Expression of genes related to plant hormone signal transduction in Jerusalem artichoke (*Helianthus tuberosus* L.) seedlings under salt stress

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The date of submission: 2021-08-29

The number of tables and figures: 4 tables (1 supplementary), 12 figures (1 supplementary)

Highlights

1. Gene functions in the classification of temporal expression patterns is uniform.

2. Hormones play a key regulatory role in the early stage of plants facing high salt stress.

3. Phytohormone genes show a similar expression pattern during the first 48 h of salt stress.

Abstract:

Background: Jerusalem artichoke (*Helianthus tuberosus* L.) is tolerant to salinity stress and has high economic value. The salt tolerance mechanisms of Jerusalem artichoke are still unclear. Especially in the early stage of Jerusalem artichoke exposure to salt stress, the plant physiology, biochemistry and gene transcription are likely to undergo

large changes. Elucidating these changes may be of great significance to understanding the salt tolerance mechanisms of it.

Results: We obtained high-quality transcriptome from leaves and roots of Jerusalem artichoke exposed to salinity (300 mM NaCl) for 0 h, 6 h, 12 h, 24 h and 48 h, with 150,129 unigenes and 9023 DEGs (Differentially Expressed Genes). The RNA-seq data were clustered into time-dependent groups (nine clusters each in leaves and roots); gene functions were distributed evenly among the groups convergence. KEGG enrichment analysis showed the genes related to plant hormone signal transduction were enriched in almost all treatment comparisons. Under salt stress, genes belongs to *PYL* (abscisic acid receptor PYR / PYL family), *PP2C* (Type 2C protein phosphatases), *GH3* (Gretchen Hagen3), *ETR* (ethylene receptor), *EIN2/3* (ethylene-insensitive protein 2/3), *JAZ* (Genes such as jasmonate ZIM-domain gene) and *MYC2* (Transcription factor MYC2) had extremely similar expression patterns. The results of qPCR of 12 randomly selected genes confirmed the accuracy of RNA-seq.

Conclusions: Under the impact of high salinity (300mM) environment, Jerusalem artichoke in the seedling stage was difficult to survive for a long time, and the phenotype was severe in the short term. Based on the expression of genes on the time scale, we found that the distribution of gene functions in time is relatively even. Upregulation of the phytohormone signal transduction had a crucial role in the response of Jerusalem artichoke seedlings to salt stress, the genes of abscisic acid, auxin, ethylene, and jasmonic acid had the most obvious change pattern.

Keywords: Salt stress, Jerusalem artichoke, Time series analysis, RNA-seq

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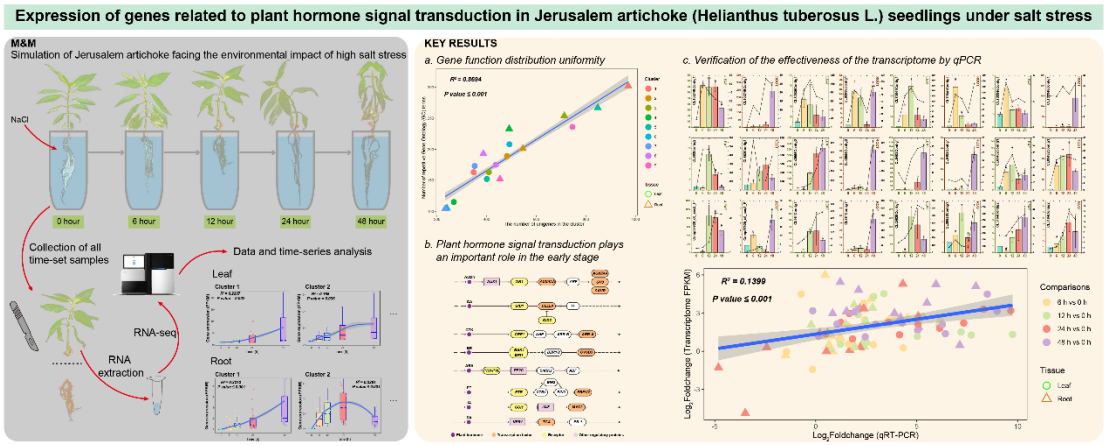
Graphical abstract



1. INTRODUCTION

More than 800 million hectares of land worldwide are affected by salinity, and the utilization of saline soil is a key issue in agriculture [1]. However, saline soils may be inhospitable to plants. Most crops are glycophytes, meaning they are relatively sensitive to salinity [2–4]; hence, finding a suitable crop to grow on saline land is difficult.

Jerusalem artichoke (*Helianthus tuberosus* L.), a plant species with variety of potential uses, is moderately salt tolerant [5] and is suitable for saline soil cultivation [6–8], which has extraordinary significance for carbon neutrality. The current research on Jerusalem artichoke focuses on breeding for greater biomass production [9–11], but less work has been done on its salinity tolerance. Different tissues of Jerusalem artichoke have differential salt tolerance, increasing from tubers to stem and leaves. Under salt exposure, no significant difference was found in Cl^- content in tubers, stems and leaves of Jerusalem artichoke, but Na^+ content decreased from tubers to leaves, suggesting restricted Na^+ transport into stem and leaves [12].

Different Jerusalem artichoke genotypes may have differential salt tolerance mechanisms. For example, genotype Nanyu No. 1 adapts to NaCl stress by reducing

net photosynthetic rate, stomatal conductance and transpiration, while maintaining a high degree of H^+-ATPase activity, whereby genotype Qingyu No. 2 accumulates proline and maintains chlorophyll concentration [13]. Although the amount of literature on salt tolerance of Jerusalem artichoke is increasing, the underlying mechanisms remain unclear.

Many studies have explained the mechanisms of plant salinity tolerance based on physiological, biochemical and molecular characterization [14]. However, with the development of high-throughput sequencing, the RNA-seq technology has increasingly been used to study transcriptome of many plant species, but there is relatively little research on Jerusalem artichoke [10,15,16], and even less so regarding salt stress [15]. Therefore, it is important to use RNA-seq to characterize salt tolerance mechanisms of Jerusalem artichoke. Comparative transcriptomic research focuses on time-course experiments to identify the expression patterns of genes. Such time-series research has been used in the exploration of various biological processes [17–20].

The important roles of plant hormones in resistance to abiotic stresses have been emphasized [21–23], including mediation of salt stress to regulate plant adaptation [24]. Many studies have showed that the expression of genes related to phytohormones depends on the severity and/or duration of abiotic stresses [25,26], indicating that changes in the expression of phytohormone-related genes needs to be characterized with respect to stress duration. Therefore, the aim of the present study was to use the transcriptome samples of tissues of Jerusalem artichoke exposed to salt stress for different durations to characterize the expression patterns of genes (especially those

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related to phytohormone signal transduction).

2. Materials and methods

2.1 Plant Material Preparation and Salt Treatment

Jerusalem artichoke [*Helianthus tuberosus* L. cv. Nanyu No. 1 (NY-1)] tubers were collected in Dafeng (Jiangsu Province, China, 33°14’ N, 120°42’ E) on February 1. Each bud eye was sliced into pieces at least 1 cm thick; the pieces were placed in quartz sand (30 cm × 20 cm enamel tray) moistened with distilled water. The initial cultivation was done at 25 ℃ in the dark using an incubator (NingBo SaiFu, PGX-350B). When leaves started to develop, cultivation continued at 25 ℃ and 12 h light/12 h dark using 1/2 Hoagland solution to moisten quartz sand. After about 2 weeks, the seedlings reached a height of at least 20 cm, and were then transplanted into pots (10 cm diameter and 10 cm height, every 5 seedlings) containing 1/2 Hoagland nutrient solution. Seedlings were grown for 2 days to to overcome any possible transplantation shock before starting the salt treatment. The salt treatment consisted of 300 mM NaCl in 1/2 Hoagland nutrient solution. Five seedlings of Jerusalem artichoke were distributed evenly in each pot. There were three replicate pots for each sampling time point. The experiment was conducted in a greenhouse at NanJing Agricultural University, College of Resource and Environmental Science (32.03N, 118.84E).

In order to investigate the transcriptional responses in different tissues, we sampled roots and leaves during exposure to salt stress at 0, 6, 12, 24, and 48 h (Table S1).

Table S1 Sample groups, tissue types and duration of exposure to 300 mM NaCl.

