

Comprehensive transcriptomic and metabolomic analyses of one-year-old and two-year-old *Polygonatum cyrtoneura* Hua

Liang Wu

Anqing Municipal Hospital

Yubin Qiu

Wenzhou Medical University

Yuting Fang

Anhui Provincial Hospital

Can Huang

Anqing Municipal Hospital

Dawei Song

Chizhou Jiuzhengshai Food Group Co., Ltd

Duohui Li

Anqing Municipal Hospital

Li Zhao

Wenzhou Medical University

Haiyang Zhao

Wenzhou University

Lamei Qi

Anqing Municipal Hospital

Lishang Dai

lishang2016@wmu.edu.cn

Wenzhou Medical University

Research Article

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Abstract

Polygonatum cyrtonema Hua is a traditional Chinese herbal medicine. It is rich in polysaccharides, flavonoids, alkaloids and other medicinal active ingredients, which plays an important role in maintaining human health. Because *P. cyrtonema* is a perennial plant, the cultivation time may affect the accumulation of functional active ingredients in the body. In purpose to explore the differences in the level of secondary metabolites, we used non-targeted metabolomics (LC-MS) technology to study the root tissues of 1-year-old and 2-year-old *P. cyrtonema*. Metabolomics results showed that the differential metabolites were enriched into 77 metabolic pathways, and the significantly enriched pathways were related to amino acid biosynthesis and carbohydrate metabolism. At the same time, phenylalanine, citric acid, cinnamic acid and other metabolites accumulated more in 2-year-old plants. Furthermore, transcriptome analysis showed that a total of 2068 differentially expressed genes (DEGs, 1026 up-regulated genes and 1042 down-regulated genes) were identified, and 102 metabolic pathways were enriched, among which glucose metabolism, phenylpropanoid biosynthesis and flavonoid biosynthesis were highly enriched metabolic pathways. These analysis results display that with the increase of cultivation time, the metabolic pathways of related active substances will be enhanced accordingly. Generally speaking, this study applied multi-omics techniques to show the differences in metabolites and gene levels of *P.* plants with different growth years, which will provide valuable clues for finding candidate genes involved in the biosynthesis of active substances.

1. Introduction

Huangjing is one of the Chinese medicines listed in the Chinese Pharmacopoeia, which is derived from the dried rhizome of *P. cyrtonema* Hua, *P. sibiricum* Red or *P. kingianum* Coll. et Hemsl, a plant of the liliaceae family [1]. The Huangjing, known as ‘immortal food’, has the effect of tonifying qi and blood, invigorate the spleen and moisten the lung, as well as benefiting the kidney in theory of traditional Chinese medicine [2]. These three kinds of medicinal plants of Polygonatum family are widely distributed and rich in resources in China, of which the *P. sibiricum* mostly grows in the northeast and north China, the *P. cyrtonema* Hua is primarily concentrated in Hunan, Zhejiang, and Anhui of the mountainous areas [3–5]. Huangjing has the characteristics of medicine and food homology. Modern pharmacological studies have found that polysaccharides, flavonoids, steroidal saponins and alkaloids are its main active ingredients, among which polysaccharides and flavonoids are hotly studied [6]. These components have been proved to have anti-tumor [7], anti-inflammatory [8], antibacterial [9], hypoglycemic [10, 11], immune regulation [12, 13] and other biofunctional effects. In addition, various kinds of Polygonatum drinks and dried fruits are sold in the market, and it can also be used as a delicious dish for people to enjoy. Generally speaking, Huangjing exhibits the same value as gold, and has a large market prospect and high research significance in the future because of its pretty medicinal and edible value.

P. cyrtonema Hua, is a perennial herb, born in a moist and shaded environment, because of its rhizome shape like ginger, it is also known as ‘ginger-type Polygonatum’ [14]. Different origins, growth ages and harvesting periods are important factors in the quality differences of *P. cyrtonema* [15]. Among them,

with the continuous growth and development of individuals, the accumulation of substances and the content of active ingredients of *P. cyrtonema* at different growth ages are more different. Some studies have found that the most important factor affecting the content of medicinal active ingredients in *P. cyrtonema* is the growth period, in which the polysaccharide content will gradually increase with the growth ages, and begin to decrease after a certain time [16]. Therefore, we took 1-year-old and 2-year-old *P. cyrtonema* as the research object to investigate the differences between them in terms of secondary metabolites and genes related to the synthesis pathway of medicinal components.

Transcriptome based on high-throughput sequencing technology can locate the molecular functions related to differentially expressed genes under different conditions, thus contributing to the mining of key genes [17, 18]. In purpose to explore the differences in gene expression of *P. cyrtonema* at different growth ages, we performed transcriptome analysis on its leaf tissues. Additionally, to further demonstrate that these differentially expressed genes may regulate the synthesis of relevant medicinal active ingredients, we also performed metabolomics analysis on their root tissues. Non-targeted metabolomics analyses use ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to identify all endogenous metabolites in an organism, and then screens for metabolites that are significantly different between groups to further investigate the metabolic pathways in which they are enriched [19]. In this study, we conducted the first joint transcriptomic and metabolomic analyses of *P. cyrtonema* of different ages, and assessed the key pathways and potential putative genes affecting the differences in active ingredients. This study will provide a reference for future research on the active components of other *polygonatum* plants with different growth periods.

