Transcriptomic Analysis of *Polygonatum sibiricum* in Response to Continuous Cropping Obstacles

Zhufang WU, Yong CHEN*

College of Agriculture and Biotechnology, Hunan University of Humanities, Science and Technology, Loudi 417000, China

Abstract [**Objectives**] This study was conducted to comprehensively understand the changes in gene expression of plants under environmental stress during different growth and development stages. [**Methods**] The effects of continuous cropping on the roots and leaves of *Polygonatum sibiricum* were investigated using transcriptome sequencing. Normally-grown first crop *P. sibiricum* was used as the control group, while continuous cropping plants served as the treatment group. Transcriptomic differences in roots and leaves under different conditions were compared. [**Results**] The leaf materials of first crop and continuous cropping *P. sibiricum* (CCLZ vs FCLZ) showed 21 916 differentially expressed genes (DEGs), while the root materials of first crop and continuous cropping *P. sibiricum* (CCRZ vs FCRZ) exhibited 12 726 DEGs (the lowest DEG count) (12 726). Among them, 1 896 DEGs were common. GO enrichment analysis revealed that DEGs were mainly enriched in metabolism, cell wall degradation, and pathogen defense. KEGG enrichment analysis indicated that DEGs in CCLZ vs FCLZ and CCRZ vs FCRZ primarily affected hormone signal transduction and pathogen interaction pathways. [**Conclusions**] This study preliminarily elucidate the regulatory mechanisms in the roots and leaves of continuous cropping *P. sibiricum* at the molecular level, providing reference for research on its adaptation to continuous cropping.

Key words *Polygonatum sibiricum*; Continuous cropping obstacle; Transcriptome; Differentially expressed gene; Functional analysis

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Polygonatum sibiricum is a perennial herbaceous medicinal plant from the Liliaceae family, commonly used in traditional Chinese medicine and is one of the most widely used medicinal materials in China[1]. However, *P. sibiricum* is a crop that suffers from continuous cropping obstacles. Like other medicinal plants such as Solomon's seal, Rehmannia, Ginseng, and Notoginseng, it exhibits significant continuous cropping problems. Especially in recent years, large-scale, continuous annual planting has exacerbated the issue, becoming the greatest threat to the industrial development of *P. sibiricum*[2]. Previous studies have shown that continuous cropping obstacles lead to an increase in the content of reactive oxygen species (ROS) in the roots and leaves of the crop, which in turn affects its antioxidant capacity[3]. In addition, continuous cropping also affects the synthesis and accumulation of important bioactive compounds in the roots and leaves, such as polysaccharides, alkaloids, and volatile oils. Therefore, investigating the effects of continuous cropping obstacles on the growth of *P. sibiricum* is of significant importance.

This study utilized high-throughput sequencing technology[5], using the first crop of *P. sibiricum* as the control and the continuous cropping *P. sibiricum* as the treatment group, to conduct transcriptomic sequencing of the roots and leaves of *P. sibiricum*. The aim was to explore the molecular mechanisms through which continuous cropping affects the growth of *P. sibiricum*, providing scientific evidence to address the issue of continuous cropping obstacles.

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Zhufang WU(1995 -), female, P. R. China, master, devoted to research about plant protection.

* Corresponding author.

Materials and Methods

Materials and experimental design

The experiment was conducted at the on-campus base of Hunan University of Humanities, Science and Technology, with two treatments: first crop (FC) and continuous cropping (CC). The treatments were randomly arranged with three replicates, each plot covering 50 m^2. In September 2021, *P. sibiricum* was planted. The soils for both treatments were sourced from the under-canopy *P. sibiricum* base of Yipuyuan Polygonatum Technology Co., Ltd. in Xinhua County. The soil for the continuous cropping treatment was from the plot where *P. sibiricum* had been planted in 2017, while the soil for the first crop treatment was from a plot that had never been planted with *P. sibiricum*. The variety planted was the local "*P. sibiricum*" from Xinhua County, Hunan Province, provided by Yipuyuan Polygonatum Technology Co., Ltd. Both treatments were managed using consistent field cultivation practices.

Test materials

During the tuber expansion stage (June 27, 2022), roots and leaves from both the first crop and continuous cropping *P. sibiricum* were collected. Four sample groups were obtained: continuous cropping *P. sibiricum* roots (CCRZ), continuous cropping *P. sibiricum* leaves (CCLZ), first crop *P. sibiricum* roots (FCRZ), and first crop *P. sibiricum* leaves (FCLZ). Each group had three biological replicates, with three plants showing consistent growth selected for each replicate. After sampling, all samples were immediately frozen in liquid nitrogen and stored at -80 ℃ for later use.