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Group Acronym	Tissue	Duration of salt exposure (h)
0R	Root	0
0L	Leaf	0
6R	Root	6
6L	Leaf	6
12R	Root	12
12L	Leaf	12
24R	Root	24
24L	Leaf	24
48R	Root	48
48L	Leaf	48

2.2 cDNA Library Establishment and Illumina RNA sequencing

To enhance the validity of the RNA sequencing data, in addition to samples detailed in Table S1, we used three more Jerusalem artichoke samples to establish the library: Sample_INF_1 and Sample_INF_2 (tuber tissues) and Sample_NY_F (all flower tissues above the calyx at the blooming stage). Total RNA of each treatment and supplementary sample was extracted using a RNAiso Plus kit (Takara, Kyoto, Japan). After digesting the DNA with DNase (Takara, Kyoto, Japan), we used magnetic beads with Oligo (dT) (Vdobiotech, Suzhou, China) to enrich eukaryotic mRNA. The RNA library was constructed using a TIANSeq Fast RNA Library Kit (Illumina) (Tiangen, Beijing, China). The library quality was assessed on an Agilent Bioanalyzer 2100 system (Agilent Technologies, California (Santa Clara), US). Finally, samples were sequenced on a single lane of an Illumina HiSeq^{TM} 2000 platform, and 125 bp paired-end reads were generated.

2.3 RNA-Seq Analysis

2.3.1 Quality Assessment of the Sequencing Data

To ensure the quality and reliability of data analysis, the raw data were filtered by

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removing reads with adapters, reads with unknown bases, and low-quality reads (Phred quality <20). Software FastQC (Babraham Bioinformatics, Cambridge, UK) (Andrews et al., 2010) was used to assess the quality of raw data before the data analysis. The low quality reads were removed by a NGS QC Toolkit (version 2.3.3) [28]. The PCA using the default parameters of the R package FactoMineR [29] and [30] showed large variability among the 10 samples (Fig. S1A). The results of raw and clean reads, Q30 values, and GC content indicated good quality of sequencing, with the data suitable for database construction (Fig. S1B).

2.3.2 De novo Assembly

Because the sequencing of the complete genome of Jerusalem artichoke is still underway, *de novo* assembly of sequences was used for the follow-up analysis. The overlapping reads were connected into longer sequences; with their continuous extending, the reads were spliced into transcripts by the pair-end method using Trinity [31] (version 20131110). Subsequently, TGICL [32] was used for fast clustering and eliminating redundancy fragments to get final unigenes. We evaluated the data after assembling, and found they met the requirements for the subsequent analysis. The distribution of unigene lengths is shown in Fig. S1C. All unigenes larger than 300 bp were used for statistical analysis. Most unigenes were in the 300-800 bp length range, with the number of unigenes decreasing with an increase in the length (Fig. S1C). After assembly, 150,129 unigenes were obtained from the transcriptome. The N50 value reached 1317, indicating the high quality of transcriptome assembly (Fig. S1D).

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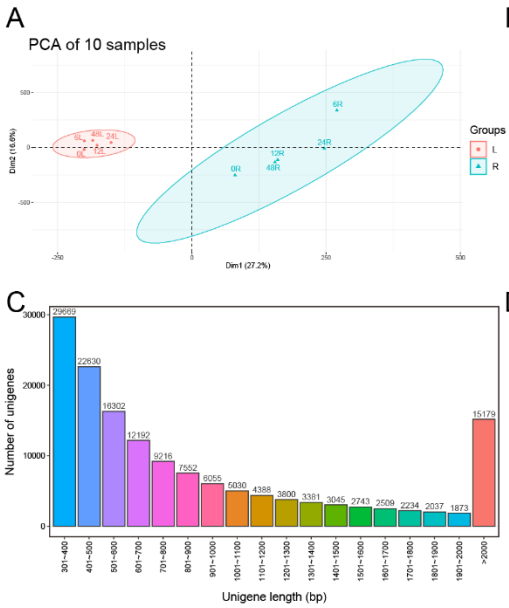

Sample	Raw reads	Clean reads	Q30 (%)	GC content (%)
0L	46,841,476	46,415,446	94.54	45.00
0R	45,107,446	44,700,256	94.15	44.00
6L	47,655,304	47,213,024	94.36	44.00
6R	48,097,190	47,652,458	94.36	45.00
12L	50,200,062	49,730,316	94.12	45.00
12R	49,373,408	48,914,668	94.56	44.00
24L	43,950,428	43,531,412	94.53	44.00
24R	42,912,264	42,504,628	94.54	45.00
48L	47,725,814	47,286,474	94.43	45.00
48R	46,737,202	46,307,118	94.52	44.00

Unigene	All	≥ 500 bp	≥ 100 bp
	150,129	97,726	46,270
	N50	**Total length (bp)**	**Max length (bp)**
	1,317	144,639,628	16,617
	Min length (bp)	**Average Length (bp)**	
	300	963.44	

Fig. S1 The results of Illumina RNA sequencing. A: PCA of 10 root (R) and leaf (L) samples. B: Details of the raw and clean data. C: Length distribution of unigenes; D: Information on assembled unigenes. N50: the sequence length of the shortest scaffold at 50% of the total genome length, representing the quality of assembly.

2.3.3 Unigene Annotation

Genetic similarity comparison was based on the BLAST method [33]. The databases RefSeq non-redundant protein sequences (NR) [34], SwissProt [35], Clusters of Orthologous Groups of Proteins (KOG) [36], Gene Ontology (GO) (The Gene Ontology Consortium, 2019), and Kyoto Encyclopedia of Genes and Genomes (KEGG) [38] were used for comparison between unigenes and known genes, with the best similarity information chosen as the final annotation.

2.3.4 Expression Abundance and Selection of Differentially Expressed Genes (DEGs)

Gene expression abundance in each sample was estimated by bowtie2 [39] and

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eXpress [40]. The level of gene expression was calculated by the FPKM method [41] (Fragments Per kb per Million reads). Differential expression analysis was carried out using the DESeq [42] R package. The method of negative binomial distribution was used for identifying differentially expressed genes (DEGs) using the $p < 0.05$ level.

2.3.5 Time-Course Sequencing Data Analysis

MasSigPro R package was used for time-course sequencing data analysis (FPKM data) to identify significant differential gene expression profiles in RNA-seq [43]. The number of clusters was tested and found to have the optimal value of 9, and the other parameters used in the software were the default values.

2.3.6 Quantitative real-time q(RT)-PCR analysis

Several DEGs that play important roles in salt stress were chosen to analyze for qRT-PCR analysis to check the consistency with the RNA-seq and qRT-PCR. Primer sequences for DEGs were designed by primer premier 6.0 (see Table 1). The 25S rRNA was quantified as an internal control, and the $2^{-\Delta\Delta Ct}$ method [44] was used to analyze the differential expression. Total RNA was extracted by a RNAprep Pure Plant Kit (Polysaccharides & Polyphenolics-rich) (Tiangen). A TaKaRa PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) was used for removing genomic DNA and reversing transcription. Quantitative RT-PCR was performed with an Applied Biosystems Step One Plus real-time PCR system with TaKaRa TB Green™ Premix Ex Taq™ (Tli RNaseH Plus). BIO-RAD Hard-Shell® PCR Plates (96-well low-profile, semi-skirted) were used.

3. Results

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3.1 Phenotype changes under high salt treatment

The phenotype of Jerusalem artichoke seedlings was recorded during the cultivation of plant material. Under the treatment with 300 mM NaCl, the change from 0 h to 12-h exposure was relatively small, with roots darkening, stem beginning to bend, and leaves starting to wilt. After 24 hours, the salt-induced changes were exacerbated. At 48 h, almost all leaves withered, and roots began to rot and become brittle (Fig. 1).

Fig. 1 Phenotypes of Jerusalem artichoke seedlings after different duration of salt treatment (300 mM NaCl). The seedlings were cultured for 14 days before imposition of the salt treatment.