2. Materials and methods

2.1 Plants source and disposal

Wild *P. cyrtonema* Hua was collected from a mountainous area in Chizhou City, Anhui Province, eastern China in June 2023. The plant types include 1-year-old and 2-year-old. Seedlings were retarded for 3 days in the laboratory of Wenzhou Medical University, and three seedlings with uniform growth were selected for sampling in 1-year-old (Pc1) and 2-year-old (Pc2) groups, respectively. Leaves and roots of 6 samples were collected and washed twice with deionized water, then washed with PBS buffer and dried. They were put into sealed bags and frozen in liquid nitrogen for 10 min, and then stored in a -80 °C refrigerator for subsequent RNA-sequencing and metabolomics analysis.

2.2 Transcriptome profiling

2.2.1 RNA collection, illumina sequencing and *de novo* assembly

RNA-sequencing of leaf tissue was performed by Shanghai Personal Biotechnology Cp. Ltd (Shanghai, China). Using the Trizol Reagent (Invitrogen Life Technologies) to extract plant total RNA, and its quality

detection is completed by a NanoDrop spectrophotometer (Thermo Scientific). Construction of a cDNA library for sequencing required three micrograms of RNA, and the library was generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Specific steps for cDNA library construction were carried out according to instruction. Finally, the libraries were quality controlled using an Agilent 2100 Bioanalyzer (Agilent, 2100) system and then using PE150 technology to paired-end sequencing on the Illumina Hi Seq platform. The sequencing platform comes with software to transform the image files generated by the sequencer to produce raw data in FASTQ format. After filtering, clean reads are obtained that can be spliced. *De novo* assembled is the splicing of clean reads using Trinity software, and the transcripts obtained were used as reference sequences for next analysis. Then the transcripts were clustered using sequence clustering software and the longest transcripts were selected to be defined as unigene.

2.2.2 Unigene functional annotation analysis and identification of DEGs

The assembled unigenes were compared with the common protein data pathway using BLAST, including non-redundant (NR) protein database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database, Swiss-Prot database, Gene Ontology (GO) database, Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggNOG), and Pfam database. From there, the functional classification and biological pathways of these genes were predicted. Transcriptome expression quantitative software RSEM was used to analyze the expression level of genes, and the expression abundance was expressed via FPKM value. Then, DESeq software was used to analyze the difference of gene expression. In this study, the threshold of absolute value of $\log_2$ Fold Change > 1 and $p < 0.05$ was design to filter observably differentially expressed genes. DEGs were then analysed for GO and KEGG enrichment.

2.3 Nontargeted metabonomic analysis

The root metabolites were analyzed by LC-MS non-targeted metabolomics analysis method, which was completed by Gene De novo Biotechnology Co., Ltd. Firstly, 100 mg of liquid nitrogen ground tissue sample was taken and put in an EP tube and adding 500 μ L of 80% methanol solution. After vortexing and shaking, the sample was allowed to stand in an ice bath for 5 min, and then centrifuged at 4 °C for 15,000 g, 20 min. A quantity of the supernatant was diluted with experimental grade pure water to a 53 percent methanol content. Subsequently, the supernatant was gathered for detection after centrifugation at 15,000 g for 20 min at 4 °C. Finally, the samples were analysed using a UHPLC-MS/MS system (UHPLC, Vanquish UHPLC; MS, Q Exactive™ HF-X). The raw data created via UHPLC-MS/MS was efficiently processed to obtain data results containing information such as mass-to-charge ratio and retention time. The results after quality control were compared with the mzCloud, mzVault and MassList databases, and finally the identification and quantification of each metabolite was obtained. Screening for differential metabolites between groups was based on VIP values from the OPLS-DA and P-values from the t-test, with difference thresholds of $VIP \geq 1$ and $P\text{-value} < 0.05$ [20].

2.4 Transcriptome validation by quantitative real-time PCR

Results of transcriptome sequencing of this study was verified via RT-qPCR. 11 DEGs were randomly selected from the database, and 18S was used as the housekeeping gene. The upstream and downstream primers were designed by Lasergene 7.1.0 software and synthesized by Shanghai Shenggong Bioengineering Co., Ltd. The primer sequence is displayed in Table S1. For the experimental of total RNA extraction, refer to the instructions of the Plant RNA Kit (Omega, USA). The RNA after quality control was used as a template for cDNA preparation using cDNA Synthesis kit (RansGen, China). RT-qPCR was next performed using the PerfectStart® Green qPCR SuperMix (RansGen, China) kit of the same brand, in which we quantified the cDNA at 200 ng/ul and used 1 ul, and the rest of the reagents were added according to the instructions. Finally, the programme was set up in the PCR instrument (ABI) according to the three-step method in the manual, and the annealing temperature was modified with reference to the primer T_m value. Using the 1-year-old sample as a control, we performed three biological replicates for each gene, and the expression level of candidate genes was computed by the $2^{-\Delta\Delta C_t}$ calculation method.

