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Original Research

Integrated transcriptome and microRNA profiles analysis reveals molecular mechanisms underlying the consecutive monoculture problem of *Polygonatum odoratum*

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Abstract: *Polygonatum odoratum* is a historically traditional Chinese medicine plant. However, the consecutive monoculture problem (CMP) widespread in other Chinese medicine limiting their cultivation on a large scale. In this study, the physiological data showed the adverse effect of CMP on the growth of *P. odoratum* under the consecutive cropping (CC) compared with the first cropping (FC). Then the high-throughput sequencing of miRNA and mRNA libraries of leaves and roots from FC and CC *P. odoratum* plants identified 671 differentially expressed genes (DEGs) and 184 differentially expressed miRNAs and revealed that the DEGs and target genes of the miRNAs were mainly involved in starch and sucrose metabolism, phenylpropanoid and brassinosteroid biosynthesis. The KEGG analysis revealed that the DEGs between CC and FC roots were enriched in the plant-pathogen interaction pathway. This study provided the expression regulation of genes related to CMP of *P. odoratum* but also suggested that CMP may result in the serious damage of pathogens to roots and cause the slow growth in the consecutive cropping plants.

Key words: *Polygonatum odoratum*; Consecutive monoculture problem (CMP); miRNA; KEGG; Plant-pathogen interaction.

Introduction

Polygonatum odoratum belonging to the *Liliaceae* family is a historically traditional Chinese medicine plant. It is mainly distributed in central and southwest China. The extracts from rhizomes of *P. odoratum* have great economic value, which could enhance human immunity, lower blood sugar, and treat diabetes (1-7). Therefore, more and more people grew *P. odoratum*. However, when *P. odoratum* consecutively cultivated in the same filed, its yields and quality will decline dramatically, which is called as the consecutive monoculture problem (CMP).

CMP is a major factor limiting the cultivation of traditional Chinese medicinal herb, including *Radix pseudostellariae* (8), *Salvia miltiorrhiza* (9), *Rehamannia glutinosa* (10). It has been well-documented that CMP can decline growth and development, and affect the yield and quality of plants mainly because of the root exudates and the rhizosphere microbial imbalance (11, 12).

Previous studies have suggested that intraspecific allelopathy was the main inducement of CMP, and some related chemicals including terpenoids, phenolics and cyanogenic glycosides have been identified (13, 14). It has been reported that a lot of genes or miRNAs in *R. glutinosa* and *S. miltiorrhiza* were responsive to CMP (9, 10). Root exudates during replanting may promote Ca^{2+} signal transduction and ethylene synthesis, which in turn disrupt gene expression (15). Transcriptome analysis of *R. glutinosa* with replanting revealed that the alteration of genes expression could reconstruct the secondary metabolism (16-21).

MicroRNAs (miRNAs) of about 22 nucleotides in length play important roles in the plant development and stress response (15, 21). In *S. miltiorrhiza*, 5 miRNAs and 7 target genes were proved to be involved in CMP in the consecutive cropping plant roots (9). However, the molecular mechanisms of miRNAs in CMP remains elucidated.

In this study, we compared the transcriptome and miRNA expression profiles in the leaves and roots of *P. odoratum* under the first cropping (FC) and consecutive cropping (CC) systems using high-throughput sequencing technology. The differentially expressed genes (DEGs) and target genes of the miRNAs were mainly involved in starch and sucrose metabolism, phenylpropanoid and brassinosteroid biosynthesis. The KEGG analysis revealed that DEGs between CC and FC roots were enriched in the plant-pathogen interaction. These results may provide important information for further understanding of the molecular mechanism in the replanting of *P. odoratum*.

Materials and Methods

Plant materials

The *P. odoratum* local cultivar “zhushiwei” was

selected as experimental material. The plants were planted in Gutang town's experimental field of Loudi city, Hunan province, China. For the consecutive cropping (CC), the local cultivar species were grown in the land where the same species had been harvested. For the first cropping (FC), the species were planted to the cabbage-peas harvested fields near to CC on the same date. The plant height (cm), polysaccharide content (%) and yield (kg/m^2) were detected from two cropping systems.

RNA extraction, library construction and sequencing

The leaf and root samples were obtained from CC and FC plants separately. For mRNA-seq, each tissue had three biological samples. Sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) according to the manufacturer's protocol. Prepared libraries were sequenced on Illumina Hiseq Platform and 150 bp paired-end reads were generated. For miRNA-seq, each tissue had two biological samples. NEB Next Multiplex Small RNA Library Prep Set for Illumina (NEB, USA) was used for libraries' construction following the manufacturer's recommendations. Illumina Hiseq platform was used to sequence small RNA libraries and 50 bp single-end reads were generated. All raw data were uploaded in the Sequence Read Archive (SRA) public database of the NCBI with accession number PRJNA507291.

