Comparative transcriptome profiling reveals the cold stress responsiveness in two contrasting Chinese jujube cultivars

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Abstract

Background

Low temperature is a major factor influences the growth and development of Chinese jujube (*Ziziphus jujuba* Mill.) in cold winter and spring. Little is known about the molecular mechanisms for coping with different freezing stress conditions in jujube. To gain insight into the freezing-related molecular changes, we conducted comparative transcriptome analyses from the cold-sensitive cultivar 'Dongzao' and cold-tolerant cultivar 'Jinsixiaozao' using RNA-Seq.

Results

In our study, more than 20,000 genes were detected at chilling (4°C) and freezing (-10°C, -20°C, -30°C and -40°C) stress between two cultivars. The numbers of differentially expressed genes (DEGs) between the two cultivars was 1831, 2030, 1993, 1845 and 2137 under the five treatments, respectively. Functional enrichment analysis suggested that metabolic pathway, response to stimulus and catalytic activity were significantly enriched at stronger freezing stress. Among them, nine DEGs participated in the pathway of Ca²⁺ signal, thirty-two DEGs were identified to take part in sucrose metabolism, and other DEGs were identified to participate in the regulation of ROS, plant hormones and antifreeze protein. In addition, important transcription factors (WRKY, AP2/ERF, NAC and bZIP) participating in the freezing stress were activated under different degrees of freezing stress.

Conclusions

Our research provides a more comprehensive understanding of DEGs involved in freezing stress at the transcriptome levels in *Z. jujuba*, especially two cultivars with different cold tolerance. These results expanding our understanding on the complex molecular mechanism of jujube, which also provide new insights and candidate genes for genetic improvement of jujube tolerance to freezing stress.

Background

Low temperature as an abiotic stress not only influences the geographical distribution of many important crops, but also negatively impacts productivity and quality of crops every year [1, 2]. Cold stresses are divided into chilling (0–15°C) and freezing (< 0°C) damage. Freezing stress also has a huge impact on the survival and geographical distribution of plants [3]. More than half of the 350,000 plant species on Earth are grown in the tropics and subtropical regions, such as rice, maize, and tomato which are chilling sensitive. And plants from temperate region are not very tolerant to freezing, they can increase their freezing tolerance by being exposed to chilling, a process known as cold acclimation [4, 5].

In previous studies, researchers have revealed that cold exposure results in a variety of alterations in physiology and gene expression patterns, and hundreds to thousands of genes even were identified that are up- and down-regulated in response to cold, including the metabolism of carbohydrates, molecular chaperones, antifreeze proteins, signal transduction (receptor kinase, protein kinase/phosphatase, Ca²⁺ binding protein) and regulatory proteins [6–9]. In addition, transcription factors could regulate genes expression [10–12]. Some of these genes were successfully transferred to cold or freezing-sensitive crops, such as *Arabidopsis thaliana*, rice, to improve yields under adverse growing condition [13, 14]. In rice, OsCDPK7 expression is induced by cold and salt stress, and overexpression OsCDPK7 increased tolerance to cold stress [15]. In other species, overexpression of some CBF genes in *Arabidopsis* and other plants conferred enhanced tolerance to cold stresses [16–21]. Also, in barley, TaCBF14 and TaCBF15 could enhanced freezing tolerance [22]. Moreover, some TFs (WRKYs, MYBs and NAC) are involved in plant cold response [23, 24]. In addition, both low and freezing temperatures result in the oxidative stress due to ROS generation, which includes hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), and hydroxyl radical (HO·) [25]. ROS signals could activated redox-responsive proteins, such as transcription factors and protein kinases [26, 5], such as mitogen-activated protein kinase (MAPK) cascade [27, 28], and activation of ZAT12 [29, 30]. *Arabidopsis* mutant, *chy1*, also accumulates high levels of ROS, which showed a reduced cold induction of CBF genes, and is defective in chilling and freezing tolerance [31].