3. Results and discussion

3.1 Metabolomics profiling of root of different years

For elucidating the differences in root metabolites of *P. cyrtoneura* under different cultivation years, this study used UPLC-MS / MS method to conduct metabolomics research on the root tissue, and successfully identified differential metabolites related to active ingredients.

Firstly, all samples were clustered using hierarchical cluster. Pc1 and Pc2 are clustered into one category respectively, indicating a high degree of data similarity within the group (Supplementary Figure S1). After qualitative and quantitative analyses of the metabolites, 1842 metabolites were obtained totally in all the samples, which were mainly classified into carboxylic acids and derivatives, organooxygen compounds, prenol lipids, fatty acyls and flavonoids (Table S2).

Multivariate statistical methods PCA and OPLS-DA were used to cluster the metabolomics data in the two samples. PCA analysis reflects the overall metabolic differences between groups of samples and the degree of variation between samples in the group by unsupervised dimensionality reduction. We normalized the peak area of the two groups of metabolites and performed PCA analysis (Fig. 1A, B). The results revealed that the control and treatment groups showed good spatial segregation on PC1, that is, there were large differences in metabolite accumulation patterns between plants of different planting years. In addition, orthogonal partial least squares-discriminant analysis (OPLS-DA) also indicated the greatest difference between the two groups (Fig. 1C, D). The R²_Y and Q² values of the model are greater than 0.5, which also reflects the reliability of the model and its applicability in subsequent research.

Under the two detection modes, a total of 161 differential metabolites (DEMs) were screened by the difference threshold of $VIP \geq 1$ and $P < 0.05$, with 80 and 81 up-regulated and down-regulated metabolites, respectively (Fig. 2). These DEMs are divided into 10 categories, of which organic acids and

derivatives are the most, with 36, followed by organic oxygen compounds, lipids and lipid-like molecules and organoheterocyclic compounds, 16, 13 and 12, respectively. The remaining categories include phenylpropanoids and polyketides, nucleosides, nucleotides, and analogues, benzenoids, alkaloids and derivatives, and organic nitrogen compounds, which together accounted for 11% (Fig. 3). Through KEGG enrichment pathway analysis, the biological function pathways involved in DEMs were further determined. In this study, the DEMs were enriched in 77 metabolic pathways totally, among which alkaloid synthesis, galactose metabolism, ABC transporters and amino acid metabolism pathways were noticeably enriched (Fig. 4, Table S3).

Additionally, variable importance in projection (VIP) can indicate the level of importance of a variable, and metabolites with top VIP scores may play a key role in this study. Figure 5 illustrates the top 20 differential metabolites of VIP score, most of them are attributed to carboxylic acids and derivatives group including citric acid and arginine, DL-arginine, L-phenylalanine, pipecolic acid, L-tyrosine and valine, followed by indoles and derivatives category including DL-tryptophan and indole-3-acrylic acid. We also found cinnamic acid to be one of the important differential metabolites. These metabolites regulate specific metabolic pathways, such as citric acid, which is a key metabolite in glucose metabolism that regulates the citric acid cycle, phenylalanine is the initial metabolite of phenylpropanoid metabolism, and cinnamic acid is an indispensable precursor for flavonoid biosynthesis. Their accumulation in 2-year-old plants increased significantly, indicating that the related metabolic pathways regulated by these DEMs may also be significantly enhanced.

3.2 Transcriptome analysis between leaves of different years

Transcriptome sequencing was performed on one-year and two-year samples. The raw data were filtered to yield 41.17 and 37.85 million clean reads, respectively, which accounted for more than 90% of the sequenced reads (Table S4). After de novo assembly, a total of 164565 transcripts were obtained, and the representative sequence of the gene (unigene) were counted as 69,373. The GC content of these sequences was greater than 43.26% and Q30 was more than 92.64% (Table S5). This result shows that the sequencing results are of pretty quality and can be used for subsequent analysis. Functional annotations were performed on 69,373 unigenes, of which the NR database had the richest number of successful annotations with 33,151, accounting for 47.79% of the overall. The remaining five databases including GO, KEGG, Pfam, eggNOG, and Swissprot annotated to 12,381, 15,693, 19,417, 31,870, and 26,226 entries, respectively. Of course, 5453 unigenes were co-annotated in all databases (Table 1).

Table 1

The success rate of gene annotation Annotated in GO: The unigene number and annotation rate in the GO database. Annotated in KEGG: The unigene number and annotation rate in the KEGG database. Annotated in eggNOG: The unigene number and annotation rate in the eggNOG database. Annotated in PFAM: The unigene number and annotation rate in the PFAM database. Annotated in NR: The unigene number and annotation rate in the NR database. Annotated in SwissProt: The unigene number and annotation rate in the SwissPort database. In all database: The unigene number annotated in all databases.

