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TRANSCRİPTOME SEQUENCİNG OF *PİNUS MASSONİANA* ROOTED AND NON-ROOTED CUTTİNGS

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Abstract

Pinus massoniana Lamb. is a unique and fat-producing tree species growing in southern China. However, cuttings of this species are difficult to root. In this study, transcriptome sequencing of rooted and non-rooted cuttings of this species was performed by Illumina sequencing technology. We obtained 138,466,788 raw reads and 132,448,884 clean reads including 124,396 unigenes after *de novo* assembly; these unigenes were annotated into seven databases. In the rooted and non-rooted cuttings, 781 differentially expressed genes (DEGs) were found, of which 368 were up-regulated and 413 were down-regulated. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis indicated that these DEGs were enriched in 73 pathways, 18 of which were significantly enriched, including 'photosynthesis', 'phenylpropanoid biosynthesis', 'starch and sucrose metabolism', and 'carbon fixation in photosynthetic organisms'. 'Plant hormone signal transduction' was involved in the regulation of endogenous hormones, including some genes related to indole acetic acid (IAA) that were significantly associated with rooting. These genes were also found in the rooting processes of other plants. Compared with the non-rooted *Pinus massoniana* seedlings, auxin-responsive protein (*AUX/IAA*) was significantly down-regulated in the rooting shoots. We selected 10 genes from the DEGs and performed real-time quantitative polymerase chain reaction (RT-qPCR) verification. The results were consistent with the gene expression based on the RNA-seq technology. Our study may be useful for improving the rooting rate of *Pinus massoniana* cuttings.

Key words: *Pinus massoniana*, Cutting, Root, Transcriptome, RT-Qpcr.

Introduction

Masson pine (*Pinus massoniana* Lamb.) is a unique and fat-producing tree species growing in southern China. It has a wide distribution and strong adaptability and plays an important role in China's forestry production (Chen *et al*., 2015; Liu *et al*., 2012). Over the last few years, many studies have been published on Masson pine, including a study on root tips (Quan & Ding, 2017) and a physiological and proteomic analysis (Xu & Wu, 2016) to determine the response to drought stress. Some researchers have investigated the growth of *Pinus massoniana* in soils with different Pi starvation levels (Fan *et al*., 2014) and in Mn-contaminated environments (Li *et al*., 2017b). However, there are few publications on the rooting and rooting physiology of *Pinus massoniana*, especially root transcriptome studies.

Transcriptome studies have been widely used in plant research since the development of RNA-seq technology. Some authors have investigated the gene expression responses of plants under stress, such as *Sophora moorcroftiana* (Li *et al.*, 2015), *Taxodium* "Zhongshansa" (Qi *et al.*, 2014) and Ramie (Liu *et al.*, 2013). Some researchers have conducted transcriptome analysis to determine the key regulated genes during adventitious root formation; for example, a gene expression analysis of *Petunia* during adventitious rooting was performed (Ahkami *et al.*, 2014) and three *PeSHRs* and one *PeSCR* were involved in the adventitious root development of *Populus* (Xuan *et al.*, 2014). Recently, several transcriptome analyses were conducted of *Pinus massoniana* (Chen *et al.*, 2016). However, transcriptome analyses of the rooting process of *Pinus massoniana* have not been reported.

In many cases, seed propagation is used for Masson pine but for certain outstanding varieties, cutting propagation is used to maintain the good characteristics of the parent generation. The rooting of the panicle is a prerequisite for the survival of the cuttings. This has been reported for the rooting of *Rhododendron scabrifolium* Franch (Li *et al*., 2017a). The gene expression during rooting is of great significance in the study of root physiology. In this study, we used Illumina sequencing technology to sequence the rooted and non-rooted cuttings of *Pinus massoniana*, screened them for DEGs, and conduct verification using RT-qPCR. The goal of this study is to clarify the rooting mechanism of *Pinus massoniana* by analyzing the DEGs and further improve the rooting rate of this tree species.

Materials and Methods

Plant material and treatment: One-year-old Masson pine seedlings were collected from a *Pinus massoniana* seed base located in the Ma village of Duyun City in Guizhou Province (P. R. China) (107°31′ E, 26°16′ N). These were potted plants from a greenhouse at the Guizhou University Forestry College. After transplanting, the tops of the seedlings were cut off and 15 cm of the stem was retained to sprout more branches. In December 2015, the semi-lignified shoots from the same tree were used as test materials. The control group (CK) was left untreated and the test group (CT) was soaked in a solution of 800 mg/L (indole-3-butyric acid (IBA): Naphthaleneacetic acid (NAA) = 3:1) for 30 min. The branches of the CK and CT were cut in the greenhouse; the substrate was fine sand. The cuttings were automatically watered 3 to 5 times a day (based on the humidity) and we observed and recorded the survival and callus growth every 10 d. After 4 months, the roots were treated with a hormone (CT) but the untreated (CK) cuttings did not root (Fig. 1). The callus and some roots below the soil surface were collected at the base of the cuttings. Prior to extracting the RNA, the material was frozen in liquid nitrogen and then stored at -70°C in a freezer.

