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Full Length Article

Transcriptome Analysis of ER Stress-related Genes and Validation of Reference Genes in Gene Expression RT-qPCR for *Cyclocarya paliurus*

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Abstract

Cyclocarya paliurus (Batal.) Iljinskaja (*C. paliurus*) is a unique tree species in China appreciated for its pharmacodynamic components. However, abiotic stresses, such as drought, salt, cold, or heat, which cause the accretion of misfolded or unfolded proteins in the endoplasmic reticulum (ER), induce a reduction in the yield of *C. paliurus*. Unfolded protein response (UPR) induced by ER stress plays a critical role in the development and adaptation of plants to adverse environmental stresses. Hence, this study aimed to investigate the genetic mechanisms of ER stress in *C. paliurus* by performing transcriptome analysis to compare the ER stress–related gene expression during tunicamycin (TM) treatment for different time durations (0, 6, and 14 h). A total of 196,207 unigenes were detected, with 1867 downregulated and 3040 upregulated in the treatment groups (TM_6 h and TM_14 h) compared with the control group (TM_0 h). In this study, nine upregulated genes were randomly selected to validate transcriptome data by real-time quantitative polymerase chain reaction (RT-qPCR). In addition, the proper number and type of reference genes to be used in the normalization of RT-qPCR were found in studies of *C. paliurus*; *ACT2* and *TUB* were the most appropriate reference options in the TM treatments. The results from this study might provide insights for the genetic basis of ER stress in *C. paliurus.* © 2019 Friends Science Publishers

Keywords: *Cyclocarya paliurus* (Batal.) Iljinskaja; DGE sequencing; ER stress; Unfolded protein response; Reference genes; Abiotic stress

Introduction

Cyclocarya (Juglandaceae) is a genus comprising only one species [*Cyclocarya paliurus* (Batal.) Iljinskaja.], which is considered an endangered tree species in China. It is distributed mainly in south China (Yao *et al.*, 2008). Previous studies found that the crude extracts , such as organic acids, alkaloids, and phytosterols, that had a variety of medicinal functions (Zhou *et al.*, 2019). Previous studies showed that the extracts of *C. paliurus* significantly decreased blood glucose levels and increased blood insulin levels. The leaves of *C. paliurus* is known to have a hypolipemic effect (Liu *et al.*, 2018). Blue light is effective for inducing the production of flavonoids in C. paliurus leaves (Fang *et al.*, 2011; Deng *et al.*, 2012). In China, *C. paliurus* has been identified as a new consumable plant product and is commonly used as an anti-glucose medicine (Wu *et al.*, 2017).

For some macromolecular proteins in eukaryotes, it is necessary to enter the endoplasmic reticulum (ER) for further folding (Marti *et al*., 2018). The protein folding process is very delicate, and is particularly susceptible to the environment, resulting in protein unfolding or misfolding. The ER has a complete system for clearing unfolded and misfolding proteins. Therefore, ER stress is induced when the protein folding demand exceeds the capacity for protein degradation ,to initiates unfolded protein response (UPR) to enhance protein folding ability to increase the cell survival rate (Liu and Howell, 2010; Yang *et al*., 2015; Wan and Jiang, 2016). Furthermore, severe ER stress conditions also promote the autophagy effect or initiate cell death (Yang *et al*., 2014).

The objectives of this study were to compare the ER stress–related gene expression under tunicamycin (TM) treatment for different time durations (0, 6, and 14 h). The 196,207 unigenes obtained were compared with data from 7 public databases as follows: Nt (sequences of NCBI nonredundant nucleotide), Nr (sequences of NCBI nonredundant protein), KOG/COG (proteins of clusters of orthologous groups), KO [a database of Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog], SwissProt (a database that includes manually annotated and

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reviewed protein sequences), PFAM (a database of protein family), and GO (a database of Gene Ontology). The genome of *C. paliurus*, an economically important plant used in China, is still unknown. To study the signaling pathway under ER stress, High-throughput sequencing was used to acquire transcriptome information in this nonmodel plant.The findings might deepen the knowledge of the ER stress pathway in *C. paliurus*. Also, the identification of ER stress–related genes might be beneficial for the studies of other abiotic stress pathways.

The proper reference genes under ER stress were first identified due to the lack of related reference genes reported in *C. paliurus*. In this study, seven reference genes were chosen based on the results of previous studies, namely, *ubiquitin* (*UBQ4*), *18S ribosomal RNA* (*18SrRNA*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *beta-tubulin* (*TUB*),*actin*(*ACT2*, *ACTF)*, and *alpha-tubulin* (*TUA*) (Maroufi *et al*., 2010; Zhu *et al*., 2012; Robledo *et al*., 2014; Wu *et al.*, 2016). Finally, proper reference genes and their ideal levels for the normalization of RT-qPCR were identified in C. paliurus under ER stress (Lin and Lai, 2010; Du *et al.*, 2017).

Abiotic stresses, such as drought, salt, cold, or heat, which cause an increase in misfolded or unfolded proteins in the ER, induce a decrease in *C. paliurus* production. ER stress plays a key role in the development of plants to adverse environmental stresses. Therefore, a transcriptome analysis was performed to compare the expression of ER stress–related gene expression under TM treatment at different time points (0, 6 and 14 h), and the genetic mechanism of ER stress was studied in *C. paliurus* (Kebede *et al.*, 2018). A total of 196,207 unigenes were detected in the treatment groups (TM_6 h and TM_14 h) compared with the control group (TM_0 h), with 1867 downregulated and 3040 upregulated genes. In this study, nine upregulated genes were randomly selected to validate transcriptome data by RT-qPCR. Further, the correct number and type of reference genes for RT-qPCR normalization in *C. paliurus* were found; *ACT2* and *TUB* were the most suitable reference choices for TM processing. The results of this study might provide insights into the genetic basis under ER stress in *C. paliurus*.

