Transcriptome Analysis of Differentially Expressed Genes in Wild Jujube Seedlings under Salt Stress

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ABSTRACT. Wild jujube (*Ziziphus acidojujuba*) and cultivated jujube (*Ziziphus jujuba*) belong to the family Rhamnaceae. Jujubes have marked drought- and salt-tolerant properties. After salt stress, wild jujube seedling growth was inhibited and photosynthetic efficiency was reduced. A bioinformatics approach was used to analyze the transcriptomics data from wild jujube seedlings grown under salt stress, and the genes differentially expressed under the salt stress were identified to provide a theoretical basis for the development and use of wild jujube plantations in salinized soil. The transcriptome sequencing from leaves of wild jujube seedlings was carried out using second-generation sequencing technology. The effects of salt stress on the differential expression of photosynthesis-related genes in wild jujube seedlings were analyzed. Transcriptome sequencing revealed a total of 5269 differentially expressed genes (DEGs), of which 2729 were up-regulated and 2540 were down-regulated. DEGs were mainly enriched with respect to photosynthesis, photosynthetic antenna proteins, glyoxylic acid and dicarboxylic acid metabolism, linolenic acid metabolism, cysteine and methionine metabolism, and porphyrin and chlorophyll metabolism. Among them, the photosynthesis pathway-related DEGs were most highly enriched. Further analysis of porphyrin and chlorophyll synthesis and photosynthesis-related pathways revealed that they were significantly enriched by 97 photosynthesis-related DEGs. The DEGs in the photosynthesis and photosynthetic antenna protein pathways were down-regulated, whereas the DEGs glutamyl-tRNA reductase (HEMA), ferrochelatase (HEMH), and pheophorbide a oxygenase (PAO) in the porphyrin and chlorophyll synthesis pathways were up-regulated, with the remainder being down-regulated. The nuclear gene encoding Rubisco, the key enzyme in the photosynthetic carbon fixation pathway, was also down-regulated. The results showed that the photosynthetic rate of wild jujube seedlings decreased following exposure to salinity stress, an effect that was related to the increased synthesis of 5-aminolevulinic acid and heme, and the up-regulation of expression of a gene encoding a chlorophyll-degrading enzyme, and was related to the down-regulation of gene expression in photosynthesis-related pathways such as light energy capture and carbon fixation. Selection of nine DEGs related to photosynthesis and chlorophyll biosynthesis by quantitative real-time-PCR confirmed that expression changes of these nine DEGs were consistent with the transcriptome sequencing results.

Salt damage is one of the main abiotic stress factors in agricultural production, seriously restricting crop production and sustainable agricultural development (Zhang et al., 2014). At present, the area of salinized arable land in China is nearly 1 billion hectares, and it is increasing year by year (Hu et al., 2016). High concentrations of salt can cause secondary damage to plants, including osmotic stress, oxidative stress, and ionic toxicity, which seriously affects the yield and quality of crops. Jujube is the first forest fruit industry in Xinjiang Province in northwestern China (Yang et al., 2016), but many jujube plantations suffer from high salinity and alkalinity, resulting

in poor seedling establishment and reduced growth. Jujube trees cultivated on saline-alkaline soils exhibit different degrees of salt damage, affecting the yield and quality of the fruit and ultimately the development of the jujube industry (Wang, 2014). Grafting is an important route by which to improve the adaptability and salt tolerance of jujube cultivars. The salt tolerance of wild jujube cultivars currently depends mainly on the use of tolerant rootstocks. Wild jujube often is used as an excellent rootstock for jujube.

Photosynthesis is an extremely important metabolic process in plants, and salt stress is an important factor affecting photosynthesis (Feng et al., 2005). Salt stress can directly affect the growth of plants and indirectly affects it by inhibiting photosynthesis; the greater the salinity stress and the longer the exposure period, the more obvious the inhibitory effect (Drew, 1992; Jeschke et al., 1992; Munns et al., 1982). Under salt stress, plant chloroplast enzyme activity increased and promoted chlorophyll degradation (Li et al., 2009). Zhang (2012) pointed out that long-term salt stress will lead to salt accumulation and photosynthesis decline in leaves. However, because of a lack of understanding of the molecular mechanism

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related to salt-induced inhibition of photosynthesis of wild jujube and the lack of effective salt-tolerant genes in wild jujube, the most appropriate strategy would appear to be the use of genetically modified (GM) jujube expressing salt tolerance. Therefore, in-depth study of the molecular mechanism of salt tolerance in jujube grown in saline-alkali areas of Xinjiang, and the screening for and mining of salt-tolerance genes, should open avenues to help to improve the salt tolerance of wild jujube by GM technology and to provide an important strategy for the development of jujube tree production and cultivation.

Plants can overcome salt stress by changing the regulation of gene expression and hence that of the relevant physiological and biochemical metabolic pathways. Atienza et al. (2004) analyzed the transcriptomics data of barley (*Hordeum vulgare*) under salt stress and showed that it caused the expression of chlorophyll-binding, protein-related genes in barley to change significantly. Song et al. (2017) analyzed the transcriptome data of leaves of sea buckthorn (*Hippophae rhamnoides*) exposed to 300 or 0 mM NaCl for 14 d and found that the DEGs involved in six pathways were mainly enriched with respect to photosynthesis; photosynthetic biological carbon fixation; pyruvate metabolism; phenylpropanol synthesis; glyoxylic acid metabolism; and serine, glycine, and threonine metabolic pathways and found that the DEGs associated with photosynthetic pathways were the most abundant of all the enrichment pathways. At present, research into the salt tolerance of wild jujube is based mainly on the physiological characteristics of wild jujube under salt stress (Ma et al., 2018; Zhao et al., 2018), and little is known of the molecular mechanism of salt resistance, as related to photosynthesis. There has been little research on transcriptome analysis of wild jujube under salt stress. In the present study, wild jujube was exposed to salt stress in the form of 150 mM NaCl, a concentration identified in a previous study (Li, 2017), and the genes differentially expressed between the salt stress [salt (150 mM NaCl)] and control [CK (0 mM NaCl)] were identified and mined to analyze salinity response in wild jujube at a molecular level. The results from these studies will provide information on wild jujube genes associated with salinity response, providing a theoretical basis for salt-tolerance mechanisms in wild jujube and their exploitation to allow the use and development of jujube in areas with salinized soils.