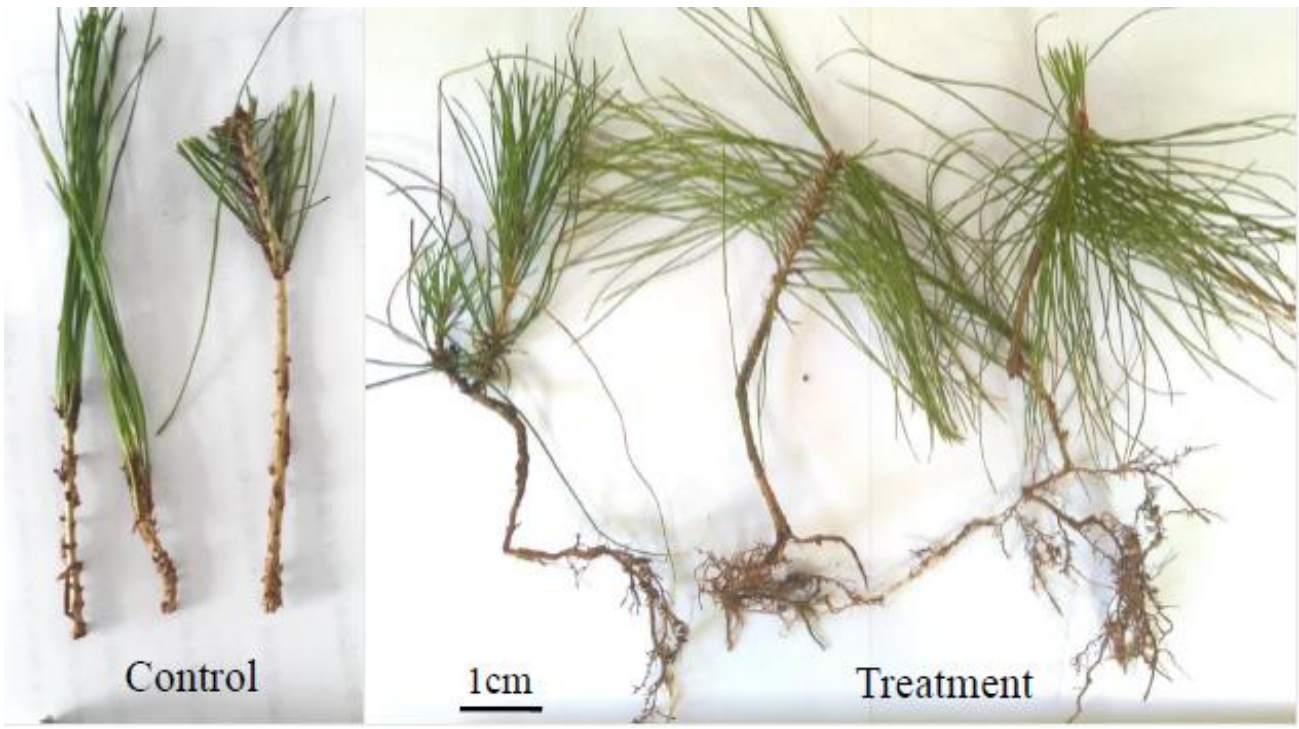

Fig. 1. Rooting of *Pinus massoniana* in the experimental group (CT) and control group (CK).

Total RNA isolation, cDNA construction, and sequence assembly: For the RNA concentration measurements, purity detection, integrity assessment, and the degradation and contamination detection, we used the method of Li *et al*., (2017c). For evaluating the library quality, we used an Agilent Bioanalyzer 2100 system. We generated a sequencing library using the method described by Li *et al*., (2015).

Quality control of clean data, transcriptome assembly, and gene functional annotation: The raw data in the fastq format were treated using in-house scripts to obtain high-quality clean reads; the Q20, Q30, and GC contents and the sequence duplication levels of the clean reads were computed. The transcriptome assembly was obtained using Trinity software (Grabherr *et al*., 2011). The gene function was annotated using seven databases: the National Center for Biotechnology Information (NCBI) non-redundant nucleotide sequences (Nt), the NCBI non-redundant protein sequences (Nr), the manually annotated and reviewed protein sequence database Swiss-Prot, the protein family database (Pfam), the Clusters of Orthologous Groups of proteins (KOG/COG), the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog database (KO) (Kanehisa *et al*., 2008), and the Gene Ontology (GO) database, using BLAST with E-value cutoffs ≦ le-5.

Gene expression and differential expression analysis: The clean data of the samples were mapped back onto the assembled transcriptome and the read count of the genes was determined from the mapping results; the levels of gene expression were assessed using the RSEM software (Li *et al*., 2011). For the adjustment of the read counts, we used the edgeR software package with one scaling normalized factor for each sequenced library prior to the differential gene expression analysis. We used the DEGseq (2010) R package to analyze the DEGs; the P-value was adjusted by the q-value and the threshold for the significant differential expression was q-value < 0.005 & |log2(fold change)| > 1. In order to adjust the gene length deviation in the DEG, the GO enrichment analysis of the DEGs was conducted using the GOseq R package (Young *et al*., 2010). KOBAS (Mao *et al*., 2005) software was used to examine the statistical enrichment of the DEGs in the KEGG (Kanehisa *et al*., 2008) pathways.

Verification of quantitative RT-qPCR: Ten genes were selected from the DEGs and detected using real-time quantitative techniques (Table 1). The test results were compared with the transcriptome data to verify the correctness of the transcriptome results. UBC was used as an internal reference (F: 5'–GATTTATTTCATTGGCA GGC–3', R: 5'–AGGATCATCAGGATTTGGGT–3') and the sample was detected with SYBR GREEN I using an Analytik Jena qTOWER 2.2 real-time PCR instrument (Germany). The expression level of the target gene in the sample (including the pre-test) was detected by SYBR GREEN I. The cDNA was synthesized using a 20 μL reaction system: total RNA 500 ng, 5 × RT reaction mix 4 μL, dT primer 1 μL, TUREscript H-RTase/RI Mix 1 μL, RNase free dH_2O 10 μL; the conditions were 42°C for 60 min and 65°C for 10 min. The real-time PCR reaction conditions were: Step 1: 95°C for 3 min, Step 2: 95°C for 10 s, Step 3: TM 58°C for 30 s + plate read, Step 5: go to step 2, 44 cycles, Step 6: melt curve analysis (60°C ~ 95°C, + 1°C/cycle, holding time 4 s). The gene relative expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantification method.