Table 1 Primers used in q(RT)-PCR

Unigene	Annotation	Sense Primer	Anti-sense Primer
CL5402Contig1	***HSP104***	GCTCTTGTCGTCGCTGCTCAA	ACTTCCAACTGCATCCGCTTCC
CL70841Contig1	***ABF1***	AATCATCCGCCTCTGCCTCCTC	TCTTCGGTGTCGCCTGTTCCTT
CL133346Contig1	***ARR-B***	AGGTGGGATGAACACGGGCTTT	AGTTGACCACTAGCGGCGAGAG
CL69482Contig1	***PP2C***	ACCGCATCCTCCATCTCCTTCC	TCTCCATCACCAGCAGCTCCTC
CL42555Contig2	***HSP20-2***	CCTCCACCTTCACCTCCTCCTT	TCCAGTGTCTTCAGCAACCTCC

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Comp37929_c0_seq2_F	***PsbP***	TCGGTGCTGCTGCTGTTGGTA	CCTCCTTGCTCGGGTTCCACTT
CL29820Contig1	***RP-L10e***	TGACTAAACACGCGGGCAAAGA	TCAGCTCCAGCACATGACAACA
CL862Contig2	***COI-1***	CACGGACACGAGCAAGCACTT	AGGGAAACCACGAGCCGCTAT
CL961Contig1	***RP-L10e-2***	GAGCCTCCTGAGCATGGTGACT	TGCGTGGTGCGTTTGGGAAA
CL82936Contig1	***ABF2***	GCTCAACCACCACCGCTGTATC	TCCCACCTAACCCACCACCATT
CL102748Contig1	None	ATCGCCGCCGCAGAACACTA	ACCTCCATGTCTCCGCTCTTCC
CL5184Contig2	***UGT75C1***	CACCACCATGACAAGCCACCAA	GTGACTTGGACGCCCGTTTGAA
Reference Gene	***25S rRNA***	CTGTCTACTATCCAGCGAAACCA	AGGGCTCCCACTTATCCTACAC

3.2 Annotation of all unigenes

Due to a lack of Jerusalem artichoke genome information and other sequencing data, the function of a large number of unigenes is still unknown. Three databases were used for unigene annotations, including clusters of orthologous groups of proteins (KOG) (Fig. 2A), Gene Ontology (GO) (Fig. 2B), and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Fig. 2C). In KOG annotation, except for the “General function prediction only”, the other most common one was “Signal transduction mechanisms”, hinting at the importance of phytohormones in the response to salt stress (Fig. 2A). In the GO function database, the most common enrichment categories in “Biological Process” were “cellular process” and “metabolic process”, in “Cellular Component” were “cell” and “cell part” and in “Molecular Function” were “binding” and “catalytic activity” (Fig. 2B). In the KEGG database, the groups with most terms were “Metabolism” and “Genetic Information Processing”. The largest number of categories in “Genetic Information Processing” was in “Transcription” and “Translation”,

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indicating that metabolic activity increased strongly in the high salt treatment (Fig. 2C).

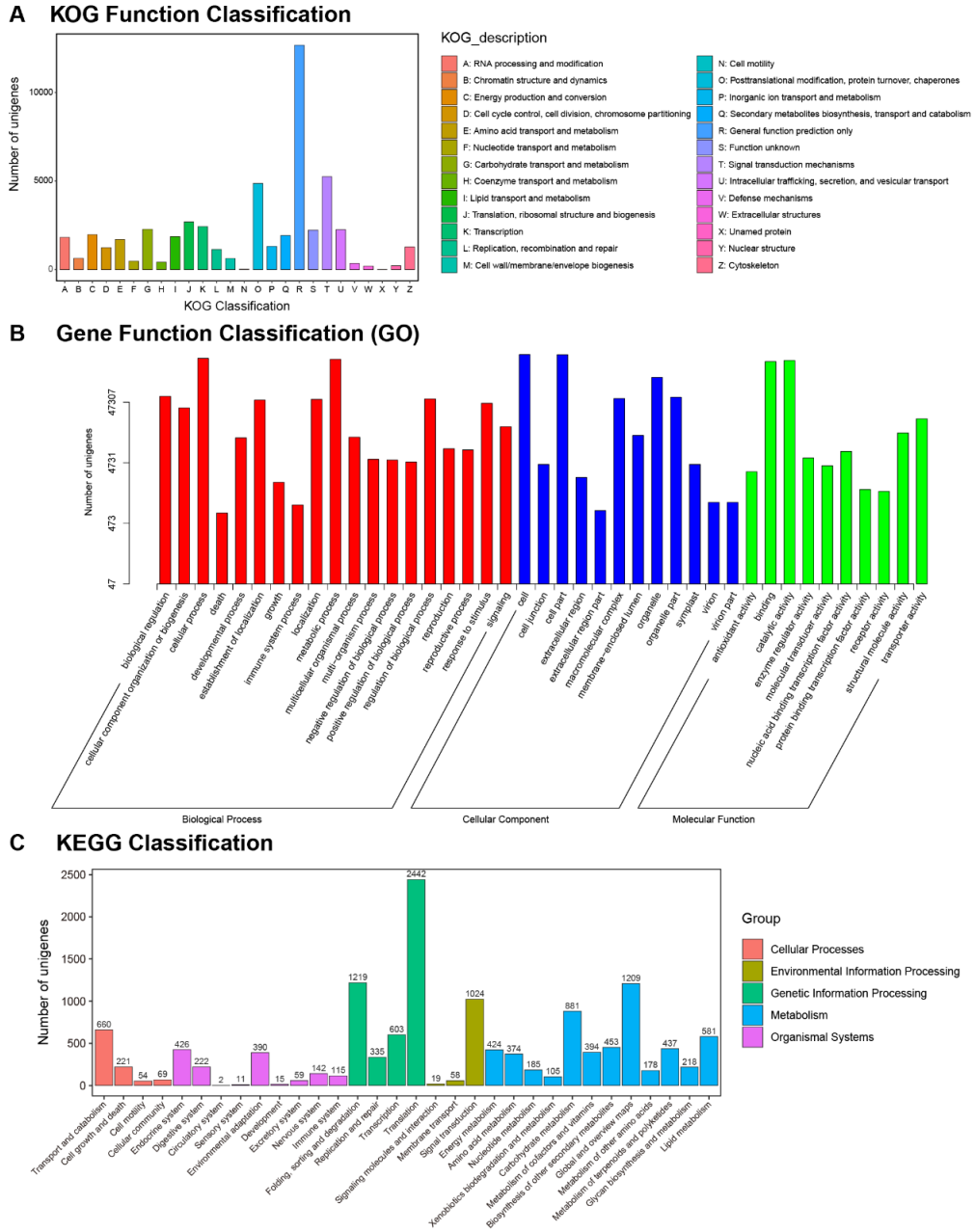

Fig. 2 Unigene annotations in various databases (KOG, GO and KEGG). A: Clusters of orthologous groups (KOG); B: Annotation in the GO (Gene Ontology) database Level2; C: Unigene annotations in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database.

3.3 Differentially expressed genes

Except the two control (0 NaCl) groups of samples, there were eight experimental groups for identifying differentially expressed genes (DEGs). The genes with *P value*

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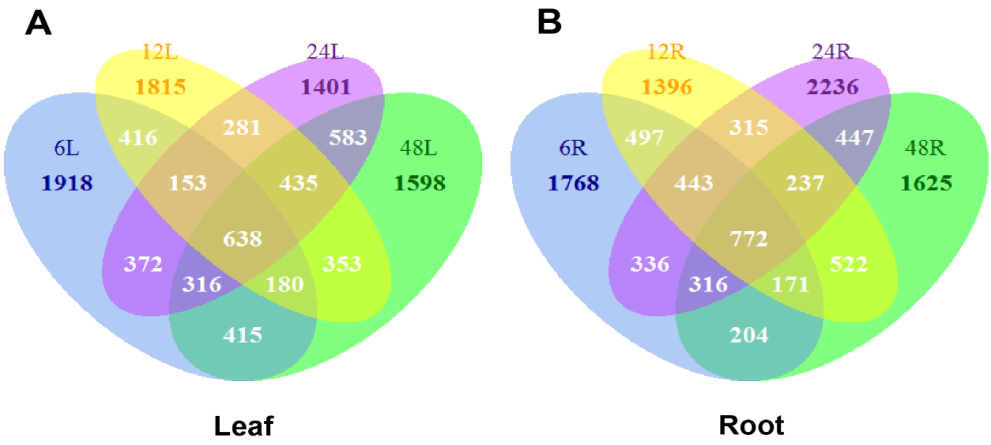
≤0.05 and $|\log2\ (\text{Fold change})| \geq 2$ were defined as differentially expressed genes (DEGs). The numbers of up-regulated and down-regulated unigenes between the experimental and control groups were identified (Fig. 3C). Due to the function of some DEGs being unknown, they needed to be filtered out; then we chose the gene ontology (GO) classification. Finally, 9023 DEGs were identified and compared in Venn diagrams (Fig. 3A & B).



