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TRANSCRIPTOME PROFILING OF COLD RESISTANT GENES IN *LIRIODENDRON CHINENSE* (HEMSL.) SARG.

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Abstract

Transcriptome sequencing technology was used to analyze differential transcript expressions of *Liriodendron chinense* of two provenances in different temperature, and a lot of unigenes were obtained. Digital gene expression profiling technology was used to select differentially expressed genes. A number of differentially expressed genes were annotated on GO classification and pathway enrichment analysis metabolic pathways. Some metabolic pathways are associated with cold resistance including plant hormone signaling, unsaturated fatty acid biosynthesis, carbohydrate metabolism etc. A group of differentially expressed unigenes related to plant cold resistance were found including calcium signaling pathway, cold-response transcription factors, and genes maintaining membrane stability and adjusting osmotic balance. Low temperature stress experiment was carried on *L. chinense* and digital gene expression profiling technology was used to select differentially expressed cold resistant genes. Twenty six unigenes were obtained including cold-response transcription factors HSP, NAC etc., and genes maintaining membrane stability and adjusting osmotic balance, for example, β-amylase. Real time PCR technology was used to verify the expression characters of differentially expressed genes. This research provides basis for study cold resistance mechanism of *Liriodendron* at gene level, and also provides theoretical basis for molecular breeding.

Introduction

There are two species in the Genus *Liriodendron*, including *L. tulipifera* naturally distributed in the east of USA, and *L. chinense* native to southern China and northern Vietnam (Scott 1993). *L. chinense* is an endangered species in the IUCN Red List, with small and isolated populations (Wang *et al.* 2012). The two species are morphologically similar, and reproductively compatible with each other. Hybrids of the two species were created in the 1960s in China. *L. tulipifera* is highly valuable as garden trees because of its unique leaf shape and tulip-like flowers (Scott 1993). It grows quickly with relatively high stress resistance (Liang *et al.* 2007, Jin *et al.* 2011). *L. chinense* is an ideal species for landscaping because of its beautiful flowers and unique shape of leaves. In recent years, the species has received increasing attention due to its rapid growth and high quality wood. Plantations of the species have been continually expanding in southern China. However, *L. chinense* has a low seed production due to ineffective pollination caused by limited pollen production (Huang and Guo 2002).

Researches on *L. chinense* mainly focused on breeding (Zhang and Zhao 2005, Liu 2011), and molecular study of this species mainly concentrated in genetic diversity by molecular markers (Luo*et al.* 2000, Li and Wang 2002, Zhan *et al.* 2010). *L. chinense* distributed naturally on mountains at an altitude of 1000 m in China's southern provinces such as Jiangxi, Guizhou, and Fujian. A major limiting factor for cultivation of *L. chinense* in northern China is poor hardiness of this species. At present, the cold resistance of *L. chinense* research mainly focused on evaluating hardiness by observing phenotypes of the plants (Li *et al.* 2010) and cold hardiness study at gene level of *L. chinense* has not yet been carried out.

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Commonly used bio-molecular techniques for gene function research has some limitation in analyzing gene function comprehensively, while traditional cloning and genetic transformation method are not only expensive but also time consuming (Wang *et al.* 2014). Compared with these technologies, transcriptome sequencing technology has advantage of the specificity and effectiveness, which could explore the function of the gene, and realize the rapid identification and function analysis of huge number of transcripts (Zhang *et al.* 2010). At present, transcriptome-sequencing technology has been used to study the gene expression as a mature technology and has become more widely used to study the cold adaptation of plants and low temperature response gene (Wang and Zhao 2013, Xu *et al.* 2014). Genome sequencing has not yet been carried out on *L. chinense*. For the first time, Yang *et al.* (2014) conducted a research on transcriptome sequencing of the flowers and leaves of *L. chinense* and obtained seven groups of carotenoids biosynthesis related differentially expressed unigenes, which showed that the transcriptome sequencing technology is feasible for studying the species at gene level. Digital gene expression (DGE) profiling and analyze gene expression of a particular organization in certain condition is a rapid, economic technology that comprises some information about the importance of cold resistance gene.

The present study established transcriptome information database of *L. chinense* on different provenances and seasons with temperature differences through high-throughput sequencing technologies, and digital gene expression profiling technology to explore differentially expressed genes. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis were carried out to find out low temperature cold resistance related gene with a aim to explore the quality genetic resources and provide theoretical basis and molecular breeding.

