Comparative transcriptome analysis of leaves of sour jujube seedlings under salt stress

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Abstract
Sour jujube is a tree species native to China, which has often been used as the rootstock for the propagation of other cultivars of jujube. In addition, its fruit can be used in the practice of traditional Chinese medicine. Thus, sour jujube has been regarded as a highly valuable plant scientifically and ecologically. Sour jujube is mostly planted in Northwest China and has extremely high salt tolerance. However, the molecular mechanism of its salt tolerance is yet to be fully understood. This study was carried out in the Laboratory of Agricultural College of Dalian Nationalities University. Two treatments were performed on the leaves of sour jujube seedlings with the experimental group (H3) being treated by 300 mM NaCl for 3 h, and the control group (CK) by sterile water for 3 h. A total of 47.02 GB of valid data and 32,730 annotated genes were obtained. Based on the gene expression in the comparison group, 2295 significantly differentially expressed genes (DEGs) were obtained, of which 807 and 1488 were upregulated and downregulated, respectively. According to gene function annotation and enrichment analysis, 148 genes were obtained, which are mainly involved in signal transduction of plant hormones (38), homeostasis of cell walls (27), secondary metabolism of organic matter (32), and redox reactions (20) in the leaves of sour jujube seedlings under salt stress. Among these DEGs, some stress-related transcription factors (31) were also identified. In addition, under salt stress, raffinose family oligosaccharide (RFO) metabolism in sour jujube seedlings was found to be greatly accelerated. By investigating the molecular responses of jujube seedlings under salt stress, our study provides a scientific basis for jujube cultivation in saline-alkali land, which is beneficial to further improvement of the salt tolerance of grafted jujube trees.

Keywords Ziziphus jujuba Mill · Seedling leaf development · Transcription factors · Raffinose family oligosaccharides

Introduction
Sour Jujube (Ziziphus jujuba Mill. var. spinosa) is a variant of jujube originated from China, which has been cultivated for more than 2,500 years. Sour Jujube is a variant of jujube which exhibits strong adaptability to extreme weather, such as infertile land, drought, and strong winds. Therefore, it has been used as the rootstock for jujube propagation. A total of 495,548 hectares of Jujube trees are planted in Xinjiang, China (Statistical Bureau of Xinjiang Uygur Autonomous Region 2009). However, land productivity is severely limited by soil salinization in Xinjiang. As the rootstock of other jujube variants, the salt tolerance of sour jujube directly affects the quality and yield of jujube. Therefore, the current studies on jujube mainly focus on tolerance difference and saline-alkali stress screening of root seedlings of sour jujube.

Studies have shown that common plants can be damaged in soil with a salinity of 0.3%. The main damage is through growth inhibition of plant tissues and organs. With
the aggravation of salt stress, plant leaves become smaller, turn yellow and even wilt, and root growth is hindered. In severe cases, it will lead to premature aging or even death of plants. Askari et al. proposed that under salt stress, due to different degrees of changes in amino acids, proteins and other hormonal substances in plants, physiological metabolic disorders of plants, including nitrogen metabolism, carbohydrate metabolism, protein metabolism, etc., have been observed, among which nitrogen metabolism disorders are thought to be the main cause of salt damage in plants (Askari et al. 2006). Salt stress also induces high osmotic stress outside plant cells, which can cause ion toxicity and promote the production of reactive oxygen species, thereby destroying ion balance and stability of biological macromolecules in cells, and affecting the normal growth of the plant (Chen et al. 2012; Miller et al. 2010). In addition, salt stress can cause secondary stresses (such as oxidative stress and drought stress) (Liang et al. 2017), and affect the morphology, physiological and biochemical levels of plant organs. Consequently, mechanical damage can be induced to plant protoplasts and cell walls, leading to decreased photosynthetic rate, dehydration and even death of cells (Osmond and Grace 1995).

Plants show resistance or adaptation to salt stresses in a variety of ways. Studies have found that plants have an osmotic balance regulation mechanism (Zhao et al. 1999). Under salt stress, plants maintain a relatively stable environment within plant cells by selectively absorbing or isolating extracellular ions (Shen et al. 2015). In addition, some defense mechanisms, such as accumulation of osmoregulation substances (e.g., soluble sugar, proline, and betaine), can be activated in plants (Qi et al. 2014). Regulation of the antioxidant defense system in plants can also cause salt stress resistance. During long-term evolution, plants have produced active oxygen scavenging systems, including antioxidant enzymes and antioxidants. Antioxidant enzymes include oxide dismutase (SOD), ascorbate (APX), and catalase (CAT) (Kaur et al. 2016). Antioxidants include glutathione, polyols, and flavonoids (Noctor et al. 1998).