C

Compared	Up-regulated	Down-regulated	Total regulated
0R vs 0L	3652	2106	5758
0R vs 6R	3543	964	4507
0R vs 12R	3247	1106	4353
0R vs 24R	4557	545	5102
0R vs 48R	2955	1339	4294
0R vs 6L	1994	2414	4408
0R vs 12L	2985	1286	4271
0R vs 24L	2549	1630	4179
0R vs 48L	2780	1738	4518

Fig. 3 Venn diagrams of all DEGs in the transcriptome of NaCl-treated plants compared with the control (0 NaCl). A: leaf transcriptome; B: root transcriptome; C: The number of filtered genes with p ≤0.05 and $|\log2\ (\text{fold change})| \geq 2$.

3.4 Time-course sequence clustering and expression patterns of genes related to plant hormone signal transduction

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Since the samples for RNA-seq were classified according to duration of salt treatment, we wanted to understand the similarities or differences in the expression patterns of the spliced unigenes. Finally, we divided the expression patterns of thousands of DEGs into nine categories for each roots and leaves using cluster analysis. After removing the lower expression branches, we finally obtained nine typical expression patterns (Fig. 4).

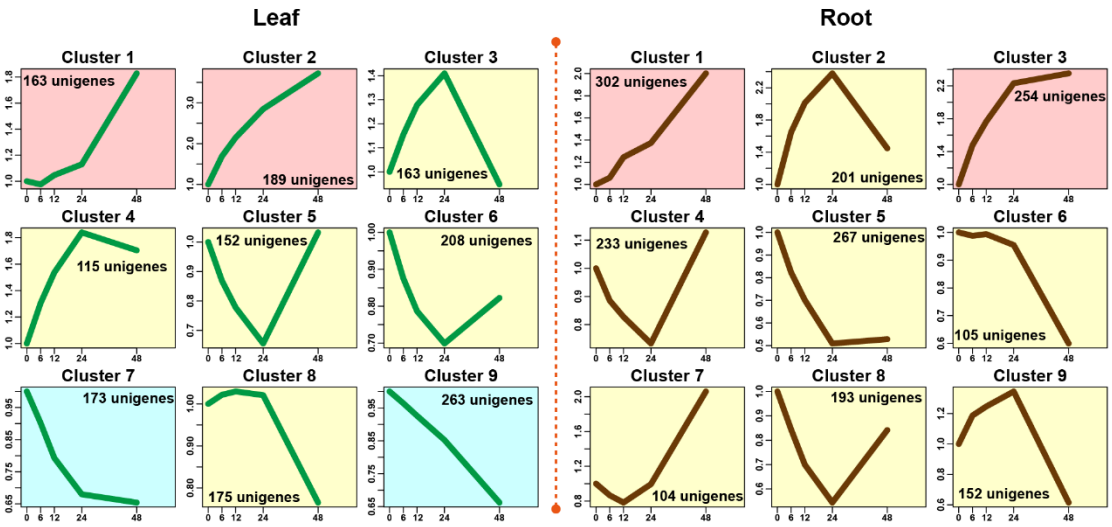

Fig. 4 Nine clusters of time-series expression patterns in roots and leaves of Jerusalem artichoke seedlings under 300 mM NaCl treatment. The FPKM values of all DEGs were used for time-course clustering. The color of line graphs represented the changes in gene expression over time (green: leaves; brown: roots). The rectangle colors represent three different expression models (red: monotonous rising; blue: monotonous falling; yellow: mixed pattern of rising and falling). The number on the graph represents the number of unigenes in the cluster.

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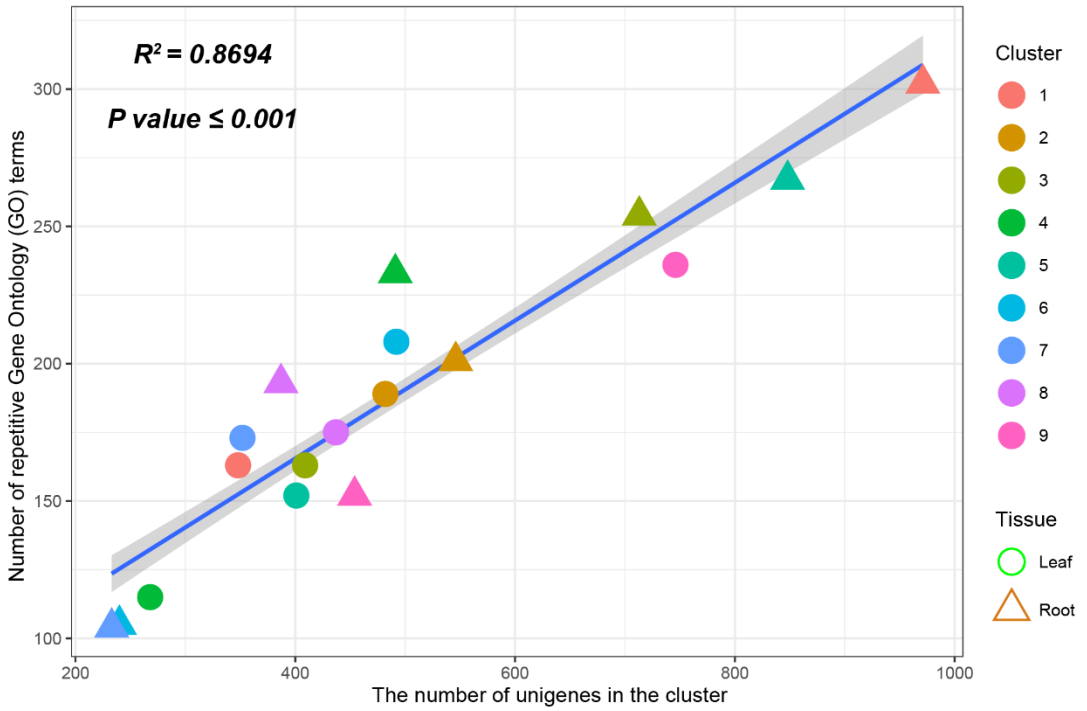

Fig. 5 Regression analysis of the number of genes in the cluster and the number of redundant Gene Ontology (GO) terms.

We chose the terms from the GO database for determining the similarity of the gene functions in the clusters. Interestingly, regardless of whether root or leaf samples were considered, redundant GO terms were positively correlated with the number of genes in the cluster, suggesting that functions were evenly distributed among modules, and modules did not have functional convergence (Fig. 5).

In order to further characterize the key genes in the clusters with the same expression pattern, we increased the screening threshold to $p < 0.01$ to observe the expression patterns and functional distribution (Fig. 6 & Fig. 7).

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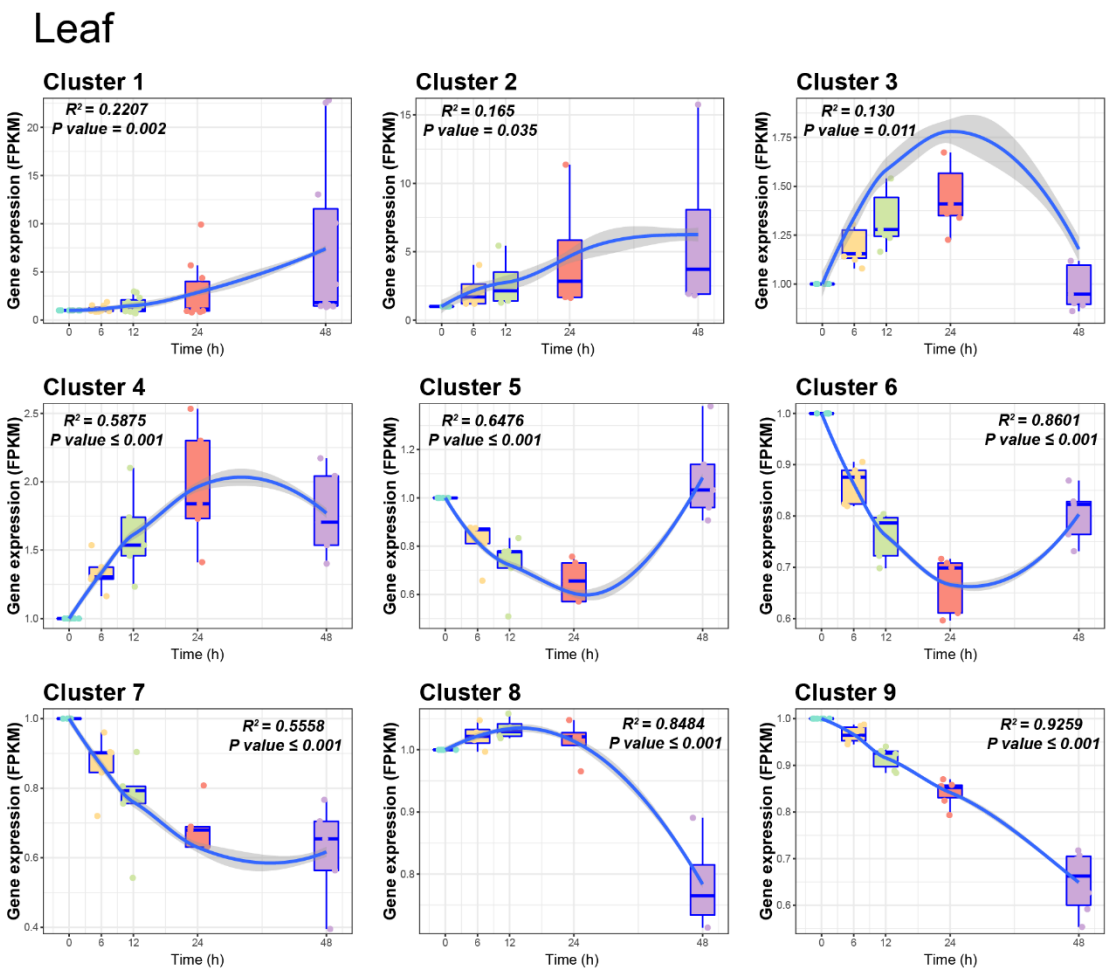