Table 1. Primers of ten genes for RT-qPCR determination.

Transcript no.	Gene description	Primer sequence
comp46811	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies	F:5'-AACAACTCAACCGCATTC-3' R:5'-ATCAGGAACGACAACAGA-3'
comp38612	Glutathione S-transferase	F:5'-CGTAATCAACTCGCTTCT-3' R:5'-ATCAAGGTACTCGGCTAT-3'
comp40016	Glutathione S-transferase	F:5'-GAACCGAGTGTTGGATAT-3' R:5'-ATGGCAGATGTTGTAGAT-3'
comp38529	Glutathione S-transferase	F:5'-CGTGTATCCTACCAGAGA-3' R:5'-TGATGACATAGAAGCATTACT-3'
comp34227	Transcription factor, Myb superfamily	F:5'-TCAAGAACTACTGGAACACT-3' R:5'-GGACAGGAACAGGAGATT-3'
comp30930	Flavonol reductase/cinnamoyl-CoA reductase	F:5'-ACTCATTCAACTTCAATCCTTCA-3' R:5'-GCCAGCCATTATCTCAACAT-3'
comp33477	UDP-glucuronosyl and UDP-glucosyl transferase	F:5'-GCTTCTTGATTCCATTATTGT-3' R:5'-TGATTGACGAACCATAGATT-3'
comp40433	Terpene synthase activity	F:5'-AGTTCCTTGGTTAAGTTATCG-3' R:5'-TTCGTCCGTATCCTGTTAT-3'
comp47788	Peroxidase	F:5'-GGCATAATAATGTGGAATCA-3' R:5'-TTCTTCATATCGCTCTCAT-3'
comp45820	Multicopper oxidases	F:5'-TTCAAGATAGCAGGACACAA-3' R:5'-CGGCAGAGATGACAAGTAT-3'

Results

Transcriptome sequencing and assembly: For the transcriptome dataset construction, we used Illumina sequencing. A total of 138,466,788 raw reads were obtained. There were 132,448,884 clean reads remaining including 124,396 unigenes after clustering generation and sequencing the library preparations. The unigene length ranged from 201 bp to 18,053 bp (Fig. 2).

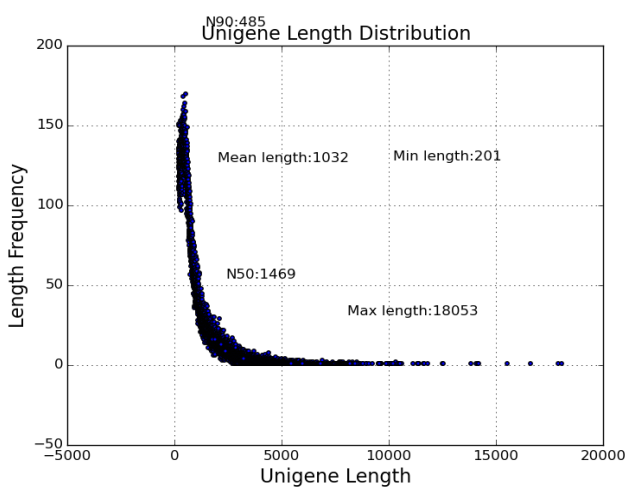

Fig. 2. Unigene length distribution after assembly.

BLAST analysis: The gene function annotations were performed using the seven databases to obtain comprehensive information on the gene function. The database with the highest number of successfully annotated genes was Nr (54.26%), followed by GO (44.37%), and KO (20.77%). The number of successful genes annotated in the 7 databases accounted for 10.46% of the total gene number. Five out of the seven database annotation results were depicted as Venn graphs. The numbers of genes successfully annotated in the five databases were Nr 67, 502, Nt 53, 753, Pfam 54, 177, GO 55, 196, KOG 30, 032, respectively (Table 2).

Functional annotation of genes: The GO allocation allows us to predict the functions of the *Pinus massoniana* L. genes; 55,196 (44.37%) unigenes were divided into the three main functional categories of biological processes (BP), cellular components (CC), and molecular functions (MF); these three categories contained 56 subclasses (Fig. 3). The number of 'cellular processes', 'metabolic processes', and 'signaling processes' of the annotated genes amounted to more than 2,000 in the BP category. 'Cell' and 'cell junction' had the highest number of genes in the CC category. Two categories with the largest number of genes were 'binding' and 'catalytic activity' in the MF category. These results were similar to the GO terms for *Sophora moorcroftiana* (Li *et al*., 2015), *Prosopis alba* (Torales *et al*., 2013), and *Anthurium* (Tian *et al*., 2013). The 30,032 (24.14%) unigenes were classified into 26 groups using the KOG database (Fig. 4). The largest percentage of genes were assigned to 'General function production only' (R), followed by 'Posttranslational modification, protein turnover, chaperones' (O), whereas 'Cell motility' (N) and 'U named protein' (X) had the lowest percentages. After the KO annotation, the genes were divided into five branches based on the KEGG metabolic pathways they were involved in (Fig. 5). The number of genes was highest in the 'Translation' pathway (2856), followed by 'Carbohydrate metabolism' (2449).