Studies have shown that the Ca$^{2+}$ concentration in plants will increase rapidly under salt stress, and Ca$^{2+}$ channels are induced to hinder Na$^{+}$ absorption in order to maintain normal plant growth (Knight et al. 1997). In addition, Ca$^{2+}$ is a signal molecule for salt stress signal transduction pathways (Shi. 1970), such as the phospholipid signaling pathway, the supersalt-sensitive (SOS) pathway (Zhu et al. 2003; Chinnusamy et al. 2006), the abscisic acid (ABA) signaling pathway (Daie et al. 1989) and the calcium-dependent protein kinase (CDPK) cascade (Harmon et al. 2000; Xu et al. 2010).

When constructing a sequencing library, a certain number of PCR amplification is usually required. However, due to the preferential PCR amplification, the amplification multiples of each target sequence are not the same. Such duplications generated by PCR amplification will lead to deviation of quantitative gene expression results. Therefore, we employ the UMI technology to mark each segment during PCR amplification, and merge reads with the same label in later processes of data analyses to effectively remove the duplications.

In the early stage of the experiment, we conducted the NaCl tolerance test on sour jujube seedlings. The results showed that the seedlings grew well under the treatment of NaCl at the concentration of 100 mM, 200 mM, 300 mM, 400 mM and 500 mM. Under the treatment of 400 mM NaCl, the seedlings showed dysplasia and the leaves were wilted. Under the treatment of 500 mM NaCl, the growth of the seedlings was obviously hindered and eventually led to death. The physiological indexes of sour jujube plants were determined under NaCl treatments with other concentrations, which showed that the activities of POD, SOD and CAT, and the contents of IAA, ABA and GA3 significantly changed three hours after the treatment. Therefore, de novo sequencing was used to analyze the transcriptomes of leaves of jujube seedlings treated with 300 mM NaCl for 3 h. This study provides a valuable theoretical basis for exploring the molecular mechanism of salt stress resistance of sour jujube.

**Materials and methods**

**Plant materials**

Sour Jujube seeds were donated by the National and Joint Engineering Laboratory of High Efficiency and Superior-Quality Cultivation and Fruit Deep Processing Technology of Characteristic Fruit Trees of South Xinjiang Tarim University. The experiments were carried out in the laboratory of Environment and Resources College of Dalian Nationalities University. Healthy sour jujube seeds with the same size were selected and disinfected with 0.5% potassium permanganate solution for 30 min at room temperature. Then, the seeds were cultured in an artificial climate box with a light intensity of 12,000 lx, 14 h of light, 10 h of darkness, and a culture humidity of 70%. The sour jujube seeds were placed on the soaked gauze to promote their germination, and the germinated seedlings were transplanted and potted after one week. Every three days, 500 ml 1/2 of Hoagland nutrient solution was applied to the plants. The seedlings were grown for 20 d, during which, sour jujube seedlings in the experimental group and the control group were watered with 300 mM NaCl and deionized water, respectively. After 3 h, three different jujube seedlings were randomly selected for sampling. A total of 0.5 g of the treated jujube leaves in each sample was placed and stored in liquid nitrogen for the preparation of the next experiment.
RNA extraction and cDNA library construction and sequencing