Fig. 6 Clusters of time-course patterns of gene expression in leaves of Jerusalem artichoke seedlings under salt stress treatment (*P value* <0.01). The box plots indicate the changes in gene expression at specific stress-duration times, and the blue curves represent the corresponding regression relationships (with confidence intervals denoted in grey).

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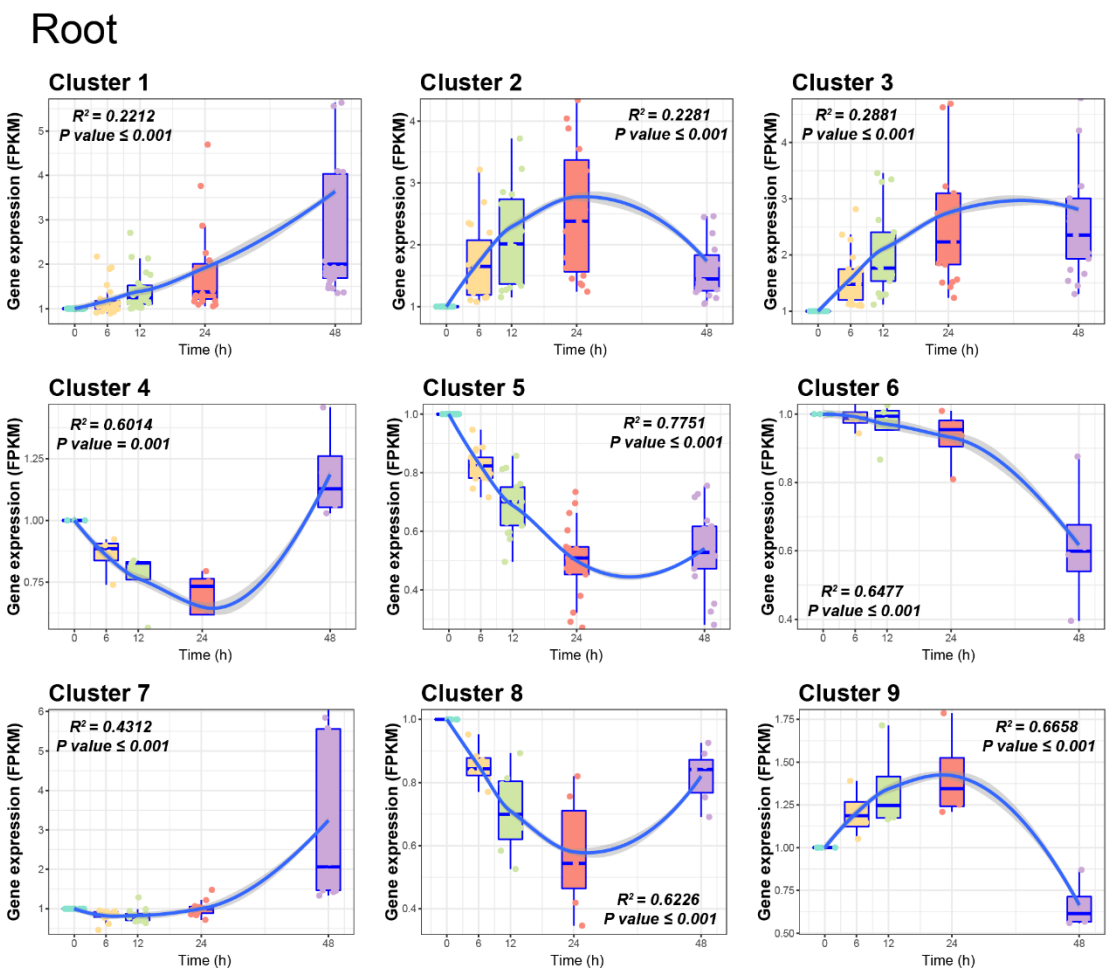

Fig. 7 Clustering of time-representation patterns of nine gene types in roots of Jerusalem artichoke seedlings under salt stress treatment (*P value*< 0.01). The box plot indicates the change in gene expression at that time, and the blue curve is the regression curve of the change in gene expression at five time points.

3.5 Changes in plant hormone signaling in the transcriptome

Changes in plant hormones are critical in a variety of abiotic stress processes. In the KEGG enrichment analysis, we examined the enrichment of plant hormone signaling in each sample (Table 2). Compared with the no-stress controls (0R, 0L), the pathway of plant hormone signal transduction was enriched in almost all samples from NaCl-stressed seedlings.

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We screened all the unigenes that were annotated to the pathways and classified them according to the annotations in the KOG database as the phytohormone type (auxin, cytokinin, gibberellin, abscisic acid, ethylene, brassinosteroid, jasmonic acid, and salicylic acid) and the participating pathways (Table 3 & Fig. 8A). Among the eight different plant hormone signal transduction pathways, unigenes were annotated to all types of phytohormones, indicating that the effects of salt stress on phytohormones were comprehensive and extensive. To explore further the expression patterns of these genes, we showed all the expression patterns of unigenes annotated to phytohormone signal transduction using heat maps (Fig. 8B).

To characterize the expression patterns of different phytohormones, we collected the expression data of all hormone-related genes and performed PCA analysis. The PCA results showed that the expression patterns of different hormones varied (Fig. 9A & Fig. 9B). In order to observe the relationship between different hormones, a co-occurrence network was constructed. Different hormones were in different positions in the network, and auxin, ethylene, jasmonic acid were in the most critical positions in the network (Fig. 9C & Fig. 9D).

Table 2 KEGG enrichment analysis (plant hormone signal transduction).

Compared	***P value***	**FDR**	**Enrichment score**
0R vs 6R	***	***	2.11
0R vs 12R	***	***	2.24
0R vs 24R	0.43	0.85	1.01
0R vs 48R	***	*	1.54
0R vs 6L	***	***	1.87
0R vs 12L	***	***	3.13
0R vs 24L	***	***	2.28
0R vs 48L	***	***	2.59

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P value was derived using a hypergeometric distribution test method to calculate the significance of differential unigene enrichments in each pathway entry. The FDR value is the corrected *P value*. *** *P value* ≤0.001; * 0.01< *P value* ≤0.05. The enrichment score represents a degree of enrichment.

Table 3 Phytohormone signal transduction gene information.