Materials and Methods

Plant Materials, Treatments and Transcriptome Sequencing

0.2 gram *cyclocarya paliurus* leaves were acquired from three individual four months old trees in Huaxi district, Guizhou province, China. To induce ER stress, the leaves were soaked in liquid half-strength Murashige and Skoog (½ MS) medium containing Tunicamycin (TM, 5 μg/mL, dissolved in DMSO) and Triton (0.5%, to improve leaf uptake) for 0 h, 6 h, and 14 h (TM_0 h, TM_6 h, and TM_14 h). Leaf samples were kept in liquid nitrogen and submitted to Novogene (Beijing, China) who performed transcriptome sequencing to obtain transcript information by using Illumina HiSeq 2000.

RT-qPCR (Real Time-quantitative Polymerase Chain Reaction, RT-qPCR) and cDNA Synthesis

As an input material in sample preparation, 1.5 µg RNA was used in each sample. To generate sequencing libraries for further Illumina® sequencing, a Next® Ultra™ RNA Library Prep Kit (NEB, U.S.A.) was used, and index parameters were applied to each sample to generate corresponding sequences, following the manufacturer's procedures (Hansen *et al.*, 2010). Magnetic beads with poly-T oligos were utilized to obtain purified mRNA from the total sample. A NEB Next® First-Strand Synthesis Reaction Buffer (5X) was used to fragment and obtain divalent cations, with increased temperature. M-MuLV Reverse Transcriptase (RNase H) and random hexamer primers were utilized to incorporate the initial stand of cDNA. Subsequently, RNase H together with DNA Polymerase I were used to generate the sec strand of cDNA. Then, the remaining overhangs were changed into blunt ends by polymerase/exonuclease operations. A NEB Next® adaptor was used to prepare the cDNA for the next hybridization process, after which the 3' ends of the DNA fragments were adenylated (a hairpin shape). cDNA fragments (150–200 bp) were preferably selected, and the AMPure XP suite was used to purify the library fragments (Beckman Coulter, U.S.A.). Then, 3 µL of USER Enzyme (NEB, U.S.A.) was added with the size-chosen, adaptor-ligated cDNA (for incubating for 15 min under 37°C), following 5 min at 95°C before performing the PCR procedure. Universal PCR primers, Index (X) Primer, and Phusion High-Fidelity DNA polymerase were used in the PCR. Finally, the PCR products were purified (AMPure XP), and the quality of the product was determined by an Agilent Bioanalyzer 2100 apparatus. Clustering and sequencing were performed by the Experimental Department of Novogene. Following the manufacturer's instructions, the parameter-programmed sample clustering was processed on a Cluster Generation (cBot) System using a Cluster Kit (TruSeq PE Cluster Kit v3-cBot-HS, Illumia). After the cluster was generated, the library was sequenced on an Illumina Hiseq apparatus and paired-end reads were developed.

Transcriptome Data Analysis

In-house perl scripts were used to obtain and analyze raw data (original readings, fastq format) (Cock *et al.*, 2009). Readings that contain ploy-Ns, adapters, and data with unsatisfactory quality were removed to generate clean data (processed readings). Q30, Q20, sequence duplication level, and GC-content from the high-quality clean data were obtained, which were then utilized in the following analyses in this study. The transcriptome assembly procedure is described as follows: the right and left files (read2 and read1

files, respectively) from all libraries were collected into a central file (right fq and left fq, respectively). Trinity was conducted to assemble transcriptome data from both right fq and left fq (Grabherr *et al.*, 2011). The default setting for min kmer cov was 2, and the other relevant parameters remained on default settings. The gene functional annotation procedure is described as follows: Nt (sequences of NCBI non-redundant nucleotide), Nr (sequences of NCBI non-redundant protein), KOG/COG (proteins of clusters of orthologous groups), KO (database of KEGG Ortholog), Swiss-Prot (a database that includes manually annotated and reviewed protein sequences), Pfam (database of protein family), and GO (database of Gene Ontology) databases were used.

Differential Expression Analysis

This experiment utilized the DESeq R package (version 1.10.1) to obtain the differential expressions of the two groups/conditions. We used statistical procedures in DESeq and its model based on negative binomial distributions to analyze differential expression in digital gene expression (Tian *et al.*, 2013). To limit false positive results, we then adjusted the P value (Davidson and Oshlak, 2014). When the adjusted P value determined by DESeq was <0.05, the genes were considered differentially expressed (Benjamini and Hockberg, 1995; Huang *et al.*, 2018).

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis

To understand biological system and high level functions of detected different expression genes, (DEGs), the KEGG (Minoru, 2008) database was applied. The KEGG database provided information on the molecular level including valuable datasets developed by genome sequencing and other high-throughput methodologies (reference website: http://www.genome.jp/kegg/). We used KOBAS software to analyze DEGs and their statistical enrichment in KEGG pathways (Mao *et al.*, 2005).

GO (Gene Ontology) Enrichment Analysis

The GOseq R software package with the non-central hypergeometric distribution of Wallenius (Young *et al.*, 2010) was used to analyze GO enrichment for DEGs. This method allowed length bias of gene adjustment in DEGs.

PPI (Protein-protein Interaction)

To obtain the predicted PPI, DEG sequences were blasted (blastx) to a genome of *Arabidopsis thaliana* (the interaction between two proteins that were in the STRING database; reference website: http://string-db.org/). The PPI of the referenced DEGs was imaged using Cytoscape (Su *et al.*, 2014).

qRT-PCR (Quantitative Real Time-polymerase Chain Reaction) Analysis

Nine unigenes were validated with qRT-PCR in this study. Gene-specific primer pairs were designed to pair with selected genes in the qRT-PCR experiment (Supplementary Table 3). Using the reference unigene sequences, Primer Premier 5.0 was used to design specific primers. SYBR Green Real-time PCR Master Mix (Takara, Osaka, Japan) and a CFX manager (Bio-Rad, U.S.A.) were used to quantify data in real-time. Three biological replicates (from three trees) were conducted for each group. Each plate was tested in triplicate independently for both selected and reference genes. The different gene expression levels were evaluated utilizing the comparative CT approach (2-CT) (Singh *et al.*, 2011).

