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Abstract

Sour jujube (*Ziziphus jujuba Mill. var. spinosa*) is a native tree species in China. It is often used as the rootstock of jujube, its fruit can also be used as medicine. So it has high scientific and ecological value. Sour jujube is mostly planted in Northwest China and has extremely high salt tolerance. But the molecular mechanism of its salt tolerance is rarely studied. This study was carried out in the laboratory of Environment and Resources College of Dalian Nationalities University, two treatments were performed on the leaves of sour jujuba seedlings, the experimental group (H3): 300 mM Nacl for 3 h, and the control group (CK): sterile water for 3 h. A total of 47.02GB of valid data and 32730 annotated genes were obtained. Based on the analysis of gene expression in the comparison group, a total of 2295 genes with significant differential expression were obtained, of which 807 were up-regulated and 1488 were down-regulated. According to gene function annotation and enrichment analysis, we finally screened 148 genes. These genes were mainly involved in signal transduction of plant hormones (38), homeostasis of cell walls (27), secondary metabolism of organic matter (32), redox reactions (20) in leaves of sour jujuba seedlings under salt stress. It was also accompanied by some stress-related transcription factors (31). In addition, under salt stress, raffinose family oligosaccharides (RFO) metabolism of sour jujube seedlings was accelerated. Studying the molecular response of sour jujuba seedlings under salt stress provides a scientific basis for its cultivation in saline areas and is beneficial to further improve the salt tolerance of grafted jujube trees.

Keywords *Ziziphus jujuba Mill,* Seedling leaf development, Transscription factors, Raffinose family oligosaccharides

Introduction

Sour Jujube (*Ziziphus jujuba Mill. var. spinosa*) is a Chinese native species having 2,500 years of cultivation history. Sour Jujube is a variant of jujube Which has strong adaptability to extreme weather, such as infertile land, drought, and strong winds. Therefore, it is often used as a good rootstock for jujube. Jujube trees are planted on 495,548 hectares in Xinjiang, China (Statistical Bureau of Xinjiang Uygur Autonomous Region 2009). However, soil salinization in Xinjiang is very serious, which directly leads to the decrease of land productivity. Therefore, as the root stock of jujube, the salt tolerance of sour jujube will directly affect the quality and yield of jujube. So at present, studies on jujube mainly focus on tolerance difference and saline-alkali stress screening with root seedlings.

Studies have shown that common plants are harmed in soil with a salinity of 0.3 percent. The main damage is to inhibit the growth of plant tissues and organs. With the aggravation of salt stress, plant leaves become smaller, turn yellow and even wilt, and root growth will be blocked. In severe cases, it will lead to premature aging or even death of plants. Hossein Askari 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., among which nitrogen metabolism disorders are thought to be the main cause of salt damage in plants(Askari et al. 2006).Salt stress can also cause high osmotic stress outside the cells of the plant, which can cause ion toxicity and induce the production of reactive oxygen species, thereby destroying the ion balance and the stability of biological macromolecules in the cell, and affecting the normal growth of the plant(Chen et al. 2012; Miller et al. 2010). In addition, salt stress can cause secondary stress (oxidative stress, drought stress) (Liang et al.2017), and the morphology, physiological and biochemical levels of plant organs will be affected twice. These can cause mechanical damage to

plant protoplasts and cell walls, decrease in photosynthetic rate, dehydration and even death of cells (Tan. 1996).

Plants can resist or adapt to salt stress in a variety of ways. Studies have found that plants themselves 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, the plant itself will activate some defense mechanisms, such as accumulation of some osmoregulation substances, such as: soluble sugar, proline, betaine, etc (Qi et al. 2014). Regulation of the plant's antioxidant defense system is also a way for plants to resist salt stress. During long-term evolution, plants have produced active oxygen scavenging systems, including antioxidant enzymes and antioxidants. Antioxidant enzymes include oxide dismutase (SOD), ascorbase (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 rise rapidly under salt stress, and Ca^{2+} channels are induced to hinder Na^{+} absorption to maintain normal plant growth (Knight et al.1997). In addition, Ca^{2+} can also be used as a signal molecule to participate in the process of salt stress signal transduction (Shi. 1970). Current studies have found that Ca^{2+} are involved in the supersalt-sensitive (SOS) pathway (Zhu et al. 2003; Chinnusamy et al. 2006), the calcium-dependent protein kinase (CDPK) cascade (Harmon et al. 2000; Xu et al. 2010), the abscisic acid (ABA) signaling pathway (Daie et al. 1989), and the phospholipid signaling pathway.

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 Duplication generated by PCR amplification will lead to deviation of quantitative gene expression results. Therefore, we employ UMI technology to mark each segment, and mark each segment during PCR amplification, and merge reads of the same label in the later processing of data so as to effectively remove Duplication.

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 100mM, 200mM and 300mM. While under the treatment of Nacl at the concentration of 400mM, the seedlings showed dysplasia and the leaves were wilting. Under the treatment of Nacl at the concentration of 500mM, the growth of the seedlings was obviously blocked until death. And the determination of physiological indexes of sour jujube plants under Nacl treatment conditions showed that the activities of POD, SOD, CAT, and the contents of IAA, ABA, GA3 and other contents of sour jujube seedlings had changed significantly after three hours of treatment. Therefore, this study used de novo sequencing to analyze the transcriptome of sour Jujube seedling leaves treated with deionized water and 300mM Nacl after 3 hours. This study provides a valuable theoretical basis for exploring the molecular mechanism of sour jujube in the process of resisting salt stress.

Materials and Methods

Plant Material

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 in South Xinjiang Tarim University. This experiment was carried out in the laboratory of Environment and Resources College of Dalian Nationalities University. Choose the same size and healthy Sour Jujube

seeds, and disinfect them with 0.5% potassium permanganate solution for 30 minutes at room temperature. After that, they were cultured in an artificial climate box with a light intensity of 12000 lx, 14 hours of light, 10 hours 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 are transplanted potted one week later. Every three days, 500ml 1/2 of Hoagland nutrient solution was applied to the plants. The seedlings were grown to 20 days, the experimental group of Sour Jujube seedlings were watered with 300 mM NaCl, and the control group was treated with deionized water. After 3 hours, randomly selected three different jujube seedlings for sampling, 0.5 g of each of the NaCl-treated jujube leaves were taken, and 0.5 g of each of the deionized water-treated Sour Jujube leaves was placed and stored in liquid nitrogen for preparation for the next experiment.