Materials and Methods

In this experiment, leaf buds of *L. chinense* were collected in January 2014 and July 2014, in the forest farms located in Jiangxi, Anhui and Yunnan provenances. Jiangxi forest farm is located in 27.80°N, long. 114.40°E, and altitude 129 m, with an annual average temperature of 19.30°C, annual rainfall of 1619.7 mm, the average 15° slope, and red brown soil. Anhui provenance is located in 31.13°N, 118.20°E, and altitude 1450 m, with an annual average temperature of 10.10°C, annual rainfall of 1280 mm. Yunnan provenance is located in 21.40°N, 101.60°E, and 700 m altitude with an annual average temperature of 20.30°C, annual rainfall of 1942 mm (Li *et al*. 2001). Three sample trees with good growth condition and similar age were selected from each provenance, and the 30 leaf buds from the top of branches of each tree were collected. The leaf buds were packed with tin foil paper, put into liquid nitrogen immediately, and then kept in 80°C cryogenic refrigerator.

During the middle of July in 2014, one-year-old branches at the top were collected from a 14 years old tree of Anhui provenance, which was planted in Chinese Academy of Forestry, Beijing. Thirty branches were selected from upper, middle and lower crown, respectively. Ten branches from each part were mixed together, and three groups of mixture were prepared for the low temperature stress experiment. The first group was used as temperature control. The leaf buds of the first group were selected, wrapped with foil paper and frozen into liquid nitrogen immediately, and then preserved in –80°C cryogenic refrigerator. The second and the third groups were packed with plastic bags. The second group was kept for 24 hrs in the 4°C refrigerator and then, wrapped with foil paper and frozen into liquid nitrogen immediately, and finally preserved in 80°C cryogenic refrigerator. Similarly, third group was kept for half an hour in 30°C refrigerator and then wrapped with foil paper and frozen into liquid nitrogen immediately, thereafter preserved in –80°C cryogenic refrigerator.

Four groups of *L. chinense* leaf buds were used as experiment samples, and each group has three biological repetitions, with 12 samples. RNA-prep Pure Plant Kitcolumn (centrifugal type) was used to extract RNA from the above samples, respectively. RNA degradation and contamination was monitored on 1% agarose gel. RNA purity was checked using the Nano-Photometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit®RNA Assay Kit in Qubit®2.0 Flurometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bio-analyzer 2100 system (Agilent Technologies, CA, USA).

Four samples in the form of mixing RNA from Anhui group (AN), and Yunnan group (YUN) were respectively built transcriptome library. A total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEB Next® Ultra™ RNA Library Prep Kit for Illumina®(NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-Toligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNaseH-). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. Remaining overhangs were converted into blunt ends via polymerase activities. After adenylation of 3' ends of DNA fragments, next Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced by an Illumina Hiseq 2500 platform and paired-end reads were generated.

As the samples tested qualified, leaf bud mRNA of *L. chinense* was enriched. Double-stranded cDNA was then synthesised. Segments were amplified by PCR, and the libraries were obtained by after PCR products were purified. Agilent 2100 and Q-PCR were used to evaluate the quality of the library. Single-ended sequencing (single-end, SE) was used here, with 50 bp length of the sequence tags. The transcriptome sequence was used as reference, and each sample of the clean reads was compared with the transcriptome sequencing information. According to this method, 4 expression profile databases were established. Each expression profile database sample has 3 biological repeats.

Raw data (raw reads) of fast q format were firstly processed and clean data (clean reads) were obtained by removing reads containing ploy-N and low-quality reads from raw data. At the same time, Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality. The left files (read1 files) from all libraries were pooled into one big left fq file, and right files (read 2 files) into one big right.fq file. Transcriptome assembly was accomplished based on the left. fq and right. fq using Trinity (Grabherr *et al.* 2011) with min_kmer_cov set to 2 by default and all other parameters set default. Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Nt(NCBI non-redundant nucleotide sequences); Pfam (Protein family);

KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot(A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database).

Every two combinations were compared to find out gene differential expression level. Differential expression analysis of 7 DGE database was performed using the DESeqR package (1.10.1). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The p- values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. In this study, genetic screening conditions is q-value < 0.005 and | log2 (foldchange)| >1. Multiple hypothesis was used to revise p- value, and the p-value was decided by controlling false discovery rate (FDR).