Chinese jujube, native to China, is one of the country's most important fruit crops. In recent years, although global temperature is increasing, abnormal weathers, such as cold extremes, have been observed to increase all over the world, especially in main gain crops and industrial crops production areas [32, 33]. These disasters seriously hinder the development of jujube industry [34, 35]. To understand the jujubes respond to freezing stress responses is crucial to provide valuable information for breeding cold tolerance jujube cultivars. While many cold stress studies have been conducted in model experimental plants, such as *Arabidopsis*, rice, potato, grape and apple [36–38], little is known about the molecular mechanisms of freezing tolerance in *Z. jujuba*. Thus, in the present study, we used RNA-seq technology to compared the difference between the two Chinese jujube, the cold-sensitive cultivar 'Dongzao' and cold-tolerant cultivar 'Jinsixiaozao', under different degrees of freezing stress. We identified the freezing stress related candidate genes and metabolic pathways involved in chilling and freezing tolerance by comparing two cultivars at different freezing stresses. Many of the genes are involved in photosynthesis, hormone signaling and plant immunity, and the key genes expression patterns are associated with greater cold tolerance. The objective of this work was to better understand the molecular regulation that occur under cold tolerance using different degrees of low temperature and specific cultivar to cultivar comparisons, which providing a rich genetic resource for further study of cold response in jujube.

Results

Quality analysis and sequence assembly of RNA-seq data

Total RNA extracted from the xylem of jujube branches was employed to build expression libraries for sequencing by using the Illumina High-Seq sequencing system. We obtained 30 libraries reads that each library has about 89 million in total. After filtering, clean reads varied from 87 million to 88 million, which occupied on average 97.47% in total reads of all libraries, were collected (Table 1). In all samples, the Q30 value was all over 86%, and the GC content was 43%. Genome map rate which accounted for more than 73%, and gene map rate could reach from 72.93–76.32%. A total about 20,000 genes were detected to be expressed in each library. In addition, more novel transcripts were both detected in DZD and JSD. Then, about alternative splicing, each sample has a similar number of event. Indel, JSD has the maximum mark, significantly more than in DZC. Three biological replicates were collected for each cultivar sample under each temperature. Pearson’s correlation coefficients among the three technical replicates replication samples were high ($\gamma = 0.91-0.98$), indicating that the sequencing quality allowed for further analysis.

Table 1

Summary of the RNA-seq data collected from ‘Dongzao’ and ‘Jinsixiaozao’.

Sample	Raw data	Clean reads	Q30(%)	GC(%)	Genome map Rate	Gene map Rate	Expressed Gene	Expressed Transcripts	Novel Transcripts	Alternative Splicing	Indel
DZCK	89647985	87382161	86.91	44.19	72.64%	74.96%	20411	25818	988	90011	4342
DZA	89648769	87565991	88.23	44.12	73.14%	75.11%	20362	25710	954	87733	8628
DZB	89648625	87947768	87.87	44.33	73.84%	76.32%	20431	25881	980	88983	5811
DZC	89648887	87910197	88.73	44.19	73.52%	75.50%	20192	25458	924	85154	3927
DZD	89649519	87899251	88.22	44.02	73.98%	73.91%	21546	26853	1066	87281	4230
JSCK	89647603	87753183	87.40	43.64	73.12%	74.68%	20416	25724	983	89086	5555
JSA	89648885	87822119	89.13	43.69	72.43%	74.23%	20264	25542	983	91659	4705
JSB	89648594	88010731	88.92	43.91	72.85%	74.63%	20154	25425	972	86901	6013
JSC	89648221	87657475	87.74	43.70	72.58%	73.31%	19871	25011	963	87767	5261
JSD	89647797	87561609	87.25	43.69	73.28%	72.93%	20648	26003	1051	99095	6220
DZCK: ‘Dongzao’ treated at 4°C; DZA: ‘Dongzao’ treated at -10°C; DZB: ‘Dongzao’ treated at -20°C; DZC: ‘Dongzao’ treated at -30°C; DZD: ‘Dongzao’ treated at -40°C; JSCK: ‘Jinsixiaozao’ treated at 4°C; JSA: ‘Jinsixiaozao’ treated at -10°C; JSB: ‘Jinsixiaozao’ treated at -20°C; JSC: ‘Jinsixiaozao’ treated at -30°C; JSD: ‘Jinsixiaozao’ treated at -40°C.											