Petal RNA Extraction, Construction, and Sequencing of cDNA Libraries

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The total RNA quantity and purity were analysis of Bioanalyzer 2100 and RNA 1000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0. Poly(A) RNA is purified from total RNA(5ug) using poly-T oligo-attached magnetic beads using two rounds of purification. Following purification, the mRNA is fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were used for first-strand cDNA synthesis using random hexamers and reverse transcriptase. Second-strand cDNA synthesis was subsequently performed using buffer, dNTPs, RNase H and DNA polymerase I. The double stranded cDNA were purified using AMPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA) and subjected to end repair process, adenylation and then ligated to modified Illumina multiplex barcode adapters, which including custom Unique Molecular Identifiers for minimizing sequence-dependent bias and amplification noise according to (Shiroguchi et al. 2012). The adaptor ligated cDNAs were size selected with AMPure XP beads and subjected to PCR amplification to enrich the adapter-ligated fragments, which were further purified using AMPure XP beads. The barcode adapters was provided by Lc-Bio Technologies (Hangzhou). At last, library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the HiSeq PE Cluster Kit v4 (Illumina, San Diego, CA, USA) following the manufacturer’s instructions. After cluster generation, RNA-Seq libraries were sequenced using the Illumina HiSeq 4000 instrument at LC Sciences (Hangzhou, China) and generated 150 bp paired-end reads.

Transcriptome Data Processing and Analysis

Raw data in fastq format were first processed using in-house perl scripts. For this step, clean data were obtained not only by removing reads containing adapter sequences, reads containing poly-N sequences, and low quality reads from the raw data, but also then by removing sequence-dependent bias and amplification noise using UMI-tools (Smith et al. 2017). when the reads containing identical Unique Molecular Identifiers. All downstream analyses were based on clean data of high quality. De novo assembly of the transcriptome was performed with Trinity 2.4.0 (Grabherr et al. 2011). Trinity groups transcripts into clusters based on shared sequence content. Such a transcript cluster is very loosely

referred to as a 'gene'. The longest transcript in the cluster was chosen as the 'gene' sequence (aka Unigene).

All assembled Unigenes were aligned against the non-redundant (Nr) protein database (http://www.ncbi.nlm.nih.gov/), Gene ontology (GO) (http://www.geneontology.org), SwissProt (http://www.expasy.ch/sprot/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) and eggnog (http://eggnogdb.embl.de/) databases using DIAMOND (Buchfink et al.2015) with a threshold of evalue<0.00001

Differentially expressed Unigene analysis

Salmon (Patro et al. 2017) was used to perform expression level for Unigenes by calculating TPM (Mortazavi et al. 2008). The differentially expressed Unigenes were selected with log2 (fold change) >1 or log2 (fold change) <-1 and with statistical significance (p value < 0.05) by R package edgeR (Robinson et al.2010). Next, GO and KEGG enrichment analysis were again performed on the differentially expressed Unigenes by perl scripts in house.

Quantitative Real-Time PCR (qRT-PCR) Verification

The PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, China) was used to reverse transcribe the total RNA of the jujube leaves in the experimental and control groups. Genes were real-time quantified using SYBR ® Premix Ex TaqTM II (Tli RNaseH Plus) (Takara, China) and the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher, China). Setting procedure: 95 °C pre-denaturation for 30 s; 95 °C denaturation for 5 s, 55 °C annealing for 30 s, 72 °C extension for 60 s, and 40 cycles. The relative expression of genes was calculated using the $2^{-\triangle\triangle CT}$ method (Livak & Schmittgen, 2001).

Results

Sequence assembly and analysis

In order to better explore the molecular mechanism of jujube in response to salt stress, we sequenced the samples from the control group and the experimental group. After quality control, 48.02GB of raw data (CK,24.8GB and H3,23.32GB) were obtained from 6 samples. Among them, 47.02GB were valid data (ck,24.18GB and H3,22.84GB). The number of effective data reads was 47288016-55184060, and the number of reads after UMI re-processing was 36050722-42458030, accounting for 76.24%-77.23% of Valid reads. The content of Q20 was above 98.42%, Q30 was above 94.78%, and GC was above 46.15%. (Table 1) After the samples were mixed and assembled,Total length of unigenes was 33117256 nt, N50 of unigenes was 1724 nt. There were 32,730 unigenes,GC content was 41.48%. among which 8,413 were 200-300nt genes, 4,620 were greater than or equal to 2000 nt genes, and 4,213 were 300-400nt genes (Fig. 1).

Since genes with similar functions were highly conserved in sequence (nucleic acid sequence or protein sequence) between different species, we selected six authoritative databases. (Table 2). A total of 32,730 genes were annotated from these six databases.The number of unigenes identified by NR was the largest (18,984, accounting for 58%).In this analysis,84% Sour Jujube unigenes showed high similarity

to *Ziziphus jujuba*, Secondly, *Anthurium amnicola* (1.72%),*Morus notabilis* (1.64%),*Prunus persica* (1.15%),*Prunus mume* (0.89%),*Juglans regia* (0.86%),Others (9.75%)(Fig.2a). For the KEGG annotation,9874 unigenes were assigned into 19 KEGG functional categories(Fig.2b).Among them, translation(1521,15.40%) , carbohydrate metabolism(1445,14.63%) , folding, sorting and degradation(1058,10.72%) , environmental adaptation(963,9.75%) , transport and catabolism (826,8.37%). After comparison with protein databases (Nr and SwissProt), GO annotation information of 18,984 unigenes was obtained (Fig.2c). These genes were divided into three main functional categories: biological process, cellular component, and molecular function. They contained 25, 15 and 10 functionally detailed categories, respectively. In the biological process category, the three types of genes were the most annotated, including biological process (2751,14.50%),regulation of transcription ,DNA-templated(1131,5.96%), transcription ,DNA-templated(836,4.40%).In the cellular component category, nucleus(4994,26.30%),cytoplasm(2749,14.48%),integral component of membrance (2683,14.13%) were most annotated. In the molecular function category, molecular function(2551,13.44%),protein binding(1693,8.92%),ATP binding(1448,7.63%) were Top 3. For the eggNOG annotation, Posttranslational modification, protein turnover, chaperones(1561) , Signal transduction mechanisms(1289), Transcription(1008),and nearly half of Unigenes' functions were unknown(Fig.2d).

Screening of significantly different genes

Based on the analysis of the sequencing data of 6 samples from the experimental group and the control group, we finally screened 2,295 differentially expressed genes, 807 up-regulated and 1,488 down-regulated (Fig. 3a). We took these 2295 genes that were significantly different and made a heat map (Fig. 3b). Different colors represented differential expression of sample genes. It can be seen that under salt stress, compared with the control group, the number of significantly different genes that are down-regulated is greater than the number of significantly different genes that are up-regulated.

Genes enrichment and functional classification

Through differential genes screening, we initially obtained 2295 significantly different unigenes, and combined with 32730 unigenes functional annotation, we obtained 1831 unigenes with functional annotation. Among them, 1,666 unigenes have GO annotations, and 948 unigenes have KEGG annotations. Firstly, 1666 unigenes were analyzed for the significant difference of GO function. Combined with GO enrichment analysis scatter plot, 1666 significantly differentially expressed genes were divided into 20 functional classifications. Among them, the enrichment degree ranked the top five: high-density lipoprotein particle remodeling, phloem development, xyloglucan: xyloglucosyl transferase activity, syncytium formation, xyloglucan metabolic process(Fig. 4a). Finally, we selected 948 unigenes with KEGG annotation from 1831 unigenes with functional annotation for KEGG enrichment analysis (Fig.4b). Among them, the enrichment classification with high enrichment degree is as follows: alpha−Linolenic acid metabolism, Pentose and glucuronate interconversions, Carotenoid biosynthesis, Plant hormone signal transduction, Phenylpropanoid biosynthesis (Remove the P value>0.05).