Trizol reagent (Invitrogen, CA, USA) was used to extract the total RNA following the manufacturer’s procedure. The purity and quantity of the RNA were measured using the RNA 1000 Nano LabChip Kit (Agilent, CA, USA). A total of 5 µg of total RNA was used to purify poly(A) RNA by passing through poly-T oligo-attached magnetic beads. The purified mRNA was fragmented and used for the synthesis of first-strand cDNA using random hexamers and reverse transcriptase. Using the first-strand cDNA as the template, the second-strand cDNA was subsequently synthesized using a system containing DNA polymerase I, RNase H, dNTPs, and buffer. AMPure XP beads (Beckman Coulter Genomics, MA, USA) were used to purify the double stranded cDNAs, which were subjected to end repair and adenylation. To minimize amplification noise and sequence-dependent bias, the cDNAs were ligated to the modified Illumina multiplex barcode adapters containing customized unique molecular identifiers (Shiroguchi et al. 2012), and then purified and amplified by AMPure XP beads and PCR, respectively. The amplified cDNAs were further purified by AMPure XP beads. The barcode adapters were provided by Lc-Bio Technologies (Hangzhou, China). The quality of the cDNA library was evaluated using an Agilent Bioanalyzer 2100 system (Agilent, CA, USA). The index-coded samples were clustered using a HiSeq PE Cluster Kit v4 (Illumina, San Diego, CA, USA) on a cBot Cluster Generation System. The RNA-Seq libraries were sequenced on an Illumina HiSeq 4000 instrument at LC Sciences (Hangzhou, China) and 150 bp paired-end reads were generated.

Transcriptome data processing and analysis

The raw data were stored in the fastq format, which were processed by our in-house perl scripts to generate the clean data. To achieve this purpose, reads containing poly-N sequences and adapter sequences, low quality reads, amplification noise and sequence-dependent bias were removed using UMI-tools (Smith et al. 2017). Trinity 2.4.0 was used for the de novo assembly of the transcriptome (Grabherr et al. 2011) by clustering the transcripts based on the shared sequence content. For each cluster of these transcripts, it can be referred to as one gene very loosely, while the longest transcript is selected as the sequence of this gene (Unigene). All assembled Unigenes were aligned against the SwissProt database (http://www.expasy.ch/sprot/), non-redundant (Nr) database (http://www.ncbi.nlm.nih.gov/), Gene ontology (GO) (http://www.geneontology.org), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) and the eggnoG database (http://eggnogdb.embl.de/) using DIAMOND with an evalue threshold of < 0.00001 (Buchfink et al. 2015).

Differentially expressed unigenes

The expression level of the Unigenes was calculated using Salmon (Patro et al. 2017) and represented by transcript per million (TPM) (Mortazavi et al. 2008). The criteria of determining the differentially expressed Unigenes were set as log2 (fold change) ≥ 1 or log2 (fold change) < −1 and the statistical difference p < 0.05 (Robinson et al. 2010). Gene Ontology (GO) terms and KEGG pathways of these Unigenes were analyzed using our in-house perl scripts.

Quantitative real-time PCR (qRT-PCR) verification

The total RNA of the jujube leaves was reverse transcribed using the PrimeScript RT reagent Kit (Takara, China) for both the experimental and control groups. qRT-PCR was performed using SYBR Premix Ex TaqTM II (Takara, China) with the following procedures: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 30 s, and extension at 72 °C for 60 s. The 2−ΔΔCT method was employed to calculate the relative expression of genes (Livak and Schmittgen 2001).

Results

Sequence assembly and analysis

To understand the molecular mechanism of jujube in response to salt stress, the transcriptome was sequenced for the samples from both the control group and the experimental group. A total of 48.02 GB of raw data (CK, 24.8 GB and H3, 23.32 GB) were obtained from 6 samples. Among them, 47.02 GB were valid data (CK, 24.18 GB and H3, 23.32 GB) were obtained from 6 samples. The number of effective data reads was 47,288,016–55,184,060, and the number of reads after UMI re-processing was 36,050,722–42,458,030, accounting for 76.24–77.23% of the valid reads. The content of Q20, Q30 and GC was above 98.42%, 94.78%, and 46.15%, respectively (Table 1). After the samples were mixed and assembled, the total length of the unigenes was 33,117,256 nt, and N50 of the unigenes was 1724 nt. There were 32,730 unigenes, with the GC content of 41.48%. Among these unigenes, 8,413 had a length of 200–300 nt, 4,620 were longer than or equal to 2000 nt, and 4,213 had a length of 300–400 nt (Fig. 1).