Materials and Methods

TEST MATERIALS. The test material was facultative wild jujube seeds, which were provided by Yulin Jia County in northern Shaanxi. The chemical 5-aminolevulinic acid (ALA) was supplied by Sigma-Aldrich (St. Louis, MO).

CULTURE CONDITIONS. Seed treatment and plant culture. First, the wild jujube seeds were surface sterilized with 0.5% potassium permanganate solution for 30 min, and then the sterilized seeds were thoroughly rinsed with sterile distilled water five to six times. Finally, the treated seeds were placed in a germination box, 40 seeds per box, with two layers of wet filter paper, and the germination box was placed in a 26 °C artificial climate chamber (RXZ intelligent type; Ningbo Jiangnan Instrument Factory, Ningbo, China) under 24 h darkness. When the seed cotyledons were exposed, the incubator lighting was adjusted to a light intensity of 12,000 lx, with light for 14 h, darkness for 10 h, relative humidity of 65% to 67%, and temperature of 26 to 28 °C (same for day and night).

When the seedlings had produced two true leaves, wild jujube seedlings with similar growth status were selected and suspended in a 2-cm-thick foam board over a hydroponic box [19 × 13 × 11 cm (length × width × height)], containing 500 mL half-strength Hoagland's nutrient solution, which was changed every 3 d to ensure the normal growth of the plant.

TREATMENT OF TEST MATERIALS. Uniformly sized wild jujube seedlings grown to the six-leaf stage were selected for experimental study. Two treatments were set up, namely, CK (Hoagland solution) and salt (Hoagland solution + 150 mM NaCl). For the salt stress, to avoid the salt stimulatory effect (Li, 2017), the salt concentration was gradually increased to the target concentration with a gradient of 50 mM NaCl per day; the day on which the NaCl concentration reached 150 mM is recorded as day 0. Each treatment was repeated three times (CK-1 to CK-3 and Salt-1 to Salt-3) and with three seedlings per replicate. The physiological index and photosynthetic rate were measured on day 3 of treatment. On the same day, leaves from each treatment were snap-frozen in liquid nitrogen and stored at -80 °C before RNA extraction and transcriptome sequencing.

MEASUREMENT OF CHLOROPHYLL AND PHOTOSYNTHESIS. Chlorophyll concentration was determined by a previously published protocol (Li, 2000). The leaves of wild jujube seedlings at the sixth leaf stage were harvested; any dirt was wiped away, and the seedling tissue was cut and mixed. A sample (0.3 g) of freshly cut tissue was taken and repeated three times for each treatment; the tissue was put into mortar, a small amount of quartz sand was added, 2 to 3 mL of 95% ethanol solution was added, and the sample was ground into a homogenate before adding 10 mL ethanol, and continuing to grind the tissue. The extract stood for 3 to 5 min, the filtrate was collected through a filter paper, and decanted into a 25-mL brown volumetric flask, and the volume was adjusted with ethanol and mixed thoroughly. The absorbance of the extract was measured using a spectrophotometer (ultraviolet-2600/2700; Shimadzu Corp., Kyoto, Japan) at wavelengths of 665 and 649 nm.

The photosynthetic rate was measured using a portable photosynthetic measurement system (CIRAS-3; PP Systems International, Amesbury, MA), and the third and fourth fully expanded leaves from the base were selected for assay. The measurements were repeated three times on different plants. Photosynthetic gas exchange parameters were determined from 1000 to 1400 hr on day 3 after salt stress.

STATISTICAL ANALYSIS. Data entry and processing were performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA), and treatment means were compared by conducting an analysis of variance using SPSS software (version 17.0; IBM Corp., Armonk, NY).

RNA EXTRACTION AND LIBRARY CONSTRUCTION. Leaves of CK and salt stress six-leaf stage wild jujube seedlings were snap-frozen in liquid nitrogen and stored at -80 °C. RNA extraction, quality control, database construction, and Illumina Hi Seq™ 2500/Mi Seq™ sequencing (Illumina, San Diego, CA) were carried out by Compass Biotechnology Inc. (Beijing, China). Total RNA was extracted using TRIzol reagent (Invitrogen, Beijing, China). The purity and concentration of RNA were determined using a spectrophotometer (NanoDrop 2000C; Thermo Fisher Scientific, Waltham, MA) and a fluorometer (Qubit; Invitrogen, Carlsbad, CA), respectively. After the sample was tested, the eukaryotic cell mRNA was enriched with Oligo (dT) using magnetic beads. After enrichment was completed, the fragmentation buffer (ABclonal Technology,

Woburn, MA) was added to break the mRNA into short fragments. Using mRNA as template, the first strand of cDNA was synthesized with six-base random primers; then, the second strand of cDNA was synthesized. The double-stranded cDNA was purified by AMPure XP beads (Beckman Coulter, Brea, CA). The purified double-stranded cDNA was end-repaired, A-tailed, and ligated to the sequencing linker. Fragment size selection of double-stranded cDNA was performed by AMPure XP beads, and the selected double-stranded cDNA was then PCR-enriched to construct a cDNA library (Yun et al., 2018).

SEQUENCE ALIGNMENT OF SEQUENCING DATA. The raw data files obtained by high-throughput sequencing (Illumina Hi Seq™2500/Mi Seq™) were converted to the original sequenced reads. The CASAVA (Illumina, San Diego, CA) base calling analysis tool was used because it can reduce the incidence of sequencing errors. It can clean readings with low quality and jointed reads to improve the quality of information analysis. Clean reads served as the basis for subsequent analysis. The clean data of each sample were compared with the *Z. jujuba* reference genome (Ziziphus jujuba Genome Project, 2015), using STAR software (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Alignment was performed, and the number of fragments was calculated for each gene.