Roche LCPDS2 software was used to design primers of the differentially expressed genes, and the primers were synthesized from Shanghai Jierui biological engineering Co. Ltd. (Table 1). 18s ribosomal RNA, which expressed stable, were selected for internal qPCR gene amplification. LightCycler® 480 SYBR Green I Master kit (Roche, the Swiss) was used, fluorescent quantitative PCR reaction was carried out in the Light Cycler® 480 type (Roche, the Swiss), and each response repeated 3 times. The PCR reaction system is as follows: 2 × Light Cycler® 480 SYBR Green I Master, 5 µl, 10 µm forward primer, 0.2 µl, 10 µm reverse primer, 0.2 µl, cDNA, 1 µl, Nuclease-free H_2O, 3.6 µl. PCR procedures for 95ºC for 10 min; 95°C for 10s, 30s, 60°C 40 cycles. After the loop, the melting curve detection product specificity was as follows: from 60°C to 97°C, every 5°C acquisition fluorescent signal. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of experimental data. Specific calculation method is the relative expression of genes = $2^{-\Delta\Delta Ct}$, among them,$\Delta\Delta Ct = \Delta Ct_{\text{(samples of experimental group)}} - \Delta Ct_{\text{(samples of control group)}}$, $\Delta Ct = Ct_{\text{(target gene)}} - Ct_{\text{(internal control gene)}}$.

Results and Discussion

Two cDNA samples were prepared from an equal mixture of total RNA isolated from leave buds for two libraries (Anhui and Yunnan),which were sequenced with paired-end using the Illumina HiSeq™ 2500. Raw reads 47.11 million were obtained from Anhui library, and 47.86 million raw reads were obtained from Yunnan library. Those reads, which had low quality, contained adapters, or unknown bases more than 10%, were discarded. After data cleaning, 44.84 million clean reads containing a total of 5.60 Gb nucleotides were obtained from Anhui library, and 44.99million clean reads containing a total of 5.62 Gb nucleotides were obtained from Yunnan library. More than 96.05% reads was larger than 20 bp, and GC percentage was 46.75%. To demonstrate the quality of sequenced data, 26 unigenes were randomly selected and ten pairs of primers were designed for qRT-PCR.

Unigenes were divided into three categories by GO enrichment analysis, including molecular functions, cells, and biological processes, which were subdivided into 55 small class (Fig. 1). There were 35535 unigenes to perform the function of molecules (molecular function), and annotate in a total of 13 categories, among them 3 categories have more than 1000 unigenes in cluding combining (16771), the catalyst activity (13620) and transferring factor activity (1717). There are 49175 unigenes to perform the function of cellular component, and annotate in the 19 categories, in which 8 categories have more than 1000 unigenes including cells (9801), cell factors (9779), organelles (6973), macromolecular compounds (5395), membrane (4861), membrane element (4175), organelles elements (3970), and membrane bound cavity (1686). There were 73636 unigenes involving in biological processes, with a total of 23 categories, and 12 categories of them were of more than 1000 unigenes.

Table 1. Special primers for qRT-PCR of genes.

Unigene ID	Forward primer	Reverse primer	Product length (bp)	Ta (°C)
Comp132916_c0	CAAACCTGCCCTTAATCTAGT	GTTGTGGTTCCCTTCGTAT	123	60
Comp133363_c0	GACTATGGATTGGAACCGTC	GGGCTGAACGGATTACTC	132	60
Comp116793_c0	CATTTCCGCTATCTTGCTT	TCGAATTCTGCAACCGTC	101	60
Comp144834_c1	CATTCTTATGCTCGGTTCTTT	TCTCGACTCGAACCATACT	114	60
Comp138028_c1	TGCTTGAAGTATTTGGGAAGG	ATTCGACATGGTTGAGGAC	123	60
Comp128040_c0	AGCGGCATCAAATCTAGC	ATGAAGAATCTCTGTAACAGCA	104	60
Comp130000_c0	CCTTCCCAAATCCACACCAATA	GAGAAACCCATTCCGAGT	130	60
Comp118790_c0	CTCCAAGGGCCTTTGATT	TCTGCTCCCATCTCCTCTA	113	60
Comp121722_c0	TCCCATTATAGAACACTAGCGT	AGAATGGTTTCTGGAAGGC	105	60
Comp137276_c0	AACAACATGAATGTCAATACCG	CCAGGGTTGGAAGATCAC	115	60
Comp133557_c0	TTCTCTCCAAATAATCGGCG	AGTCAAGCCGTTCGTTCA	109	60
Comp142941_c0	GTACATGCGGATGGAGTC	CTCATCCTACGGTCAGCAA	120	60
Comp138270_c0	CCCTCCAATTCGATGACC	ACACCTGTTTCCTTTCTTCATA	113	60
Comp142503_c0	GTGGTGGTGAGAAACGAG	TCAGCCGTCGGATTAAACA	102	60
Comp143973_c0	TATGGTCACCCACAACCG	ACTAAAGTTCACACATGGCTA	109	60
Comp138196_c0	GCATTGTCACGGTCACTTA	CAACAAGTTGAAGCTCCTC	118	60
Comp121820_c0	TCGGGTGTCTTATCAGTGC	CCAAGTTCTGGAACTATGGTC	118	60
Comp137356_c0	TCTGACTATCCGGCAACG	GAAGGATATGCGCTTCGATTTA	128	60
Comp142849_c0	GCAACAAAGTATGCCAAGAC	TCCATTGCCGGAATCTGA	111	60
Comp142526_c0	CATAACAGTATCTGGCAAGGTC	TCTGTGTGCTGTGTTGTAGT	100	60
Comp141644_c0	ATCAATGGATGGGTTAATGCAA	AATTCTCCTTCGACGAGAC	107	60
Comp140242_c0	ATATTCTGGCGATTTAGACAGT	ATAGAACAAGAGGTGTGCTTGA	128	60
Comp137127_c0	AGCTTGTTAGCGGTTCTAT	CCATTACATTGAGAATCGCC	112	60
Comp138653_c0	AAATCGGTCCCATATTCCTAC	ACAAGGGAGGGAGAACTTAGA	118	60
Comp111763_c0	AAGAAATCCCATCCTCTGC	AGAAAGGATCGTAGACGTTG	100	60
Comp89802_c0	TCGAAACAAATGCACGCTT	TCAAACGGTCCAGACACGA	114	60
18S rRNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	187	60