Since genes with similar functions are highly conserved in sequence (nucleic acid sequence or protein sequence) between different species, we selected six authoritative databases (Table 2), from which, 32,730 genes were annotated.
The number of unigenes identified by NR was the highest (18,984, accounting for 58%). A total of 84% Sour Jujube unigenes showed high similarity to those of *Ziziphus jujuba*, while the similarity was relatively low to others: *Anthurium amnicola* (1.72%), *Morus notabilis* (1.64%), *Prunus persica* (1.15%), *Prunus mume* (0.89%), *Juglans regia* (0.86%), and the others (9.75%) (Fig. 2a). For the KEGG annotation, 9874 unigenes were assigned into 19 KEGG functional categories (Fig. 2b), including translation (1521, 15.40%), carbohydrate metabolism (1445, 14.63%), folding, sorting and degradation (1058, 10.72%), environmental adaptation (963, 9.75%), and transport and catabolism (826, 8.37%). Compared with protein databases (Nr and SwissProt), GO annotation of 18,984 unigenes was obtained (Fig. 2c), including molecular function, biological process and cellular component. These GO terms can be further divided into 25, 15 and 10 functional categories, respectively. For the molecular function category, the top 3 terms include molecular function (2551, 13.44%), protein binding (1693, 8.92%), and ATP binding (1448, 7.63%). For the biological process category, the top 3 terms include biological process (2751, 14.50%), regulation of transcription, DNA-templated (1131, 5.96%), and transcription, DNA-templated (836, 4.40%). For the cellular component category, The top 3 terms are nucleus (4994, 26.30%), cytoplasm (2749, 14.48%), and integral component of membrane (2683, 14.13%). For the eggNOG annotation, the top 3 terms are transcription (1008), signal transduction mechanisms (1289), and posttranslational modification, protein turnover, chaperones (1561), and nearly half of the unigenes has unknown functions (Fig. 2d).

**Table 1** Statistics of transcriptome sequencing results

| Sample | Raw reads | Valid reads | Dedup reads | Dedup2Valid (%) | Q20 (%) | Q30 (%) | GC (%) |
|--------|-----------|-------------|-------------|----------------|---------|---------|--------|
| CK-1   | 53,429,444| 52,868,562  | 40,658,098  | 76.90          | 98.52   | 95.04   | 46.37  |
| CK-2   | 55,472,602| 54,741,400  | 42,033,246  | 76.79          | 98.51   | 94.99   | 46.15  |
| CK-3   | 55,796,578| 55,184,060  | 42,458,030  | 76.94          | 98.50   | 94.95   | 46.22  |
| H3-1   | 54,130,800| 53,483,562  | 41,307,688  | 77.23          | 98.42   | 94.78   | 46.17  |
| H3-2   | 47,828,528| 47,288,016  | 36,050,722  | 76.24          | 98.45   | 94.87   | 46.19  |
| H3-3   | 53,552,170| 52,978,462  | 40,506,686  | 76.46          | 98.47   | 94.91   | 46.17  |

**Table 2** Unigene annotation against six public databases

| Database | Numbers | Ratio (%) |
|----------|---------|-----------|
| All      | 32,730  | 100       |
| GO       | 18,984  | 58        |
| KEGG     | 9874    | 30.17     |
| Pfam     | 17,325  | 52.93     |
| swissprot| 15,690  | 47.94     |
| eggNOG   | 20,608  | 62.96     |
| NR       | 21,629  | 66.08     |

**Screening of significantly differential genes**

From the sequencing data of 6 samples of the experimental and control groups, 2,295 differentially expressed genes were identified, including 807 up regulated and 1,488...
down-regulated genes (Fig. 3a). A heat map was constructed (Fig. 3b), in which different colors represent the differential expression of sample genes. The results show that the downregulated significantly differential genes outnumbered those up-regulated genes.

**Gene enrichment and functional classification**

We initially obtained 2295 significantly differentially expressed unigenes, and 32,730 unigene functional annotations, from which, we obtained 1831 unigenes with functional annotation. Among these unigenes, 1,666 and 948 unigenes have GO annotations, and KEGG annotations, respectively. The Go terms of these 1666 unigenes were analyzed, which can be classified into 20 functional catalogs. The top five terms include high-density lipoprotein particle remodeling, phloem development, xyloglucan: xyloglucosyl transferase activity, syncytium formation, and xyloglucan metabolic process (Fig. 4a). A total of 948 unigenes were assigned with KEGG annotations (Fig. 4b). The top terms include pentose and glucuronate interconversions, alpha – Linolenic acid metabolism, carotenoid biosynthesis, plant hormone signal transduction, and phenylpropanoid biosynthesis.