RNA extraction, RNA-seq library construction, and sequencing

The total RNA extraction, RNA-seq library construction, and sequencing of the roots and leaves of *P. sibiricum* were outsourced to Beijing Novogene Bioinformatics Technology Co., Ltd. Total RNA was extracted using the TianGen Polysaccharide Polyphenol Kit (QIAGEN, Germany). After removing ribosomal RNA, the obtained mRNA was randomly fragmented using divalent cations in NEB Fragmentation Buffer. Library construction was performed according to the standard NEB protocol. After library quality checks, sequencing was conducted using the Illumina NovaSeq 6000 platform (Illumina, USA) for paired-end sequencing.

Sequencing data processing and bioinformatics analysis

The raw sequencing data were filtered, sequencing error rates were checked, and GC content distribution was assessed to obtain clean reads. The reads were assembled using Trinity, followed by hierarchical clustering with Corset to obtain genes for subsequent analysis.

Functional annotation of genes was carried out using the Nr, Nt, Pfam, KOG/COG, Swiss-Prot, KEGG, and GO databases to obtain functional information for the genes.

The transcriptome assembled by Trinity was used as the reference sequence. RSEM software was employed to align the clean reads of each sample with the reference sequence to analyze gene expression levels. Differentially expressed genes (DEGs) were identified using DESeq2 ($|\log2(\text{FoldChange})| > 1$ & P-value < 0.05). GO enrichment analysis of DEGs was performed using GOseq, and KEGG pathway enrichment analysis of DEGs was conducted using KOBAS (2.0).

Data statistics and analysis

Data were statistically analyzed using Excel and SPSS 16.0 software.

Results and Analysis

Sequencing data and functional annotation

The raw data obtained from Illumina NovaSeq 6000 sequencing were filtered to remove low-quality sequences, resulting in 356 775 590 clean reads (98.10%). After assembly, 868 824 transcript sequences and 400 854 Unigenes were obtained.

The functional annotation results showed that 142 008 Unigenes (35.42%) were successfully annotated in the NR database (Table 1). The number of Unigenes annotated in the GO and KO databases were 99 631 (24.85%) and 45 655 (11.38%), respectively (Table 1). As shown in Fig. 1, the 99 631 Unigenes annotated in the GO database participated in 43 GO functional categories, including molecular function, biological processes, and cellular components. The 45 655 Unigenes successfully annotated in the KO database were involved in 34 KEGG pathways, including 301 metabolic pathways (Fig. 2).

Differential expression gene analysis of continuous cropping *P. sibiricum*

Using $|\log2(\text{FoldChange})| > 1$ and P-value < 0.05 as the criteria, DESeq2 was used to screen for differentially expressed genes. Compared to the first crop of *P. sibiricum*, there were 21 916 differentially expressed genes in the leaves of continuous cropping *P. sibiricum*, of which 11 145 were upregulated and 10 771 were downregulated (Fig. 3). In the roots of continuous cropping *P. sibiricum*, there were 12 726 differentially expressed genes, with 6 493 upregulated and 6 233 downregulated (Fig. 3). There were 3 937 commonly differentially expressed genes in both the roots and leaves, with 1 742 upregulated and 1 896 downregulated (Fig. 4).

Table 1 *P. sibiricum* Unigenes annotation statistics

Annotation databases	Number of Unigenes	Percentage//%
Annotated in NR	142 008	35.42
Annotated in NT	76 180	19.00
Annotated in KO	45 655	11.38
Annotated in SwissProt	91 264	22.76
Annotated in PFAM	99 641	24.85
Annotated in GO	99 631	24.85
Annotated in KOG	34 412	8.58
Annotated in all Databases	14 954	3.73
Annotated in at least one Database	184 986	46.14
Total Unigenes	400 854	100.00

GO functional enrichment of differentially expressed genes in continuous cropping *P. sibiricum*