Phytohormone	Function abbreviation	Function full name	Unigene numbers
Auxin	***AUX1, LAX***	auxin influx carrier (AUX1 LAX family)	5
	TIR1	transport inhibitor response 1	2
	IAA	auxin-responsive protein IAA	12
	ARF	auxin response factor	7
	GH3	auxin responsive GH3 gene family	9
	SAUR	SAUR family protein	14
Cytokinin	***AHK2_3_4 (CRE1)***	arabidopsis histidine kinase 2/3/4 (cytokinin receptor)	4
	AHP	histidine-containing phosphotransfer protein	2
	ARR-B	two-component response regulator ARR-B family	2
	ARR-A	two-component response regulator ARR-A family	4
Gibberellin	***GID1***	gibberellin receptor GID1	4
	GID2, SLY1	F-box protein GID2	1
Abscisic acid	***PYL***	abscisic acid receptor PYR/PYL family	9
	PP2C	protein phosphatase 2C	15
	SnRK2	serine/threonine-protein kinase SRK2	7
	ABF	ABA responsive element binding factor	7
Ethylene	***ETR***	ethylene receptor	4
	CTR1	serine/threonine-protein kinase CTR1	3
	MPK6	mitogen-activated protein kinase 6	2
	EIN2	ethylene-insensitive protein 2	2
	EIN3	ethylene-insensitive protein 3	3
	EBF1_2	EIN3-binding F-box protein	6
	ERF1	ethylene-responsive transcription factor 1	10
Brassinosteroid	***BAK1***	brassinosteroid insensitive 1-associated receptor kinase 1	2
	BSK	BR-signaling kinase	6
	CYCD3	cyclin D3, plant	3
Jasmonic acid	***COI-1***	coronatine-insensitive protein 1	1
	JAZ	jasmonate ZIM domain-containing protein	16
	MYC2	transcription factor MYC2	7
Salicylic acid	***NPR1***	regulatory protein NPR1	1
	TGA	transcription factor TGA	4
	PR1	pathogenesis-related protein 1	2

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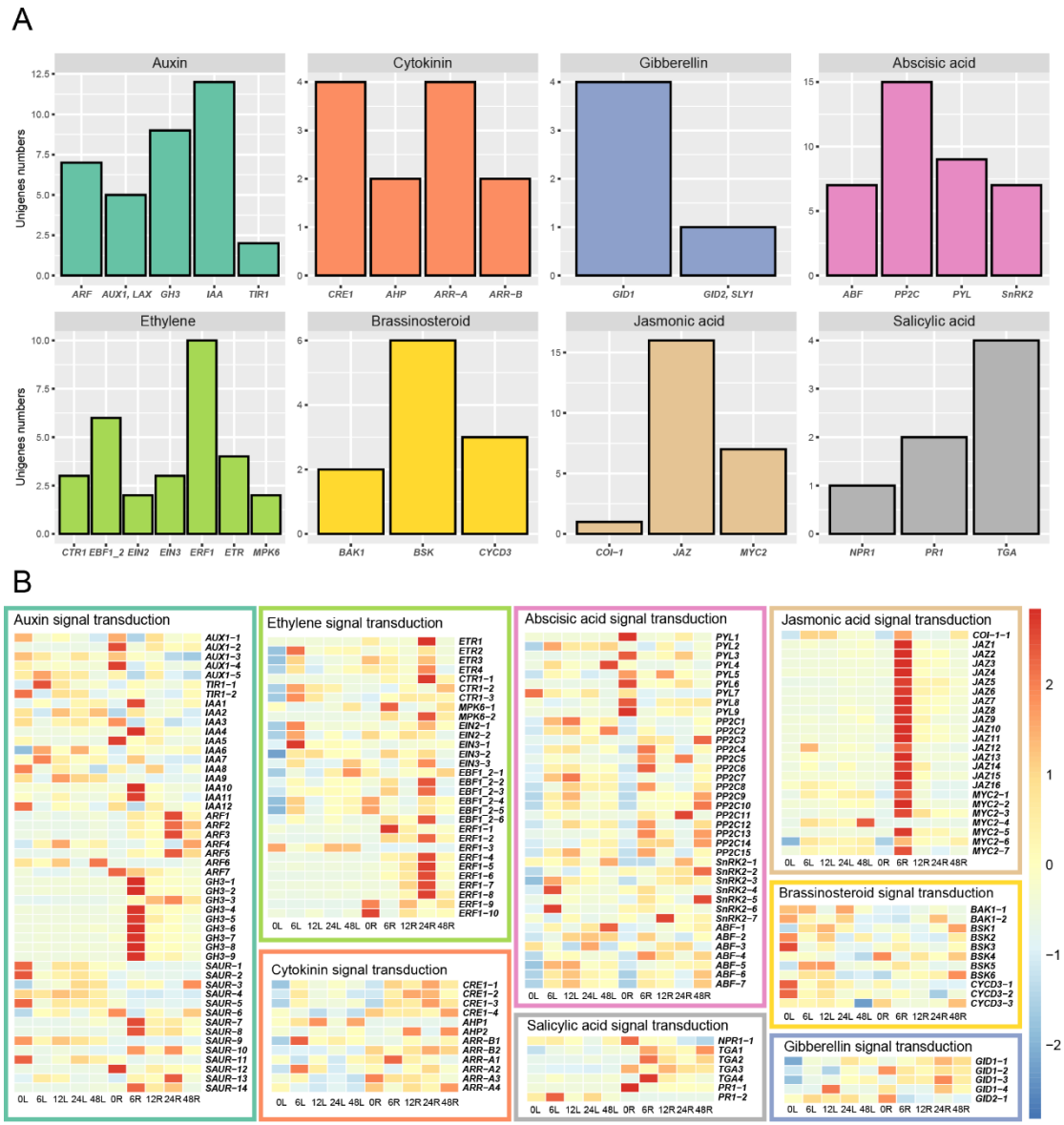

Fig. 8 A: The number of unigenes involved in various plant hormone signal transduction pathways. The x-axis represents function abbreviation in the KEGG database (for details see Table 2); B: An expression heat map of all unigenes involved in plant hormone signaling.

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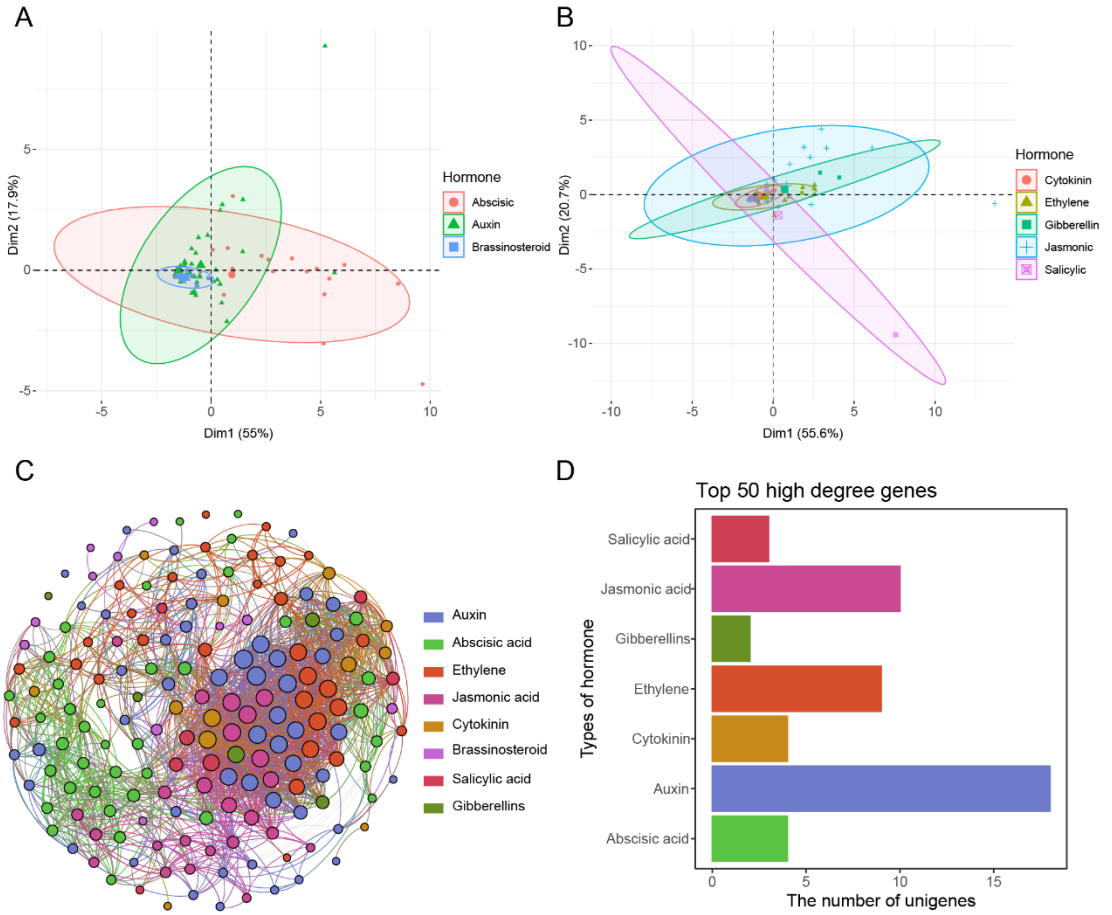

Fig. 9 PCA of all phytohormone-related genes. A includes abscisic acid, auxin and brassinosteroid; B includes cytokinin, ethylene, gibberellin, and jasmonic and salicylic acids. C: Co-occurrence network of eight hormones; D: Top 50 high degree (network) genes distributed in different hormones.