DIFFERENTIAL GENE SCREENING AND ENRICHMENT ANALYSIS. The raw data were normalized using the standardized method in DESeq2 in R (Love et al., 2014). Gene expression abundance was calculated using the “reads per kilobase of transcript per million mapped reads” (RPKM) method (Reiner et al., 2003), that is, the number of reads matched to the exon region of length 1 kb of a specific gene in each 1 million sequencing sequences (reads). The formula used for its calculation is as follows:

$$\text{RPKM} = \frac{R \times 10^9}{T \times L},$$

where RPKM is the expression level of gene A, *R* is the number of reads aligned to gene A, *T* is the total number of reads aligned to all genes, and *L* is the base number of gene A. The RPKM method is used to calculate gene expression abundance, which can eliminate the influence of the sequencing amount difference and gene length. The gene expression amount calculated by this method can directly realize the comparison of gene expression differences among different samples. According to the gene expression abundance, namely, RPKM value, the fold change (FC) of the gene among different samples was calculated. In this study, according to bioinformatics, genes with a *P* < 0.05 and FC [the ratio of FC = RPKM (Stress)/RPKM (CK)] expression of ≥2 times were defined as DEGs, namely, |log₂ (FC)| > 1 and corrected *P* < 0.05. Volcanic maps are used to show the overall distribution of DEGs.

DIFFERENTIAL GENE GO ENRICHMENT ANALYSIS. *Gene Ontology* (GO) is a database of international standard classifications of gene functions established by the Gene Ontology Consortium (Ashburner et al., 2000). GO has three ontologies in total, one describing the molecular function of the gene, second is the biological process involved, and third is the cellular component. The software used by us in the GO enrichment analysis is clusterProfiler (Yu et al., 2012). The principle of GO enrichment analysis is hypergeometric distribution. The hypergeometric distribution relationship among these differential genes and some specific branches in the GO classification are calculated according to the selected differential genes. A probability

is obtained by a hypothesis test to determine whether the differential gene is enriched in the GO, and functional classification statistics are performed for the genes. In this experiment, the data sets of differential genes screened were used as test data sets, and a *P* < 0.05 was used as the screening standard. Using 5269 DEGs as background data sets, GO function enrichment analysis was performed to obtain a GO functional classification with significant enrichment in DEGs.

DIFFERENTIAL GENE KEGG ENRICHMENT ANALYSIS. *Kyoto Encyclopedia of Genes and Genomes* (KEGG) is a systematic analysis of gene function and genomic information database collection. It helps researchers to study genes and expressions as a whole network (Wixon and Kell, 2000). Significant enrichment analysis of pathways involves the use of the KEGG pathway as a unit to apply a hypergeometric distribution test to find a pathway that is significantly enriched in DEGs compared with the transcriptome background. The DEGs were compared with the KEGG database for path analysis by using DAVID (Laboratory of Human Retrovirology and Immunoinformatics, Frederick, MD) software (Huang et al., 2009). The number of DEGs contained in each pathway was counted, and the hypothesis test was calculated by hypergeometric test; the smaller the *P*, the higher was the degree of enrichment. The specific calculation formula used was as follows:

$$p = 1 - \sum_{x=0}^{x-1} \frac{\binom{M}{x} \binom{N-M}{K-x}}{\binom{N}{K}}$$

where *N* represents the number of all genes, *K* represents the number of all DEGs in *N*, *M* represents the number of genes aligned to a particular KEGG metabolic pathway, and *x* is the number of DEGs aligned to a particular KEGG metabolic pathway. The KEGG enrichment screening standard was *P* < 0.05. Among the DEGs between the two samples, the KEGG metabolic pathway corresponding to this condition is a significantly enriched metabolic pathway. Pathway saliency enrichment analysis was performed, which indicated that the DEGs were mainly involved in photosynthesis and porphyrin and chlorophyll metabolism.

QUANTITATIVE REAL-TIME-PCR (qPCR) ANALYSIS. To verify the reliability of the photosynthetic transcriptome data of jujube seedlings under salt stress, we selected nine differential genes that relate to photosynthesis and porphyrin and chlorophyll metabolic pathways. They are LHCB4, LHCB2, and LHCA4 in the photosynthetic antenna protein pathway, PsbO in photosystem II, PetF in the light and electron transport chain, and por, HEMH, PAO, and HEME in the porphyrin and chlorophyll synthesis pathways. The expression of nine differential genes in CK and salt stress groups was verified by qPCR. According to the selected coding sequences of the nine candidate genes, qPCR primers were designed using Vector NTI 10 software (Informax, Frederick, MD). ACT1 is an internal reference gene (Bu et al., 2016). The designed primers were synthesized by Bioengineering Co., Ltd. (Shanghai, China). The primer sequences are shown in Table 1.

RNA REVERSE TRANSCRIPTION AND REAL-TIME PCR. An aliquot (1 μg) of RNA was taken from each sample, and the cDNA of each sample was obtained using the ABM Reverse Transcription Kit (ABM, Zhenjiang, China), following the

manufacturer's instructions. We used qPCR with the Green Realtime PCR Master MIX kit (TOYOBO, Osaka, Japan), having a 20- μ L reaction total volume, including 2 μ L of cDNA template, 10 μ L of Green Realtime PCR Master Mix, 1 μ L Forward primers, 1 μ L Reverse primers, and 6 μ L of double-distilled water. We performed qPCR on a CFX96 Optical Reaction Module for Real-Time PCR Systems (Bio-Rad Laboratories, Hercules, CA). The reaction procedure was 95 °C pre-denaturation for 1 min, 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s, using 40 cycles. The expression level of the gene was measured by the relative quantification method, the $2^{-\Delta\Delta CT}$ method (Tang and Jia, 2008).

Results

Effects of salt stress on plant growth, chlorophyll concentration, and photosynthetic rate of wild jujube seedlings

Wild jujube seedlings under the salt stress were significantly smaller than those of the CK (Fig. 1). The plant height, chlorophyll a content, chlorophyll b content, and photosynthetic rate of the wild jujube seedlings under the salt stress were significantly lower than those of the CK, being decreased by 17.5%, 36%, 33.84%, and 65%, respectively (Table 2), indicating that salt stress reduced the chlorophyll concentrations, the photosynthetic rate, and the growth of wild jujube seedlings.