Differential expression of rooting and non-rooting of *Pinus massoniana*: The input data of the differential expression of genes are the read count data obtained from the analysis of the gene expression levels. The differential gene screening conditions are: q-value < 0.005 & |log2(fold change)| > 1. The volcano graph depicts the relationship between the q-value and the log2 (fold change) (Fig. 6). There were 781 genes for rooting and non-rooting differential expression in *Pinus massoniana* seedlings, of which 368 were up-regulated and 413 were down-regulated.

Table 2. Gene annotation success rate statistics.

Databases	Number of unigenes	Percentage (%)
Annotated in NCBI non - redundant protein sequences (Nr)	67502	54.26
Annotated in NCBI non - redundant nucleotide sequences (Nt)	53753	43.21
Annotated in KEGG Ortholog database (KO)	25846	20.77
Annotated in A manually annotated and reviewed protein sequence database (SwissProt)	54551	43.85
Annotated in Protein family (PFAM)	54177	43.55
Annotated in Gene Ontology (GO)	55196	44.37
Annotated in Clusters of Orthologous Groups of proteins (KOG)	30032	24.14
Annotated in all Databases	13017	10.46
Annotated in at least one Database	84585	67.99
Total Unigenes	124396	100

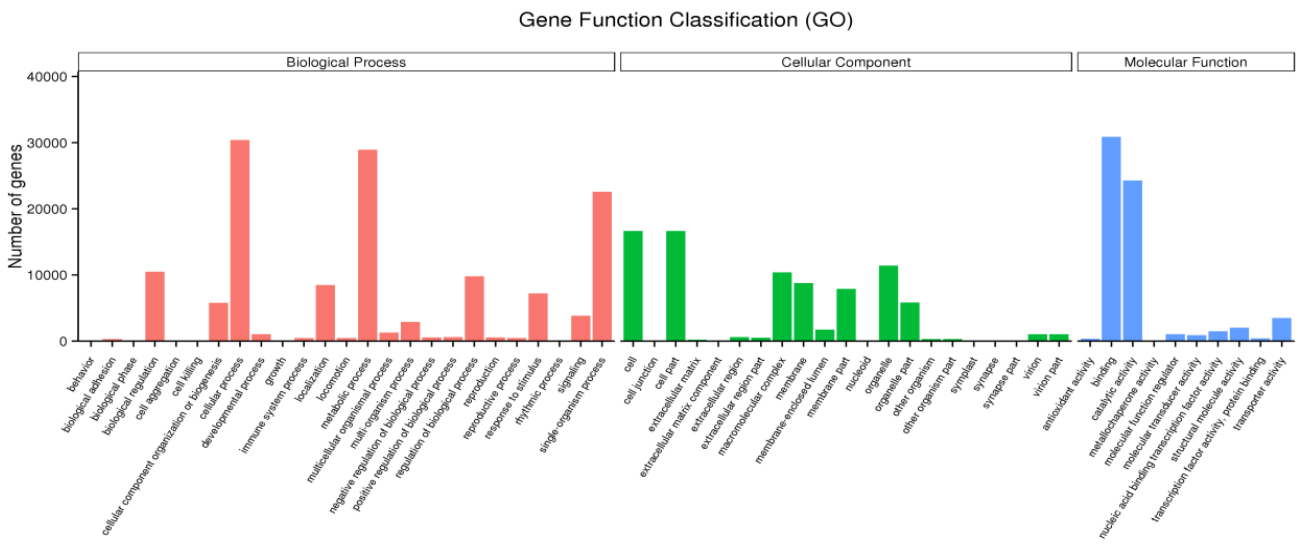

Fig. 3. GO annotation of unigenes. The abscissa indicates the GO term of the next level of the three major classes of GO and the ordinate represents the number of genes annotated to the term (including the sub-term of the term).

