinase phosphorylation cascade to monitor or maintain ROS homeostasis (Takahashi et al. 2011). In this study, the expression of *HPCA1* was down-regulated, indicating that the content of H_2O_2 has decreased to an unharmful level at 24 h after the ending of salt treatment (Fig. 8). The up-regulation of *RCD1* and *MPK8* also showed that *N. sibirica* reduced the content of ROS and maintained its homeostasis at that time (Fig. 8).

Expression of cell wall genes in *N. sibirica*

Many studies have shown that the cell wall is an important factor to determine cell shape and function, and it is the first layer of defense against salt stress (van Zelm et al. 2020; Zhao et al. 2021a). Cell walls provide mechanical strength to withstand the changes of cell swelling pressure caused by salt stress, prevent ions from entering protoplasts and play the role of defense and ion detoxification (Monniaux and Hay 2016). The cell wall sensor can sense the damage to cell wall caused by salt stress (Liu et al. 2021a). At present, various cell wall sensors have been found (Engelsdorf et al. 2018; Liu et al. 2021a). FER can perceive the softening of cell wall structure caused by salt stress, which may be caused by FER's perception of the decrease of pectin crosslinking (Feng et al. 2018). Moreover, some studies have found that FER detects cell wall damage at the growth recovery stage of plants, rather than stress-induced wall damage immediately after salt stress (Nongpiur et al. 2020). The double mutants of THE1 and HERKUCES1 (HERK1) showed the same phenotype as fer4 in terms of growth and salt stress, indicating that they can act together to sense cell wall signals (Gigli-Bisceglia et al. 2020). WAK1 has an extracellular domain linked to pectin, which can sense the change of cell wall (Decreux and Messiaen 2005), and its expression is induced at the late stage of abiotic stress signal response (Nongpiur et al. 2020). The expression levels of *FER*, *THE1* and *WAK1* were all up-regulated in this study (Fig. 9), suggesting that these three kinds of cell wall sensors may perceive the changes of cell wall at the later stage of salt stress. In addition, four other *WAK*s DEGs were identified, indicating that they also play an important role in cell wall perception.

Plant cell wall is composed of cellulose, hemicellulose, pectin, lignin and various types of structural proteins (Munns and Tester 2008; Xu et al. 2021; Zhao et al. 2020). In the green module, the terms related to hemicellulose, such as "xylan 1,4-beta-xylosidase activity", "(1- > 3)-β-D-glucan biosynthetic process" and "hemicellulose metabolic process" were significantly enriched (Fig. 5a). CesA, CSI1, Cellulose synthase-like protein (CSL), KOR1 and chitinase-like protein (CTL) are the key proteins in cellulose biosynthesis (Liu et al. 2021a; Xu et al. 2021). PME are related to the methyl esterification of pectin and maintaining the integrity of cell wall under salt stress (Liu et al. 2021a). PAE is related to pectin modification (Xu et al. 2021). In this study, these genes related to cell wall components were identified and most of them were up-regulated (Fig. 9). Therefore, *N. sibirica* can strengthen the structure of cell wall through the synthesis of related components of cell wall and withstand the changes of cell swelling pressure caused by salt stress at the recovery stage.

There are many cell wall proteins (CWPs) in cell wall, which play a key role in the cell wall modification and stress signal transduction (Liu et al. 2021a). FLA, XTH and EXP are all key factors regulating cell wall and participate in the salt stress response of plants (Liu et al. 2021a; Tiika et al. 2021). In the present study, most of these genes were down-regulated under salt stress (Fig. 9), directly resulting in the dwarf plant phenotype and slow growth.

Expression of signal transduction genes in *N. sibirica*

Previous studies have shown that plants respond to salt stress by regulating complex signal networks (Ma et al. 2022). Plant hormones not only regulate plant growth and development, but also play an important role in plant molecular signals in response to salt stress (Ma et al. 2022; Zhu et al. 2021). ABA, ETH, SA and JA are considered as stress response hormones, while IAA, CKs and BRs are classified as growth promotion hormones (Yu et al. 2020). It has been found that phospholipids including phosphatidylinositol (PI) regulate salt stress response by participating in salt stress signal transduction (Han and Yang 2021). Salt stress triggers the activation and enhances the gene expression of MAPK signaling cascades (Chinnusamy et al. 2006). ROS signaling and osmotic signaling in plants under salt stresses is mediated by MAPK signaling pathways (Lin et al. 2021; Takahashi et al. 2011).