Transcriptome sample sequencing data quality control

For the sequencing of the six cDNA libraries (three CK + three salt stress), the clean reads statistics of each sample is shown in Table 3. After sequencing quality control, a total of 73.05 Gb clean reads were obtained, and the Q30 base percentage of each sample was not less than 93.14%, indicating that the sequencing results met the requirements.

DEG ANALYSIS. The gene expression folds between samples were calculated according to the number of RPKM reads, which was $|\log_2 FC| > 1$ and $P < 0.05$. Genes that were significantly differentially expressed were screened. Of the 5269 DEGs identified, 2729 were up-regulated and 2540 were down-regulated. Among these DEGs, 97 genes were related to photosynthesis (Fig. 2)

DEG ENRICHMENT ANALYSIS. The DEGs in the 150 mM salt and CK were compared, and the GO database was used to achieve the GO annotation of the DEGs, and then the clusterProfiler software was used to classify each DEG with respect to GO function. Screening the corrected term with a $P < 0.05$ (Fig. 3), nine biological processes were enriched into a single process, a single-organism metabolic process, an establishment of localization, and porphyrin-containing items such as biosynthesis of porphyrin-containing compounds and carbohydrate transport. There were three items in the cell component, the proportion of the cytoplasmic part being the highest,

Table 1. Nine differentially expressed genes (DEGs) and an internal reference gene quantitative real-time-PCR (qPCR) primer sequence for the wild jujube seedlings under the control (Hoagland solution) and salt stress (Hoagland solution + 150 mM NaCl).

Primer	Gene	Sequence of the primers (5' to 3') ²
ACT1		F:AGCCTTCCTGCCAACGAGT R:TTGCTTCTCACCCTTGATGC
por	LOC107432449	F:GCTGCATTGCTACAACCTGGCTTG R:AACGAAGCAGAGTCCTTGTTCAG
LHCB4	LOC107413851	F:ACGGCACCATGATCGGAGACC R:ACCTCAGTGTACGGCTGGAACG
PsbO	LOC107424756	F:GTGCCATCGTACCGTGGTTTCG R:AAGTTCCTCCTCGTCTCCTCTTCC
HEMH	LOC107420114	F:ACCTCCTGCTCCAGTGTCTTCTCC R:GCATTGCCAGTGGTTGAGTTGTTG
PAO	LOC107405855	F:GCCTCCAACCACACCACCAAC R:CTCATCCTCAACGCCGTCATCTTC
PetF	LOC107415178	F:ATGAGGAGGCAGCCAGTGACC R:TGTGGACCATCTGGTGTAAACAAGC
LHCA4	LOC107419233	F:GTCACCACGCAAGCCTCAGC R:GAAGGCAAGCCAGGTAACCACCTC
LHCB2	LOC107416999	F:CTGAGACCTTCGCCAAGAACCG R:GAAGACACAGCCTAGTGCTCCAAG
HEME	LOC107413960	F:AGCTGCTGATGTTGATCGAGTGAG R:GCCACCTTCAACCACATATGATGC

²F = forward primer (5' to 3'), R = reverse primer (5' to 3').

Fig. 1. Wild jujube seedlings plants are tolerant to salt stress. Representative photos are shown of wild jujube seedling plants when they were exposed to control [CK (Hoagland solution)] and salt stress [salt (Hoagland solution + 150 mM NaCl)] for 3 d. The growth of wild jujube seedlings under salt stress was inhibited, compared with CK plants without salt stress.

followed by the plastid stroma. In the molecular function classification, nine were enriched, with the oxidoreductase activity accounting for the highest proportion, followed by metal ion binding.

Pathway enrichment analysis of DEGs between the salt stress and CK samples was performed using the KEGG database to visualize the functions of the DEGs and the biological processes and signaling pathways operating under salt stress. The pathways with $P < 0.05$ were defined as the pathways of significant enrichment of wild jujube DEGs under salt stress. The enriched metabolic pathways included photosynthesis, photosynthesis-antenna proteins, glyoxylate and dicarboxylate metabolism, alpha-linolenic acid metabolism, cysteine and methionine metabolism, and porphyrin and chlorophyll metabolism (Fig. 4).

ENRICHMENT PATHWAY ANALYSIS. Analysis of photosynthesis pathway in leaves of wild jujube seedlings exposed to salt stress. There were 14 DEGs in the photosynthetic antenna protein pathway and all were down-regulated (Table 4). There were 15 DEGs in photosystem II (PSII) and 10 DEGs in photosystem I (PSI). The cytochrome b6/f complex (cytochrome b6f, cyt b6f) contained one DEG, and there were six DEGs in the photosynthetic electron transport chain. There was one DEG in F-type ATPase. Photosynthesis pathway had a total of 33 DEGs, and all showed down-regulation in the salt-stressed seedlings (Table 5). According to the transcriptome data analysis in the current study, PSI, PSII, and ATP synthase all contained DEGs, indicating that salt stress may cause changes in the structure and function of these three complexes, thus affecting wild jujube photosynthesis. There were 25 DEGs in photosynthetic carbon fixation, of which three were up-regulated and 22 down-regulated under salt stress conditions (Table 6). Compared with the CK group, the up-regulated

DEGs in the salt-stressed sample were pyruvate, phospho-dikinase (ppdK), and aspartate aminotransferase (GOT1)—whereas down-regulated DEGs were malate dehydrogenase (MDH), ribose-5-phosphate isomerase (RPIA), ribulose kinase (PRK), phosphoglycerate kinase (PGK), triose phosphate isomerase (TPI), fructose-1,6-bisphosphatase (FBP), sedoheptulose-1,7-diphosphate, glutamic acid-glyoxylate aminotransferase and fructose-bisphosphate aldolase (ALDO), and ribulose-bisphosphate carboxylase/oxygenase (Rubisco). The gene that up-regulates expression and down-regulates expression is glyceraldehyde-3-phosphate dehydrogenase. These results indicate that the DEGs in the photosynthetic pathway may be DEGs with greater influence on the salt stress response.