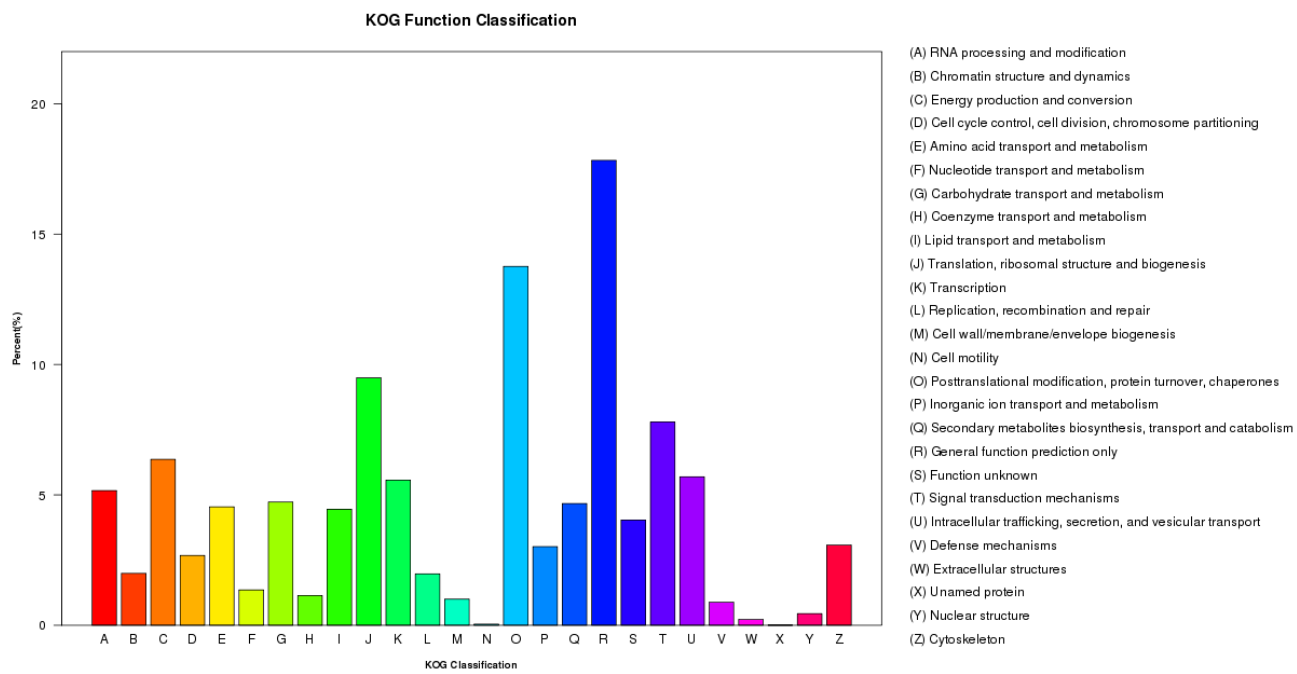

Fig. 4. KOG classification of putative proteins. The abscissa shows the name of the 26 groups of KOG and the ordinate indicates the percentage of the number of genes annotated to the group.

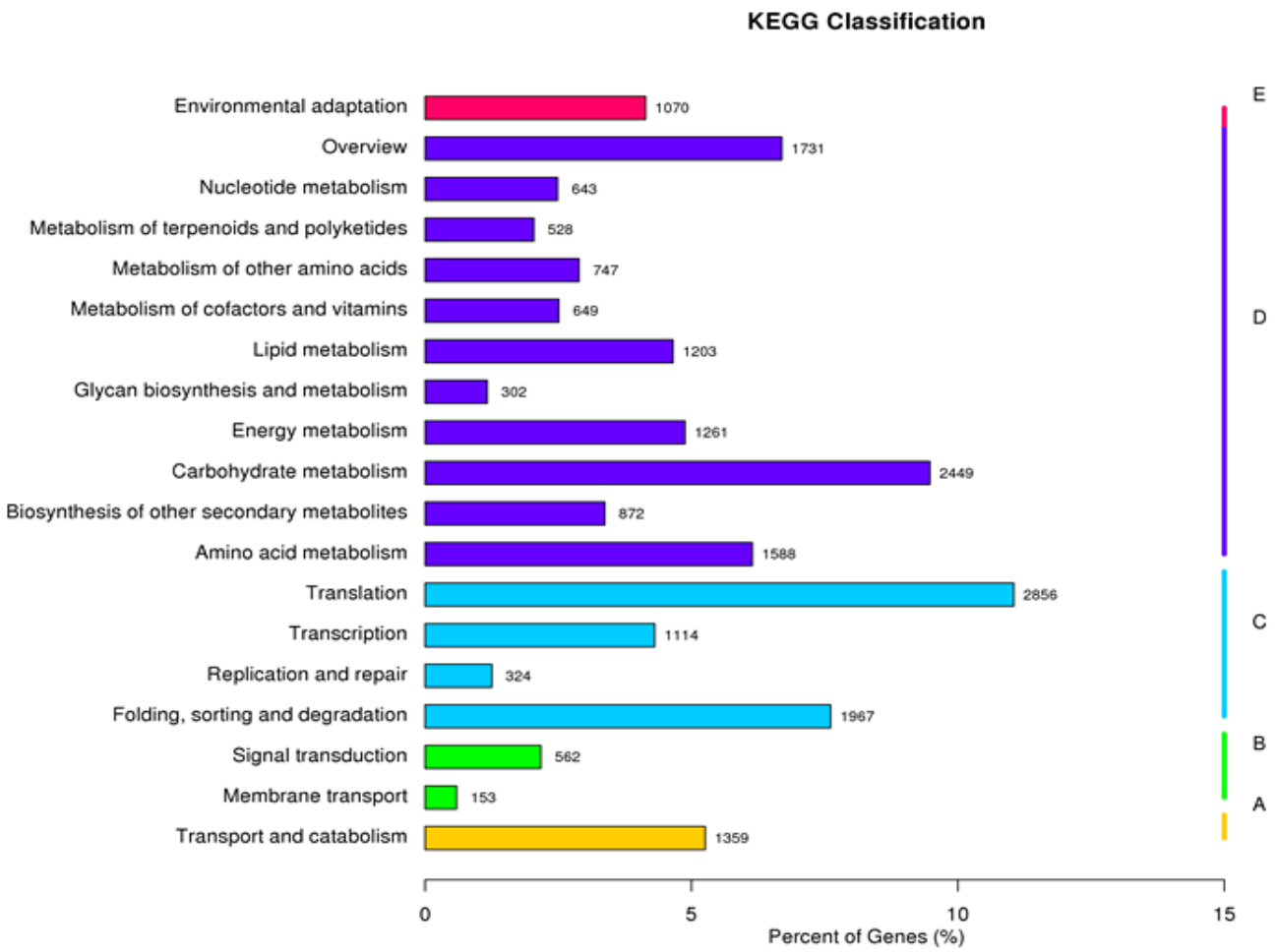

Fig. 5. KEGG function annotation of transcriptome. The left ordinate is the name of the KEGG metabolic pathway and the abscissa is the percentage of the number of genes annotated to the pathway. A-E are the five branches of the gene involved in the KEGG metabolic pathway and correspond to 'cellular process', 'environmental information processing', 'genetic information processing', 'metabolism', and 'organismal systems'.