For the seven reference genes that were selected, the levels of gene expression acquired by qRT-PCR were measured with crossing point (Cp). With the geNorm algorithm applied, the stability value (M) and pairwise variation (V) for each gene were obtained. M indicates the most appropriate reference genes, and V represents the optimal number of reference genes. For each gene, the stability was obtained by the NormFinder algorithm: the lower the stability value, the steadier the reference gene (Wang *et al.*, 2012). All experimental procedures were done in triplicate. SPSS software (V16.0. Chicago, I.L., U.S.A.) was used for statistical analysis, and a Student's *t*-test using S.P.S.S. was conducted. The outcomes were considered significant when $P < 0.01$.

Results

Sequencing and Assembly Transcriptome

To identify and analyze the expression of genes related to ER stress in *C. paliurus*, cDNA samples were collected from leaf tissues treated with tunicamycin (TM) during a robust growth phase. In total, 470,173,828 original raw reads were generated using Illumina sequencing [as shown in Supplementary Table 1; all data were submitted to NCBI GEO (GSE133027)]. After removing low-quality sequences, a total of 452,300,260 clean reads remained. An aggregate of 268,621 transcripts were amassed with N50 lengths with an average of 1659 and 950 bp. The length of 68.92% of the transcripts ranged from 200 to 1000 bp. The transcripts were amassed into unigenes, resulting in 196,207 unigenes with an average length of 1196 bp and an N50 length of 1811 bp (Supplementary Fig. 1). Due to the lack of a reference genome sequence for *C. paliurus*, the *de novo* amassed transcriptome sequence was viewed as the reference sequence. The quality of the assembled unigenes was evaluated using Corset; all clean reads were programmed to the assembled unigenes. A sum of 255,084,157 clean reads (56.39%) was then effectively realigned to the reference sequence, demonstrating a sufficient quality of assembled unigenes for further analysis.

Table 1: Statistics of digital gene expression (DGE) sequencing

Sample	Raw reads	Clean Reads	Clear Bases	Error(%)	Q20(%)	Q30(%)	GC Content(%)
TM_0h1	49051962	47323406	7.1G	0.02	97.12	92.66	45.01
TM_0h2	53622654	51432524	7.71G	0.02	97.33	93.1	44.87
TM_0h3	51143430	49168870	7.38G	0.02	97.16	92.76	44.88
TM_6h1	48059140	49269170	6.94G	0.02	97.14	92.73	44.9
TM_6h2	50625418	48752916	7.31G	0.02	97.35	93.18	44.76
TM_6h3	49902314	47872316	7.18G	0.02	97.31	93.09	44.51
TM_14h1	49286802	47267348	7.09G	0.02	96.68	92.37	45.18
TM_14h2	63284274	60976736	9.15G	0.02	97.29	92.89	44.8
TM_14h3	55197834	53236974	7.99G	0.02	97.4	93.05	44.74

TM_0 h(Control): mock treatment, TM_6 h:TM treatment for 6 h, TM_14 h: TM treatment for 14 h). Sample name_1/2/3, Three biological repeats. Clean reads: The number of reads after removing low-quality sequences. The subsequent analysis is based on clean reads. Error rate: Base error rate. Q20 and Q30, the percentage of bases with Phred values >20 and >30, respectively. GC content: the GC ratio of the total base number

Table 2: DGE reads mapped to the reference sequences

Sample name	Total reads	Total mapped
TM_0h1	47323406	34420044(72.73%)
TM_0h2	51432524	37849208(73.59%)
TM_0h3	49168870	35575418(72.35%)
TM_6h1	46269170	33346798(72.07%)
TM_6h2	48752916	35515510(72.85%)
TM_6h3	47872316	35169010(73.46%)
TM_14h1	47267348	34457314(72.90%)
TM_14h2	60976736	43981682(72.13%)
TM_14h3	53236974	38830734(72.94%)

Total reads: Number of reads after removing low-quality sequences (clean data). Total mapped: Number of reads that could be mapped back to the reference sequences. Values within the parenthesis represent (total mapped divided by total reads) × 100%

Table 3: 17 putative genes related to known ER stress gene exhibited differential expression