**Screening of salt stress-related genes**

A total of 148 significantly differentially expressed genes were selected based on the functional classification and enrichment, which are mainly involved in signal transduction of plant hormones, homeostasis of cell walls, secondary metabolism of organic matter, and redox reactions; some of these genes are assigned to stress-related transcription factors (Fig. 5).

Cell wall plays a critical role in stress responses (Farrokhi et al. 2006). Studies have shown that cell wall-related
proteins, such as xyloglucan endotransglucosylase/hydrolase (XTH), expansin (EXP), pectinesterase (PME), and inositol oxygenase (MIOX), function in cell homeostasis by altering cell ductility and permeability (O’Donoghue et al. 2012; Geilfus et al. 2011; Miedes et al. 2013). Compared with the control group, our results show that 3 out of 27 cell-wall-related genes identified in this study were up regulated (XTH23A, XTH23B, and EXPA1B).

As signal molecules, plant hormones play an indispensable role in stress responses by regulating various metabolic processes in plants. For example, ABA is a key regulatory factor in response to abiotic stress, which can enhance plant tolerance by inducing many factors, such as H\(^+\)-ATPase, Na\(^+\)/H\(^+\) antiporter, PRO, betaine synthase, SOD, CAT, etc. (Zhang et al. 2006; Atia et al. 2009). A total of 38 hormone-related genes were identified. Fourteen of these genes are auxin-responsive genes (including three family genes: IAA/AUX, GH3, and SAUR), which are down regulated under salt stress. Thirteen genes are ABA response genes, of which 10 were up regulated and 3 were down regulated. Nine genes are regulated in response to gibberellin (GA), among which 7 were down regulated (mainly related to GASA and CXE genes), and 2 were up regulated (CXE15, and At5g05600). Only two genes were regulated in response to cytokinin (CTK, CYP72A14), and both were upregulated.

Osmotic regulation of plant metabolism by salt stress can lead to severe dehydration of plants and production of reactive oxygen species, such as KO\(_2\), H\(_2\)O\(_2\) and O\(_2\). Superoxide dismutase plays a key role in cleaning excess reactive oxygen species in cells (Hsu et al. 1997). We screened 20 oxidoreductase-related genes, including eighteen peroxidase-related genes, among which four were up regulated (PAP17,
PER25, POD, and HIP26) and 14 were down regulated. Only two of these oxidoreductase-related genes are superoxide dismutase-related genes, with one being up regulated (Fe/Mn-SOD) and one down regulated (FSD2).

Enrichment analysis shows that genes related to the metabolic reaction of sour jujube are important in response to salt stress. A total of 32 metabolism-related genes were identified, including genes in phenylpropanoid biosynthesis (8 up-regulated genes and 6 down-regulated genes), alpha-Linolenic acid metabolism (3 up-regulated genes and 5 down-regulated genes) and galactose metabolism (7 up-regulated genes and 3 down-regulated genes).

Under environmental stresses, transcription factors bind corresponding cis-acting elements to initiate stress-related gene transcription and expression (Jiang et al. 2006). Transcriptomics studies have shown that a large number of transcription factors are induced under salt stress (Kawaura et al. 2008). A total of 31 salt stress-related transcription factors were identified, including genes in five families: AP2/EREBP (3 up-regulated genes and 1 down-regulated gene), HD-ZIP (1 up-regulated gene and 6 down-regulated genes), MYB (3 up-regulated genes and 1 down-regulated gene), NAC (6 up-regulated genes), and WRKY (4 up-regulated genes and 2 down-regulated genes).

Screening of raffinose family oligosaccharide (RFO) metabolism-related genes

RFO oligosaccharides play an important role in response to different environmental stresses such as low temperature, high temperature, drought, and high salt (Peters and Keller 2009). Studies have shown that RFOs, as a typical compatible substance, can replace water molecules and bind to the hydrophilic surface of proteins in a dehydrated environment to form a protective film to protect proteins and maintain their natural active conformation (Hincha et al. 2003). In addition, RFOs bind to the phospholipid bimolecular membrane to protect it from dehydration, and the protective ability of RFOs to membrane lipid is positively correlated with the galactosyl polymerization degree (Cacela and Hincha, 2006). Combined with GO and KEGG enrichment of unigenes, a total of 6 structural genes that may be involved in RFO metabolism under salt stress were identified, including GOLS2A (TRINITY_DN16169_c1_g6), GOLS2B (TRINITY_DN11553_c0_g1), GOLS1 (TRINITY_DN16169_c1_g10), STS (TRINITY_DN11993_c0_g1), RFS2 (TRINITY_DN13747_c0_g3) and RFS5 (TRINITY_DN11601_c0_g1). Under salt stress, their expression levels were up regulated, which may lead to the accumulation of RFO oligosaccharides in jujube leaves and enhance the tolerance of plants (Fig. 6).