There were 8083 unigenes classified as 32 categories by Pathway enrichment analysis, and the 32 categories were divided into 5 groups according to the KEGG metabolic pathway by which they participate in: cellular processing, environmental information processing, based information processing, metabolism and organic systems (Fig. 2). In the top 10 metabolic pathways with the most number of unigenes, four were considered to be related with plant cold hardiness, such as plant hormone signal transduction with a total of 170 unigenes, accounted for 1.56%; fatty acid metabolism with a total of 90 unigenes, accounted for 0.83%; peroxidase with a total of 88 unigenes, accounted for 0.81%; photosynthesis with a total of 68 unigenes, accounted for 0.63% (Table 2).

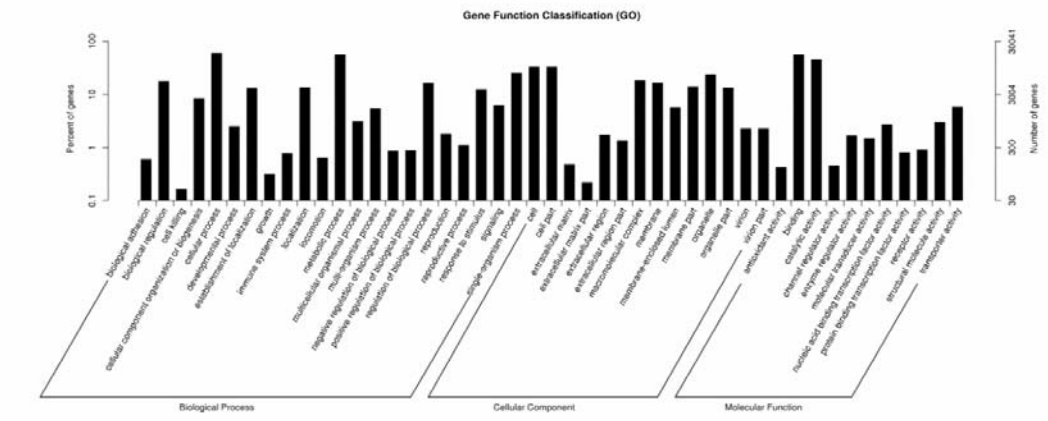

Fig.1. GO function annotation of uigenes.