DEGs obtained by comparing two cultivars

In order to understand the differences between different cultivars in response to different low temperature stress, gene expression profiles in ‘Dongzao’ and ‘Jinsixiaozao’ at the same freezing stress were further analyzed though FPKM values. The detailed statistic is shown in Additional file 2. First, we investigated DEGs specific to each freezing stress by comparing ‘Dongzao’ to ‘Jinsixiaozao’. There were 1831 (885 up-regulated genes and 946 down-regulated genes), 2030 (979 up-regulated genes and 1051 down-regulated genes), 1993 (949 up-regulated and 1044 down-regulated genes), 1845(923 up-regulated and 922 down-regulated genes) and 2137(1158 up-regulated and 979 down-regulated genes) DEGs were identified at 4°C, -10°C, -20°C, -30°C and - 40°C, respectively, suggesting responsive genes to freezing and cold resistance (Fig. 1 and Additional file 3).

In five different degrees of cold stress, as shown in Fig. 2, 854 common DEGs were detected in both cultivars in response to five treatments, that may be suggests these DEGs are involved in the process of dealing with low temperature under different low temperature, and both participate to the same pathways in response to freezing stress. In addition, at -40°C, the number of unique DEGs (600) are significantly higher than other group, indicating that these genes responded differently and thus are candidate contributors to the difference in cold tolerant between ‘Dongzao’ and ‘Jinsixiaozao’.

In this research, RNA sampled from branch for qRT-PCR validation of the sequence based transcription profiles for six DEGs. As shown in Fig.S1, all the genes showed a similar expression pattern between the RNA-seq data and the qRT-PCR results, which indicated that these genes are really expressed and the changes at different temperature are real. Also the results confirmed that the RNA-seq data are highly reliable for further analysis.

Function annotation and enrichment analysis of DEGs

To understand the functions of discovered DEGs between two cultivars, all DEGs were searched against the protein database, followed by Gene Ontology (GO) analysis to assess the genes' putative functions. The top GO terms in each treatment, ranked by the number of DEGs, the stronger freezing stress, varied with freezing treatment stages. The more genes were annotated, and the metabolic processes were modified and changed (Fig. 3). In the comparative analysis of these five groups, GO terms of shared DEGs between the two cultivars could reflect common responses to freezing stress. Of these GO terms, the majority of DEGs were classified into 'metabolic process', 'cellular process' and 'response to stimulus'; and 'cell', 'cell part' and 'membrane' in cell components; 'catalytic activity', 'binding', 'transporter activity' in molecular function.

In addition, to gain insights into the metabolic or signal pathways involved in freezing stress, 20 common metabolism processes are most enriched at five freezing treatments in two cultivars (Fig. 4). We also found different numbers of DEGs in the same pathway between two cultivars. Among these top pathways, carbohydrate metabolism pathway, galactose metabolism, fructose and mannose metabolism were associated with many up/down regulated genes. Furthermore, in the comparison of the five groups, there are some pathway enrichment in the stronger freezing stress in 'Jinsixiaozao', such as peroxide of metabolic pathways, which is only at -30°C significant enrichment, ABC transporter process is at -40°C. In brief, in the functional annotation of DEGs, many genes of different metabolic pathways were involved in the regulation of freezing stress in jujube, and further study on the differential expression pattern in these pathways has a great significance for revealing the cold resistance of jujube.

Whether jujube trees can safely pass the winter is an important factor affecting the yield and quality of the next year. Therefore, in order to further analyze the difference in jujube at extreme temperatures, we conducted a comparative analysis of the two cultivars with temperature of -30°C and -40°C. In comparison, we found that more significantly DEGs were found in the stronger freezing stress. Peroxisome pathway were enriched in DZC vs. JSC, and ABC transporters pathway enriched in DZD vs. JSD. In addition, 107430353 annotated to the WRKY family was significantly changed at lower temperature, and in ABC family Genes 107429561, 107408533, 107404223, 107414655, and 107416457 simultaneously exhibited significant up-regulation. The number of DEGs related to kinase activity and carbohydrate metabolism were increased at the stronger freezing stress than the weak.

KEGG pathway classification of DEGs under cold stress in 'Dongzao' and 'Jinsixiaozao', respectively. The Y axis corresponds to KEGG pathway, the X axis shows the enrichment ratio between the number of DEGs enriched in a particular pathway. The color of the dot represents q-value, and the size of the dot represents the number of DEGs mapped to the referent pathway.