3.6 Real-time Quantitative PCR verification

In order to verify the entire transcriptome experiment and time-course analysis, we randomly selected 12 genes for verification by qPCR. Similarly to the sample set for transcriptome sequencing, we prepared samples of Jerusalem artichoke roots and leaves for gene expression at different durations of 300 mM NaCl stress treatment (0 h, 6 h, 12 h, 24 h, and 48 h). The results showed that the qPCR experiment had good reproducibility, and the trends in gene expression were consistent with those revealed

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by the transcriptome sequencing, confirming the effectiveness of transcriptome sequencing (Fig. 10).

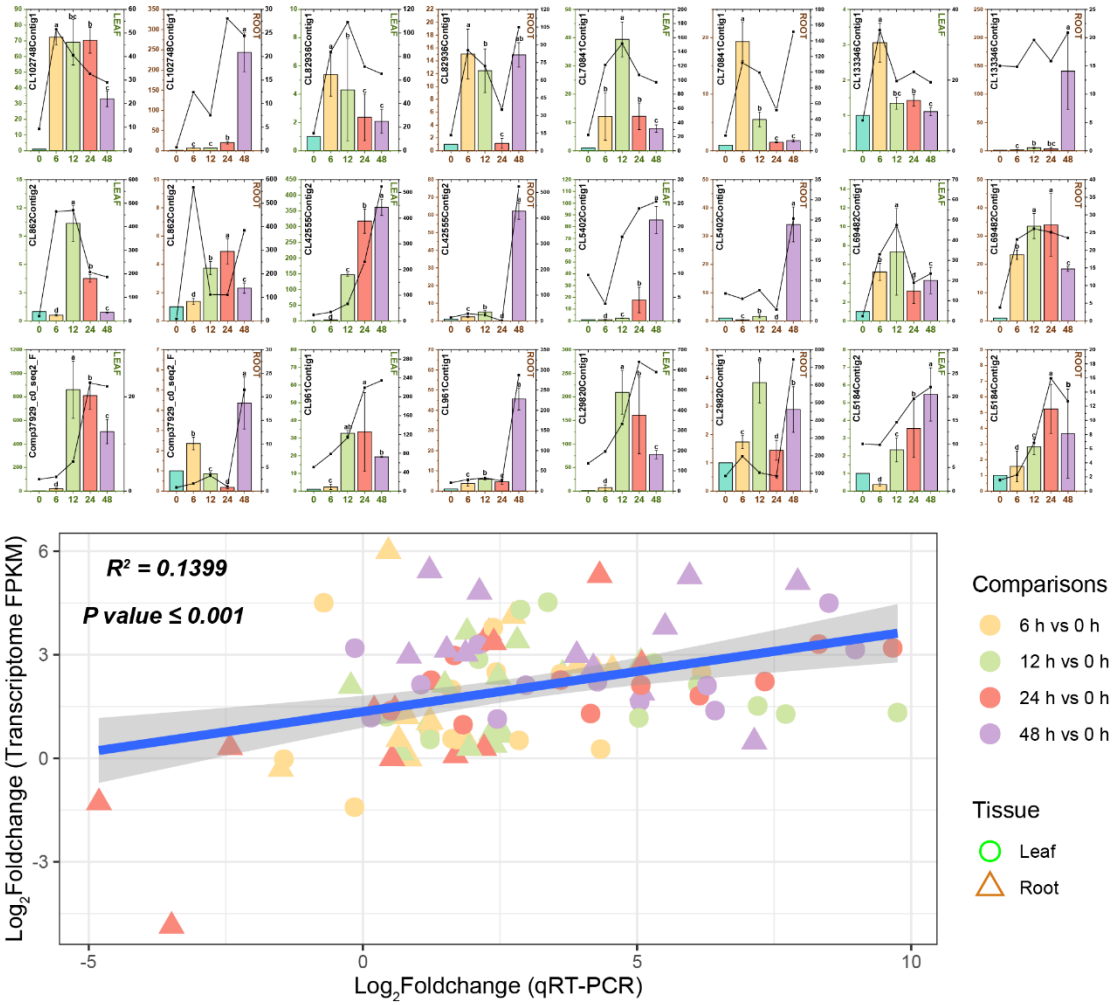

Fig. 10 The qPCR validation of 12 randomly selected genes. The histograms in each plot represent the results of real-time qPCR, and the line graph represents the results of RNA-seq. The test method for the difference of qPCR results at different times used one-way ANOVA ($p < 0.05$). Differences among samples are represented by different letters.

4. Discussion

4.1 Time-course expression clustering and functional similarity

Traditional gene expression studies often focus on the difference between the control samples and the treatment samples. However, gene expression is strongly dependent on the duration of the treatment. Our time-course results showed that the expression of many genes was not linear with increased duration of salt stress (Fig. 3 & Fig. 6 & Fig. 7).

Many algorithms and programs have been developed to analyze time series of gene expression [45], enabling characterization of the dynamics of gene expression with increased treatment duration [43,46,47]. In the present study, we chose masigPro to cluster the expression data [43], and the parameter k=9 to fully display the different expression patterns of all genes in either root or leaf samples. Among the nine expression clusters, some exhibited continuously rising or falling expression, whereas others reached the highest or lowest expression at a specific stress duration (6, 12 or 24 h) (Figs. 5, 7 & 8).

The idea of predicting gene function through gene expression clustering to imply a possible relationship between temporal expression and gene function similarity [48] was not supported in the present study on Jerusalem artichoke (Fig. 5), but it is foreseeable that future studies of time-gradient transcriptomes may increase the number of sampling time points to explore changes in expression due to stress treatment in finer detail, even change discrete time point observations into continuous time observations.

4.2 Changes in signal transduction pathways of eight plant hormones

Plant hormones play a key role in the growth and development as well as in coping with various abiotic stresses [49–53]. In the present study, the pathways of plant

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hormone signal transduction under salt stress were enriched in eight comparison groups (based on plant organ and stress duration), showing complexities of the phytohormonal responses to salt stress (Fig. 3) [54]. In this research, genes related to signal transduction of all eight types of plant hormones were involved, suggesting that phytohormones regulate the transcription dynamics in Jerusalem artichoke in the early stage of high-salt stress (Fig. 11).