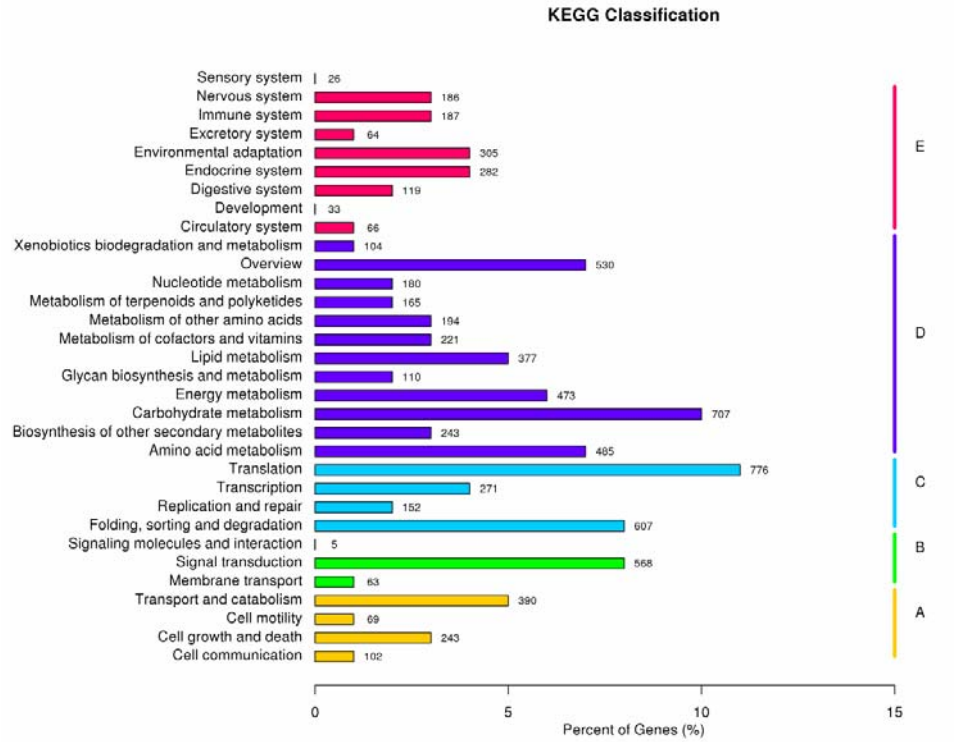

Fig. 2. KEGG classification of transcriptome.

Unigenes obtained from *L. chinense* transcriptome were compared with KOG (Eukaryotic ortholog groups) database, and a total of 9944 unigenes matched with KOG database, which were divided into 26 functional classifications. Among them, the unigenes number of the general function prediction (R) with 2215 unigenes was the most, and then was protein post-translational modification (O)with 1226 unigenes, and Signal transduction mechanisms (T) with 815 unigenes (Fig. 3).

Table 2. Top 10 metabolic pathways with most unigenes in KEGG.

Pathway ID	Pathway name	Unigenes number	Percent (%)
ko03010	Ribosome	372	3.42
ko01200	Carbon metabolism	303	2.79
ko01230	Amino acid biosynthesis	269	2.47
ko04626	Plant - pathogen interaction	227	2.09
ko04141	Protein processing In the endoplasmic reticulum	205	1.88
ko03040	Spliceosome	197	1.81
ko00500	Starch and sucrose metabolism	191	1.76
ko04075	Plant hormone signal transduction	170	1.56
ko03018	RNA degradation	166	1.53
ko03013	RNA transport	165	1.52

Table 3. Data quality of DGE.

Sample	Raw reads	Clean reads	Clean bases (G)	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)	Match Percent (%)
YUN_nor1	12230746	12027164	0.60	0.01	98.95	96.66	48.01	93.21%
YUN_nor2	12781816	12700011	0.64	0.01	99.01	96.80	46.78	92.89%
YUN_nor3	11979067	11896583	0.59	0.01	98.99	96.72	46.94	93.73%
YUN_col1	11506184	11444157	0.57	0.01	99.03	96.83	46.88	92.61%
YUN_col2	12376295	12292617	0.61	0.01	99.00	96.82	46.62	92.60%
YUN_col3	11019472	10965261	0.55	0.01	99.01	96.78	46.51	91.65%
AN_nor1	12547934	12109264	0.61	0.01	99.24	97.52	46.72	92.52%
AN_nor2	10565358	10178246	0.51	0.01	99.16	97.39	46.52	92.47%
AN_nor3	10651528	10269035	0.51	0.01	99.17	97.30	46.45	92.12%
AN_col1	11477138	11047048	0.55	0.01	99.09	97.19	45.74	90.94%
AN_col2	11089001	10677036	0.53	0.01	99.05	97.06	45.74	90.82%
AN_col3	10705049	10284022	0.51	0.01	99.10	97.22	45.78	90.48%
AN_25_1	11560846	11125313	0.56	0.01	99.09	97.06	47.06	93.21%
AN_25_2	11597108	11158384	0.56	0.01	99.10	97.10	46.27	92.89%
AN_25_3	10706615	10242343	0.51	0.01	99.12	97.21	46.96	93.73%
AN_4_1	11323359	10870470	0.54	0.01	99.05	96.99	46.60	92.61%
AN_4_2	10882719	10473402	0.52	0.01	99.09	97.11	45.65	92.60%
AN_4_3	10787546	10308974	0.52	0.01	99.09	97.10	46.26	91.65%
AN_30_1	10508686	10130883	0.51	0.01	99.02	97.04	44.97	92.52%
AN_30_2	10383784	10010891	0.50	0.01	99.11	97.23	45.74	92.47%
AN_30_3	11677850	11240380	0.56	0.01	99.07	97.13	45.96	92.12%