Gene_Id	Gene Description
Cluster-29186.105919	DnaJ protein ERDJ2A OS=*Arabidopsis thaliana* GN=ERDJ2A PE=1 SV=1
Cluster-29186.94830	Heat stress transcription factor B-3 OS=*Arabidopsis thaliana* GN=HSFB3 PE=2 SV=1
Cluster-29186.94955	Endoplasmic reticulum oxidoreductin-1 OS=*Arabidopsis thaliana* GN=AERO1 PE=1 SV=1
Cluster-29186.112715	Serine/threonine-protein kinase/endoribonuclease IRE1b OS=*Arabidopsis thaliana* GN=IRE1B PE=2 SV=1
Cluster-29186.21342	Protein transport protein Sec61 subunit gamma OS=Oryza sativa subsp. japonica GN=Os02g0178400 PE=3 SV=1
Cluster-29186.56416	Protein transport protein SEC23 OS=Ustilago maydis (strain 521 / FGSC 9021) GN=SEC23 PE=3 SV=1
Cluster-29186.75574	GTP-binding protein SAR1B OS=*Arabidopsis thaliana* GN=SAR1B PE=1 SV=1
Cluster-29186.90927	Protein disulfide-isomerase OS=Datisca glomerata GN=PDI PE=2 SV=1
Cluster-29186.91893	Protein disulfide isomerase-like 2-3 OS=Oryza sativa subsp. japonica GN=PDIL2-3 PE=2 SV=1
Cluster-29186.93033	Calreticulin OS=Ricinus communis PE=2 SV=1
Cluster-29186.99554	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3B OS=*Arabidopsis thaliana* GN=STT3B PE=2 SV=1
Cluster-29186.73075	Mannosyl-oligosaccharide 1,2-alpha-mannosidase MNS3 OS=*Arabidopsis thaliana* GN=MNS3 PE=2 SV=1
Cluster-29186.71208	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1A OS=*Arabidopsis thaliana* GN=OST1A PE=2 SV=1
Cluster-29186.37582	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit DAD1 OS=Citrus unshiu GN=DAD1 PE=3 SV=1
Cluster-29186.126287	Alpha-1,3/1,6-mannosyltransferase ALG2 OS=Dictyostelium discoideum GN=alg2 PE=3 SV=1
Cluster-29186.121831	Probable dolichyl pyrophosphate Glc1Man9GlcNAc2 alpha-1,3-glucosyltransferase OS=*Arabidopsis thaliana* GN=At2g44660 PE=2 SV=3
Cluster-29186.108208	UDP-glycosyltransferase 74F2 OS=*Arabidopsis thaliana* GN=UGT74F2 PE=1 SV=1

Sequence Annotation

The unigenes obtained from previous steps were compared with data from seven public databases, including Nt, Nr, KOG/COG, SwissProt, PFAM, GO, and KEGG. The results indicated significant matches for 120,165 unigenes (61.24%) to the Nr database, 91,838 unigenes (46.8%) to the Nt database, and 87,173 unigenes (44.42%) to the SwissProt database. Altogether, 131,652 unigenes (67.09%) were found in one or more databases, with 19,657 unigenes (10.01%) present in every database studied (Supplementary Table 2). The GO analysis was conducted with a total of 82,680 unigenes (Fig. 1). Under cellular component (CC), genes involved in “organelle” (17,095), “cell part” (25,390), and “cell” (25,404) were dominant. Under molecular function, genes involved in “transporter activity” (1176), “catalytic activity” (8571), and “binding” (10,493) were found. Additionally, under biological process (BP), “single-organism process” (35,782), “metabolic process” (44,862), and “cellular process” (48,113) were identified. For the KOG classification, 32,662 unigenes were found, which were divided into 26 groups (Fig. 2). The “chaperones, protein turnover, post-translational modification” (4518) was the largest group, followed by “general function prediction” (3967), “translation, biogenesis, and ribosomal structure” (3521), “RNA processing and modification”

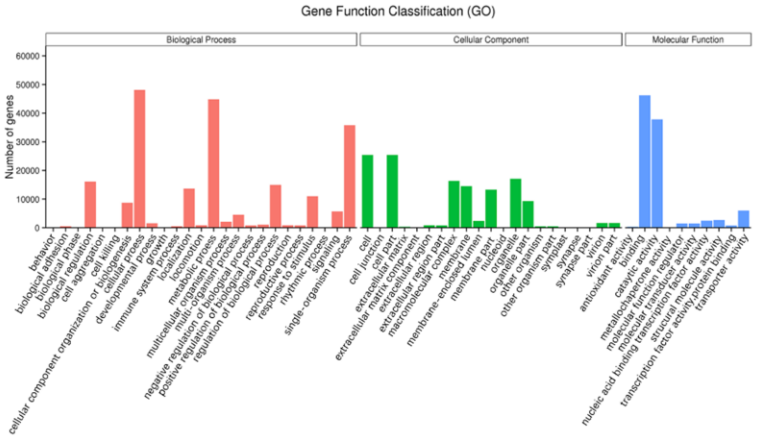

Fig. 1: The Gene Ontology (GO) categorization of unigenes

This figure shows that *'*metabolic process,*'* *'*single-organism process,*'* and *'*cellular process*'* were dominant in biological process (BP); *'*organelle,*'* *'*cell part,*'* and *'*cell*'* genes were highly represented in cellular component (CC); genes about *'*transporter activity*'* and *'*binding catalytic activity*'* (8,571) were highly represented in molecular function

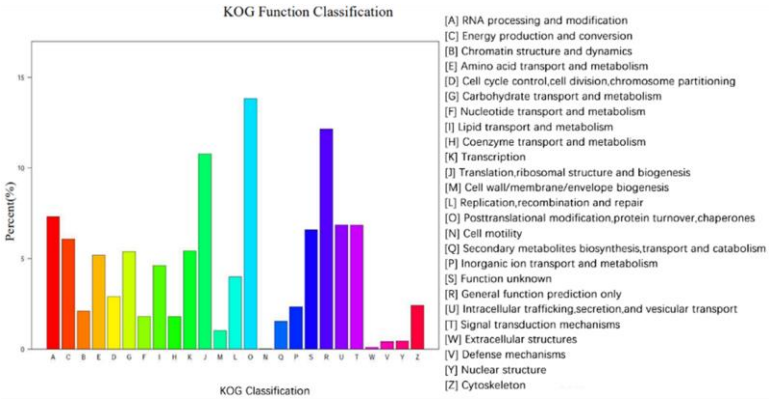

Fig. 2: KOG annotation of putative proteins

A total of 32,662 unigenes were found in KOG and these were divided into twenty-six groups, the three groups of *'*posttranslational modification, protein turnover, chaperones*'*, *'*translation, ribosomal structure and biogenesis*'*, *'*general function prediction only*'* had the highest percentage in the annotation genes, followed by *'*signal transduction mechanism*'*, *'*intracellular trafficking, secretion, and vesicular transport*'*, *'*RNA processing and modification

(2389), "intracellular vesicular transport, secretion, and trafficking" (2241), "mechanisms of signal transduction" (2235), and "energy conversion and production" groups (1988). Only a very limited number of unigenes were attributed to "cell motility" (8) and "extracellular structures" (35). The analysis of the metabolic pathway for unigenes was also conducted using KEGG; a total of 132 pathways were predicted using this method, indicating 49,517 unigenes (Fig. 3). "Translation" contained the most unique transcripts (4637), followed by "carbohydrate metabolism" (4434) and "folding, sorting, and degradation" (3652). These results might provide insights into the understanding of ER stress in *C. paliurus*.