Based on the functional characteristics of DREB transcription factors in other plants and the expression patterns of transcriptome genes, a regulatory model as shown in Fig. 7 was proposed. Under salt stress, the DREB2A gene and the unknown transcription factors of jujube positively regulate the expression of GOLS1 and GOLS2 genes, which is beneficial to the enhancement of raffinose metabolism.
Fig. 5 Heat map of genes related to salt stress. H3 indicates the control group, and CK indicates the experimental group. The TPM values of the unigenes were log2 transformed. Genes in orange and blue are up regulated and down regulated, respectively.
Ten differentially expressed genes (5 up-regulated genes and 5 down-regulated genes) were randomly selected from the transcriptomic database for RT-PCR validation. The experimental template was the same as the transcriptome sequencing template. The expression patterns of these 10 genes were consistent with those obtained from RNA-Seq (Fig. 8).

**Discussion**

Soil salinization in Xinjiang has become a severe problem. As of 2015, the total arable land area in Xinjiang was 4,124,563 hectares, among which the saline-alkali land was 204,501 hectares accounting for 4.96% of the total arable land. Soil salinization not only reduces soil productivity, but also causes many ecological problems, which is one of the key factors to limit agricultural production in Xinjiang. Jujube trees cultivated on saline-alkali soil have different degrees of salt damage symptoms such as lack of seedlings, reduced growth, and decline in yield and quality, which slows down the development of the jujube industry in Xinjiang. Jujube trees with great traits usually use sour jujube as the rootstock, because of its high drought and saline tolerance. However, studies on the salt-tolerance mechanism of sour jujube are still lacking. In this study, two treatments were performed on the leaves of sour jujube seedlings. In the experimental group (H3), 300 mM NaCl was applied for 0.5 h, and in the control group (CK) sterile water was applied instead. A total of 47.02 GB of valid data and 32,730 annotated genes were obtained. A total of 2295 genes with significantly differential expression were identified, including 807 up-regulated genes and 1488 down-regulated genes. Based on gene function annotation and enrichment analysis, 148 genes were obtained, which are mainly involved in signal transduction of plant hormones, homeostasis of cell walls, secondary metabolism of organic matter, and redox reactions in leaves of sour jujube seedlings under salt stress. Some stress-related transcription factors were also identified.

Under high salt stress, the extensibility of plant cell walls is significantly enhanced, which helps reduce the dehydration effect of plants and ion persecution (Jones et al. 2004). Studies have shown that a number of genes, such as XTH (xyloglucan endotransglucosylase), EXP (Expansins), and PME (pectinesterase), play important roles in cell wall development and metabolism. Cho et al. found that CaXTH1, CaXTH2, and CaXTH3 genes in pepper can respond to high salt stress (Cho et al. 2006). Heterologous expression of maize ZmXTH23 in E. coli can increase the salt tolerance of the host bacteria (Chen et al. 2019). A total of 10 XTH genes were identified, of which only the XTH23A and XTH23B genes were up regulated under salt stress. Expansins are also closely related to plant tolerance to environmental stresses. Studies have shown that expansins promote plant cell wall relaxation (Cosgrove et al. 2014). When corn seedlings are under salt stress, the ZnEXP6 protein on the cell wall of the leaves disappears, which hinders the growth of corn leaves (Geilfus et al. 2015). Seven EXP genes of sour jujube were identified, among which only one gene (EXPA1B) was up regulated. Pectin is one of the main components of the cell wall, and PME is a key enzyme for pectin metabolism, which are closely related to the extension and metabolism of the cell wall. PME is also involved in abiotic stress responses (Levesque-Tremblay et al. 2015; Senechal F et al. 2014). A total of eight PME genes were identified, all of which were down regulated. We speculated that the expression of XTH23A, XTH23B, and EXPA1B genes may increase under...
salt stress, thereby enhancing the ductility of the cell wall of sour jujube leaves to combat the damage caused by salt stress. The PME family genes decrease cell wall pectin decomposition by reducing the expression level to maintain the cell wall stability of sour jujube leaves. One of the main hazards of salt stress is the increase of cell membrane permeability due to membrane lipid peroxidation. POD and SOD-related genes can effectively inhibit the removal of reactive oxygen species and reduce ionic damage caused by NaCl (Roxas et al. 2000). Physiological experiments showed that the SOD and POD activities of sour jujube seedlings would first increase and then decrease when subjected to salt stress (Ma YX et al. 2018). Among the 20 redox-related genes we identified, 3 POD-related genes (PAP17, PER25, POD) and one SOD-related gene (TNT) were significantly up regulated when subjected to salt stress. Therefore, we speculate that these genes can reduce the damage caused by salt stress by increasing the activity of superoxide dismutase and peroxidase.