Analysis of porphyrin and chlorophyll synthesis pathways in leaves of wild jujube seedlings under salt stress. Chlorophyll is the main pigment involved in photosynthesis in higher plants. Chlorophyll concentration directly affects the strength of photosynthesis, which affects the plant's tolerance of stress. Under salt stress, 21 DEGs were detected in the porphyrin and chlorophyll synthesis pathways of the leaves of wild jujube seedlings, with 18 DEGs being down-regulated and three DEGs up-regulated. The up-regulated genes were associated with ALA, heme, and red chlorophyll metabolites. LOC107426811 (HEMA), LOC107420114 (HEMH), and LOC107405855 (PAO), combined with the chlorophyll of wild jujube, achieved negative feedback loop regulation, which promoted the degradation of chlorophyll and reduced chlorophyll concentration. The pathway map in Fig. 5 shows that expression of chlorophyll synthesis-related genes is down-regulated under salt stress, indicating that salt stress has a negative correlation with porphyrin and chlorophyll biosynthesis pathways. These enzymes cover almost all the chlorophyll biosynthetic pathways conversion process (Fig. 5).

qPCR RESULTS. From the transcriptome analysis results, nine candidate genes related to photosynthesis and porphyrin and chlorophyll synthesis pathways were selected (LOC107432449), LHCB4 (LOC107413851), PsbO (LOC107424756), HEMH (LOC107420114), PAO (LOC107405855), PetF (LOC107415178), LHCA4 (LOC107419233), LHCB2 (LOC107416999), and HEME (LOC107413960). The expression levels in the two samples were analyzed by qPCR and compared with the results of transcriptome analysis. The results showed that the expression trends of qPCR and transcriptome sequencing genes gave basically similar results (Fig. 6), which confirmed the reliability of transcriptome sequencing results.

Table 2. Changes in growth index and photosynthetic indexes of wild jujube seedlings under control [CK (Hoagland solution)] and salt stress [salt (Hoagland solution + 150 mM NaCl)]. The experiment included three biological replicates.

Parameter	Treatments (mean $\pm$ SD)	
	CK	Salt
Plant height (cm)	9.2 $\pm$ 0.42*	7.6 $\pm$ 0.6
Chlorophyll a content (mg·g ⁻¹)	18.8 $\pm$ 0.41*	12.03 $\pm$ 0.34
Chlorophyll b content (mg·g ⁻¹)	5.88 $\pm$ 0.57*	3.89 $\pm$ 0.1
Photosynthetic rate (μmol·m ⁻² ·s ⁻¹)	3 $\pm$ 0.17*	1.05 $\pm$ 0.13

*Significantly different at $P < 0.05$.

Table 3. Sequencing data of six samples of wild jujube seedlings under control [CK (Hoagland solution)] and salt stress [salt (Hoagland solution + 150 mM NaCl)].

Sample	Raw reads	Clean reads	Raw base (G) ^z	Clean base (G) ^y	Q20 (%) ^x	Q30 (%) ^x	GC content (%) ^w
CK-1	38544211	38214548	11.56	11.46	97.46	93.14	41.75
CK-2	45742283	45512838	13.72	13.65	97.85	94.01	44.05
CK-3	40179700	39967417	12.05	11.99	97.66	93.54	41.58
Salt-1	39331915	38979318	11.8	11.69	97.55	93.46	41.78
Salt-2	43314420	43072244	12.99	12.92	97.72	93.78	42.79
Salt-3	37980837	37799291	11.39	11.34	97.75	93.85	42.83

^zTotal number of bases for sequencing raw data; i.e., number of raw reads multiplied by the length of the sequencing.

^yTotal number of bases of the filtered data.

^xQ20 and Q30 = percentage of bases with Phred values greater than 20 and 30, respectively, as a percentage of total bases.

^wGC: G/C bases as a percentage of total bases.

Fig. 2. The volcano plot of differentially expressed genes (DEGs) under salt stress [salt (Hoagland solution + 150 mM NaCl)] and control [CK (Hoagland solution)] conditions. Genes with significant differential expression are indicated by red dots (up-regulated) and blue dots (down-regulated). Genes with no significant differential expression are indicated by green dots; abscissas represent fold change of genes in different samples; ordinate represent the statistical significance of the difference in gene expression changes.

Fig. 3. Gene Ontology [GO (Ashburner et al., 2000)] term enrichment in differentially expressed genes (DEGs) in wild jujube seedlings between salt stress [salt (Hoagland solution + 150 mM NaCl)] and control [CK (Hoagland solution)] samples. The abscissa is the number of enriched genes and the ordinate is the GO enrichment entry. GO terms with $P < 0.05$ after correction are shown, with yellow denoting molecular function, green denoting cellular components, and blue denoting biological processes.

EFFECTS OF SALT STRESS ON PHOTOSYNTHESIS PATHWAY OF WILD JUJUBE SEEDLINGS. Photosynthesis is the basis of plant growth and increasing crop yields and is one of the most stress-sensitive physiological processes in plants (Jie et al., 2015). The photosynthetic pathway is divided into three phases: absorption and conversion of light energy, photosynthetic electron transport, and photosynthetic carbon fixation. The first stage is to absorb light energy by photosynthetic antenna proteins. The photosynthetic antenna protein subclass is divided into two subcategories by assisting PSI and PSII, namely, the light-harvesting complexes LHCA and LHCB, respectively (Jansson, 1999). In the second stage, electrons generated by photolysis of water are transferred by PSI, PSII, ATP synthase, and Cyt b6f. The third stage is fixation of photosynthetic carbon to produce organic matter for use by the organism. The following occurs under salt stress: the structure of the light-harvesting pigment protein complex of the plant leaves is degraded and destroyed, and the function is impaired; the electron transport ability of the PSII receptor side is decreased; the activity and content of Rubisco are decreased; the chlorophyll absorption capacity is weakened; and the carbon assimilation is suppressed. The intermediate products of phosphoglycerate and triose phosphate glyceraldehyde are not conducive to the normal growth of plants (Chi, 2008). In this experiment, compared with the CK, genes encoding LHCA1–5 and LHCB1–6 were in down-regulated status in the photosynthetic antenna protein pathway under salt stress. This change will affect the structure or function of the plant's light-harvesting complex, thus affecting the light absorption process of the plant. The higher plant photoreaction process is mainly achieved by the cooperation of four transmembrane multi-subunit protein complexes (PSII, Cyt b6f, PSI, and ATP synthase) located on the photosynthetic membranes. Among them, PSII uses the absorbed light energy to carry out the photochemical reaction, so that water molecules are cleaved to generate oxygen and the generated protons are released into the thylakoid cavity. PSI absorbs light energy and assists the electron transfer from PSII to convert β -Nicotinamide adenine dinucleotide 2'-phosphate (NADP⁺) into reduced nicotinamide adenine dinucleotide phosphate (NADPH). Cyt b6f complex has the function of linking electron transfer chain between the two complexes of PSI and PSII. ATP synthase synthesizes ATP using the transmembrane proton gradient generated by electron transport from PSII to PSI (Li, 2014).