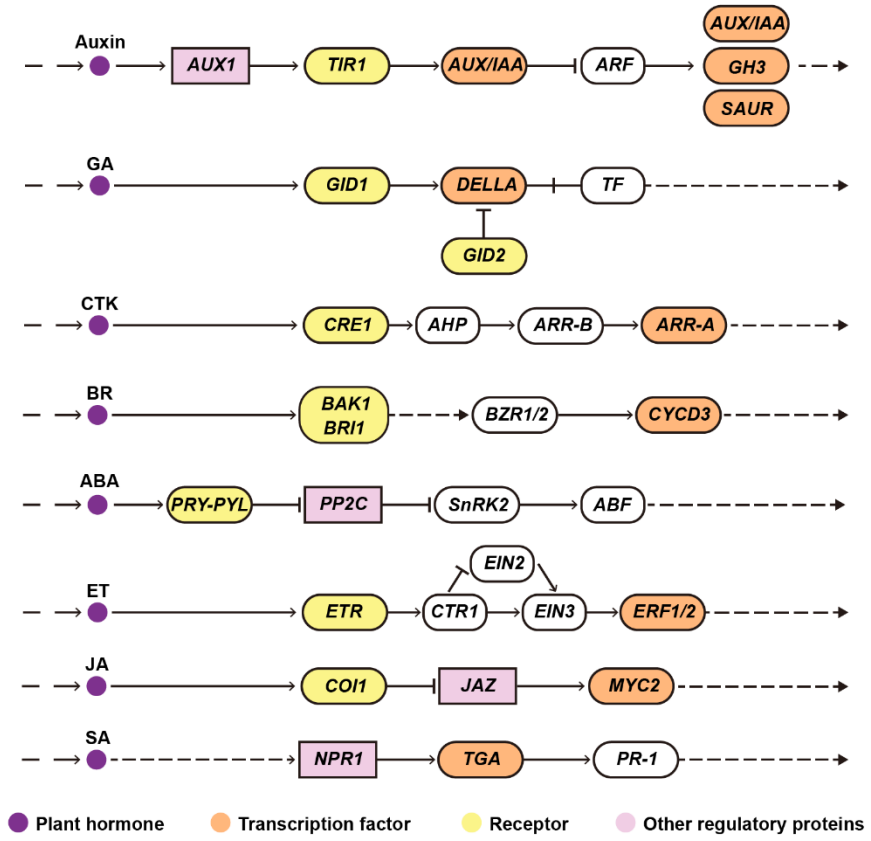

Fig. 11 Eight major plant hormone signal transduction pathways. Different component types were shown in different colors. Purple dots: different phytohormones; Squared boxes: regulatory function genes; Round boxes: genes with no regulatory function. Solid line: direct effect; Dashed line: indirect effect; Pointed arrows: molecular interaction or relationship; Blocked arrows: inhibitory effect; Crossed line: protein separation action. GA: gibberellic acid; CTK: cytokinin; BR: brassinosteroid; ABA: abscisic acid, ET: ethylene; SA: salicylic acid; JA: jasmonate.

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Among the plant hormones, ABA is one of the most widely studied ones. *PYL* (from the abscisic acid receptor *PYR / PYL* family) is a protein that mediates ABA signaling, acting as a receptor for ABA that regulates a series of downstream responses. In the present study, seven of the nine genes belonging to the *PYL* family were highly expressed in roots at 0 h, and the expression was decreased with increasing duration of salt stress, indicating that the *PYL* expression was suppressed by the salt treatment. *PP2C* (Type 2C protein phosphatases) is also a key link in the ABA signaling pathway, with *PYR / PYL* controlling ABA signaling by inhibiting *PP2C* [55]. In our results, it was found that the expression of *PYL* in the early stage of root stress (0-6 h) was opposite to the expression of *PP2C*, which also confirmed the existence of this inhibitory effect, which gradually disappeared over time. Hence, the inhibition of *PP2C* occurred in the early stage of salt stress (Fig. 8B), which is consistent with the results of the *Arabidopsis* salt stress expression profile [26].

In the auxin signaling pathway, *GH3* (Gretchen Hagen3) was highly expressed only in roots in the early stage of salt stress (6 h) (Fig. 8B). The *GH3* gene is involved in auxin homeostasis by catalyzing auxin conjugates and binding free indole-3-acetic acid (IAA) to amino acids. The *GH3* mRNA has been shown to be one of the most relevant early molecular markers of primary auxin response [56,57]. The *GH3* gene family in maize responded to several abiotic stresses and stress-related phytohormones [58].

Ethylene has long been recognized as a stress-related phytohormone that regulates different levels of salt response, including membrane receptors, components in the

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cytoplasm, and nuclear transcription factors [59], with the ethylene receptor (*ETR*) being the first component of ethylene signaling. Overexpression of the *Nicotiana tabacum* type II *ETR* homologue *NTHK1* in *Arabidopsis* increased salt sensitivity, and the mutants of *EIN2* (ethylene-insensitive protein 2) also had increased sensitivity to salt stress [60], suggesting that *EIN* and *ETR* were important in a response to salt stress in the ethylene signaling pathway. In non-model plant mulberry (*Morus* sp.), the expression of the gene encoding *EIN3* protein was induced by salt stress. Over-expression of that gene in Arabidopsis enhanced its salt resistance [61]. Although there are many reports on ethylene and salt stress, the temporal and tissue-specific expression of genes involved in ethylene signal transduction under salt stress is still poorly understood. In the present study, the expression of most genes in the ethylene signaling pathway was increased in roots after 24 h of salt exposure. In leaves, except for *ERF*, most genes involved in ethylene signaling were expressed in the early stage of stress treatment (6 h), and there was a tendency of increased expression with the duration of salt stress (peaking for most genes at 24 h) (Fig. 8B).

The expression of *Pohlia nutans* L. jasmonate ZIM-domain gene *PnJAZ1* was induced by salt stress [62]. Transgenic *Arabidopsis thaliana* overexpressing *PnJAZ1* had increased tolerance to salt stress and decreased ABA sensitivity during early development [63]. In the present study, up to 16 *JAZ* genes were annotated into the jasmonic acid signaling pathway (Fig. 8B). The Arabidopsis *JIN1 / MYC2* mutant *jin1* (jasmonate insensitive 1) had significantly lower resistance to salt stress and lower proline and sugar content than the wild type. The concentration of flavonoids

demonstrated that *MYC2* was involved in controlling the plant protection systems under salt stress [64]. In the present study, the expression patterns of differentially expressed genes (DEG) in the jasmonic acid signaling pathway were similar, being highly expressed in roots in the early stage (6 h) of salt stress, but not at other times (Fig. 8B).

The expression of genes related to other four plant hormone (brassinosteroids, gibberellins, cytokinins, and salicylic acid) signal transduction did not show distinct patterns (Fig. 8B)

4.3 Time-course expression of phytohormone signal transduction genes in Jerusalem artichoke

Phytohormonal changes were large in the initial stage of plant stress. The results of the enrichment analysis (Table 3) were important in explaining the role of phytohormone signal transduction during exposure to high-salt stress. We characterized the expression patterns of the genes related to phytohormonal signal transduction in the time-course of salt stress exposure. This type of study is rare [65,66].

In our salt stress study of Jerusalem artichoke, the expression pattern of the phytohormone signal transduction-related genes was highly dependent on the type of phytohormone. Similar findings were reported in *Arabidopsis* subjected to moderate dehydration stress [67]. The complex signal transduction network generated by the interaction of different phytohormones was also found under copper toxicity treatment, explaining the differential expression patterns of various phytohormones [68]. The variable temporal expression patterns of the phytohormone metabolism genes were reported during the process of water absorption in *Arabidopsis* seeds [69]. The above-

mentioned literature, together with our own findings, indicate the relevance of characterizing the time-course of the expression of phytohormone-related genes and functions.

5. Conclusions

This was the first time-series analysis of the Jerusalem artichoke transcriptome under salt treatments. Under the impact of high salinity (300mM) environment, Jerusalem artichoke in the seedling stage was difficult to survive for a long time, and the phenotype was severe in the short term. Based on the expression of genes on the time scale, we found that the distribution of gene functions in time is relatively even. Upregulation of the phytohormone signal transduction had a crucial role in the response of Jerusalem artichoke seedlings to salt stress, with genes related to each phytohormone showing a similar expression pattern during the first 48 h of exposure to salt stress. The genes of four of the eight hormones (abscisic acid, auxin, ethylene, and jasmonic acid) had the most obvious change pattern. The revealed dynamics of genes related to plant hormone signal transduction under salt stress represent a basis for further characterization of the downstream gene regulation.

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Acknowledgements

This work was financially supported by Forestry Science and Technology Innovation and Extension Project in Jiangsu Province (No. LYKJ[2019]07), the National Key Research and Development Program of China (2016YFC0501207), the National Key Project of Scientific and Technical Supporting Programs funded by the Ministry of Science & Technology of Jiangsu Province (BE2018387 and BE2017310-2), and the Inner Mongolia Autonomous Region Science and Technology Achievement Transformation Special Fund Project [2020CG0057]. Server resources required for bioinformatics analyses were supported by the high-performance computing platform of Bioinformatics Center, Nanjing Agricultural University.

Author contributions

ZSZ, XHL and YY designed the experiments. YY, XMG, WCR and YY performed the experiments. YY, JYW, ZSZ and XHL analyzed the data. YY, ZSZ, XHL and ZR wrote and revised the manuscript. All authors read and approved the fifinal manuscript.

Data availability statement

The raw data of RNA-seq have been uploaded to NCBI. The SRA data have not yet been released.

Supplementary data

 doi:10.20944/preprints202111.0565.v1

Table S1 Sample groups, tissue types and duration of exposure to 300 mM NaCl.

Fig. S1 The results of Illumina RNA sequencing. A: PCA of 10 root (R) and leaf (L) samples.