Screening of salt stress related genes

According to the functional classification and enrichment analysis of genes, we finally selected 148 significantly different genes, which are mainly involved in signal transduction of plant hormones, homeostasis of cell walls, secondary metabolism of organic matter, redox reactions, It is also accompanied by some stress-related transcription factors (Fig. 5).

The cell wall plays an important role in plant growth and development and in response to environmental stress (Farrokhi et al. 2006). Cell wall-related proteins such as xyloglucan endotransglucosylas-e/hydrolase(XTH),expansin(EXP),pectinesterase(PME),inositol oxygenase(MIOX) maintain 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, out of the 27cell wall-related genes screened, only 3 genes were up-regulated (*XTH23A*, *XTH23B*, *EXPA1B*).

Plant hormones play an indispensable role in regulating plant growth and development and stress response. They can be involved in regulating various metabolic processes in plants as signal molecules. Especially as a key regulatory factor in response to abiotic stress, ABA has an irreplaceable role, inducing H^+-ATPase, Na^+/H^+ antiporter, PRO, betaine synthase, SOD`, CAT, etc. to enhance plants through physiological and biochemical mechanisms Tolerance to salt stress (Zhang et al. 2006; Atia et al. 2009). We screened a total of 38 hormone-related genes. Fourteen of them are auxin-responsive genes (including three family genes: IAA / AUX, GH3, SAUR), and their expression levels were all down-regulated after salt stress. There were 13 ABA response genes, of which 10 genes were up-regulated and 3 genes were down-regulated (involved in all kinds of genes), indicating that under salt stress, ABA was involved in inducing many responses as a signaling molecule. There were 9 genes response to gibberellin (GA), 7 of which were down-regulated (mainly related to GASA and CXE genes), and 2 genes were up-regulated (*CXE15*, *At5g05600*). Only two genes were involved in cytokinin (*CTK*, *CYP72A14*), and both were upregulated.

Salt stress osmotic regulation of plant metabolism will cause the plant to be severely dehydrated and form reactive oxygen species, such as KO_2, H_2O_2 and O_2. Superoxide dismutase and catalase in the body play a key role in cleaning excess reactive oxygen species in cells (Hsu et al. 1997). We screened 20 oxidoreductase-related genes. Eighteen genes were peroxidase-related genes, four of which were up-regulated (*PAP17*, *PER25*, *POD*, *HIPP26*) and 14 genes were down-regulated. Only two genes are superoxide dismutase-related genes, one up-regulated (Fe/Mn-SOD) and one down-regulated (FSD2).

The enrichment analysis found that the genes related to metabolic reaction of sour jujuba under the salt stress conditions were very significant. We selected 32 metabolism-related genes, which were mainly involved in Phenylpropanoid biosynthesis (8 genes up-regulated and 6 genes down-regulated), alpha-Linolenic acid metabolism (3 genes up-regulated and 5 genes down-regulated) and galactose metabolism (7 genes up-regulated and 3 genes down-regulated).

When plants are under stress, transcription factors will combine with corresponding cis-acting elements to initiate the corresponding gene transcription and expression to reduce the damage caused by stress to plants (Jiang et al. 2006). Transcriptomics studies found that a large number of transcription factors were induced to express under salt stress (Kawaura et al. 2008). Through analysis and screening, we obtained 31 transcription factors related to salt stress, which are involved in 5 families: *AP2* / *EREBP* (3 genes were up-regulated, 1 gene was down-regulated), HD-ZIP(1 gene was up-regulated, 6 genes were down-reg ulated), MYB(3 genes were up-regulated, 1 genes were down-regulated), NAC (all 6 genes were up-regulated), WRKY(4 genes were up-regulated, 2 genes were down-regulated).

Screening of Raffinose family oligosaccharides (RFO) metabolism related genes

RFOs oligosaccharides play an important role in the tolerance to stress such as low temperature, drought, high temperature and high salt (Peters S and Keller F, 2009). Studies have shown that RFOs, as

a typical compatible substance, replaces water molecules and binds to the hydrophilic surface of the protein in the dehydration environment to form a protective film to protect the protein and maintain its natural active conformation (Hincha et al.2003). In addition, RFOs binds 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 enrichment and KEGG enrichment of unigenes, a total of 6 structural genes that may be involved in RFOs metabolism under salt stress were screened.,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). After these 6 Unigenes were subjected to salt stress, their expression levels showed a significant up-regulation trend. Their high expression may lead to the accumulation of RFOs oligosaccharides in jujube leaves and enhance the tolerance of plants.

Based on DREB transcription factors functional characteristics in other plants and the expression pattern of transcriptome genes, a regulatory model as shown in Figure 7 is proposed. Under salt stress, the sour jujube DREB2A gene and unknown transcription factors will positively regulate the expression of GOLS1 and GOLS2 gene, which helps raffinose metabolism process enhanced.

Real-time quantitative PCR to verify gene expression

Ten differentially expressed genes (5 genes were up-regulated,5 genes were down-regulated) 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 10 genes were consistent with that of RNA-Seq (Fig. 8).

Discussion

The soil salinization in Xinjiang is serious. As of 2015, the total arable land area in Xinjiang was 4,124,563 hectares, and the area of saline-alkali land was 204,501 hectares. The area of saline-alkali land accounted 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 keys to restricting 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 advancement of the jujube industry in Xinjiang. Excellent jujube tree usually use sour jujube as rootstock, because it has drought tolerance, saline characteristics. However, the research on the salt-tolerance mechanism of sour jujube is still lacking, Therefore, it is important to explore the salt tolerance of sour jujube to improve the quality of jujube. In this study, two treatments were performed on the leaves of sour jujuba seedlings, the experimental group (H3): 300 mM NaCl for 0.5 h, and the control group (CK): sterile water for 0.5 h. A total of 47.02GB of valid data and 32730 annotated genes were obtained. Based on the analysis of gene expression in the comparison group, a total of 2295 genes with significant differential expression were obtained, of which 807 were up-regulated and 1488 were down-regulated. According to gene function annotation and enrichment analysis, we finally screened 148 genes. These genes were mainly involved in signal transduction of plant hormones, homeostasis of cell walls, secondary metabolism of organic matter, redox reactions in leaves of sour jujuba seedlings under salt stress. It was also accompanied by some stress-related transcription factors.