Much research has been carried out on the effects of salt stress on plant photosynthesis. Zhao et al. (2017) studied the photosynthetic characteristics of wild jujube seedlings induced by salt stress. The results showed that salt stress caused damage to the photosynthetic apparatus of the PSII reaction center, causing decreases in the initial capture ability and photosynthetic electron transport rate of light energy, which ultimately led to the weakening of photosynthesis ability of the wild jujube seedlings. PSII is an important structure for photoreaction in photosynthesis. Salt stress leads to a decrease in the efficiency of light energy and PSII electron transport, resulting in a significant decrease in net photosynthetic rate, stomatal conductance, and transpiration rate of plants (Liu and Cao, 2018; Meng et al., 2019; Sun et al., 2019).

The present study found that many genes were annotated into photosynthesis-related pathways, indicating that these

Fig. 4. Kyoto Encyclopedia of Genes and Genomes [KEGG (Wixon and Kell, 2000)] pathway analysis of the wild jujube transcriptome in the presence of salt stress. The vertical axis refers to pathways; the horizontal axis refers to enrichment factors. The size of the dot refers to the number of differential expression genes in this pathway. The color of the dot corresponds to the scope of the probability value.

Table 4. Distribution of differentially expressed genes (DEGs) in the photosynthesis-antenna proteins pathways in salt stress [salt (Hoagland solution + 150 mM NaCl)] versus control [CK (Hoagland solution)] conditions.

DEG	Description	Log ₂ FC ^z	P ^y	Expression
LOC107413638	chlorophyll a-b binding protein 6 (LHCA1)	-1.887	< 0.0001	down
LOC107404976	photosystem I chlorophyll a-b binding protein 6 (LHCA2)	-2.329	<0.001	down
LOC107423894	photosystem I chlorophyll a-b binding protein 6 (LHCA2)	-1.7478	0.002	down
LOC107416800	chlorophyll a-b binding protein 8 (LHCA3)	-1.84	<0.0001	down
LOC107419233	chlorophyll a-b binding protein P4 (LHCA4)	-1.8	<0.001	down
LOC107419977	photosystem I chlorophyll a-b binding protein 5 (LHCA5)	-1.843	<0.0001	down
LOC107413540	chlorophyll a-b binding protein of LHCII type 1 (LHCB1)	-1.655	0.004	down
LOC107416999	chlorophyll a-b binding protein 151 (LHCB2)	-2.248	<0.0001	down
LOC107430393	chlorophyll a-b binding protein 13 (LHCB3)	-1.67	0.001	down
LOC107430831	chlorophyll a-b binding protein CP29.2 (LHCB4)	-1.561	0.009	down
LOC107413851	chlorophyll a-b binding protein CP29.3 (LHCB4)	-1.898	<0.001	down
LOC107405503	chlorophyll a-b binding protein CP26 (LHCB5)	-1.727	0.005	down
LOC107424276	chlorophyll a-b binding protein CP26 (LHCB5)	-2.112	<0.001	down
LOC107427642	chlorophyll a-b binding protein CP24 10A (LHCB6)	-1.888	<0.001	down

^zFold change [fold = RPKM (salt)/RPKM (CK)]; RPKM = reads per kilobase of transcript per million mapped reads.

^ySignificantly expressed DEGs were determined using a corrected corresponding probability value, $P < 0.05$.

photosynthesis-related genes were very sensitive to salt. The DEGs in the photosynthetic pathway after salt seedling stress were expressed in five components: PSI, PSII, cytochrome b6/f complex, photosynthetic electron transport chain, and ATP

synthase, all of which were affected by salt stress. The photosynthetic DEGs were grouped into three aspects: PSII, PSI, and photosynthetic electron transport chain. The photoreceptor enhancer proteins (PsbO, PsbP, and PsbQ) of PSII participate

Table 5. Distribution of differentially expressed genes (DEGs) in the photosynthetic pathways in salt stress [salt (Hoagland solution + 150 mM NaCl)] versus control [CK (Hoagland solution)] conditions.