Comparison analysis at different degree freezing stress in 'Dongzao' or 'Jinsixiaozao'

To further understand the effect of different degrees freezing stress on jujube, we also compared analysis five stresses in 'Dongzao' and 'Jinsixiaozao', respectively. In 'Dongzao', DZCK as a control, 1291 DEGs were identified at the different degree of freezing stress, though only 55 common DEGs were identified in all freezing stress (Fig. 5, Fig. S2 and Additional file 4). Then, these DEGs were further screened and 327 significant DEGs ($P\text{-value} < 0.05$ and $|\text{Log}_2| \geq 1$) were identified. Of these significant DEGs, some genes related to Ca^{2+} signal pathway, including up-regulated gene (107413690) and down-regulated genes (107404917, 107423107, 107425924, 107417689, 107421107, 107422102, 107430853 and 107424939). some related to sucrose metabolism were classified into biological process among the four groups, which including 7 up-regulated and 25 down-regulated. Furthermore, about transcription factor, through the analysis of DEGs, we found that the genes belonging to the NAC, WRKY, MYB, AP2/ERF and bHLH families all showed a significant up or down changed. 107424282 homologous with grape MYB transcription factor is significantly down-regulated in cold stress, suggesting that it may negatively regulate cold resistance of jujube trees. In addition, NAC transcription factor (107435293) had a higher fold changes at -30°C, indicating that this transcription factor may be regulating the cold resistance of jujube trees in response to low temperature.

In the comparison of four groups of 'Jinsixiaozao', JSCK as a control, a total of 1668 DEGs were identified (Fig. 5, Fig. S3 and Additional file 5), and 86 common DEGs were present in all comparisons. Then we focused analysis on the significant DEGs ($P\text{-value} < 0.05$ and $|\text{Log}_2| \geq 1$), a total of 71 significantly DEGs were identified at -10°C, 39 genes were up-regulated and 32 were down-regulated. In the case of -20°C, 108 DEGs were identified, including 31 up-regulated genes and 77 down-regulated genes. With the decrease of temperature, 31 genes were up-regulated and 70 genes were down-regulated at -30°C. At -40°C, only 43 genes had bigger changed. Similar to the results of 'Dongzao', many genes associated with abiotic stress have been annotated in 'Jinsixiaozao', such as sucrose metabolism, Ca^{2+} signal pathway and so on. Interestingly, among these DEGs, 107422102 was significantly down-regulated at -10°C in 'Jinsixiaozao', however, while it was not changed until the temperature dropped to -40°C in 'Dongzao'. That may the different regulatory mechanisms of the two cultivars in response to freezing stress, thus reflecting different cold resistance.

Key transcription factors associated with freezing stress

A number of genes are induced by these stresses, and their products are thought to function in stress tolerance and response. In this study, we further analyzed the expression of key transcription factors (AP2/ERF, WRKY, NAC and bZIP), which have been reported to be involved in response

to freezing stress. All samples FPKM values of these genes are list in Additional files 6. In all libraries, we identified a total of 28 WRKY members, 52 AP2/ERF members, 22 NAC members and 15 bZIP members in two cultivars (Fig. 6). Comparison of these TFs expressions between the two cultivars revealed that all identified WRKY members, some are highly expressed in 'Dongzao' and others are highly expressed in 'Jinsixiaozao', indicating different regulation in different pathway. Among them, the expression level of WRKY04 (Gene ID: 107417519), WRKY11 (Gene ID: 107414569), WRKY20 (Gene ID: 107419756) in 'Jinsixiaozao' have totally different from 'Dongzao'. In AP2/ERF gene family, three of 52 AP2/ERF members (Gene ID: 107418844, 107432807 and 107422868) were up-regulated under the light cold stress in 'Dongzao', but began to change under the lower temperature 'Jinsixiaozao'. In terms of NAC and bZIP, NAC19 (Gene ID: 107415997) has higher expression level in DZD than other samples, while in 'Jinsixiaozao' the expression level was higher at all five temperatures; bZIP members are generally divided into two expression patterns. The observed expression differences of these transcription factors between the two cultivars suggest that TFs may play important roles in response to different freezing stress in jujube.