Studies have shown that under high salt stress, the extensibility of plant cell walls is significantly enhanced, which helps to reduce the dehydration effect of plants and ion persecution to protect the plants

themselves (Jones et al. 2004). Studies have found that: XTH (xyloglucan endotransglucosylase), EXP (Expansins), and PME (pectinesterase) genes all 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 will increase the salt tolerance of the host bacteria (Chen DB et al.2009). We screened a total of *10 XTH* genes, of which only the *XTH23A* and *XTH23B* genes were up-regulated under salt stress. Expansins are also closely related to plant tolerance to environmental stress. Studies have shown that expansins promote plant cell wall relaxation (Cosgrove et al.2014). When corn seedlings are under salt stress, the *ZmEXPB6* protein on the cell wall of the leaves will disappear, which will cause the growth of corn leaves to be blocked (Geilfus et al. 2015). The seven *EXP* genes of sour jujube we screened, 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 is closely related to the extension and metabolism of the cell wall. PME is also involved in abiotic stress response processes (Levesque-Tremblay et al. 2015; Senechal F et al. 2014). The eight PME genes we screened were all down-regulated. We speculated that the expression of *XTH23A*, *XTH23B*, and *EXPA1B* genes may increase during 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 reduce the cell wall pectin decomposition process by reducing the expression level to maintain the cell wall stability of sour Jujube leaves. One of the main hazards of salt stress is that cell membrane permeability increases due to membrane lipid peroxidation. POD and SOD-related genes can effectively inhibit the removal of reactive oxygen species and reduce the ionic damage caused by NaCl (Roxas et al.2000). Physiological experiments showed that the SOD and POD activities of sour jujuba seedlings would first increase and then decrease when subjected to salt stress (Ma YX et al. 2018). Among the 20 redox-related genes we screened, we found that 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 they can reduce the damage caused to plants by salt stress by increasing the activity of superoxide dismutase and peroxidase.

The secondary metabolites of plants have the functions of anti-oxidation, scavenging free radicals and reducing lipid peroxidation in the process of plants responding to biotic and abiotic stresses, protecting the plants from ultraviolet radiation, and conducive to plant growth in harsh environments (Treutter. 2006). Multiple genes of sour jujuba were involved in secondary metabolism under salt stress. After analysis, we found that the process of higher enrichment were Phenylpropanoid biosynthesis，Galactose metabolism，alpha-Linolenic acid metabolism. The expression of β-glucosidase-related genes (*At4g27290*, *KIN14R*, *BACOVA_02659*, *F26G*, *BGLU17*), cinnamoyl-related genes (*TKPR2*, *EO*, *DCR*, *ACT*), 4-coumarate-CoA ligase-related genes (*AAE6*, *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. 2013). Jasmonic acid (JAs) synthesis-related genes (*At5g37990*, *OPR1*, *OPR2*) were significantly expressed during alpha-Linolenic acid metabolism. As important signal transduction molecule, JAs play an important role in plant growth and development, disease tolerance, and induction of defense-related genes(Browse. 2009). ADH and LOX genes are also involved in plant salt tolerance mechanisms (Strommer et al. 2009; Blokhina et al. 2003). Raffinose family oligosaccharides (RFOs) are a type of functional oligosaccharides unique to plants. RFOs metabolism starts from inositol galactose synthase (GOLs) and catalyzes the synthesis of different RFOs members through raffinose synthase (RFSs),

stachyose synthase (STSs) and other enzymes (Kuo et al. 1988; Janecek et al. 2011). RFOs 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*, *GOLS2B*) and raffinose synthetase genes (*RFS2*, *RFS5*) were significantly up-regulated, which suggests that the RFOs metabolic process of sour jujube was strengthened to resist the damage caused by salt stress. In addition, β-galactosidase gene (*BGAL3*, *BGAL10*, *IDD14*) expression of galactose-related genes in jujube was significantly decreased.

Phytohormones are important substances that regulate plant growth and development and participate in stress response. Transcriptome analysis showed that IAA, ABA, GA, and CTK genes of *Ziziphus jujuba* all responded to salt stress, and their expression patterns were different. After the jujube seedlings were treated with more than 150mM NaCl, within 6 hours, the IAA and GA content 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 in the early stage of salt stress, while multiple genes in the ABA synthesis pathway and signal transduction pathway were up-regulated, which indicated that in the early stage of salt stress, the ABA pathway was used to respond to 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 conditions of salt stress, which are combined with corresponding cis-acting elements to initiate the corresponding gene transcription and expression, regulate and reduce the damage caused to plants by salt stress. After analysis, we found that five transcription factors of sour jujuba were significantly expressed to resist salt stress. They were *AP2/EREPB*, *HD-ZIP*, *MYB*, *NAC*, *WRKY* family genes. Among them, we screened four AP2/EREPB family transcription factors, three up-regulated (*EFR5*, *DREB2A*, *DREB3*) and one down-regulated (*ERF1B*). Dehydration response element binding protein and ethylene response element binding factor are two important subfamilies of the AP2 / EREBP family (Sakuma Y et al. 2002). Over-expression of Arabidopsis *AtDREB2A*, rice *OsDREB2A*, and lettuce *LsDREB2A* genes will increase salt tolerance in plants. Therefore, it was speculated that AP2 / EREPB family transcription factors played an important role in resisting salt stress. HD-ZIP family is a kind of plant-specific transcription factors, which plays an important role in responding to stress signals. Sour jujube *ATHB-12* expression was significantly increased under salt stress. Go analysis found that ATHB-12 is involved in the response of abscisic acid. Therefore, we hypothesized that under the conditions of salt stress, the *ATHB-12* gene of sour jujuba may combine 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 plants responding to salt stress. We also screened six *NAC* family genes, all of which were significantly up-regulated. It was found that a salt stress response gene in Arabidopsis was induced by multiple *NAC* transcription factors (Tran et al 2004). Therefore, we speculated that multiple genes in the *NAC* family of sour jujuba 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 in a short time or transiently under salt stress. The results of our analysis fully support these views.

The metabolism of RFOs oligosaccharides plays a vital role in resisting abiotic stress. The related research on RFOs metabolism involved in the regulation of plant stress tolerance mainly focuses on the regulation of GOLS gene expression and its function. The transcription levels of *AtGolS1* and *AtGolS2*

were up-regulated by drought and high salt stress. Overexpression of *AtGolS2* significantly increased the content of galactinol and raffinose in Arabidopsis thaliana, and enhancing the drought tolerance of plants (Taji et al.2002). Overexpression of heat shock transcription factor (HSF) and dehydration responsive element-binding (DREB) transcription factors in Arabidopsis significantly increased the transcription level of endogenous GOLS genes in Arabidopsis lines. The tolerance of plants to abiotic stress was also significantly increased (Buchfink et al.2005; Panikulangara rt al.2004). Maize *ZmGOLS2* was regulated by the *DREB2A* transcription factor, and overexpression of *ZmGOLS2* and *DREB2A* in Arabidopsis improved Arabidopsis abiotic stress tolerance (Gu et al.2016). Combining the expression patterns of various genes in sour jujube, we proposed the regulatory pattern shown in Figure 7. When sour jujube was under salt stress, its *DREB2A* gene might positively regulate *GOLS1* and *GOLS2* genes. Their high expression might directly lead to the accumulation of raffinose in the leaves of wild jujube and promotes the normal growth of plants.

Overall, we performed a comprehensive analysis of RNA-seq in leaves of sour jujuba seedlings under salt stress by transcriptome sequencing. Most genes were found to be involved in signal transduction of plant hormones, homeostasis of cell walls, secondary metabolism of organic matter, redox reactions and stress-related transcription factors. It laid a foundation for further studying the molecular mechanism of salt tolerance of sour jujube and improving the salt tolerance of sour jujube.