The 21 DGE samples were sequenced, and more than 0.5 G clean reads were obtained from each sample, the error rate of bases was 0.01%.Q20 values were more than 99%. Q30 values were between 96.66 and 97.52%. The GC percentages were from 44.97 to 48.01%, with the average of 46.39%. More than 90% unigenes of each sample matched the transcriptome sequences (Table 3). According to the results, quantity of the differentially expressed genes between seasons was larger than the differentially expressed genes between provenances, which indicates, for *L. chinense*, the factor of seasonal change affects more than that of provenance change (Fig. 4). Quantity of the differentially expressed genes in different seasons of Anhui provenance was more than Yunnan provenance, and this might be related to the different cold resistance of the two provenances (Fig. 4). Differentially expressed genes were annotated to a group of the metabolic pathways associated with plant cold regulation, and these metabolic pathways were mainly divided into three types. The first is the cold regulation signal transduction, including Plant hormone signaling, Calcium signaling pathways and MAPK (mitogen-activated protein kinase) signal transduction pathways. The second is photosynthesis, including photosynthesis antenna proteins and photosynthesis. The third is cold hardiness physiology metabolism, including fatty acid metabolism, biosynthesis of unsaturated fatty acid, argnine and proline metabolism, peroxsiome, starch and sucrose metabolism, and β-amylase metabolism, etc.

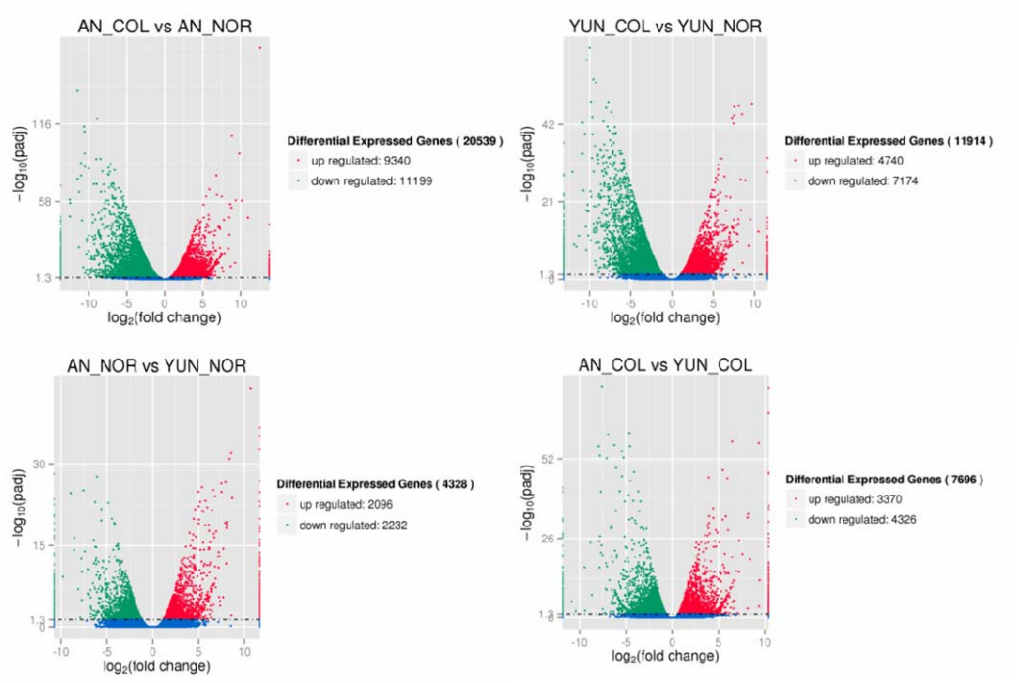

Fig. 4. Analysis of differentially expressed genes in nature environment.

Signal transduction pathway plays a key role in plant in the response to low temperature (Janská *et al.* 2010). Calcium ion is an important messenger in the process of plant growth and development, and also plays an important role in the cold stress (Reddy *et al.* 2011). In the 7 DGE combinations, differentially expressed genes which enriched in calcium signaling pathway of KEGG pathways were analyzed. In four combinations, six differentially expressed unigenes were annotated as calmodulin. Higher expression of calmodulin is conducive to play the role of calcium

sensors in signal transduction molecules, and the concentration of intracellular calcium ions could be regulated, which reflected the response in *L. chinense* to low temperature environment Comp131675_c0 and comp99556_c0 were annotated as phosphoinositide phospholipase C (Table 4). Comp138526_c0 and comp133602_c0 were annotated as protein phospholipase 3. Results showed that, for the same provenance of *L. chinense*, cold season compared to normal temperature season (Yun-nor vs Yun-col, An-nor vs An-col), down-regulated unigenes annotated to calcium ion signaling pathway were more than up-regulated unigenes.

Table 4. Putative cold-response unigenes in *Liriodendron* transcriptome.