	Number of genes	Percentage (%)
Annotated in GO	12,381	17.85
Annotated in KEGG	15,693	22.62
Annotated in eggNOG	31,870	45.94
Annotated in PFAM	19,417	27.99
Annotated in NR	33,151	47.79
Annotated in SwissProt	26,226	37.80
In all database	5,453	7.86

Figure 6 allows assessment of global gene expression between the two groups, with a threshold of $|\log_2FC| \geq 1$ and $P\text{-value} < 0.05$, 2068 differential genes were totally identified. The number of genes with increased and decreased expression levels was similar, 1026 and 1042, respectively. Next, we used the R package Pheatmap to cluster these DEGs (Fig. 7). The horizontal axis was classified by the expression level of the equal gene in different samples, and vertical classification was based on the expression patterns of different genes in the same sample. The results showed that the expression levels of these genes in the two samples are indeed quite different and that they were grouped into multiple categories, with genes in the same category having similar biological functions.

To determine the major biological functions exercised by these DEGs, they were analysed for GO and KEGG enrichment. First, Fig. 8A shows the top 10 terms with the most significant enrichment in each GO category. We found that among the cellular component categories, 'chloroplast thylakoid membrane', 'plastid thylakoid membrane', and 'thylakoid membrane' were the more enriched terms for DEGs. In the molecular functions, the most abundant items were 'oxidoreductase activity', 'lyase activity' and 'catalytic activity'. In biological processes, DEGs are mainly concentrated in 'carbohydrate metabolic process', 'oxidation-reduction process' and 'small molecule metabolic process' terms. The results of GO analysis showed that photosynthesis, enzyme catalytic activity and metabolic process played important role in the difference of plants.

Meanwhile, KEGG enrichment analysis of all DEGs showed that these DEGs were successfully assigned to 102 pathways (Fig. 8B, Table S6). Among them, glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism, pyruvate metabolism and glycolysis / gluconeogenesis metabolism pathways were

significantly enriched for DEGs, and these pathways are the important branches constituting the process of sugar metabolism. We also found that phenylpropanoid biosynthesis pathway is also one of the significantly enriched pathways, which is the initial stage of flavonoid biosynthesis. These results indicate a correlation between DEGs related to biosynthesis of sugars, flavonoids, and other active ingredients in *P. cyrtonema* and the growth years, which is consistent with the results of metabolomic analyses.

3.3 RT-qPCR validation

In purpose to verify the accuracy of sequencing data, eleven of all EDGs were stochastically chose for RT-qPCR experiments. Figure 9 indicates that the expression level of these DEGs verified by RT-qPCR was not in perfect agreement with the RNA-seq results, but the expression trend was consistent for all genes. Of these, six genes were co-upregulated and five genes were co-downregulated. This indicates that the transcriptome data in this study are relatively credible and can be used for further analysis.

3.4 Combined analysis identifies important metabolic pathways

Integration of metabolomic and transcriptomic analyses suggests that there may be large differences in flavonoid accumulation between different years of *P. cyrtonema* (Fig. 5, 8). The differential metabolite phenylalanine, the initiator of the phenylpropane biosynthesis pathway, generates trans-cinnamic acid by the action of phenylalanine deaminase, which is the first reaction of the phenylpropane metabolic pathway [21]. The level of phenylalanine and cinnamic acid influences the production of several downstream secondary metabolites, and the results show that they accumulate significantly more in biennial plants. Whereas one of the metabolites of phenylpropanoid is flavonoids, and flavonoid biosynthesis initiates phenylpropane public metabolic pathway [22], the results by transcriptome indicate that DEGs show enrichment in phenylpropanoid biosynthesis and flavonoid biosynthesis pathways. These results suggest that significant differences between annual and biennial plants are reflected in the medicinal constituents, especially flavonoid synthesis, and that biennial *P. cyrtonema* possesses a greater abundance of upstream metabolites for flavonoid synthesis.

5. Conclusion

We used a multi-omics analysis to identify differences between 1-year-old and 2-year-old *P. cyrtonema* at the genetic level and major secondary metabolites in the present study. In metabolomics, citric acid, phenylalanine, cinnamic acid, and other secondary metabolites accumulated significantly more in the 2-year-old *P. cyrtonema*, and their involvement in sugar metabolism and phenylpropane metabolism pathways may be the key pathways. At the same time, DEGs identified in transcriptomics were also significantly enriched in phenylpropanoid biosynthesis, flavonoid biosynthesis and other glucose metabolic pathways, which were similar to the results of metabolomics. These related pathways are key pathways for the synthesis of medicinal components of *P. cyrtonema*, and with these results we found that the differences between annual and biennial plants were significantly reflected in the accumulation

of medicinal components. It is the first time to research the transcriptomics and metabolomics of *P. cyrtonema* in different years. This study will provide us with some new insights on the germplasm resources of *P. cyrtonema*, and lay the foundation for later researchers to identify candidate genes.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of Interests

The authors have declared that no competing interests exist.