Digital Gene Expression (DGE) Sequencing

To compare gene expression levels in TM_0 h, TM_6 h, and TM_14 h, a DGE analysis was performed. Low-quality sequences were removed prior to the analysis; 47,323,406, 51,432,524, 49,168,870, 46,269,170, 48,752,916, 47,872,316, 47,267,348, 60,976,736, and 53,236,974 clean reads were collected at TM_0 h 1, TM_0 h 2, TM_0 h 3, TM_6 h 1, TM_6 h 2, TM_6 h 3, TM_14 h 1, TM_14 h 2, and TM_14 h3, respectively (Table 1). The clean reads were then added to the RSEM (RNASeq by Expectation Maximization) program for the transcriptome assembly. Zero mismatch in the bowtie parameter was found. In the DGE sequencing results, minimum 72.13% could be programmed into the initial reference gene sequences (Table 2).

Differential Expression Analysis

To identify the gene expression of the downstream of UPR under ER stress conditions, the time profiles of ER stress–related gene expression were studied after *C. paliurus* was treated with TM (5 μg/mL). A DEG is a greatly depleted or enriched gene in one treatment compared with the rest of the samples (|log2 (fold-change) | >1; $q < 0.005$). Further, 6743 and 11,943 nonredundant DEGs (differentially expressed genes) were generated in TM_6 h and TM_14 h, respectively.

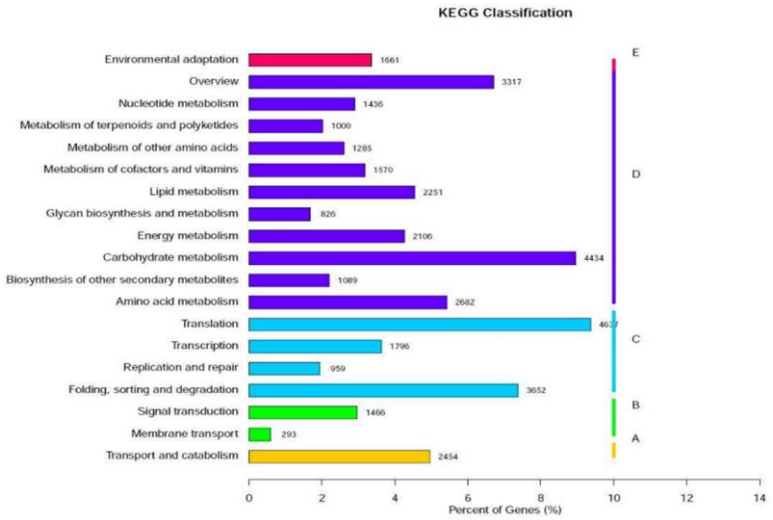

Fig. 3: KEGG annotation of putative proteins

This process suggests 132 pathways, involving 49,517 unigenes. The *'*chaperones, protein turnover, post-translational modification*'* (4,518) was the largest group, followed by *'*general function prediction*'* (3,967), *'*translation, biogenesis, and ribosomal structure*'* (3,521), *'*RNA processing and modification*'* (2,389), *'*intracellular vesicular transport, secretion, and trafficking*'* (2,241), *'*mechanisms of signal transduction*'* (2,235), and *'*energy conversion and production*'* groups (1,988). Only a very limited number of unigenes were attributed to *'*cell motility*'* (8) and *'*extracellular structures*'* (35). The analysis of the metabolic pathway for unigenes was also conducted using KEGG; a total of 132 pathways were predicted using this method, indicating 49,517 unigenes (Fig. 3). *'*Translation*'* contained the most unique transcripts (4,637), followed by *'*carbohydrate metabolism*'* (4,434) and *'*folding, sorting, and degradation*'* (3,652)

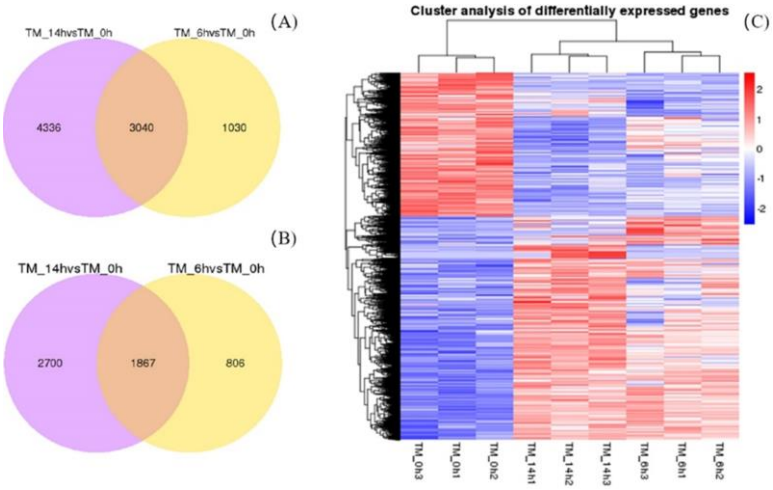

Fig. 4: Up- and down-regulated differentially expressed genes (DEGs) with TM treatments vs. the control