DEG	Description	Log ₂ FC ^z	P ^y	Expression
LOC107424756	oxygen-evolving enhancer protein 1 (PsbO)	-1.658	<0.001	down
LOC107416619	oxygen-evolving enhancer protein 2 (PsbP)	-3.137	<0.0001	down
LOC107416616	oxygen-evolving enhancer protein 2 (PsbP)	-1.878	<0.001	down
LOC107413542	psbP-like protein 1 (PsbP)	-1.43	<0.001	down
LOC107428949	photosynthetic NDH subunit of luminal location 2 (PsbQ)	-2.411	<0.0001	down
LOC107431631	photosynthetic NDH subunit of luminal location 3 (PsbQ)	-2.185	0.004	down
LOC107413524	photosynthetic NDH subunit of luminal location 1 (PsbQ)	-2.359	<0.0001	down
LOC107421293	oxygen-evolving enhancer protein 3-2 (PsbQ)	-1.769	<0.001	down
LOC107423325	psbP-like protein 1 (PsbR)	-1.304	0.001	down
LOC107421047	photosystem II 22 kDa protein (PsbS)	-1.134	0.005	down
LOC107405691	photosystem II reaction center W protein (PsbW)	-2.734	<0.0001	down
LOC107425881	photosystem II reaction center W protein (PsbW)	-2.003	<0.0001	down
LOC107428525	photosystem II core complex proteins psbY (PsbY)	-1.747	<0.0001	down
LOC107416633	photosystem II repair protein PSB27-H1 (Psb27)	-1.589	<0.001	down
LOC107434253	photosystem II reaction center PSB28 protein (Psb28)	-2.172	<0.0001	down
LOC107423198	photosystem I reaction center subunit II (PsaD)	-1.965	<0.001	down
LOC107423019	photosystem I reaction center subunit II (PsaD)	-1.7017	0.005	down
LOC107426460	photosystem I reaction center subunit IV A (PsaE)	-1.834	0.001	down
LOC107417894	photosystem I reaction center subunit IV (PsaE)	-1.22	0.001	down
LOC107418592	psbP-like protein 1 (PsaF)	-1.589	0.003	down
LOC107417186	photosystem I reaction center subunit V (PsaG)	-1.781	<0.001	down
LOC107404842	photosystem I reaction center subunit VI-2 (PsaH)	-1.987	<0.001	down
LOC107415237	photosystem I reaction center subunit PsaK (PsaK)	-1.782	0.002	down
LOC107412513	photosystem I reaction center subunit XI (PsaL)	-2.014	<0.001	down
LOC107425674	photosystem I reaction center subunit N (PsaN)	-2.101	<0.0001	down
LOC107430257	cytochrome b6-f complex iron-sulfur subunit 2 (PetC)	-1.491	<0.001	down
LOC107422616	plastocyanin (PetE)	-2.686	<0.0001	down
LOC107415178	ferredoxin (PetF)	-1.914	<0.0001	down
LOC107403595	ferredoxin C 2 (PetF)	-1.112	<0.001	down
LOC107423176	ferredoxin C 1 (PetF)	-1.71	<0.001	down
LOC107424409	ferredoxin-NADP reductase, leaf-type isozyme (PetH)	-1.719	<0.0001	down
LOC107420103	cytochrome c6 (PetJ)	-1.663	0.001	down
LOC107421010	ATP synthase gamma chain (garama)	-2.173	<0.0001	down

^zFold change [fold = RPKM (salt)/RPKM (CK)]; RPKM = reads per kilobase of transcript per million mapped reads.

^ySignificantly expressed DEGs were determined using a corrected corresponding probability value, $P < 0.05$.

in the photoreaction of PSII. Particularly sensitive to stress response (Vani et al., 2001), ferredoxin-coenzyme II reductase (PetH) is the last catalytic enzyme in the photosynthetic electron transport chain, mainly transferring electrons from PSI to NADP⁺, with the resulting NADPH participating in CO₂ assimilation (Fukuyama, 2004). PsbR is a docking protein on the surface of the thylakoid membrane. PsbS is capable of excess light energy dissipation and is involved in the interaction between the LHCII and PSII cores. PsbW is a nonlocalized, low-molecular-weight protein in the PSII core complex, with a role in photoprotection. PsbY is the PSII core protein. All these DEGs enriched in the photosynthetic pathways were down-regulated under salt stress, indicating that salt stress may reduce photosynthetic rate by inhibiting light energy conversion, electron transport, photosynthetic phosphorylation, and the photosynthesis dark reaction process in photosynthesis photo-reaction.

In the photosynthetic carbon fixation pathway, this experiment reported that MDH, RPIA, PRK, PGK, TPI, FBP, sedoheptulose-1,7-diphosphate, glutamic acid-glyoxylate aminotransferase, and fructose-biphosphate aldolase (ALDO), and ribulose-bisphosphate carboxylase/oxygenase (Rubisco) genes

were all down-regulated. Rubisco is the rate-limiting enzyme in the process of photosynthetic carbon assimilation (Andrews, 1988; Gutteridge, 1991). The down-regulation of the Rubisco gene indicates that Rubisco catalyzes the key step of the carboxylation of ribulose-1,5-diphosphate, and CO₂; this is consistent with the results of Lin (2017). The stress-induced, down-regulated genes in the photosynthetic carbon assimilation pathway were mostly genes encoding key enzymes in the pentose phosphate pathway, indicating that salt stress inhibits the pentose pathway and reduces the synthesis of organic carbon. This result indicated that salt stress may change the structure and function of the photosynthetic complex by down-regulating the photosynthesis-related pathway genes and metabolism-related genes to change the carbon metabolism pathways. Subsequently, this affects the photosynthesis of wild jujube, reduces the synthesis of organic carbon, and finally inhibits the growth of plants and their development.

EFFECTS OF SALT STRESS ON PORPHYRIN AND CHLOROPHYLL SYNTHESIS PATHWAY IN WILD JUJUBE SEEDLINGS. The level of chlorophyll directly affects the process of photosynthesis, which subsequently affects the growth and development of

Table 6. Distribution of differentially expressed genes (DEGs) in carbon fixation pathways in photosynthetic organisms in salt stress [salt (Hoagland solution + 150 mM NaCl)] versus control [CK (Hoagland solution)] conditions.

DEG	Description	Log ₂ FC ^z	P ^y	Expression
LOC107423358	pyruvate, phosphate dikinase (ppdK)	1.312	<0.0001	up
LOC107420693	aspartate aminotransferase (GOT1)	2.229	<0.0001	up
LOC107424221	malate dehydrogenase (MDH1)	-2.808	<0.001	down
LOC107424201	malate dehydrogenase (MDH1)	-3.38	<0.001	down
LOC107427480	malate dehydrogenase (MDH2)	-1.813	<0.0001	down
LOC107434568	phosphoribulokinase (PRK)	-1.433	<0.0001	down
LOC107417353	ribulose biphosphate carboxylase small chain (rbcS)	-2.029	<0.0001	down
LOC107417243	ribulose biphosphate carboxylase small chain (rbcS)	-2.687	<0.0001	down
LOC107429454	ribulose biphosphate carboxylase small chain (rbcS)	-1.98	<0.0001	down
LOC107431911	phosphoglycerate kinase (PGK)	-1.56	<0.001	down
LOC107417916	phosphoglycerate kinase (PGK)	-2.191	<0.0001	down
LOC107412780	glyceraldehyde-3-phosphate dehydrogenase GAPCP2 (GAPDH)	1.613	<0.0001	up
LOC107427982	glyceraldehyde-3-phosphate dehydrogenase A (GAP)	-1.433	0.003	down
LOC107419772	glyceraldehyde-3-phosphate dehydrogenase B (GAP)	-1.454	0.004	down
LOC107427583	triosephosphate isomerase (TPI)	-1.26	<0.001	down
LOC107407446	fructose-bisphosphate aldolase (ALDO)	-1.915	0.001	down
LOC107421671	fructose-1,6-bisphosphatase (FBP)	-1.294	0.002	down
LOC107430709	fructose-1,6-bisphosphatase 1 (FBP)	-2.598	<0.001	down
LOC107427681	fructose-1,6-bisphosphatase (FBP)	-1.051	<0.001	down
LOC107430723	fructose-1,6-bisphosphatase (FBP)	-2.239	<0.0001	down
LOC107413771	sedoheptulose-1,7-bisphosphatase	-1.456	<0.0001	down
LOC107428611	glutamate-glyoxylate aminotransferase 2	-1.002	<0.001	down
LOC107412211	probable ribose-5-phosphate isomerase 3 (RPIA)	-1.834	<0.001	down
LOC107433843	probable ribose-5-phosphate isomerase 4 (RPIA)	-1.757	0.01	down
LOC107418684	probable ribose-5-phosphate isomerase 4 (RPIA)	-1.973	<0.0001	down