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Authors' contributions

Wu and Qiu wrote the main manuscript text, Fang and Huang performed the research, Song and Li analyzed the data, Wu and Zhao contributed reagents/materials/analysis tools. Dai and Qi wrote the paper with other authors.

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Figures

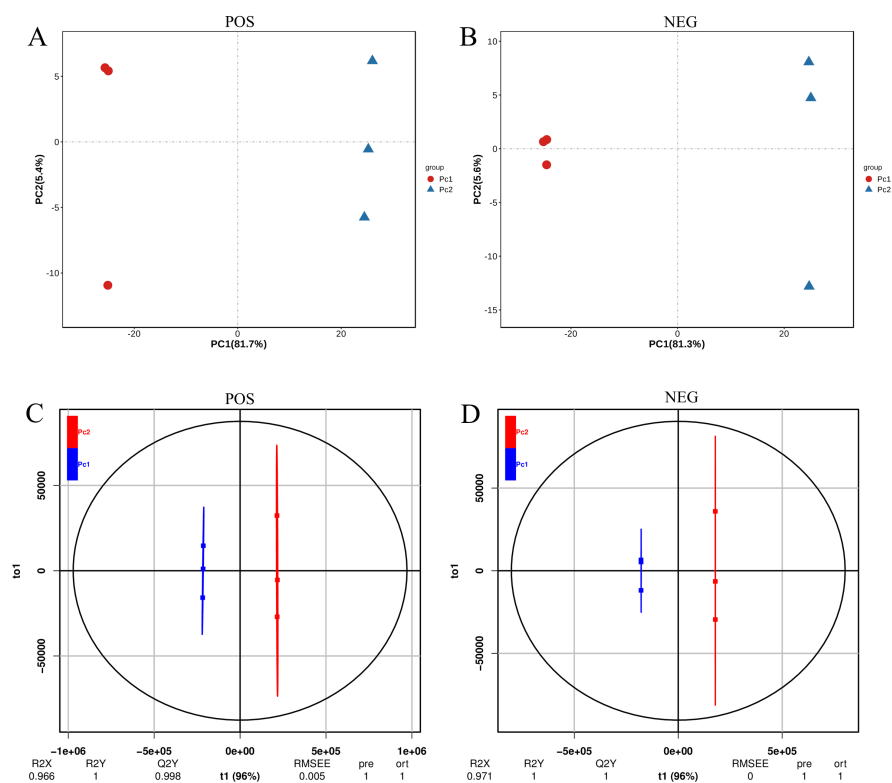


Figure 1

Multivariate statistical analysis of metabolite data. **(A, B)** The scores plot of principal component analysis (PCA) in positive and negative ion modes. **(C, D)** Orthogonal partial least squares discriminant analysis (OPLS-DA) in two modes. Note: Dots of the same color indicate different samples of the same treatment.

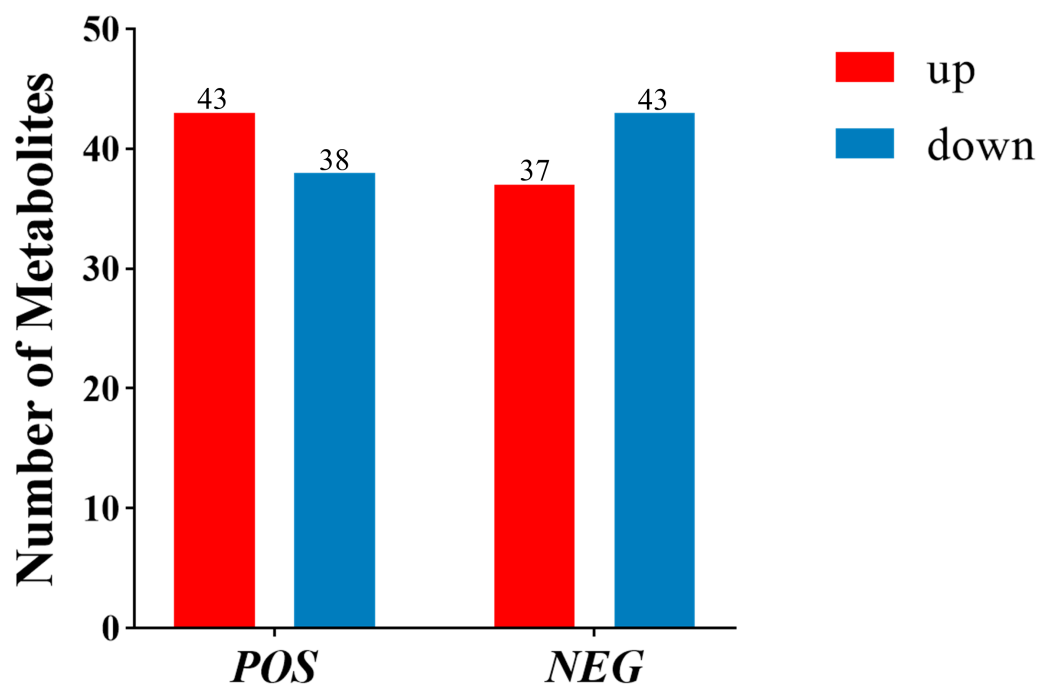


Figure 2

Statistics of differentially expressed metabolites identified in positive ion and negative ion modes. Red color indicates up-regulation and blue color represents down-regulation.