(**A**) Up-regulated DEGs (Heat map); (**B**)Down-regulated DEGs (Heat map); (**C**)Hierarchical cluster analysis of gene expression using log ratios. Gene expression levels are identified by the following colors: blue for reduced transcript abundance and red for increased transcript abundance. TM_0 h: no TM treatment as control, TM_6 h:TM treatment for 6 h, TM_14 h:TM treatment for 14 h

By comparing the DEG at TM_6 h versus TM_0 h, 6743 DEGs were found, with 2673 downregulated and 4070 upregulated genes. On comparing TM_14 h versus TM_0 h, a total of 11,943 DEGs were found, with 4567 downregulated and 7376 upregulated genes. Among these, 1867 downregulated and 3040 upregulated DEGs were consistent between both TM_6 h versus TM_0 h and TM_14 h versus TM_0 h (Fig. 4A and 4B). To distinguish functional enrichment clusters, hierarchical clustering was performed following the patterns in the gene expression analysis (Fig. 4C).

RT-qPCR Validation for Reference Genes

To confirm the reliability of DEGs, real-time PCR validation was performed. Recent studies have shown that even commonly used and studied reference genes can differ under various environments. However, no reference gene has been reported for *C. paliurus*. In the present study, seven reference genes *(UBQ4, TUB, TUA, GAPDH, 18SrRNA, ACT2,* and *ACTF)* were chosen by evaluating the results from previous studies, since they demonstrated stable expression levels.

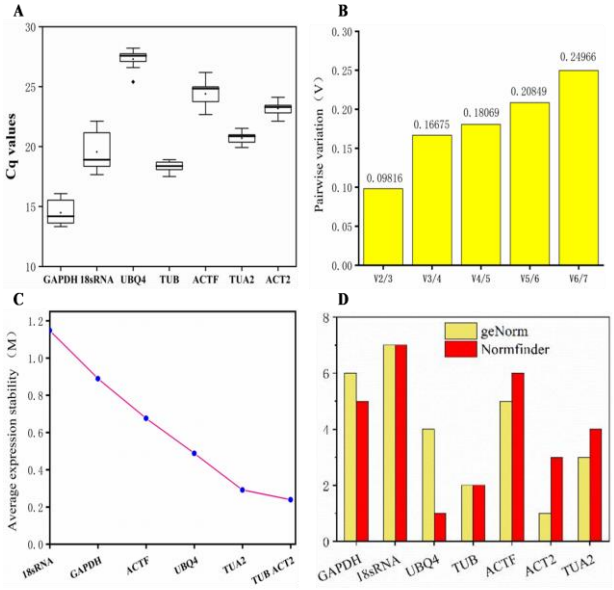

Fig. 5: Reference gene validation under ER stress

(**A**) All the Cq values of candidate reference genes. Median values were indicated by lines across boxes. 25/75 percentiles are shown by the boxes. The maximum and minimum values were shown by whisker caps. Outliers are marked by circles. (**B**) geNorm software program was used to do pairwise variation (Vn/n +1) analysis and to determine the normalization factors (NFn and NFn +1) in all samples. (**C**) M values expressed the average expression stability in the reference genes tested. They were determined by removing the least stable reference genes step by step. The lower the M value, the more stable the expression under ER stress (geNorm). (**D**) Ranking comparison of candidate reference genes according to their stability values obtained by geNorm and NormFinder software

All of the crossing point (Cq) values of the reference genes are exhibited in Supplemental Table 4. The Cq values of the seven reference genes were variable (Fig. 5A), ranging between 13.33 *(GAPDH)* and 28.21 *(UBQ4)*, while the mean values of Cq were in the range of 14.48 (*GAPDH*) to 27.29 (*UBQ4*). In addition, the levels of 18SrRNA were shown to be the most fluctuating (4.46 Cq; the min and max values 17.66 and 22.12, respectively), and *TUB* had the least fluctuations (1.41 Cq; the min and max values 17.5 and 18.91, respectively). Since the gene expression level negatively correlates with the Cq value, the experimental results indicated that *UBQ4* had low expression and *GAPDH* had high expression.

By introducing a new reference gene, software geNorm was used to calculate the pairwise change (*V*) in the normalization factor and determine the optimal amount based on the ratio of *Vn*/*Vn* + 1. Fig. 5B shows that the V2/3 values of the two reference genes were below 0.15, and therefore they were sufficient for use in normalizing gene expression in the TM treatments.

An analysis using geNorm was performed on the expression stability to further evaluate the reference genes of interest. The outcomes are shown in Fig. 5C and Supplemental Table 5. *TUB* and *ACT2* (M = 0.2391) were shown to be the most promising option for ER-stress–related normalization. The NormFinder program was used to further verify the results obtained from the geNorm analysis. Some differences were observed in the results of these two analyses. The most stable genes were basically similar, but the ranking orders of the two algorithms were different (Fig. 5D).

RT-qPCR under ER Stress Conditions

To validate the gene expression under ER stress conditions, RT-qPCR normalized by two reference genes (*TUB* and *ACT2*) was performed. Eight highly upregulated and two minimally upregulated DEGs were found by both DGE and sequencing of the transcriptome (Fig. 6). These DEGs contained two genes relevant to transcription factors [bHLH28; OS=*A. thaliana* GN=BHLH28 PE=2 SV=1 (Cluster-29186.16026), heat stress transcription factor B-3 OS=*A. thaliana* GN=HSFB3 PE=2 SV=1 (Cluster-29186.98807)]. Five genes were related to oxidoreductase family protein [*Populus trichocarpa* (Cluster-29186.148872), oxygenase superfamily protein SRG1 OS=*A. thaliana* GN=SRG1 PE=2 SV=1 (Cluster-29186.21713), methylenetetrahydrofolate reductase 1 OS=*Zea mays* PE=2 SV=1 (Cluster-29186.99722), acyl-protein thioesterase [*Ricinus communis*] (Cluster-29186.149462), and cyclin-dependent kinase B2-1 OS=*A. thaliana* GN=CDKB2-1 PE=1 SV=2 (Cluster-29186.30046)]. Two genes were related to chaperonin and cytochrome [chaperonin CPN60-2, mitochondrial OS=*Cucurbita maxima* GN=CPN60-2 PE=1 SV=1 (Cluster-29186.152587) and cytochrome P450 82G1 OS=*A. thaliana* GN=CYP82G1 PE=1 SV=1 (Cluster-29186.21846)]. One gene was related to the NAC family