Data were normalized based on the mean expression value of each gene in all samples analysed. Red and green boxes indicate high and low expression levels, respectively, for each gene.

Key DEGs in galactose metabolism pathway

The accumulation of stachyose plays an important role in plant resistance to low temperature stress, while galactonl is a key enzyme in the synthesis of stachyose, and α -galactosidase is important in the decomposition process of stachyose. Thus, we further characterized the identified DEGs involved in the metabolism of galactose. In the comparison of five groups, KEGG pathway analysis that the galactose metabolic pathway was significantly enriched under each temperature condition. During the metabolism of galactose, some genes associated with galactose, stachyose, raffinose, and sucrose pathway showed significant differences to cope with the adverse effects of low temperatures. Through annotation of the target genes and comparison with KEGG database, some genes were shown involving in metabolic pathway and biosynthesis of galactose. As listed in Table S2, among them, 107411641 is a key gene in the process of UDP-galactose to form galactonl, which is significantly up-regulated in stronger freezing stress, that will enhance the synthesis pathway of stachyose. However, in the pathway of stachyose decomposes into galactose and raffinose, FPKM value for 107425264, 107430302, 107414011 and 107428511 showed decrease, which reduces the decomposition of stachyose (Fig. S4).

Discussion

Over the past years, extreme weather occurs frequently, the uncontrollable climate affected the growth and development of jujube, even caused their death. Multiple responses to cold stress have been reported in many plant species, reflected by the variations in transcription or metabolism [39, 30]. The cold tolerance of plants depends on cellular signal transduction pathways. Recently, significant advances have been made regarding the understanding of plant cold-signaling perception and transduction [40–42]. In our study, the systematic transcriptome profile between 'Jinsixiaozao' and 'Dongzao' was reported to identify the genes responsible for freezing resistance, which could help the breeding for future development of improved cold-tolerance in jujube cultivars.

Identification of DEGs in two cultivars

In two cultivars, some DEGs were investigated and hundreds of freezing-related genes were identified in ten libraries of jujube branch (about 87 million reads in 'Dongzao' and 'Jinsixiaozao'), without difference between the global number of genes expressed at the four stages of freezing stress. The majority of DEGs was involved in metabolic process, a total of 362 significantly DEGs (323 DEGs in 'Jinsixiaozao') were found to be differentially expressed in 'Dongzao' at the different degree of freezing stress. The stage DZCK was associated with the largest number of significant DEGs with respect to DZD. Compared with the control, most significant DEGs showed down regulation. This result is similar to Kou et al. [43] research in potato, which suggested that different cultivars have similar responses to cold resistance. Moreover, GO classification of 'Dongzao' and 'Jinsixiaozao' was compared and enriched in functional categories, mainly 'catalytic activity', 'metabolic process', 'cell part' and 'binding'. And KEGG pathway analysis revealed that the principal enriched pathways comprised plant hormone signal transduction, metabolic pathways, ribosome and secondary metabolic pathways. With the data provided by the comprehensive analysis using transcriptomic profiling, important genes involved in the development and regulation of these freezing stress attributes were characterized, which can further contribute to understanding *Z. jujuba* biology.

Physiological responses related genes under freezing stress

Freezing stress may cause plant death. Some research shows that, under this conditions, plants could increase their tolerance by synthesizing numerous protective substances and proteins [44–47]. Raffinose family oligosaccharides (RFOs) are a class of functional oligosaccharides unique to plants, mainly including raffinose, stachyose and verbascose. Some study found that RFO can improve the ability of plants to cope with adversity. The more RFOs accumulate, the stronger the ability of plants to be exposed to low temperatures. Galactose and related enzymes are involved in the entire RFO metabolic pathway [48]. In our study, galactose metabolism was significantly enriched, and relative genes in this pathway have up or down regulation to cope with cold stress. Among these genes, 107426117 (stachyose synthetase), 107415484 (raffinose synthase) and 107411641 (inositol 3- α -galactosyltransferase) were significantly up-regulated under stronger freezing treatment in 'Jinsixiaozao', however,

some of them did not significant change under the weak freezing stress. That may be suggested these genes play more important role in galactose metabolism in response to cold, especially under stronger freezing stresses. In addition, late embryogenesis abundant protein (LEA) could respond to abiotic stresses such as low temperature and drought [49]. In our study, the up-regulation of LEA (Gene ID:107409127) indicates that LEA genes are involved in freezing tolerance in 'Jinsixiaozao' (-40°C). The function of LEA protein in this study should be further studied as a regulatory mechanism of freezing response.