Gene ID	Blastn hit	Abbreviation	Length of unigenes (bp)
comp132916_c0	WRKY transcription factor 6 [*Glycine soja*]	WRKY	2116
comp133363_c0	PREDICTED: probable WRKY transcription factor 46 [*Vitis vinifera*]	WRKY	2442
comp116793_c0	WRKY transcription factor 5 [*Dimocarpus longan*]	WRKY	614
comp144834_c1	heat shock factor protein HSF30 [*Vitis vinifera*]	HSP	2668
comp138653_c0	heat shock protein, putative [*Ricinus communis*]	HSP	1065
comp111763_c0	cytosolic class I small heat shock protein [*Chimonanthus praecox*]	HSP	264
comp89802_c0	heat shock protein HSP70-2, partial [*Arabidopsis thaliana*]	HSP	447
comp130000_c0	PREDICTED: myb-related protein Myb4-like [*Populus euphratica*]	MYB	1085
comp118790_c0	MYB transcription factor MYB124 [*Glycine max*]	MYB	866
comp121722_c0	NAC domain-containing protein, putative [*Ricinus communis*]	NAC	988
comp137276_c0	predicted protein [*Physcomitrella patens*]	NAC	1216
comp133557_c0	NAC domain class transcription factor [*Malus domestica*]	NAC	1141
comp142941_c0	hypothetical protein POPTR_0003s07910g [*Populus trichocarpa*]	AP2	996
comp138270_c0	AP2/ERF domain-containing transcription factor, putative [*Theobroma cacao*]	AP2	1491
comp142503_c0	PREDICTED: ethylene-responsive transcription factor 11-like [*Vitisvinifera*]	AP2	400
comp143973_c0	contains similarity to retrovirus-related polyproteins and to CCHC zinc finger protein [*Arabidopsis thaliana*]	Zinc finger	3633
comp138196_c0	PREDICTED: cyclic dof factor 2 [*Vitis vinifera*]	Zinc finger	3599
comp121820_c0	hypothetical protein PDE_02293 [*Penicillium oxalicum 114-2*]	Zinc finger	849
comp137356_c0	unnamed protein product, partial [*Vitis vinifera*]	Zinc finger	2358
comp138028_c1	hypothetical protein VITISV_003842 [*Vitis vinifera*]	HSF	2322
comp128040_c0	Heat stress transcription factor B-2b –like protein [*Gossypium arboreum*]	HSF	1814
comp142849_c0	PREDICTED: ATP synthase delta chain,chloroplastic-like [*Elaeis guineensis*]	Beta-amylase	2177
comp142526_c0	PREDICTED: inactive beta-amylase 9 [*Vitis vinifera*]	Beta-amylase	2176
comp141644_c0	PREDICTED: uncharacterized protein LOC100245192 [*Vitis vinifera*]	Fatty acid desaturase	753
comp140242_c0	hypothetical protein VITISV_035465 [*Vitis vinifera*]	Phospholipase	1777
comp137127_c0	hypothetical protein VITISV_043921 [*Vitis vinifera*]	Phospholipase	1232

Table 5. Correlation analysis between $2^{-\Delta\Delta CT}$obtained from qRT-PCR and fold changeobtained from transcriptome.

Number of group	Combination of DGE database	Correlation between $2^{-\Delta\Delta CT}$ and fold change
1	YUN_COL vs YUN_NOR	0.79
2	AN_COL vs AN_NOR	0.83
3	AN_COL vs YUN_COL	0.89
4	AN_NOR vs YUN_NOR	0.81
5	AN_4 vs AN_25	0.80
6	AN_sub30vs AN_4	0.91
7	AN_sub30vs AN_25	0.89

In response to cold stress, plants have developed a variety of complex physiological and biochemical defense mechanisms methods. These reactions need large-scale cold response gene expression (Tsuda *et al.* 2000, Vágújfalvi *et al.* 2003, Zhang *et al.* 2009). Transcription factors played an important function role in plant development and stress adaptation, which are both the switch in the reactions and the end of the signaling in the specific responses to cold stress (Zhang *et al.* 2010). Transcription factors which have up-regulated expressed are likely to play an important role in plant defense and stress reaction (Singh *et al.* 2002). In the present research, 23 differentially expressed unigenes were considered to be potential transcription factors, and they were annotated into 7 groups, such as WRKY, AP2, MYB, NAC, HSP, Zinc finger and HSF (Table 4), according to the homologous comparison with genetic information in the database.