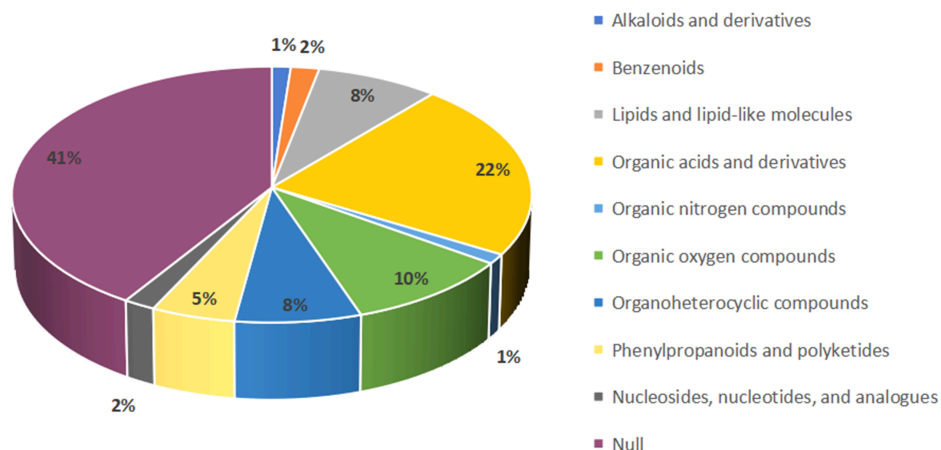


Figure 3

Primary classification of all differentially expressed metabolites (DEMs).

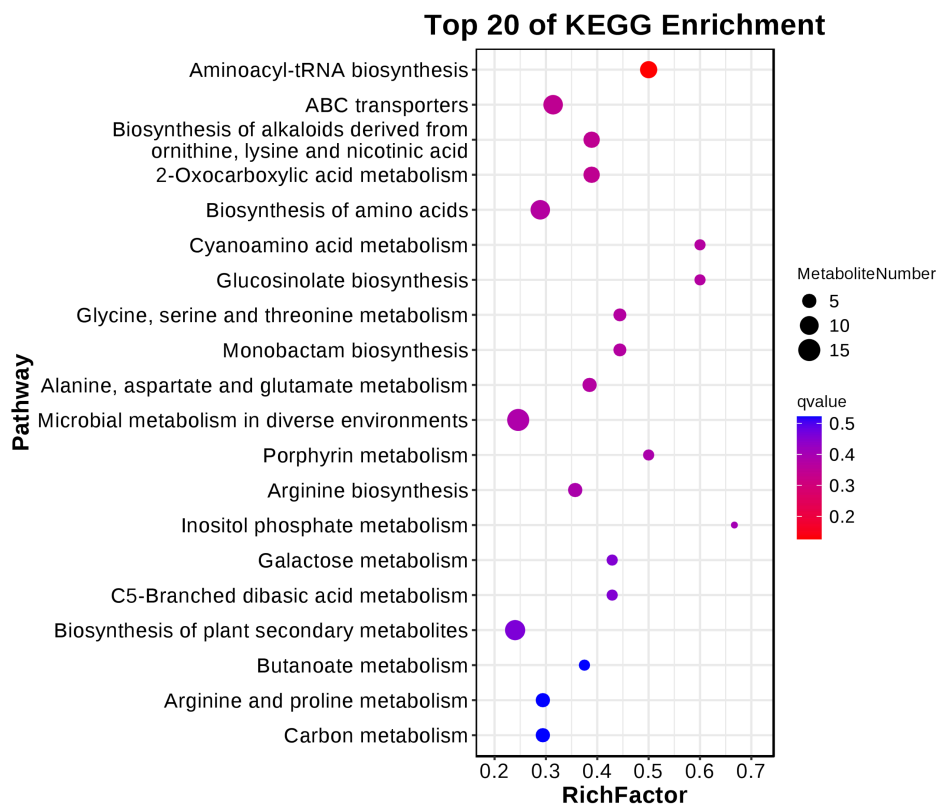


Figure 4

KEGG enrichment pathway of differentially expressed metabolites (DEMs) in *P. cyrtonema*.