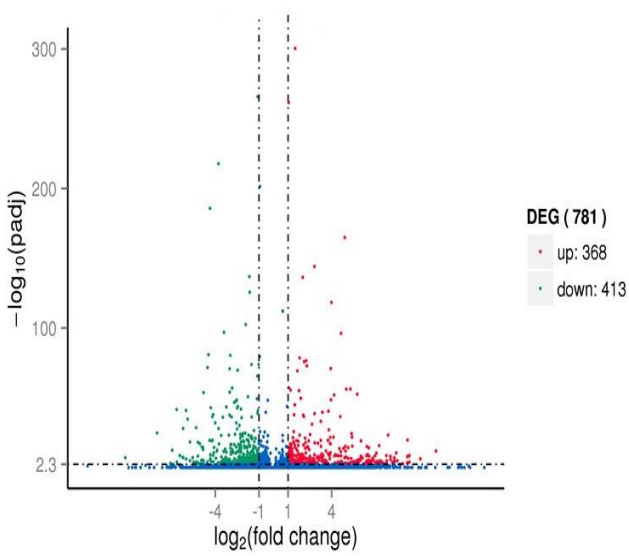

Fig. 6. Gene differential expression. The abscissa represents the change in the expression (fold change) of the gene in the CK and CT. The ordinate represents the significant degree of change in the gene expression (statistical). The scatter points indicate the individual genes, Blue, red, and green dots indicate genes with no significant differences, significantly different up-regulated genes, and significantly different down-regulated genes respectively.

In living organisms, different genes affect biological functions on the basis of mutual coordination. Metabolic pathways in which differential genes are involved, i.e., metabolic pathways that are significantly enriched, generally determine the most important physiological metabolic and signal transduction pathways. KEGG is the main public database for determining pathways (Kanehisa, 2008). A pathway significant enrichment analysis determines the paths of differential genes that are significantly enriched relative to all annotated genes using the KEGG Pathway database. The sequencing data indicate that there are 73 metabolic pathways that express KEGG enrichment in the rooting and non-rooting genes of *Pinus massoniana* and 18 are significantly enriched. We created a convergence plot based on the number of pathways of the differential genes (Fig. 7). The most significant pathways are 'Photosynthesis' with 22 DEGs, 21 of which are down-regulated, followed by 'Phenylpropanoid biosynthesis' with 17 DEGs (5 down-regulated), 'Starch and sucrose metabolism, carbon fixation in photosynthetic organisms' with 17 DEGs (9 up-regulated), 'Carbon fixation in photosynthetic organisms (16 DEGs), 'Protein processing in endoplasmic' (14 DEGs), 'Plant-pathogen interaction' (13 DEGs), 'Photosynthesis-antenna proteins' (12 DEGs), and 'Plant hormone signal transduction' (7 DEGs).