Author contribution statement Ruiheng Lyu, Rui Wang conducted the experiments and prepared the manuscript, Cuiyun Wu analyzed the experimental data, and Yajing Bao, Peng Guo designed the experiment.

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Complaince with ethical standards

Conflict of interest The authors declare no conflict of interest.

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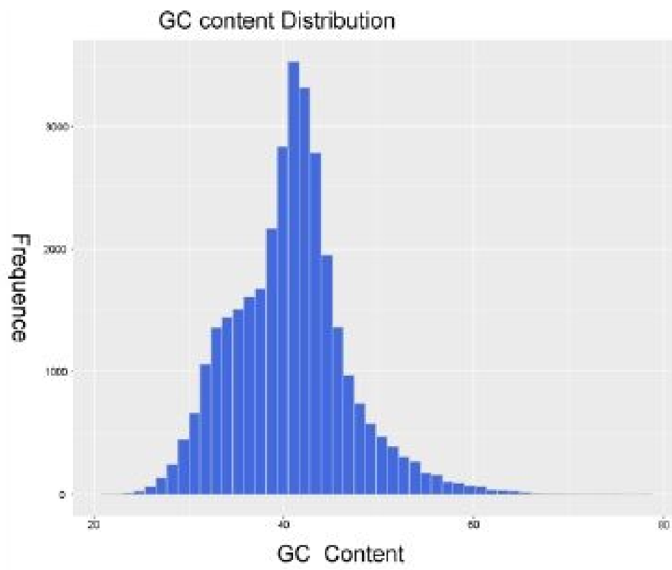
Figures



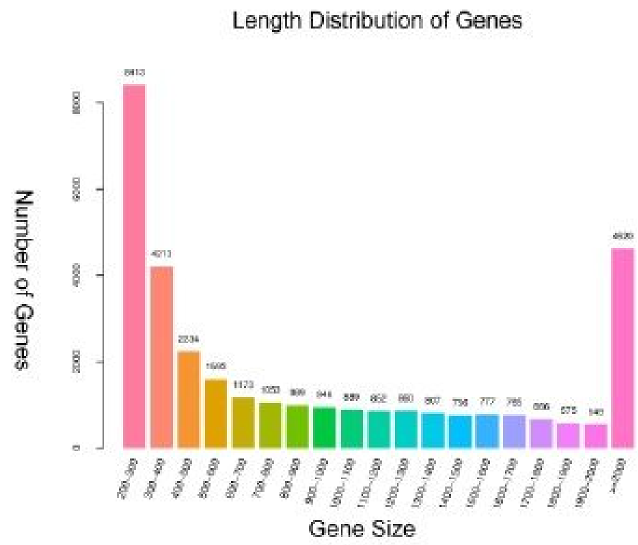


Figure 1

Gene assembly length distribution map and GC content statistics of assembly results

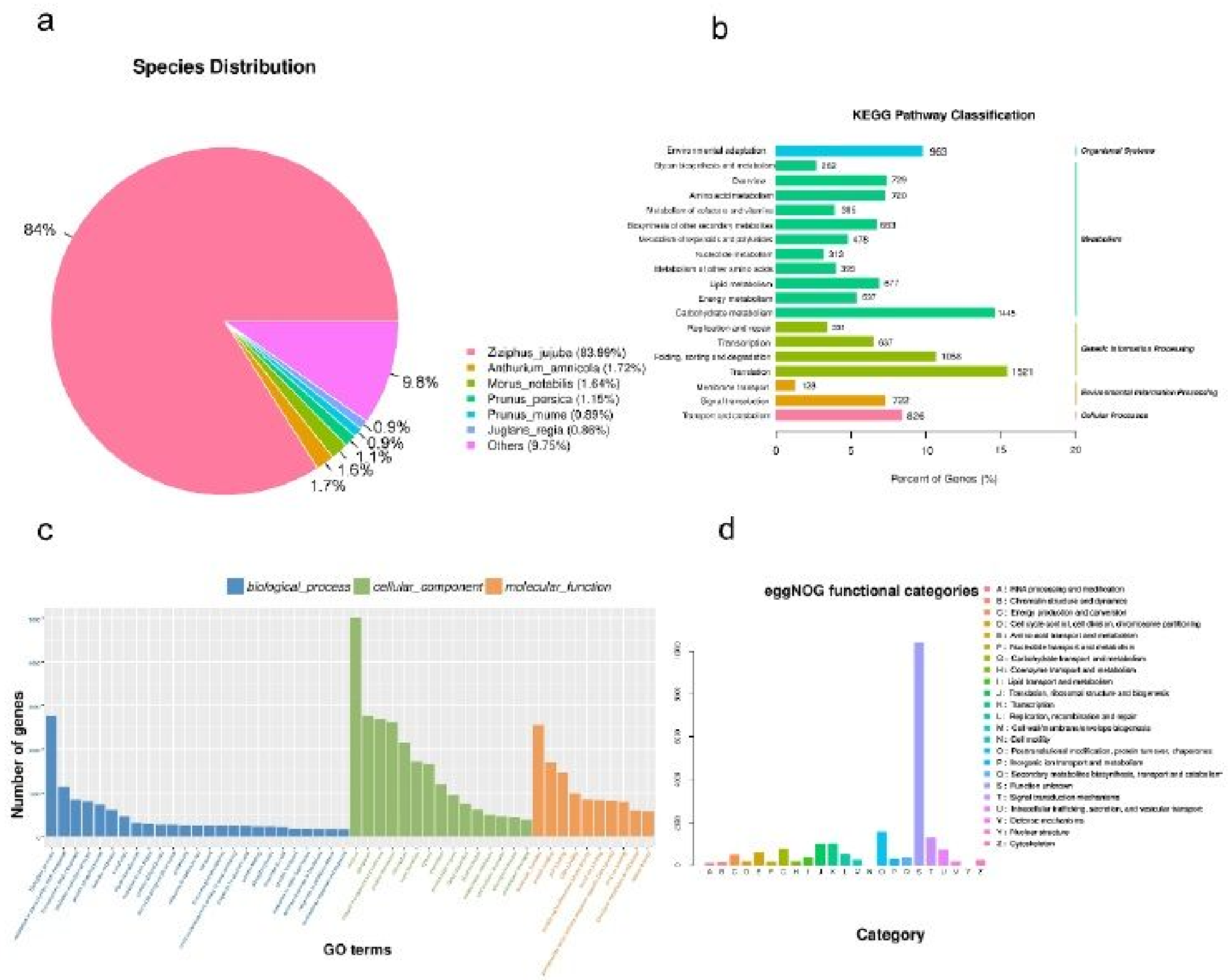


Figure 2

Gene function classification map. a Species-based distributions of blast results. Different color shows the percentage of unigenes hit to genes in different species according to Nr blast results. b KEGG classification annotation map. The horizontal direction represents the percentage of genes annotated to each functional pathway. c sour jujube transcriptome sequence Go annotation. d eggNOG annotation of transcriptome sequence in sour jujube. The letters on the horizontal axis indicates 26 categories of eggNOG. The vertical axis shows the number of the genes displayed in the categories