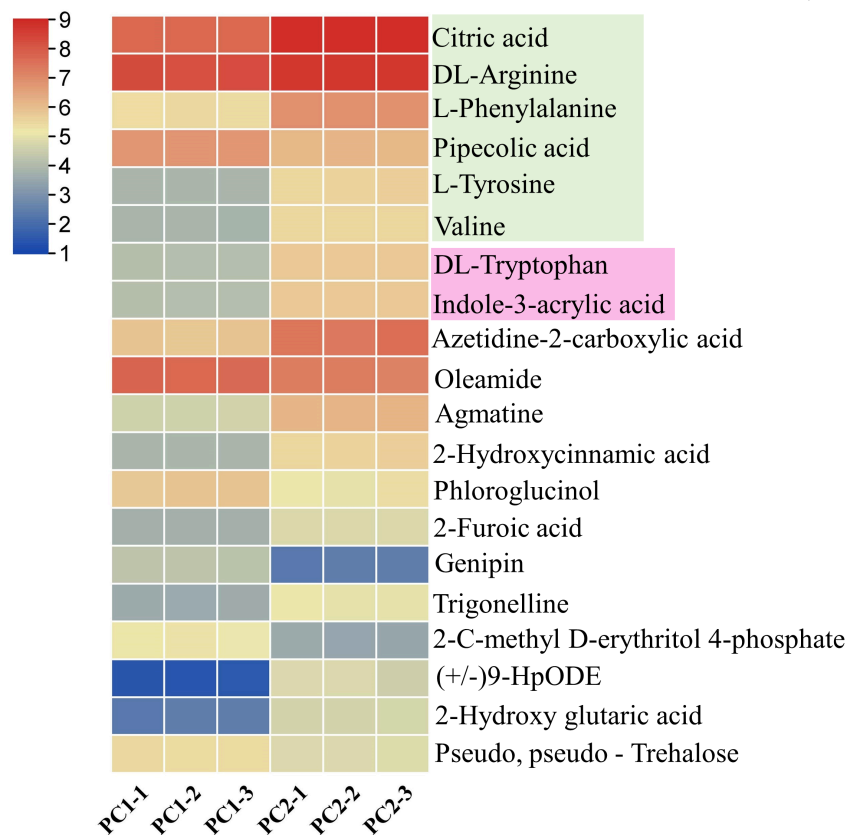


Figure 5

Accumulation changes of the top 20 differential metabolites of variable importance in projection (VIP).

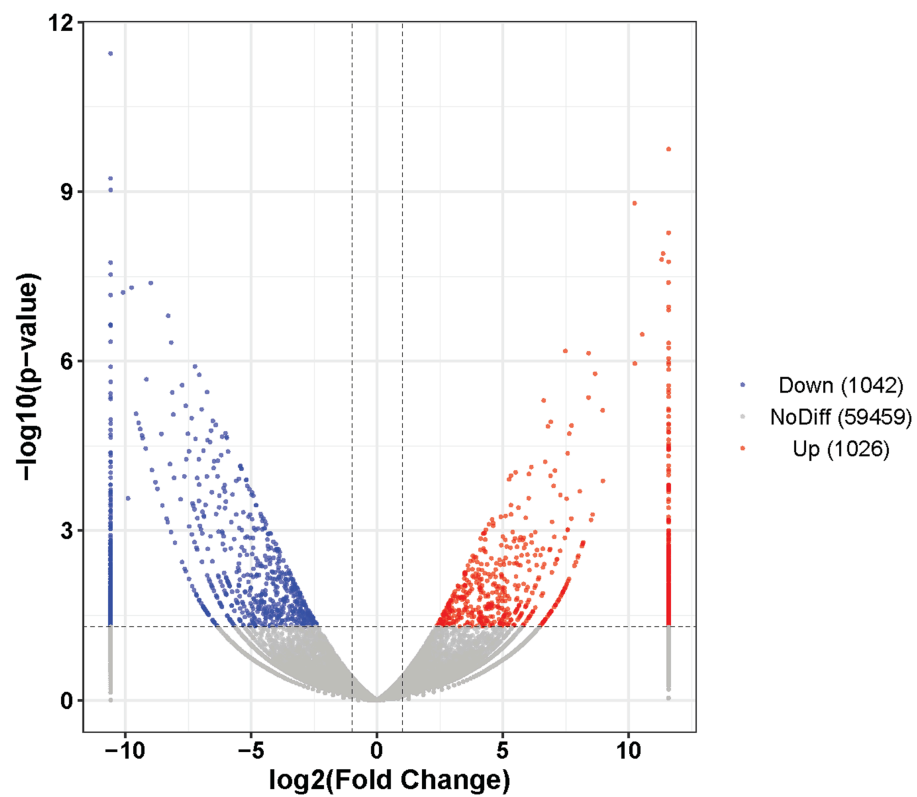


Figure 6

The differentially expressed genes (DEGs) were plotted for *P. cyrtonema*.