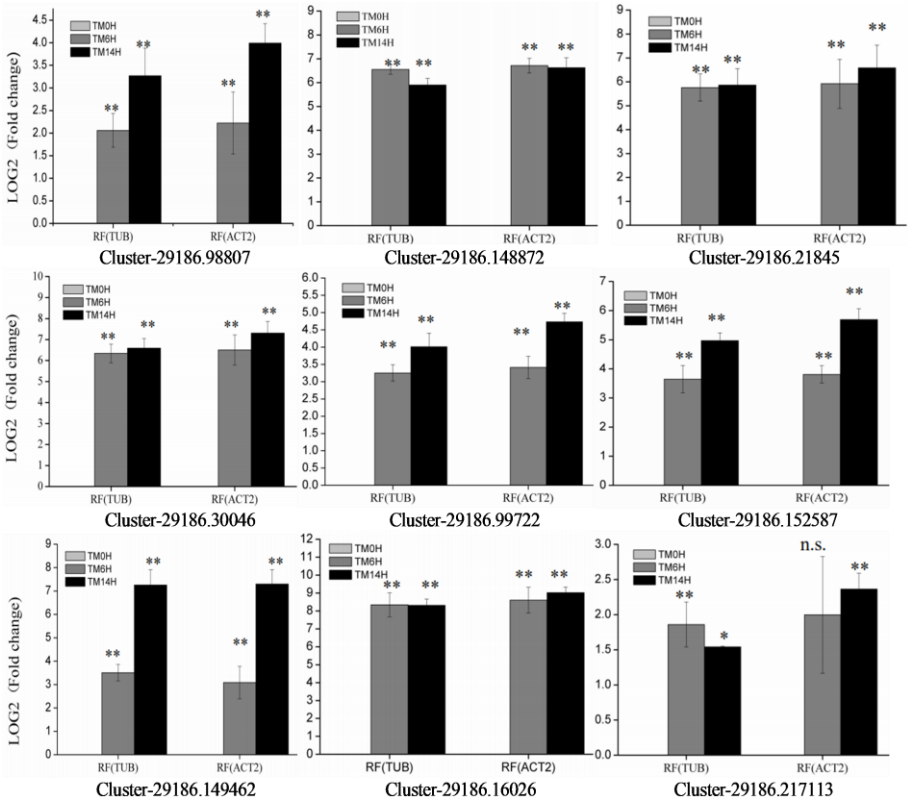

Fig. 6: Double reference genes used to quantify relative expression under ER stress

Log2 fold induction refers to the gene expression value in the TM treatments. Plants treated for 6 hours and 14 hours normalized to that of the control plants, and all expression values were assigned to the level of the *TUB* or *ACT2* gene. RF(TUB): Reference gene *TUB;* RF(ACT2): Reference gene *ACT2*. Error bars represent SE(n=3). Asterisks indicate significance levels when comparing to the control in t-test. (*, $P < 0.05$; **, $P < 0.01$; n.s., not significant at $P < 0.05$)

[NAC domain-containing protein 83 OS=*A. thaliana* GN=NAC083 PE=1 SV=1 (Cluster-29186.100002)]. Previously reported RNA-seq results agreed with the expression pattern of the total of 10 genes collected from the qRT-PCR experiment. Therefore, RNA-seq results were validated.

Putative Genes Related to the Response of ER Stress

For ER stress, UPR is of great significance. The results from the gene sequencing analysis indicated that 17 putative genes associated with known ER stress marker genes exhibited differential expression (Table 3). The DEGs obtained from transcriptome sequencing were investigated in the KEGG and GO databases, where four heat shock factor proteins, one kinase IRE1-like protein, two genes related to protein disulfide isomerase, one calreticulin, two protein transport proteins, and seven proteins involved in the glycosylation process were found among the DEGs in the TM treatments versus the control.

Discussion

The accumulation of a large amount of misfolded or unfolded proteins in the ER can have a toxic effect on cells and is the main cause of ER stress. Therefore, proper folding of regulatory proteins is the key to maintaining cell homeostasis (Zhang and Wang, 2016). The nascent peptide chain enters the ER protein folding regulatory pathway via the Sec61 translocator complex (Zhang *et al.*, 2013). Depending on whether the nascent protein has a glycosylation site, it is divided into two protein folding pathways: the glycosylated protein is recognized by oligosaccharide transferase and enters in the calnexin/calreticulin cycle for folding; the nonglycosylated protein depends mainly on the molecular chaperone binding protein (BiP) and other cofactors in the ER for folding (Crout and Vic, 2010).