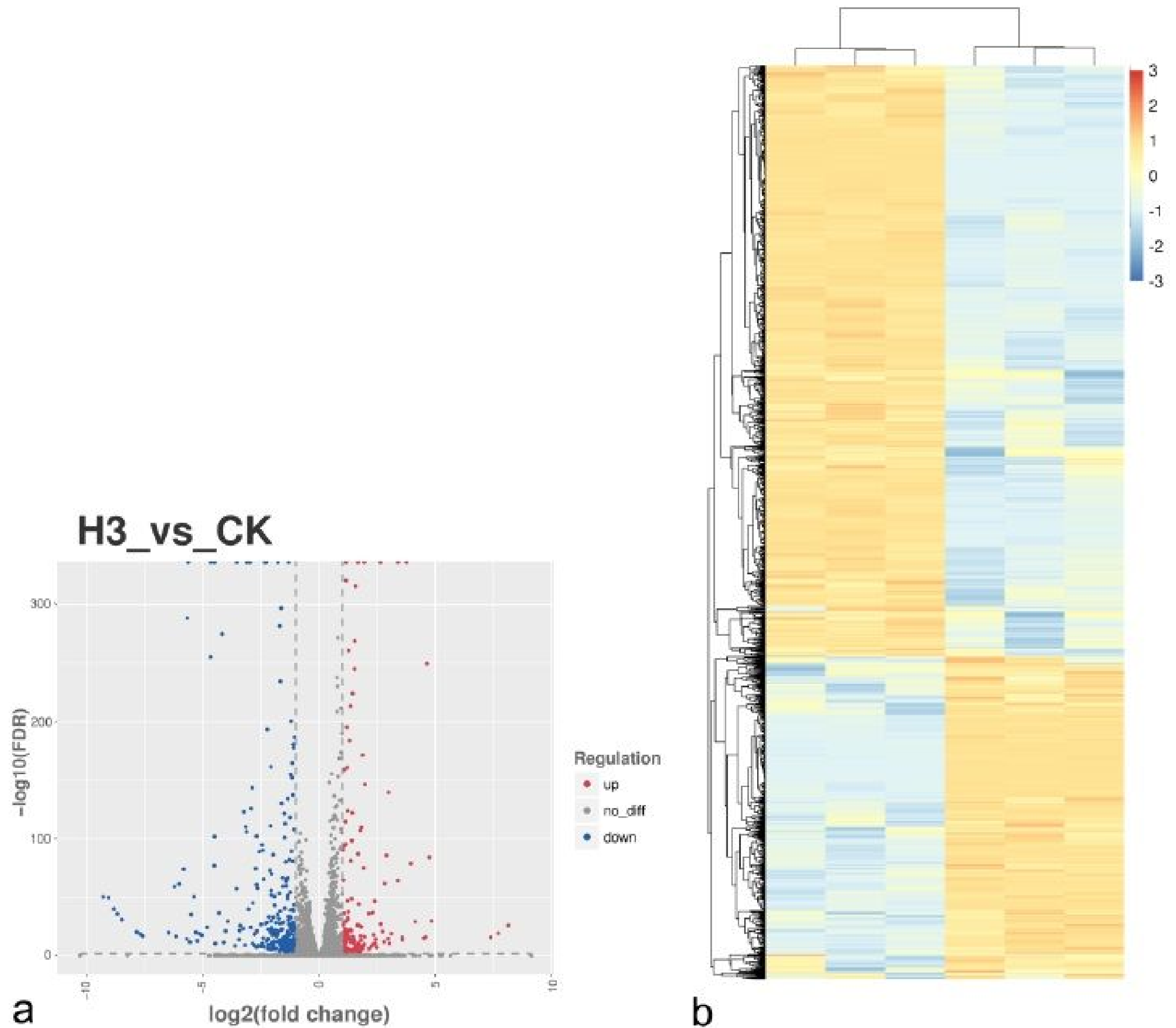


Figure 3

Statistical Analysis and Hierarchical Clustering of Differential Genes. a Difference gene volcano map. The abscissa represents the fold change in differential expression of genes in different samples; the ordinate represents the statistical significance of the difference in gene expression; the up color represents the significantly differently expressed genes that are up-regulated, the down color represents the significantly differently expressed genes that are down-regulated, and no represents non- Significantly differentially expressed genes. b Heat map of clustering of differential genes. The abscissa is the sample and the ordinate is the gene. Different colors indicate different gene expression levels.

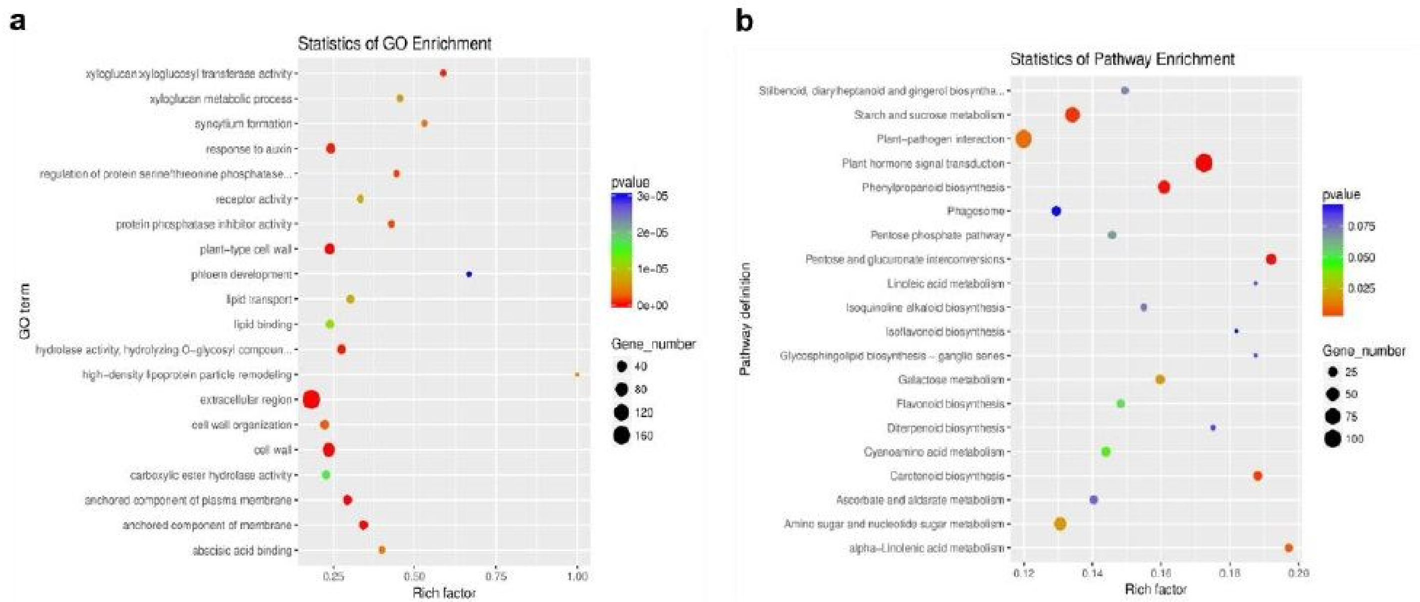


Figure 4

GO enrichment and KEGG enrichment of unigenes. a GO enrichment analysis of scatter plots. Ggplot2 was used to analyze the GO enrichment results, which were shown as scatter plots. The Rich factor represented the number of differential genes located in the GO/the total number of genes located in the GO. The larger the Rich factor was, the higher the GO enrichment degree was. b KEGG enrichment analysis of scatter plots.