GO functional enrichment analysis revealed that 3 527 differentially expressed genes in the roots of continuous cropping *P. sibiricum* were enriched in 134 GO functional categories. Significance enrichment analysis of the annotated GO functional categories for differentially expressed genes was performed with a threshold of Padj < 0.05. The results showed that these genes were significantly enriched in three GO functional categories, including cellular components such as "cell" (GO: 0005623) and "intracellular" (GO: 0005622), as well as molecular functions such as "DNA-binding transcription factor activity" (GO: 0003700) (Fig. 5). In the leaves of *P. sibiricum*, 6 452 differentially expressed genes were enriched in 134 GO functional categories, significantly enriched in three GO functional categories, including molecular functions such as "hydrolase activity, acting on glycosyl bonds" (GO:0016798), "DNA-binding transcription factor activity" (GO: 0003700), "ion binding" (GO: 0043167), and "transferase activity, transferring glycosyl groups" (GO: 0016757) (Fig. 6). The results indicate that continuous cropping may affect the differential expression of genes encoding DNA-binding transcription factors, glycoside hydrolases, glycosyltransferases, and ion-binding proteins in *P. sibiricum*.

KEGG pathway enrichment of differentially expressed genes in continuously-cropped *P. sibiricum*

KEGG annotation of differentially expressed genes showed that 1 317 differentially expressed genes were enriched in 356 metabolic pathways in *P. sibiricum* root and 2 583 differentially expressed genes were enriched in 376 metabolic pathways in *P. sibiricum* leaves. As shown in Fig. 7, KEGG significance enrichment analysis under the condition of Padj < 0.05 found that the differentially expressed genes of CCRZvsFCRZ were significantly

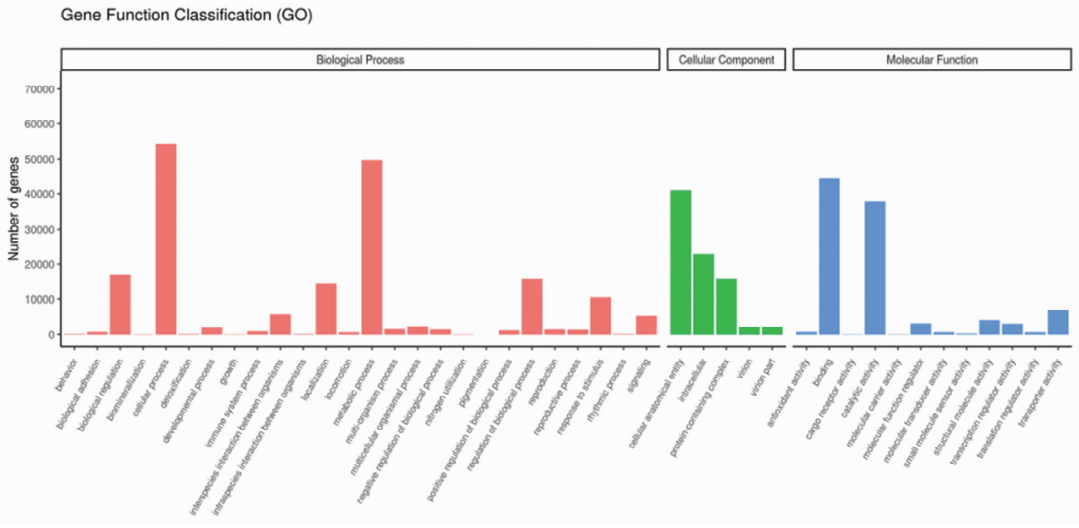
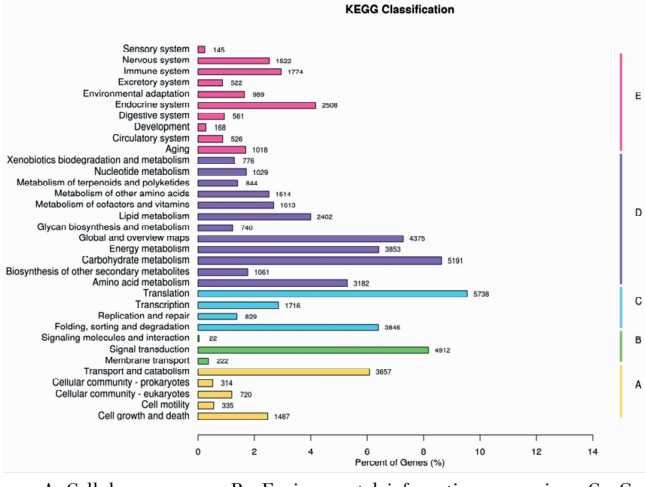