C. paliurus is regarded as a special plant resource for anti-glucose medicine in China. It is very important to mine the transcriptome data of *C. paliurus* to improve its yield, especially when *C. paliurus* is grown under adverse conditions. Abiotic stress can lead to the buildup of unfolded or incorrectly folded proteins and in turn causes damage to the ER (Zhu, 2016). In the present study, 17 putative genes were identified in *C. paliurus*, including 2 protein disulfide isomerases (*PDI*) (Cluster-29186.90927 and Cluster-29186.91893), 1 IRE1 (Cluster-29186.112715),

1 CRT (Cluster-29186.93033), 2 transport proteins (Cluster-29186.56416 and Cluster-29186.21342), 4 chaperones (Cluster-29186.105919, Cluster-29186.94830, Cluster-29186.94955, and Cluster-29186.75574), and 7 glycosyltransferases (Cluster-29186.99554, Cluster-29186.73075, Cluster-29186.71208, Cluster-29186.37582, Cluster-29186.126287, Cluster-29186.121831, and Cluster-29186.108208). Previous reports showed that the glycosyltransferase OST in *Arabidopsis* consisted of 5 subunits: DAD1, DAD2, STT3A/STT3B, DGL1, and HAP6. The overexpression of *DAD1* and *DAD2* can inhibit DNA fragmentation caused by ultraviolet damage in *Arabidopsis* and also repress the apoptosis in tobacco (Wei *et al.*, 2003; Danon *et al.*, 2004). The *Arabidopsis* double mutant *stt3a-1stt3b-1* plants are embryonic lethal, and defective glycosylation1-1 (DGL-1) is an important subunit of glycosyltransferase. If *DGL1* is mutated in *Arabidopsis*, it weakens the glycosylation of proteins and affects cell differentiation and growth (Lerouxel *et al.*, 2010). The overexpression of *BiP* in soybean and tobacco increased plant drought tolerance (Alvim *et al.*, 2001; Valente *et al.*, 2009). After knocking down *BiP2*, the plants become very sensitive to pests and infection. Wheat *(Triticum aestivum) CRT (Ta-CRT)* is upregulated by drought induction, and the overexpression of *Ta-CRT* in tobacco enhances plant drought tolerance (Wang *et al.*, 2017). BrCRT2 is a candidate gene for controlling tipburn in Chinese cabbage. The overexpression of *BrCRT2* (calreticulin family protein) increases Ca^{2+} storage in the *Arabidopsis crt2* mutant and also reduces cell death at the tip and margin under Ca^{2+} depletion conditions (Su *et al.*, 2019). *A. thaliana* THERMOSENSTITIVE MALE （STERILE1）containing DnaJ and ERdj5C domains plays a role in heat stress in pollen tubes. *Chlamydomonas reinhardtii IRE1 (CrIRE1)* mutants *crire1-1* and *crire1-2* reduced the expression of the ER stress marker gene and increased the expression of the reactive oxygen species marker gene, suggesting that CrIRE1 is an important component of ER stress in *Chlamydomonas* and the ER stress sensor IRE1 is highly conserved in the evolutionary history (Yamaoka *et al.*, 2018). The overexpression of the *PDI* gene from *Methanothermobacter thermautotrophicus* enhances heat stress tolerance in rice (Wang *et al.*, 2018). The aforementioned results demonstrated that the genes identified in the present transcriptome data were associated with abiotic stresses. The study of the ER stress signaling pathway and related factors might help in the research of other abiotic stress pathways in *C. paliurus.*

RNA high-throughput sequencing (RNA-Seq) has been effectively connected in *Litchi chinensis* Sonn, *Vitis vinifera* cv. Shiraz, *Vaccinium* section *Cyanococcus*, *Citrus sinensis*, *Myrica rubra*, *Malus domestica*, *Diospyros kaki*, *Pyrus bretschneideri* Rehd, *Prunus persica*, and *Musa acuminata* and longan fruits (Gleave *et al.*, 2008; Feng *et al.*, 2012; Rowland *et al.*, 2012; Zhang *et al.*, 2012a, b; Li *et al.*, 2013). This study was designed to identify candidate genes associated with ER stress in *C. paliurus* using DGE and transcriptome sequencing techniques. The study pioneered the systematic selection of proper reference genes for RT-qPCR in *C. paliurus*. The results suggested that *18SrRNA* was the least stable in the TM treatments. This gene was considered to be unstable by a previous study on zucchini (Obrero *et al.*, 2011), pear (Wu *et al.*, 2012), cucumber (Wan *et al.*, 2010), peach (Tong *et al.*, 2009), and melon (Leida *et al.*, 2015). Therefore, *18SrRNA* is not suitable for RT-qPCR normalization studies with mid- or low-level expression of target genes. Due to the negative correlation between Cq values and expression levels, *UBQ4* was found to be the least suitable reference gene in *C. paliurus*, with gene expression either significantly lower or higher than the rest of the reference genes tested. In this study, the geNorm and NormFinder algorithms were applied to determine the stability of candidate reference genes. *TUB* and *ACT2* were shown to be the least viable genes, and *18SrRNA* was the least stable reference gene during TM treatments. However, previous reports showed that *TUB* was the least stable reference gene and *18SrRNA* was the most stable reference gene under temperature stress in longan (Wu *et al.*, 2016). These results suggested that an appropriate reference should be chosen for each experimental condition.

In summary, to obtain high-yield and high-quality *C. paliurus*, it is important to study the molecular regulation mechanism under ER stress. This transcriptomics study classified and identified the function of differentially accumulated proteins, providing insights for studying abiotic stresses.

Conclusion

UPR induced by ER stress plays a critical role in plants under adverse environmental stress. This study was novel in reporting on transcriptome sequencing of *C. paliurus* under ER stress and screening appropriate reference genes for RT-qPCR in *C. paliurus* under ER stress. The most appropriate reference genes under TM stress were *TUB* and *ACT2.* The study of the ER stress signaling pathway and related factors might benefit future researches of other abiotic stress pathways in *C. paliurus.* Meanwhile, the identification of 17 putative genes might provide insights for molecular breeding or transgenics and help increase plant yield.

Accession Numbers

The following genes are associated with this article: GAPDH (Cluster-29186.90009),18S rRNA (Cluster-29186.38298), UBQ4 (Cluster-29186.24892), ACT2 (Cluster-29186.146029), TUA (Cluster-12578.0), TUB (Cluster-29186.78362), and ACTF (Cluster-29186.107737).

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