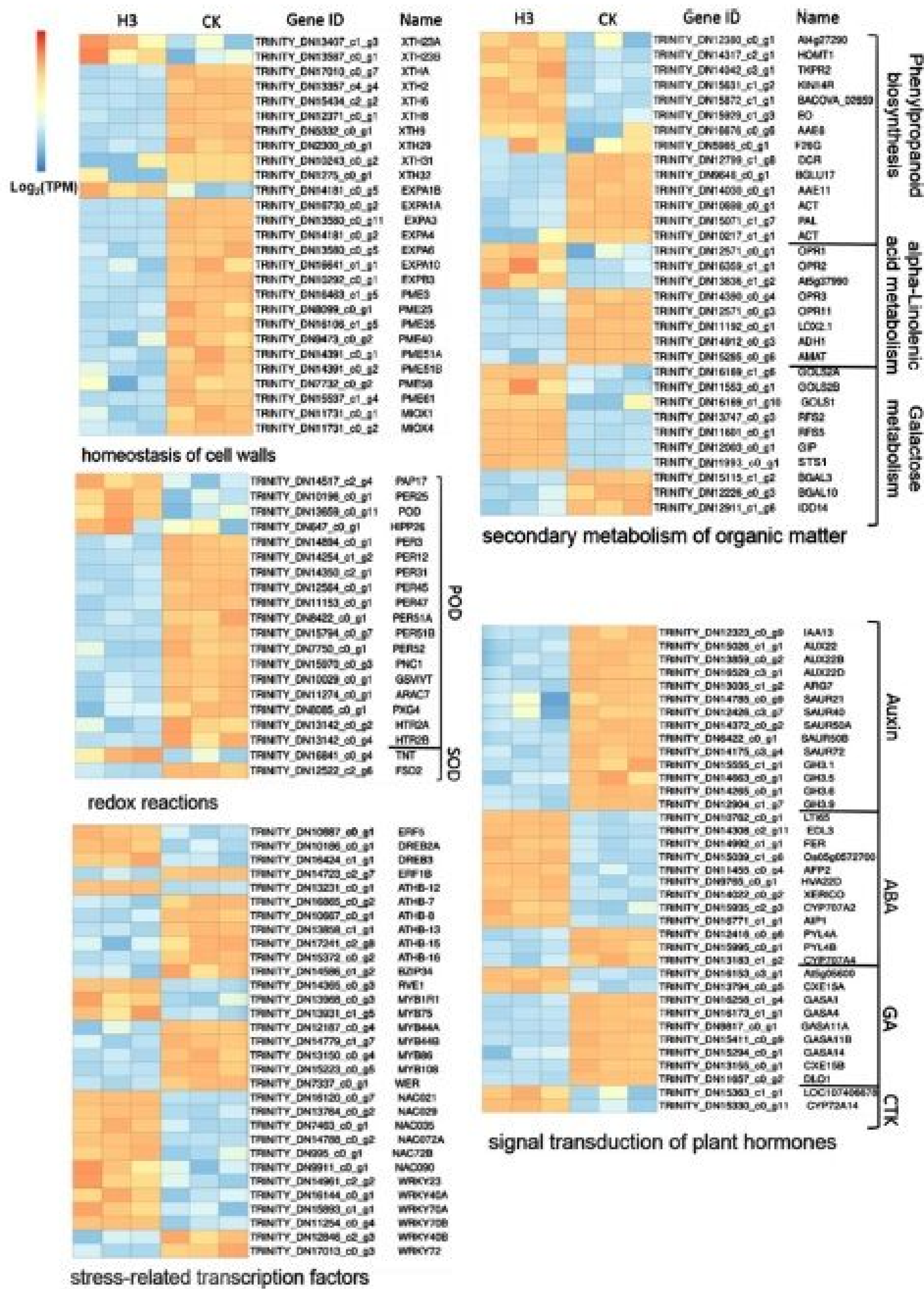


Figure 5

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. Orange represents up-regulation of gene expression and blue represents down-regulation.

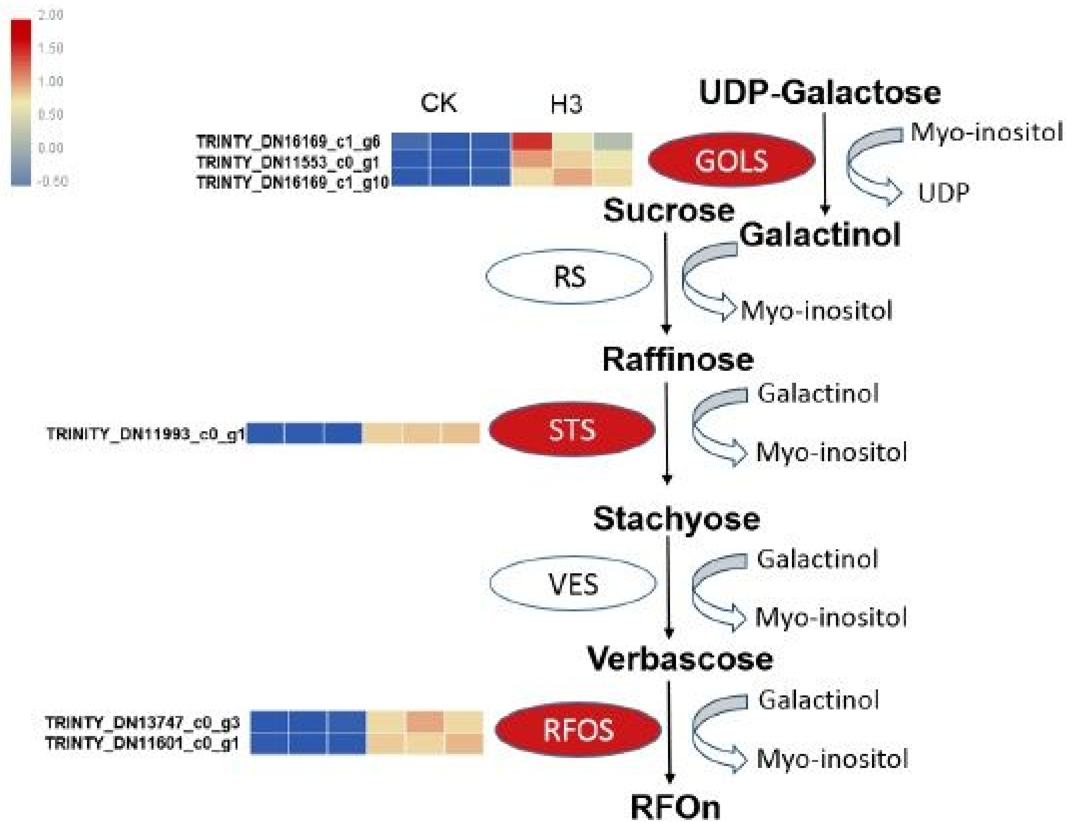


Figure 6

Differentially expressed genes and their expression levels in RFOs metabolic pathways. Heatmaps were constructed based on log2 (TPM) of leaves in control group and experimental group.