Chilling injury caused cell dehydration for plant membrane system firstly. In the process of cold acclimation in plants, cold resistance rises gradually along with cell metabolic activity enhancing and concentration of unsaturated fatty acids and phospholipids increasing (Moellering *et al.* 2010). These changes are associated with the increase of low temperature stress intensity. In this study, fatty acid desaturase (FAD) gene was found. This gene could regulate unsaturated fatty acids, thereby further regulates cell membrane fluidity. The stability of cell membrane also associated with osmotic balance. Proline is one of the most important cell permeability materials, participating in a variety of environmental stresses response. It can relieve the osmotic pressure due to cold injury caused by dehydration because it is a hydrophilic protein (Ashraf and Foolad 2007). The accumulation of sugar and other simple carbohydrates could also increase the stability of plant membrane under cold stress. DGE data analysis showed that the unigene (ID: comp141644_c0) which differentially expressed under low temperature stress was annotated for fatty acids saturated enzyme, two differentially expressed unigenes (comp140242_c0, comp137127_c0) were annotated for phospholipase (Table 4). β-mylase could degrade starch into soluble sugar, which is a kind of enzymes related to metabolic synthesis. Two differentially expressed unigenes (comp142849_c0 comp142526_c0) were annotated for genes related to β-amylase synthesis.

Selected genes related to cold resistance were detected by real-time fluorescent quantitative PCR (qPCR). Optimal reaction system was used, each samples repeated 3 times. Relative expression of the test data was calculated by $2^{-\Delta\Delta Ct}$ method. Gene expression level obtained from the transcriptome sequencing [log2 (fold change)] was compared with the relative expression. Correlation analysis showed that, the correlation of two groups from the 7 combinations ranged between 0.79 to 0.91, which indicated the transcriptome sequencing results were accurate and reliable (Table 5).

In this study, three transcript factors including AP2, NAC and Zinc finger, were up-regulated with the temperature reducing in the low temperature stress experiment. NAC gene was reported to

participate in cold stress response in plants such as sugarcane (*Saccharum officinarum*), Arabidopsis (*Arabidopsis thaliana*), wheat (*Triticum aestivum*) etc. This gene not only regulates the flowering process but also induces the expression of genes which could improve the resistance of cold and salt for plants (Chen 2011). NAC gene was of the maximum relative expression value and more than five in the process of low temperature stress. It indicated that this gene was probably closely related with winter hardiness of *L. chinense*. β-amylase could make starch into soluble sugar by degradation. Real-time fluorescent qPCR results showed that, under the stress of low temperature, beta amylase gene expressed up-regulated during the period of 25 to 4°C and down-regulated during the period of 4 to 30°C. Phospholipase is a kind of important phospholipid hydrolase affecting cold resistant ability of plants (Zeng *et al*. 2009). In this study, this gene was up-regulated during the period of 25 to 4°C in the low temperature stress experiment, which indicated the enhanced cold resistant ability response to the environment change. While, this gene expressed was down-regulated during the period of 4 to –30°C. It is predicted that *L. chinense* tissue were injured by extreme cold environment, and this gene could not express usually.

The seeds of 15 provenances in *L. chinense* were collected and planted in the forest center of Jiangxi province by the present research team 25 years ago (Li *et al.* 2001). Anhui provenance and Yunnan provenance are located in northern and southern of the species' natural distribution area, respectively, and Jiangxi forestry farm is between the two provenances on the latitude. Samples of the two provenances were planted in the same area, while long-term observation showed that the seedlings of Anhui provenance had stronger cold resistance, higher survival rate after chilling, and adult individuals grew slower than Yunnan provenance. Quantities of differentially expressed genes in the cold environment of two provenances were quite different, which may be associated with cold hardiness difference between provenances.

To study the cold resistance of evergreen tree species, leaves in different period or different environment are usually considered as suitable experiment material, while branches, flower bud and persistent leaf bud are used for deciduous trees study (Chen 2011). In order to understand the changes of gene expression levels of *L. chinense* in natural environment, the persistent tissue of this plant is necessary as an object to study. As deciduous trees, the leaves of *L. Chinense* fall off during the winter, which cannot be used as the material for transcriptome sequencing experiment. While the leave buds on the top of the stems persist in winter, with brown membrane protecting the internal young leaf bud. Leaf buds experience the change in climate, therefore, the gene expression level within the tissue changes assistant with the external environment changing. To study the cold resistance of different seasons and different provenances in *L. chinense*, samples in natural environment were selected as experiment materials. Three trees of each provenance were selected as experiment materials, and leaf buds obtained from each tree were sequenced respectively, the aim is to reduce experimental error through biological repetition. However, there is difference in the genetic background between plants, and this research still has some limitations. Further study should be taken by obtaining samples with the same genotypes through tissue culture in the future, and cold hardiness of differentially expressed genes were analyzed after doing low temperature stress treatment on the samples.

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