Fig. 1 GO functional annotation of *P. sibiricum* Unigenes

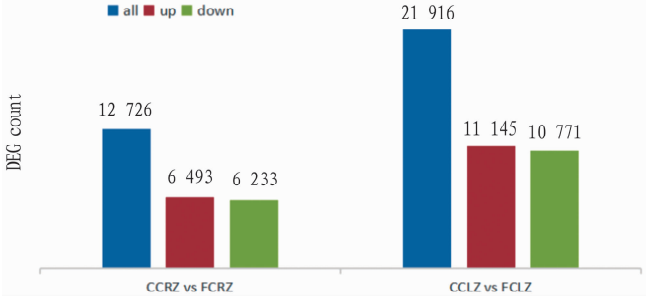


A. Cellular processes; B. Environmental information processing; C. Genetic information processing; D. Metabolism; E. Organismal systems.

Fig. 2 KEGG functional annotation of *P. sibiricum* Unigenes

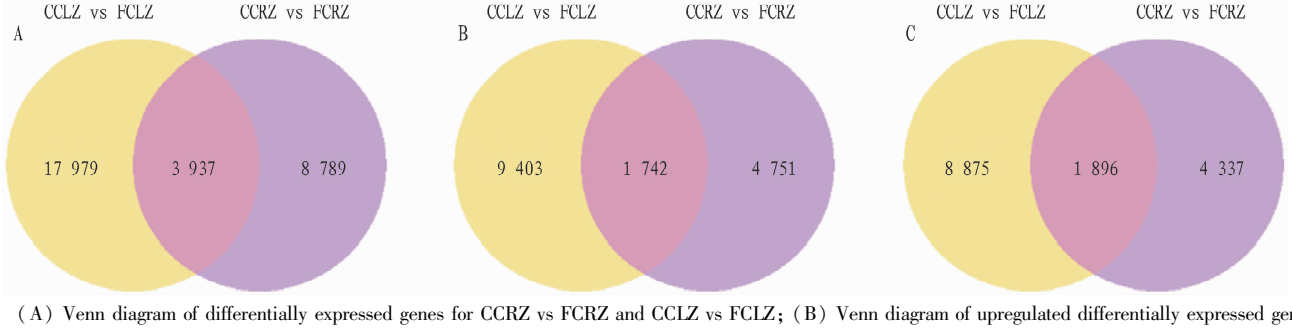
enriched in plant hormone signal transduction, stilbene, diarylheptanoid and gingerol biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, NF-kappa B signaling pathway metabolic pathways such as, Zeatin biosynthesis, plant pathogen interaction, *etc.* The differentially expressed genes of cclzvsfclz were significantly enriched in plant pathogen interaction, plant hormone signal transduction, MAPK signaling pathway-plant, stilbene, diarylheptanoid and gingerol biosynthesis, alpha linolenic acid metabolism, phenylpropanoid biosynthesis, and flavonoid biosynthesis. Biosynthesis, monoterpenoid biosynthesis and other metabolic pathways.

The results showed that continuous cropping significantly affected the signal transduction in the roots and leaves of *P. sibiricum*, the interaction between plants and protoplasm, and the biosynthesis of secondary metabolites, resulting in abnormal growth and development of *P. sibiricum* and continuous cropping obstacles.



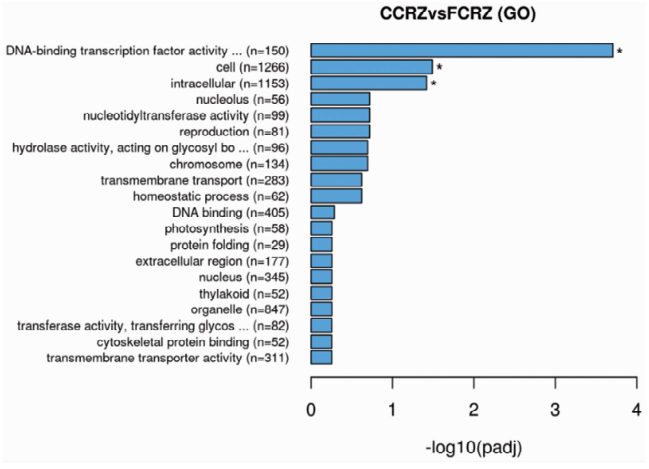
CCRZ, CCLZ, FCRZ, FCLZ represent continuous cropping *P. sibiricum* roots, continuous cropping *P. sibiricum* leaves, first crop *P. sibiricum* roots, and first crop *P. sibiricum* leaves, respectively. The same applies hereafter.