In this study, it was also found that plant hormone signal transduction, phosphatidylinositol signaling system and MAPK signal pathway were enriched to regulate signal transduction under salt stress. As shown in Fig. 10, there were many single genes involved in these three signaling pathways, and 7 single genes of the phosphatidylinositol signaling system were all up-regulated. It has been found that many single genes involved in phosphatidylinositol signaling system play an important role in growth and development. For instance, the impairment of *FAB1A/B* function in *A. thaliana* may lead to the deficiency of membrane circulation capacity of auxin transporters and inhibit the correct transport of auxin to cells (Hirano and Sato 2011). PTEN2 was involved in the regulation of xylem differentiation in *A. thaliana* and the assembly and/or transport of cellulose synthase complexes needed to construct secondary cell walls (Hunkeler 2021); DGK5 was involved in the extreme growth of *tobacco* (Scholz et al. 2022). These results suggested that these signaling pathways played an important role in resisting salt stress through the regulation of growth and development during the growth recovery period.

Genes with the top 10% of connectivity regulate salt tolerance in *N. sibirica*

In order to further determine the key genes responding to salt stress in *N. sibirica*, all differential genes were analyzed by WGCNA. The connectivity of all genes in three modules related to salt stress was analyzed by cytoscape, and the genes with top 10% connectivity were selected as key genes (Tables 2, 3 and 4) (Ni et al. 2021). Among them, many genes have been proved to be involved in salt stress. Salt stress can be recognized by HPCA1 (Pantha and Dassanayake 2020). THE1 was a sensor for maintaining and sensing the structural integrity of cell wall under salt stress (Bacete et al. 2022; Gigli-Bisceglia et al. 2020). Potassium ion transporter HAK7 absorbed K^+ and regulated the balance of Na^+/K^+ (van Zelm et al. 2020). The activation of CDC48 gene increased the tolerance of rice under salt stress (Raja et al. 2021). Phospholipase Da1 (PLD) was up-regulated in salt-treated tomato cell suspension culture, and participated in the production of phosphatidic acid (Bargmann et al. 2009). Over-expression of PMA4 increased the salt tolerance of tobacco during germination and seedling growth (Li et al. 2022). LDOX participated in flavonoid biosynthesis and enhanced the response of plants to NaCl stress (Zhang et al. 2016a). LOX reduced the oxidative damage of cell membrane and improved the salt tolerance of rapeseed (Menga and Trono 2020). These results showed that these key genes play an important role in response to salt stress. In addition, there are still many genes that have not been reported in regulating salt stress, which are the focus of our following research (Tables 2, 3 and 4).

NsRabE1c regulates salt tolerance by regulating plant growth in *N. sibirica*

Ras-related protein RabE1c belongs to the Rab protein family of small GTP binding proteins and is mainly involved in vesicle transport (Chen et al. 2021). A previous study have found that *PtRabE1b*, which is in the same family as *RabE1c*, plays an important role in the salt resistance of *populus* (Zhang et al. 2018). In the present study, we found that the germination potential, survival rate and growth of transgenic plants were worse than those of Col-0 and *AtRabE1c* mutants under salt stress (Fig. 11). Some studies have found that the growth rate of plants under salt stress can be divided into three stages: firstly, the growth rate decreases at the halting stage (from 5 min to 5 h after salt application in the main root); Secondly, low levels of plant growth were maintained during the quiescent phase (5–9 h after salting the main root); Finally, partial recovery was achieved during the growth recovery phase (starting from 9 h after salting the main root) (van Zelm et al. 2020). Since the transcriptome data came from 24 h after the stopping of salt stress and *N. sibirica* was at the growth recovery stage, we speculated that the *NsRabE1c* gene mainly regulated plant growth, leading to its high expression. Furthermore, some other studies have also found that the transformation of *A. thaliana RabE1c* gene into *Physcomitrella patens* rescues the morphological defects of Rab-E mutant, which also proved that *RabE1c* gene is related to plant growth and development (Orr et al. 2021).