^zFold change [fold = RPKM (salt)/RPKM (CK)]; RPKM = reads per kilobase of transcript per million mapped reads.

^ySignificantly expressed DEGs were determined using a corrected corresponding probability value, $P < 0.05$.

plants. For example, consider the biosynthesis process of chlorophyll from L-glutamyl-tRNA to chlorophyll a. A total of 15 enzymes are involved in the reaction (Li et al., 2019; Wang et al., 2009). If a step occurs out of place in the synthesis of chlorophyll, it will hinder the accumulation of precursors, and the subsequent precursors will decrease (Xu et al., 2006). In tomato (*Solanum lycopersicum*), the synthesis of chlorophyll is regulated by genes encoding chloroplast development, thereby altering photosynthesis and affecting the intrinsic quality of tomato (Meng et al., 2018). Under seawater salt stress, ALA and porphobilinogen accumulated in the leaves of two spinach (*Spinacia oleracea*) cultivars, whereas the concentrations of urogen III and its precursors were significantly reduced (Chen et al., 2012). HEMA is a key enzyme that regulates the pathway of chlorophyll synthesis (Liu et al., 2007). The biosynthesis of heme and chlorophyll has the same pathway, from urogen III to protoporphyrin IX (Zeng, 1959). HEMH is a key enzyme that regulates the pathway of heme synthesis. The up-regulated expression of HEMH promotes the accumulation of heme. Studies have shown that the accumulation of heme affects the synthesis of chlorophyll (Pontoppidan and Gaminikannangara, 1994). The oxidative cleavage reaction of pheophorbide monooxygenase (PAO) causes chlorophyll a to form red chlorophyll metabolites, PAO being the key rate-limiting enzyme in chlorophyll degradation.

In the present study, the expression profiles of the genes involved in the chlorophyll metabolic pathway in the presence of salt stress showed significant differences. In this pathway, 21 DEGs were enriched, of which 18 genes were down-regulated;

and 3, namely HEMA, HEMH, and PAO, were up-regulated. The accumulation of ALA and heme leads to a decrease in the levels of chlorophyll synthesis precursors and accelerates the degradation of chlorophyll, which ultimately leads to a significant decrease in chlorophyll concentration and affects plant photosynthesis.

Conclusions

Salt tolerance of plants is a complex trait in which various genes coordinate and function together. The photosynthesis of wild jujube seedlings under salt stress in our study was significantly inhibited. By analyzing the transcriptome sequencing data related to photosynthesis and porphyrin and chlorophyll synthesis pathways, it was found that the DEGs enriched in photosynthesis and photosynthetic antenna protein pathways were all down-regulated. The DEGs HEMA, HEMH, and PAO in the porphyrin and chlorophyll synthesis pathways were up-regulated, and the rest were down-regulated, with the key genes encoding Rubisco in the photosynthetic carbon fixation pathway also being down-regulated. These results indicated that the accumulation of chlorophyll synthesis precursors and the degradation rate of chlorophyll were accelerated because of the accumulation of ALA and heme, which ultimately led to a significant decrease in chlorophyll concentration, affecting plant photosynthesis and ultimately inhibiting plant growth. A total of nine differential genes involved in photosynthesis and chlorophyll biosynthesis were selected. The qPCR analysis showed that the expression changes of these nine differential

Fig. 5. Kyoto Encyclopedia of Genes and Genomes [KEGG (Wixon and Kell, 2000)] pathway of porphyrin and chlorophyll metabolism: HEMA = glutamyl-tRNA reductase; HEML = glutamate-1-semialdehyde 2,1 aminomutase; HEMB = 5-aminolevulinic acid dehydratase; HEMC = porphobilinogendeaminase; HEMD = uroporphyrinogen IX synthetase; HEME = uroporphyrinogen IX decarboxylase; HEMF = coproporphyrinogen IX oxidase; HEMV = protoporphyrinogen oxidase; HEMH = ferrous chelatase; MCH = Mg-ruthenium synthase; CHLM = Mg-protoporphyrin IX methyltransferase; CRD1 = Mg-protoporphyrin IX monomethyl ester cyclase; DVR = divinyl reductase; POR = protochlorophyllinoxidoreductase; CHLG = chlorophyll synthase; CAO = chlorophyllin alpha oxidase; PAO = pheophorbide monooxygenase. Red represents up-regulation of differentially expressed genes (DEGs), and green represents down-regulation of DEGs.

Fig. 6. Comparison of nine quantitative real-time-PCR (qPCR) and transcriptome sequencing (RNA-Seq) results for candidate genes for photosynthesis and porphyrin and chlorophyll synthesis pathways. The bar graph represents the relative expression of qPCR for candidate genes, and the line graph is the reads per kilobase of transcript per million mapped reads (RPKM) value for transcriptome sequencing. The abscissa is control [CK (Hoagland solution)] and salt stress [salt (Hoagland solution + 150 mM NaCl)], the left ordinate is the relative expression of the differential gene, and the right is the transcriptome sequencing data RPKM value.

genes under salt stress were consistent with the results of transcriptome sequencing.

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