Fig. 3 Differential expression gene statistics for the roots and leaves of *P. sibiricum*



(A) Venn diagram of differentially expressed genes for CCRZ vs FCRZ and CCLZ vs FCLZ; (B) Venn diagram of upregulated differentially expressed genes for CCRZ vs FCRZ and CCLZ vs FCLZ; (C) Venn diagram of downregulated differentially expressed genes for CCRZ vs FCRZ and CCLZ vs FCLZ.

Fig. 4 Venn diagram of differentially expressed genes in the roots and leaves of *P. sibiricum*



∗ indicates significant differences ($P<0.05$). The same applies hereafter.

Fig. 5 GO functional enrichment of differentially expressed genes in the roots of *P. sibiricum*

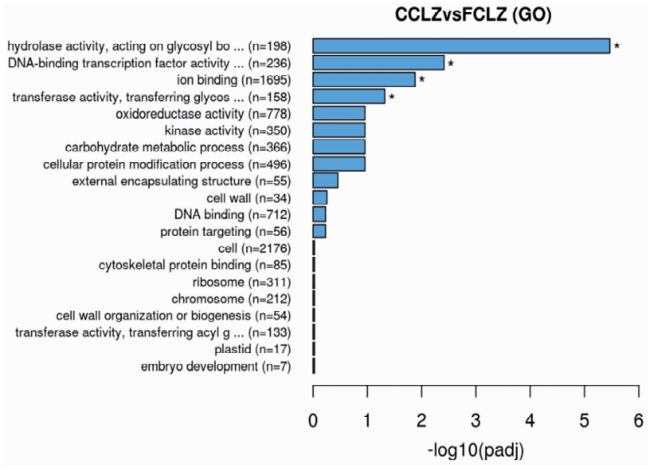
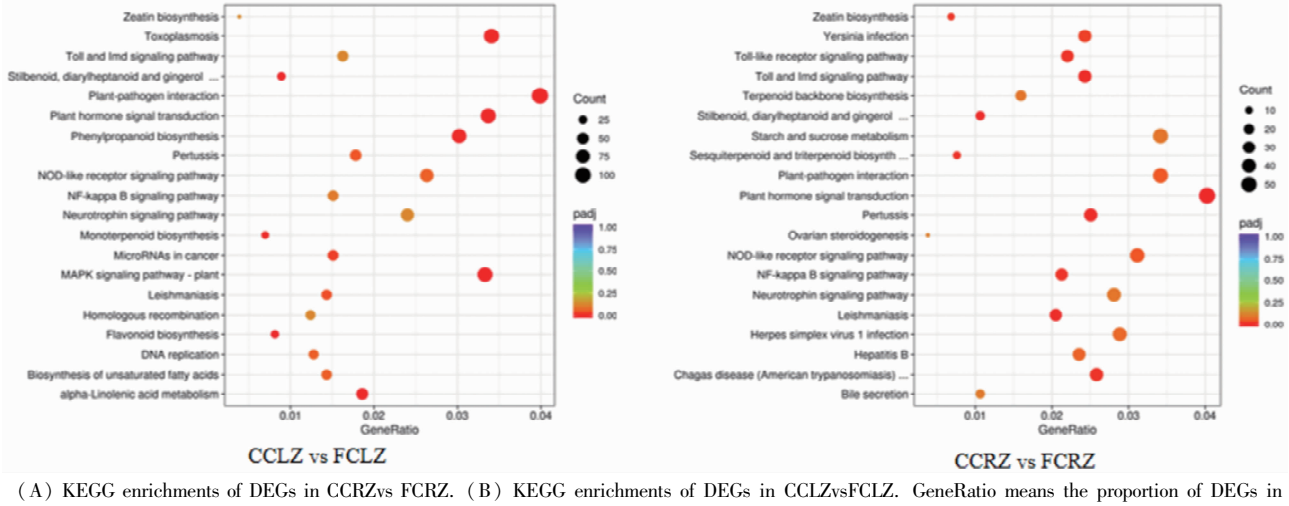


Fig. 6 GO functional enrichment of differentially expressed genes in the leaves of *P. sibiricum*



(A) KEGG enrichments of DEGs in CCRZvs FCRZ. (B) KEGG enrichments of DEGs in CCLZvsFCLZ. GeneRatio means the proportion of DEGs in the functional annotation. Padj means the corrected *P*-value. Count represents the number of genes.