Cold signal transduction related genes under freezing stress

Signal transduction pathway is known to play crucial roles in response to the stress of freezing, among these, Ca^{2+} is an important second messenger in plant [50, 51]. In our research, 34 DEGs were identified in response genes related to calcium-binding proteins, calmodulin and Ca^{2+} signal. Among them, gene (Gene ID: 107413690) showed a significant up-regulation in 'Dongzao', which indicating these genes play important role in jujube resistance to freezing stress and the function of these genes should be further studied.

In addition to Ca^{2+} , ROS also play important roles [52]. On the one hand, they induce genes expression and protein synthesis to protect cells from stress; on the other hand, they induce oxidative stress [53, 54]. Meanwhile, ROS also can directly stimulate the expression of genes in activating stress-related pathways, such as MAPK cascade pathway, hormone signaling pathway and the calcium signaling pathway. In our study, some genes with antioxidant activity are involved in freezing stress. Among these DEGs, six genes (one up-regulated gene and five down-regulated genes) were involved in the redox signaling, such as gene 107403552, which are differentially expressed in four degrees of freezing stress in 'Jinsixiaozao', indicating they played different roles in cold signal transduction under freezing stress.

Transcriptional regulation related genes under freezing stress

Transcription factors (TFs) play important functions in plant development and stress tolerance. Many transcriptomic data depicted some TFs that govern the massive and highly coordinated transcriptional changes under different freezing stress. Some studies show that most cold-regulated transcription factor genes are impaired in response to cold stress in Arabidopsis [55–57]. A lot of research shows that some transcription factors play important role in plant freezing tolerance [58–60]. Such as AP2/ERF factors RAP2.1 and RAP2.6, and the C_2H_2 -type zinc finger STZ/ZAT10 belong to the CBF regulon [61–62]. Furthermore, NAC was also shown to enhance expression during loquat fruit chilling-induced, however, it could not bind directly to promoters of lignin biosynthesis genes [63]. Many MYB-type transcription factors, for example AtMYB85 [64], AtMYB88 [65, 66], increases cold hardiness by CBF-dependent and CBF-independent pathways in apple. In other species, OsMYB4 transgenic lines of Arabidopsis exhibited improved chilling and freezing tolerance with a dwarf phenotype [67]. In this study, we validated AP2/ERF, WRKY, NAC and bZIP gene families and analyzed the expression patterns, found that different members play different roles in two cultivars. For example, in AP2/ERF family, different members of the family are involved in response to low temperatures. Among them, gene (Gene ID: 107414104) expression showed significant changes at -10°C; 107432697 and 107433390 were at -20°C; 107426263 and 107433390 at -30°C, however, 107414104, 107432697 and 107433390 changed significantly at the lowest temperatures. Similarity, other TF members are generally divided into two expression patterns. These differences suggest that the two cultivars have different responses for cold stress, even for different degree of low temperature. We did not go further to verify the function of these genes, but listed the significant differences in this work. Thus, the emphasis of future research could focus on the role of these genes in metabolic pathways, and understand the regulatory mechanism of genes.

In addition, some studies have found that alternative splicing (AS) events are important post-transcriptional regulation and are required for reprogramming gene expression under environmental stress. Previous studies showed that the number of AS events was significantly higher compared to control plants when plants exposed to different stresses, especially low temperatures [68]. To analyze the variation of AS under cold stress, in this study, we detected that all genes containing intron were alternately spliced under cold stress (Fig. S5). On the one hand, compared with the control, the number of AS events increases slightly with the temperature drops, we hypothesize that plants can fine-tune gene expression by AS events in response to temperature changes. On the other hand, most AS genes were not differentially regulated by stress, suggesting that AS may represent an independent gene regulatory layer that responds to cold stress.