In physiological verification test, we also found that when the salt concentration was 0, the transgenic plants germinated the fastest and had the longest root length (Fig. 11a and e). To further prove the above speculation, qPCR was used to study the expression levels of *NsRabE1c* in *N. sibirica* treated with 300 mmol·L^{-1} NaCl at different periods. As shown in Fig. 13, its expression level decreased at the growth stop stage and increased gradually at the growth recovery stage. The results showed that *NsRabE1c* gene plays a role in regulating the growth of *N. sibirica* during salt resistance, but the regulatory mechanism needs further study.

In the present study, the salt tolerance mechanism of *N. sibirica* was studied combining Pacbio full-length transcriptome sequencing and Illumina sequencing technologies. Nine *NSCC*s, 8 *HAK*s, *SOS*1, *NHX*2 and *NHX*6 were identified as DEGs in *N. sibirica* under salt stress. The up-regulation or down-regulation on the expression levels of these genes reduces the harm of Na^+ to *N. sibirica* by reducing the inflow of Na^+, increasing the outflow of Na^+, compartmentalizing the excess Na^+ into vacuoles and maintaining the ion balance of K^+/Na^+. The expression levels of 3 cell wall sensors, 17 cell wall structural proteins and 14 cell wall proteins significantly differed between CK and NaCl-stressed *N. sibirica.* These genes strengthen the defense of cell wall to

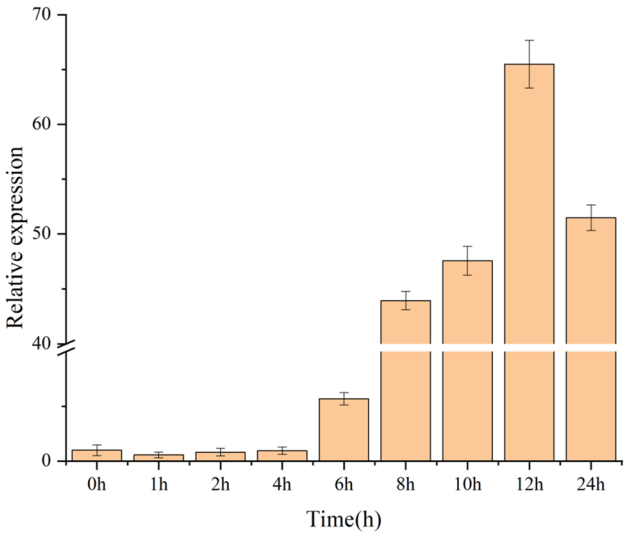

Fig. 13 Expression levels of *NsRabE1c* in *N. sibirica* treated with 300 mmol·L^{-1} salt at different periods. The relative expression levels were calculated according to the $2^{-\Delta\Delta Ct}$ method, with the actin reference gene serving as a control

NaCl stress through perception of the changes of cell wall, synthesis of cell wall components as well as cell wall modification and stress signal transduction. In addition, it was found that 39 genes involved in plant hormone signal transduction, phosphatidylinositol signaling system and MAPK signal pathway were enriched in *N. sibirica* under salt stress. Especially, 7 genes in phosphatidylinositol signaling system, which play a key role in plant growth and development, were all significantly up-regulated under salt stress. Hence, we speculate that *N. sibirica* regulates salt tolerance mainly by adjusting ion balance, modifying cell wall structure and activating signal transduction pathways.

Conclusion

In this study, the leaves of control and NaCl-stressed *N. sibirica* were sequenced using Iso-Seq and NGS techniques. These leaf samples were collected from plants at 24 h after the ending of 300 mmol·L^{-1} NaCl stress. After removing redundancy and clustering, the number of full-length transcripts is 89,017, of which 84,632 sequences were annotated. In addition, the 86,482 CDS sequences were predicted. DeSeq2 identified that 6561 differential transcripts were involved in salt stress. Moreover, 69 genes were found to be related to salt stress by WGCNA. In *N. sibirica*, these newly discovered genes not only deal with salt stress by regulating ion balance, alleviating osmotic stress, scavenging ROS and stabilizing cell wall structure, but also reduce salt stress damage by means of signal transduction, plant hormones and photosynthesis. These results increase our understanding of the salt tolerance mechanism of *N. sibirica*. Moreover, this study lays the foundation to further study the salt resistance mechanism of *N. sibirica*.

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Author contributions statement PZ and YY designed the experiments. PZ and FZ performed most of the experiments. ZW and SC extracted the RNA and conducted the transgenic experiments. PZ and WL wrote the original draft. FZ, WL and YY revised the paper. All authors have read and approved to the published version of the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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