Fig. 7 KEGG enrichments of differentially expressed genes in roots and leaves of *P. sibiricum*

Discussion

Under the condition of continuous cropping obstacle, the root and leaf organs of *P. sibiricum* showed significant characteristics of gene expression profile reconstruction. Previous studies have shown that continuous cropping disorder can trigger cascade responses of multiple physiological metabolic pathways in plants, and then drive the dynamic regulation of gene expression network[6-7]. Transcriptome sequencing showed that 12 726 and 21 916 differentially expressed genes were detected in the root and leaf tissues of *P. sibiricum*.

It is worth noting that go function enrichment analysis revealed that continuous cropping stress mainly affected GO:0016798 (hydrolase activity), GO:0003700 (DNA binding transcription factor activity), and GO:0043167 (ion binding) GO:0016757 (glycosyltransferase activity) and other key functional modules. From the perspective of organ specific response, the differentially expressed genes in the roots of *P. sibiricum* are mainly involved in the regulation of antioxidant defense system (such as SOD, pod and other enzyme coding genes) and signal transduction pathways (such as ABA, NF kappa B related signal elements), while the differentially expressed genes in the leaves are significantly enriched in the photosystem related pathways (psbA, psbd and other photosystem II components) and carbohydrate metabolism processes (such as amylase, sucrose synthase coding genes). Further analysis showed that differentially expressed genes in leaves were involved in cell wall remodeling, pathogen defense and other biological processes by regulating hydrolase activity (especially glycosidic bond hydrolase) and transcription factor activity. These transcription factors can play a central role in cell differentiation, development and stress signal response by specifically recognizing DNA cis acting elements (such as promoter or enhancer region).

At the molecular mechanism level, root differential genes are mainly involved in DNA binding transcription factor network, dynamic integration of cytoskeleton and glycosidic bond metabolic pathway. These genes decompose starch into soluble sugars such as

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which makes it difficult for relevant staff to choose the detection indicators for nutritional and hygienic quality of vegetables. At present, there is little research on the index selection and method evaluation of vegetable nutrition and health quality in China, and there is a lack of a comprehensive, systematic and standard evaluation system. Therefore, it is urgent to carry out systematic research on the selection of indicators and evaluation methods for the nutritional and hygienic quality of vegetables, so as to ensure the nutrition, safety and hygiene of vegetables eaten by people and provide a theoretical basis for the substantial improvement of nutritional and hygienic quality of vegetables and the scientific and rational development and utilization of vegetable resources. In the future, the research work should be carried out in two aspects, of which the first is to formulate a comprehensive and scientific evaluation system, which will give a reasonable choice basis for vegetable nutrition and health quality indicators, and the second is to evaluate the nutritional and hygienic quality of different vegetable species and varieties, which will provide theoretical guidance for the evaluation of vegetable nutrition and safety.

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maltose and glucose by regulating amylase activity, and then participate in cell cycle regulation, energy metabolism reprogramming and other processes. In addition, the expression of cytoskeleton related genes may affect cell morphogenesis, cytokinesis and signal transduction efficiency. It is noteworthy that the root and leaf organs of *P. sibiricum* under continuous cropping showed significant stress adaptation characteristics, which can be attributed to the fact that plants build a multi-level stress defense system to cope with continuous cropping pressure by activating antioxidant genes such as APX and cat and metabolic regulatory genes such as HXK and Sus[8].

Conclusion

Through high-throughput sequencing technology, the root and leaf of *P. sibiricum* were sequenced to obtain 356 775 590 clean Reads without reference genome, and 400 854 unigenes were obtained after assembly. The analysis of differentially expressed genes showed that there were 21 916 differentially expressed genes in the leaves of *P. sibiricum* and 12 726 differentially expressed genes in the roots of *P. sibiricum*. The enrichment of go function and KEGG function found that continuous cropping may mainly affect the biological functions of *P. sibiricum* DNA binding transcription factor, glycosylhydrolase, glycosyltransferase, and other metabolic pathways such as plant hormone signal transduction, plant pathogen interaction, and biosynthesis of secondary metabolites. It provides a theoretical basis for further study on the molecular mechanism of Polygonatum continuous cropping obstacle.

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