Post-translational regulation related genes under freezing stress

In addition to the above, post-translational modifications also are important for plant cold responses. Dong et al. [69] found that HOS1 can degrade ICE1 and negatively regulating CBFs and downstream cold response genes by ubiquitination under low temperature stress. In this study, the homologous gene (Gene ID: 107429115) was found to be significantly up-regulated. In addition, with the enhancement of freezing stress, the pathways of phenylpropanoid biosynthesis and flavonoid biosynthesis were also strengthened. Among the significantly DEGs, genes (Gene ID: 107429115 and 107415362) are glycosyl-transferase related genes, and the former is significantly up-regulated at -40°C, and the latter is down-regulated, which may mean that they are positively and negatively regulated factor in the process of regulating the cold resistance of jujube, which involved in different regulatory signaling pathways. Moreover, MLO proteins are a unique proteins family to plants, and the expression of many MLO genes is affected by various biotic and abiotic stresses [70]. There is a calmodulin binding region at the C-terminus of the MLO protein, which allows the MLO protein to bind to calmodulin as a second messenger to transmit signals to the downstream pathway. The CaM protein binds to the CaMBD of the MLO protein to increase the activity of the MLO protein. In our data, we found that the gene 107409553 encoding the MLO protein

showed a significant up-regulation when 'Jinsixiaozao' was at -40°C, but showed down-regulation in 'Dongzao'. That implies that the gene may regulated by the calcium are varietal differences between two cultivars in response to freezing stress.

Conclusions

Our present study presented a comprehensively analysis of cold responsive genes via comparative transcriptome analysis using two contrasting Chinese jujube cultivars ('Dongzao' and 'Jinsixiaozao') under different freezing stress. Further analysis of data identified some important DEGs, which involved in galactose metabolism, signal transduction, transcriptional and post-translational regulation, were differentially expressed between two cultivars to resist freezing stress. And the involvement of TFs leads to a series of physiological and cell response, and improved tolerance to freezing stress. The transcriptional level of 'Jinsixiaozao' changed greatly during freezing stress may explain they were better cold tolerance compared to 'Dongzao'. These results provide a reference for our understanding of Chinese jujube adaptation to different freezing stress and provide new insights into the molecular mechanisms of different freezing tolerance in jujube cultivars.

Methods

Plant materials and cold treatment

The current year's branches of jujube cultivars were collected from the National Key Base for Improved Chinese Jujube Cultivar (Cangzhou, China). The branches of cold-sensitive cultivar 'Dongzao' (DZ) and cold-tolerant cultivar 'Jinsixiaozao' (JS) were placed at 4°C for 10 h and used as the controls (DZCK and JSCK), the other target temperatures were -10°C (DZA and JSA), -20°C (DZB and JSB), -30°C (DZC and JSC) and -40°C (DZD and JSD). After reaching the target temperature to maintain 10 h, then collect the xylem of the branches and snap-frozen in liquid nitrogen. Three biologic replicates for each treatment were used for RNA-seq.

RNA-seq library construction and sequencing

Total RNA were extracted from the 30 samples using the Plant RNA Kit (Omega) according to the manufacturer's instructions until required. Three biologic replicates for each treatment were used for analyzing transcriptome. The library products were ready for sequencing analysis using an Illumina Genome Analyzer at Beijing Genomics Institute (Shenzhen, China) in 2018. Raw data of fastq format were firstly processed through in-house perl scripts. In this step, clean data were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files were downloaded from genome website directly: <https://www.ncbi.nlm.nih.gov/genome/?term=Ziziphus>. The Z-score method using the p-value as a statistical significance index [71] was applied to identify differentially expressed genes.

Identification of DEGs, GO and KEGG enrichment analysis

A cluster analysis was performed according to Eisen et al. [72]: the log2 of TPM for each gene was used for the hierarchical clustering analysis. Gene Ontology (GO) categorization was carried out as described previously. We used KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways (<http://www.genome.jp/kegg/>). All AS events, including cassette exons, alternative 5'SSs, alternative 3'SSs, mutually exclusive exons, coordinate cassette exons, alternative first exons, alternative last exons and intron retention, which takes as input the genome coordinates of all annotated exons and all confidently identified splice junctions.