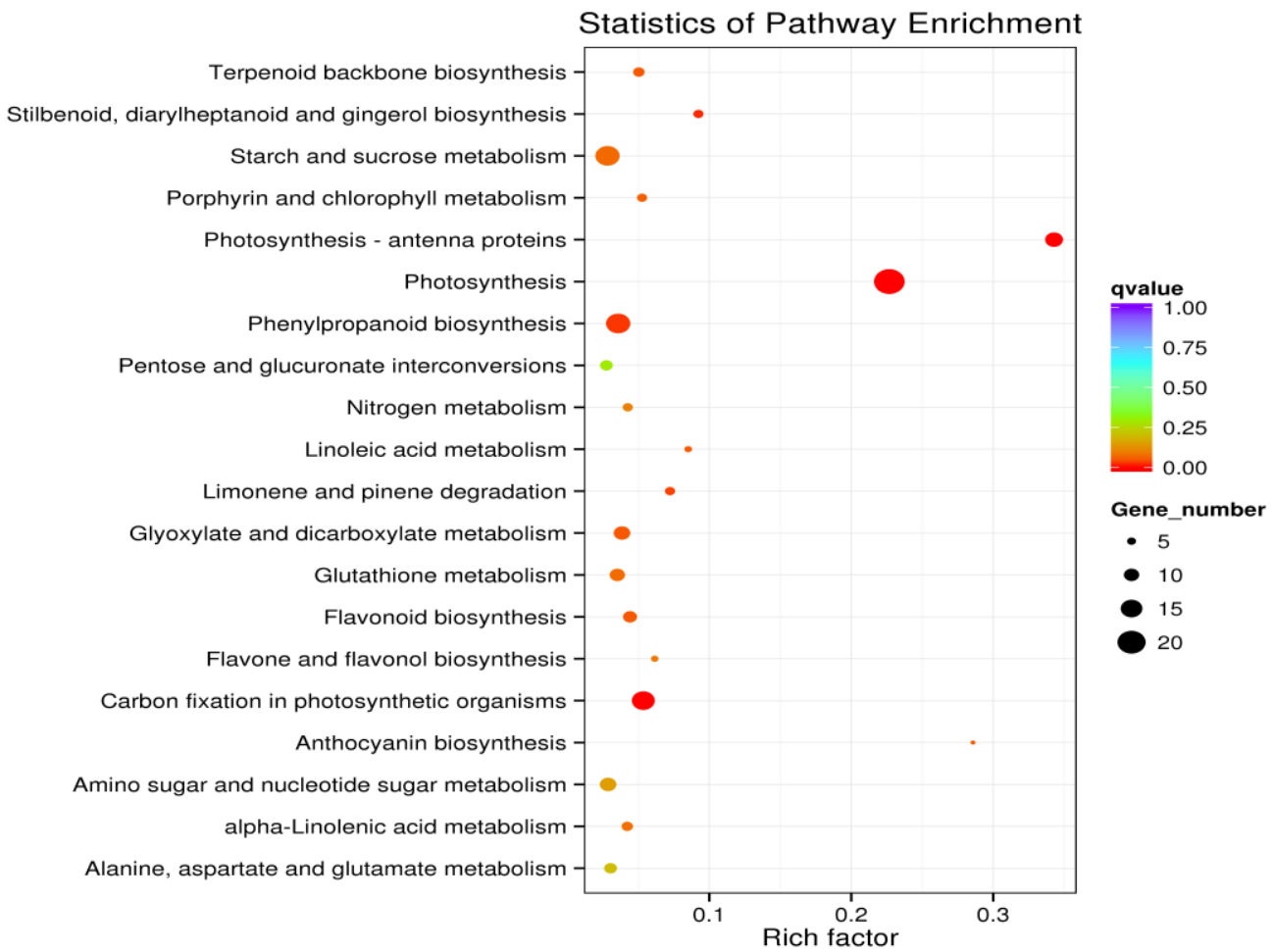

Fig. 7. Scatterplot of differential gene pathway enrichment. The abscissa indicates the path name and the ordinate indicates the Rich factor corresponding to the path. The color of the dot represents the q-value; the closer the color is to red, the smaller the q-value is. The size of the dot indicates the number of differential genes in a pathway.

The rooting of *Pinus massoniana* is related to many factors and the plant endogenous hormone is the most important factor. The differential gene enrichment in this metabolic pathway is shown in Fig. 8. The pathway of 'Plant hormone signal transduction' was involved in the regulation of endogenous hormones, including some genes related to IAA that are significantly associated with rooting such as *AUX/IAA*, auxin response factor (*ARF*), auxin responsive GH3 gene family (*GH3*), and small auxin up-regulated RNA (*SAUR*). There were 377 genes involved in the pathway including 7 differential genes. The up-regulated genes included ethylene-responsive transcription factor 1/2 (*ERF1/2*) and transcription factor *MYC2*, whereas *AUX/IAA*, two-component response regulator ARR-A family (*A-ARR*), and ethylene receptor (*ETR*) were down-regulated. The genes that were both up-regulated and down-regulated were Gibberellin insensitive dwarf 1 (*GID1*), protein phosphatase 2C (*PP2C*), and pathogenesis-related protein 1 (*PR-1*). This indicates that there are many genes involved in the expression in each metabolic pathway and the gene expressions in the metabolic pathways are interrelated; this indicates that the internal gene regulation of the rooting of *Pinus massoniana* is a very complex process.

Verification by RT-qPCR: The transcriptome sequencing results were verified using RT-qPCR (Fig. 9). The difference in gene expression is the same for real-time quantification and RNA-seq. Although the expression of some genes is slightly different, the same general trend is observed. Therefore, the transcriptome sequencing results are acceptable.

Discussion

Pinus massoniana as a tree species with important ecological and economic significance in southern China (Zhang *et al.*, 2006) and its asexual reproduction has important research value. However, it is difficult to root *P. massoniana* seedlings and requires a long time. Many internal factors and external factors affect the rooting behavior. In this study, transcriptome sequencing of the root and seedlings of *Pinus massoniana* L. was performed to evaluate the differential expression of the genes with regard to the rooting behavior.

The KEGG pathway enrichment analysis of the DEGs indicated physiological activities during which the gene expression of *Pinus massoniana* was active during rooting. 'Phenylpropanoid biosynthesis' and 'Plant-pathogen interaction' were found to be highly enriched in the KEGG pathway in the roots of *Taxodium*

'Zhongshansa' (Qi *et al*., 2014). Although the 'photosynthesis' pathway had more DEGs, more genes were down-regulated in the CT than in the CK.

During the growth and development of plants, auxin is a very important regulatory hormone (Zhao *et al*., 2010; Seo *et al*. 2016; Sorce *et al*., 2017). In the rooting induction process of plants, the application of exogenous auxin and the endogenous auxin content were 2 factors that played a decisive role (Chu *et al*., 2017). Auxin promotes the formation of adventitious roots in plants (Wiesman *et al*., 1989; Li *et al*., 2017a; Verstraeten et *al*., 2013). In our study, the genes that regulate the auxin levels were *AUX/IAA*, *ARF*, *CH3*, and *SAUR* (Fig. 8). Among them, the upstream and downstream of *ARF* was *AUX/IAA*, demonstrating its importance. This result was similar to the hormonal signal transduction pathway in the "Red Earth" grape (Mao *et al*., 2018).

When *Pinus massoniana* L. encountered adverse conditions such as drought, salt, low temperature, and abiotic stress treatment, the expression of *ARF* was stable (Chen *et al*., 2016). In addition, the *ARF* expressions of other plants have been shown to be active under abiotic stress conditions (Yoshida *et al*., 2015; McLoughlin *et al*., 2012). In this study, *AUX/IAA* showed a significant down-regulation in the differential expression of the roots of *Pinus massoniana*; this result is in agreement with the results reported for the rooting of *Triticum aestivum* (Chu *et al*., 2017). In a related study, *ARF* was combined with *AUX/IAA* protein and its function was suppressed when the plant's auxin content was low (Wang *et al*., 2014).

Some genes such as *IAA*, *GH3*, and *SAUR* were not significant in terms of the differential rooting and non-rooting expression of *Pinus massoniana*. However, they were considered the main response gene of auxin (Hagen *et al*., 2002). Some studies have reported that *SAUR* promoted the auxin-stimulated elongation in *Arabidopsis* (Chae *et al*., 2012). These genes were also found in the rooting expression of *Morus alba* L. (Du *et al*., 2016).