The secondary metabolites of plants may exhibit antioxidant functions by scavenging free radicals and reducing lipid peroxidation in the process of plant responses to biotic and abiotic stresses, thereby protecting the plants from ultraviolet radiation, and promoting plant growth in harsh environments (Treutter, 2006). Multiple genes of sour jujube were involved in secondary metabolism processes under salt stress, including phenylpropanoid biosynthesis, galactose metabolism, and alpha-Linolenic acid metabolism. The expression of β-glucosidase-related genes (At4g27290, KIN14R, BACOVA_02659, F26G, and BGLU17), cinnamoyl-related genes (TKPR2, EO, DCR, and ACT), 4-coumarate-CoA ligase-related genes (AAE6, and AAE11), and Caffeic acid 3-O-methyltransferase-related genes (HOMT1) in phenylpropanoid biosynthesis showed significant differences. Studies have found that HOMT1 gene is also a key gene for melatonin synthesis, which can effectively remove active oxygen under stress conditions and improve plant antioxidant capacity (Bajwa et al. 2014; Park et al. 2012). Expression of Jasmonic acid (JA) synthesis-related genes (At5g37990, OPR1, and OPR2) was significantly increased during the alpha-Linolenic acid metabolism. As an important signal transduction molecule, JA plays an important role in disease tolerance, induction of defense-related genes and plant growth and development, (Browse. 2009). ADH and LOX genes are also involved in plant salt tolerance (Strommer et al. 2009; Blokhina et al. 2003). Raffinose family oligosaccharides (RFOs) are unique functional oligosaccharides in plants. RFO metabolism starts from inositol.

Fig. 8 RT-qPCR results and RNA-seq expression of 10 genes from both the experimental and control groups
galactose synthases (GOLSs) and catalyzes the synthesis of different RFO members through raffinose synthases (RFSs), stachyose synthases (STs) and other enzymes (Kuo et al. 2011). RFO oligosaccharides accumulate in large amounts in photosynthetic tissues under stress such as low temperature, drought, high temperature, and high salt. Sour jujube inositol galactosyl synthase genes (GOLS1, GOLS2A, and GOLS2B) and raffinose synthetase genes (RFS2, and RFS3) were significantly up regulated, indicating that the RFO metabolic process of sour jujube was enhanced to resist the damage caused by salt stress. In addition, the expression of β-galactosidase genes (BGAL3, BGAL10, and IDD14) in jujube was significantly decreased.

Phytohormones are important substances that not only regulate plant growth and development but also participate in stress responses. Transcriptome analysis showed that IAA, ABA, GA, and CTK genes of Ziziphus jujuba were involved in salt stress responses. However, their expression patterns were different. After the jujube seedlings were treated with more than 150 mM NaCl for 6 h, the IAA and GA contents in the leaves would decrease instantaneously, and the ABA content would increase instantly (Tu et al. 2018). Transcriptome analysis showed that ABA and GA-related genes were down regulated at the beginning of salt stress, while multiple genes in the ABA synthesis pathway and signal transduction pathway were up regulated, indicating that the ABA pathway was used to respond to salt stress in the early stage of salt stress. AFP2, AHG1, HVA22D, XERICO, and AIP1 genes are all important genes for ABA in response to stress.