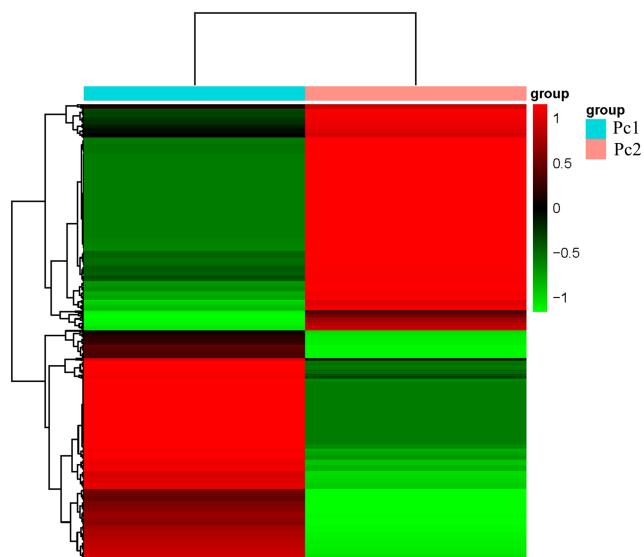


Figure 7

The heat map generated by cluster analysis of DEG.

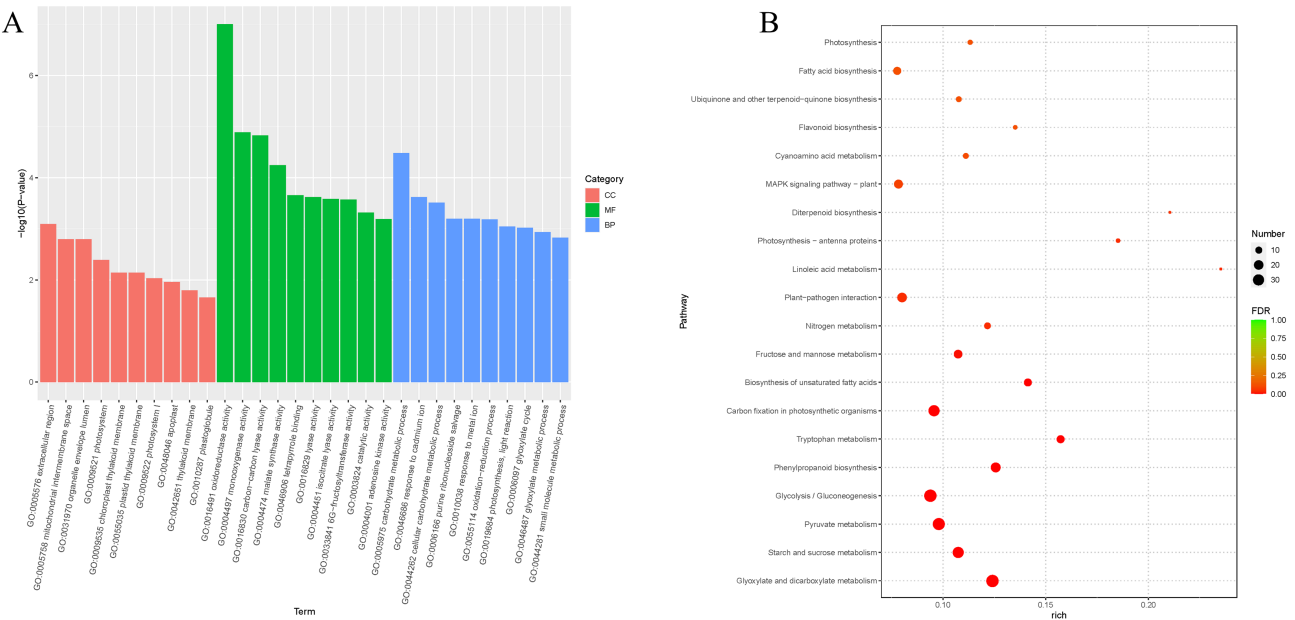


Figure 8

Analysis of differentially expressed genes (DEGs) between 1-year-old and 2-year-old plants. **(A)** GO enrichment analysis of DEGs. **(B)** KEGG pathway analysis of DEGs.

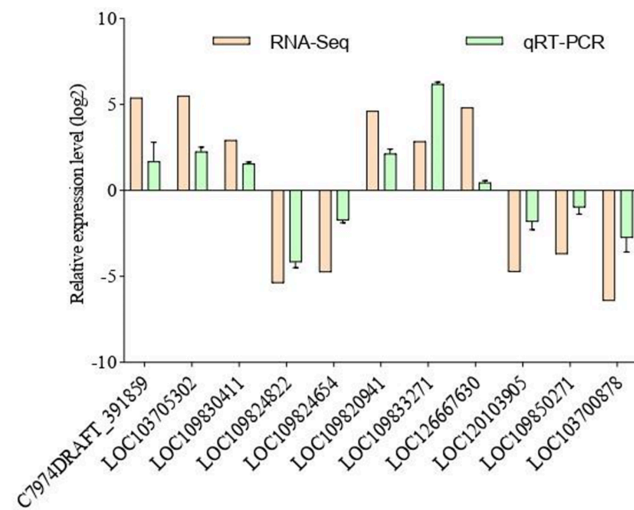


Figure 9

Comparison of RNA-seq and qRT-PCR results of 11 genes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable.docx](#)
- [SupplementaryFigure.docx](#)