Real-time quantitative PCR

Six randomly selected DEGs were chosen for validation by real-time quantitative PCR (qRT-PCR). The sequences of the primer pairs (designed using Beacon Designer 7.2), the primer sequences were listed in Table S1. Real-time PCR was performed in the presence of TB Green Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Dalian, China) and read on an IQ5 real-time PCR instrument (Bio-Rad, Hercules, CA, USA). Each reaction was carried out in a 20 µL volume consisting of 10 µL of TB Green Premix Ex Taq II, 7.0 µL of ddH₂O, 2 µL template (1 µL of the generated first-strand cDNA diluted by 9 µL ddH₂O) and 0.5 µL of each primer. The following was used for qRT-PCR: 95°C for 10 min followed by 40 cycles at 95°C for 20 sec, 55°C for 20 sec, and 72°C for 20 sec. The baseline and threshold cycles (Ct) were automatically determined by the system's software. All the experiments were performed in biological triplicate. Transcript levels were normalized against the average expression of the Zjactin gene (GenBank: EU916201).

Abbreviations

DEGs: Differentially Expressed Genes; qRT-PCR: Quantitative real-time PCR; TF: Transcription Factor; MAPK: Mitogen-Activated Protein Kinase; FPKM: Fragments Per Kilobase Per Million Mapped Reads

Declarations

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Not applicable.

Authors' contributions

XMP and HYZ conceived and designed research; HYZ and YH conducted experiments; HYZ and MYL collected and analyzed the data. HYZ wrote the manuscript; SS and YSZ provided valuable suggestions on the manuscript; XMP, WHB and YYL revised the manuscript; XMP obtained funding and is responsible for this article. All authors read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supplemental Information

Additional file1: Figure S1-S6 and Table S1-S2. Figure S1, Validation by qRT-PCR of DEGs isolated from the different samples in 'Dongzao' and 'Jinsixiaozao'. Figure S2, GO analysis of DEGs under different degree cold stress in 'Dongzao'. The X and Y axes correspond to GO terms and the number of DEGs. Figure S3, GO analysis of DEGs under different degree cold stress in 'Jinsixiaozao'. The X and Y axes correspond to GO terms and the number of DEGs. Figure S4, DEGs in galactose metabolism. Different boxes represent different genes in galactose metabolism. The red boxes represent up-regulated genes, and the green boxes represents down-regulated genes. Figure S5, Alternative Splicing Event and gene number at the same degree cold stress between two cultivars. Table S1, Primers for qRT-PCR. Table S1, DEGs involved in galactose metabolism pathways.

Additional file2 : FPKM values in 'Dongzao' and 'Jinsixiaozao'

Additional file3 : DEGs between 'Dongzao' and 'Jinsixiaozao' at the same freezing stress

Additional file4 : DEGs at different degree freezing stress in 'Dongzao'

Additional file5 : DEGs at different degree freezing stress in 'Jinsixiaozao'

Additional file6 : FPKM values of Key transcription factors

Figures

Figure 1

Numbers of DEGs in two cultivars at the same freezing stress.

Figure 2

Venn diagram of DEGs under the same freezing stress in two cultivars. DZ-JS(CK), treated at 4°C; DZ-JS(A), treated at -10°C; DZ-JS(B), treated at -20°C; DZ-JS(C), treated at -30°C; DZ-JS(D), treated at -40°C.

Figure 3

GO analysis of DEGs at the same freezing stress between two cultivars. GO classification of DEGs under cold stress in 'Dongzao' and 'Jinsixiaozao', respectively. The Y and X axes correspond to GO terms and the number of DEGs.

Figure 4

KEGG enrichment analysis of DEGs at the same freezing stress between two cultivars. KEGG pathway classification of DEGs under cold stress in 'Dongzao' and 'Jinsixiaozao', respectively. The Y axis corresponds to KEGG pathway, the X axis shows the enrichment ratio between the number of DEGs enriched in a particular pathway. The color of the dot represents q-value, and the size of the dot represents the number of DEGs mapped to the referent pathway.

Figure 5

Venn diagram of DEGs in 'Dongzao' (left) and 'Jinsixiaozao' (right).

Figure 6

Expression profiles of the jujube AP2/ERF, WRKY, bZIP and NAC genes in two cultivars. Data were normalized based on the mean expression value of each gene in all samples analysed. Red and green boxes indicate high and low expression levels, respectively, for each gene.

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