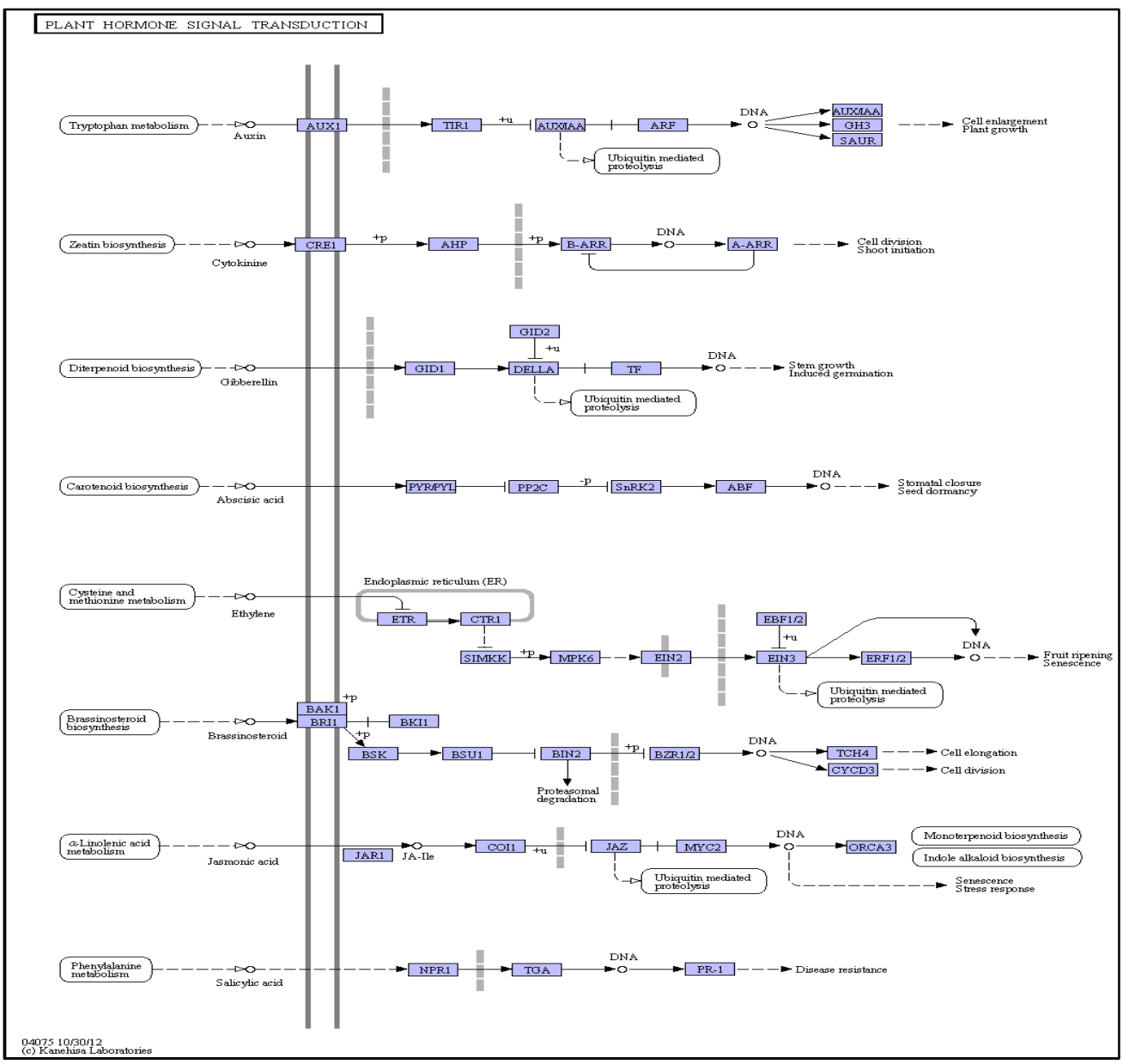

Fig. 8. DEG analysis of plant hormone signal transduction.

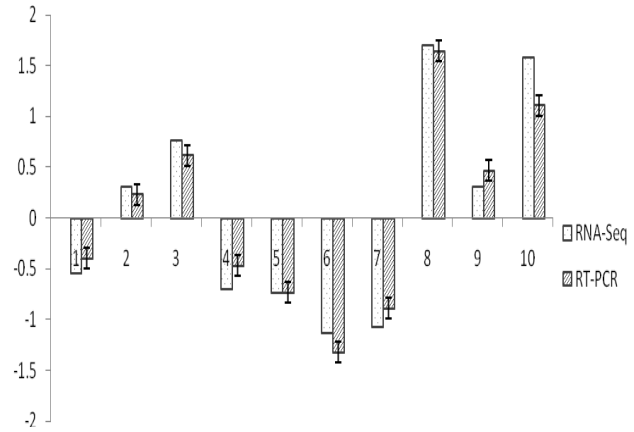

Fig. 9. Verification of the DEGs by RT-qPCR.

Conclusion

In this study, 781 DEGs were detected for the rooting and non-rooting behavior in *Pinus massoniana* seedlings using RNA-seq; 18 KEGG pathways were significantly enriched. The pathways that contained more DEGs were closely associated with the rooting of *Pinus massoniana*, such as 'Plant hormone signal transduction'. In the pathways, some genes control the rooting of *Pinus massoniana* seedlings by significant up- or down-regulation, such as *AUX/IAA* and *ARF*. The results of this study provide guidance for improving the rooting rate of pine cuttings.

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