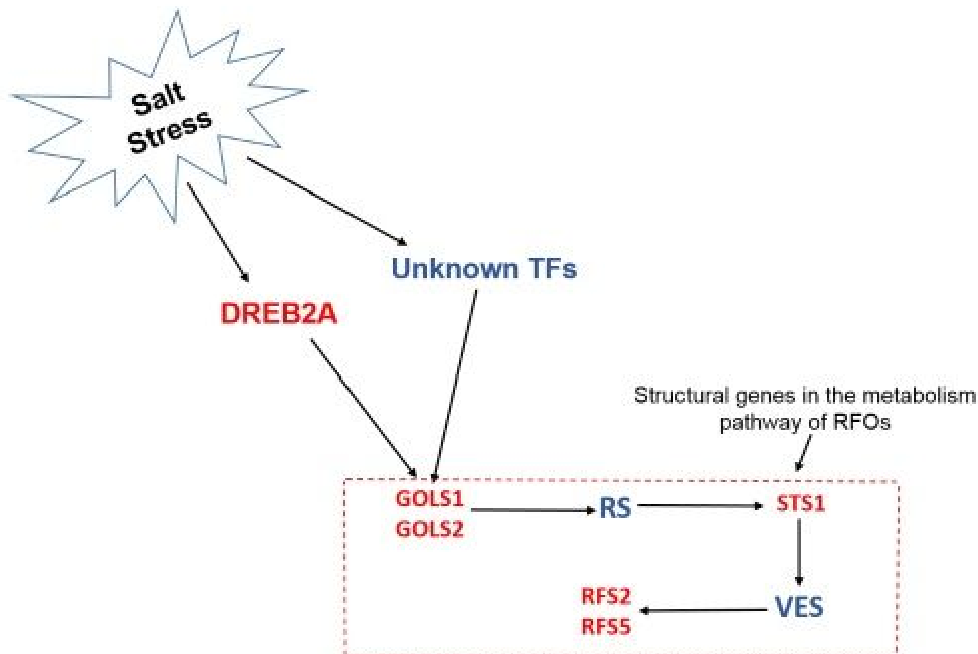


Figure 7

Proposed regulatory model between transcription factors and structural genes of RFOs metabolism pathway in sour jujube under salt stress

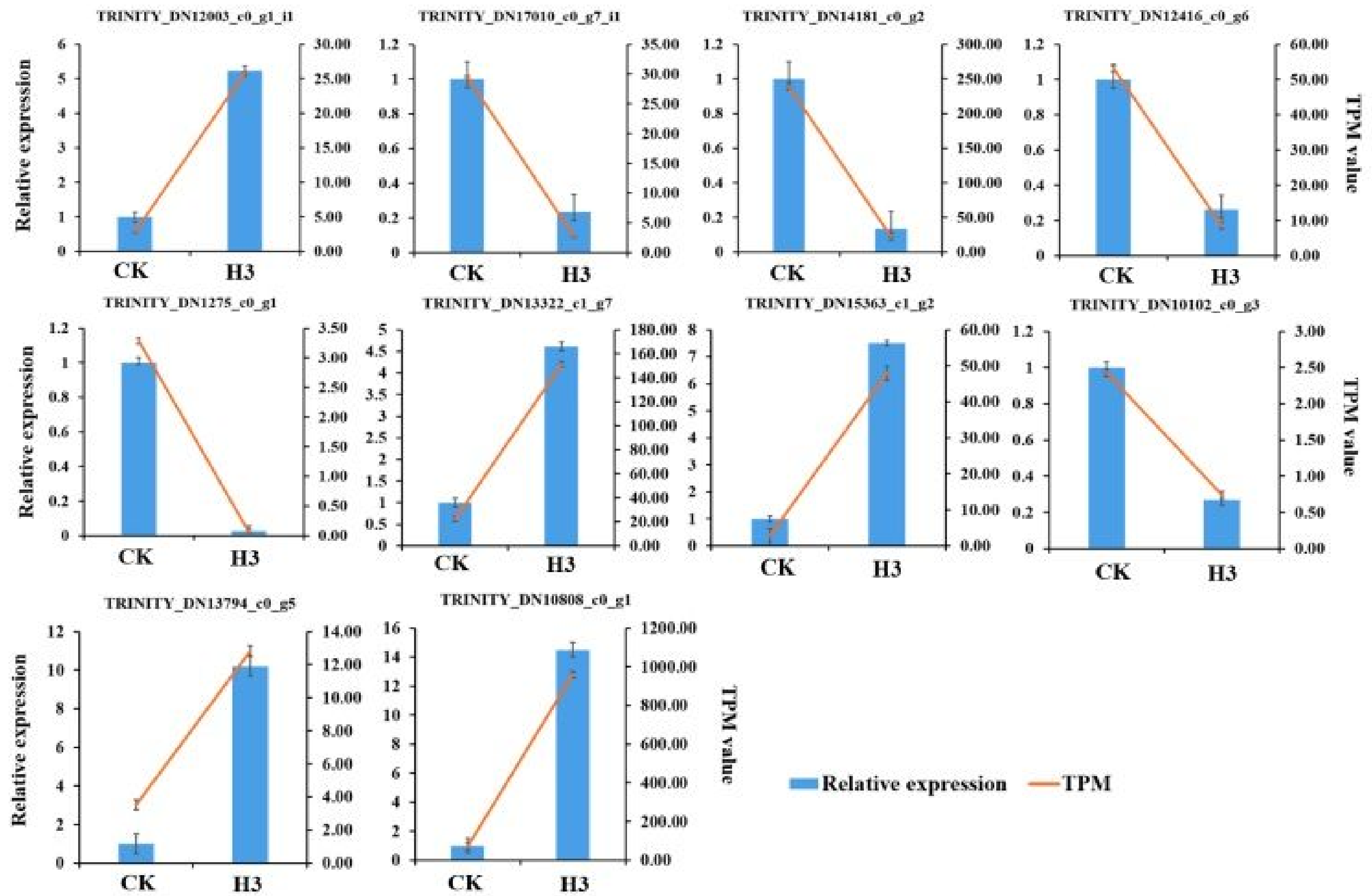


Figure 8

RT-qPCR results and RNA-seq expression of 10 genes in the experimental group and control group.

Supplementary Files

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