Transcriptional regulation is a key step for plants to respond to salt stress. Transcription factors are abundantly expressed under salt stress, which can regulate and reduce the damage caused to plants by salt stress. Five transcription factors of sour jujube were significantly expressed to resist salt stress, including AP2/EREPB, HD-ZIP, MYB, NAC, and WRKY family genes. Four AP2/EREPB transcription factors were identified, including three being up regulated (EFR5, DREB2A, and DREB3) and one down regulated (ERF1B). Dehydration response element-binding protein and ethylene response element-binding factor are two important members of the AP2/EREPB family (Sakuma et al. 2002). Overexpression of Arabidopsis AtDREB2A, rice OsDREB2A, and lettuce LsDREB2A genes can increase salt tolerance in plants. Therefore, it was speculated that AP2/EREPB transcription factors played a critical role in resisting salt stress. The HD-ZIP family contains plant-specific transcription factors, which also functions in stress resistance. Sour jujube ATHB-12 expression was significantly increased under salt stress. Go analysis found that ATHB-12 was involved in the response of abscisic acid. Therefore, we hypothesized that under salt stress, the ATHB-12 gene of sour jujube might bind with certain proteins to initiate an ABA-mediated response to salt stress. MYB (Chen et al. 2006) and WRKY (Dong et al. 2003) family genes are also involved in the process of plant responses to salt stress. Six significantly up-regulated NAC family genes were identified in this study. Studies have shown that a salt stress response gene in Arabidopsis is induced by multiple NAC transcription factors (Tran et al 2004). Therefore, we speculated that multiple genes in the NAC family of sour jujube may participate in the same salt stress process. Microarray analysis showed that the transcription regulation of Arabidopsis under abiotic stress must be completed by multiple transcription factors (Fowler et al. 2002). Moreover, AP2/EREPB, MYB, NAC and other family transcription factors were expressed within a short period of time or transiently under salt stress, which may support our hypothesis.

RFO oligosaccharides play a vital role in resisting abiotic stress. Previous studies on the regulatory effect of RFOs on plant stress mainly focus on the regulation of GOLS gene expression and its function. The transcription levels of AtGolS1 and AtGolS2 were up regulated under drought and high salt stresses. Overexpression of AtGolS2 significantly increases the content of galactinol and raffinose in Arabidopsis thaliana, thereby enhancing drought tolerance of plants (Taji et al. 2002). Overexpression of heat shock transcription factor (HSF) and dehydration responsive element-binding (DREB) transcription factors significantly increases the transcription level of endogenous GOLS genes in Arabidopsis. The tolerance of plants to abiotic stress can also be significantly increased (Busch et al. 2005; Panikulangara et al. 2004). Maize ZmGOLS2 is regulated by the DREB2A transcription factor, and overexpression of ZmGOLS2 and DREB2A in Arabidopsis improves Arabidopsis abiotic stress tolerance (Gu et al. 2016). By combining the expression patterns of various genes in sour jujube, we proposed the regulatory patterns of the genes shown in Fig. 7. When sour jujube was under salt stress, the DREB2A gene might increase the expression of GOLS1 and GOLS2 genes, which directly led to the accumulation of raffinose in the leaves of wild jujube and promoted the normal growth of plants.

In summary, we performed a comprehensive analysis of RNA-seq in leaves of sour jujube seedlings under salt stress by transcriptome sequencing. Most of the identified genes were involved in signal transduction of plant hormones, homeostasis of cell walls, secondary metabolism of organic matter, and redox reactions, and some were identified as stress-related transcription factors. Our study will lay a foundation for further studies of the molecular mechanism of salt tolerance of sour jujube and guidance for cultivation of salt tolerant jujube variants.

**Author contribution statement** RL, RW conducted the experiments and prepared the manuscript, CW analyzed the data, and YB and PG designed the experiment.
Acknowledgements This work was supported by the Key Project of Science and Technology of Xinjiang Production and Construction Corps (Grant No.2017D8006), the National Natural Science Foundation of China (Grant 31771695), the National and Local Joint Engineering Laboratory of High Efficiency and Superior-Quality Cultivation and Fruit Deep Processing Technology of Characteristic Fruits in South Xinjiang (Grant No. FE201902), Fundamental Research Funds for the Central Universities (Program for ecology research group). And this manuscript was posted as a preprint on Research Square (https://www.researchsquare.com/) on 26 July, 2021 (https://doi.org/10.21203/rs.3.rs-401000/v1).

Declarations

Conflict of interest The authors declare no conflict of interest.

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