Transcriptome de novo assembly and functional annotation

First, raw pair-end reads of mRNA sequencing data were filtered to obtain high-quality clean-reads by removing adapters, low-quality reads and ambiguous reads. Then transcriptome assembly was implemented using Trinity (22). Finally, multitudes of contigs produced by transcriptome were clustered into genes by Corset software (23). For functional annotation, the assembled unigenes were aligned to the public databases including NR (NCBI non-redundant protein sequences database), Nt (NCBI nucleotide sequences database), Pfam (Protein family database), KOG (eukaryotic orthologous groups), COG (Clusters of Orthologous Groups), Swiss-Prot (Swiss Institute of Bioinformatics databases) and KEGG (Kyoto Encyclopedia of Genes and Genomes databases). The representative Gene Ontology (GO) annotation was performed by the software Blast2GO (24).

Identification of the conserved and novel miRNAs

The raw single-end reads of miRNA were firstly used to obtain high-quality clean reads by removing 5' adapter-contaminated reads, low quality reads ambiguous reads. The sequence of 3' adapter in reads was trimmed. Only reads with length from 18 to 30 nt were used to map to transcriptome using bowtie (25). The mapped reads were used to identify conserved miRNAs by BLASTN against miRbase 20.0 databases (http://www.mirbase.org) (26). Potentially novel miRNAs were predicted with miREvo (27).

Differential expression analysis of mRNA and miRNAs

To obtain read counts of each sample, clean data of each mRNA samples were mapped to *de novo* assembled transcriptome with RSEM (28). For gene-level expression profile analysis, read counts were converted to FPKM (expected number of Fragments per Kilobase of transcript sequence per Millions of base pairs). For miRNAs, TPM (transcripts per kilobase million) was used to calculate and normalize the expression of miRNAs. DESeq2 was used to detect differentially transcribed genes and miRNAs in leaf (CCL and FCL) and root (CCR and FCR) libraries (29). The differentially expressed genes and miRNAs were identified with the fold change of $\log_2$(CCL/FCL) and $\log_2$(CCR/FCR) >= 1 or <=-1 together with adjusted P< 0.05 (false discovery rate, FDR).

Functional enrichment analysis and miRNA target genes prediction

The differentially expressed miRNAs were then used to predict their target genes using psRNATarget (30). The corresponding genes of deferentially expressed miRNA were selected to perform GO and KEGG enrichment analysis with GOseq (31) and KOBAS 2.0 (32), respectively.

Quantitative real-time PCR (qRT-PCR) analysis

The RNA for sequencing was used to detect the expression levels of miRNAs and their targets. Then 500 ng of total RNA was used for cDNA synthesis with an oligo (dT) primer. The qRT-PCR on mature miRNAs was performed according to the published protocol (33).

Results

The growth of P. odoratum was influenced by the replanting.

As shown in Figure 1, under CC condition, *P. odoratum* plants grew slower and worse than FC, and the leaves were scorched and tuberous roots became much smaller. Compared with the FC plants, the CC plants were characteristic of a smaller leaf area, shorter plant height and fewer leaf numbers obviously (Figure 1A), and their chlorophyll content, polysaccharide content

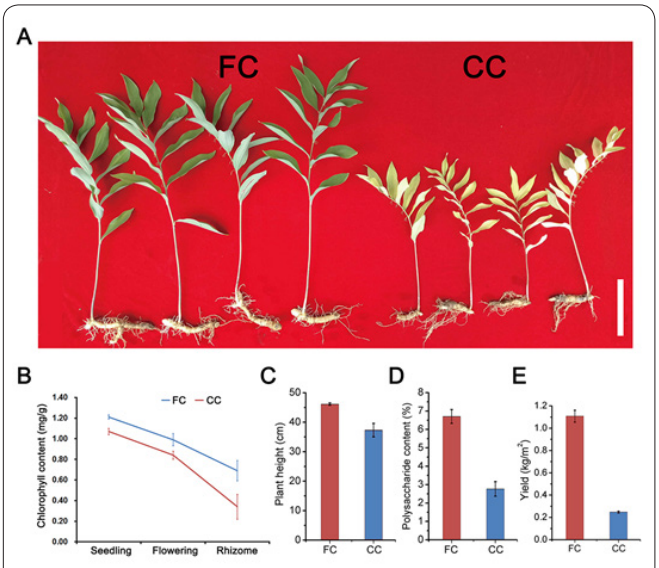

Figure 1. The different experimental samples during the replanting. **A:** The 80 days old *P. odoratum* plants of first cropping (FC, left) and consecutive cropping (CC, right), Bar=10 cm. **B:** Plant height (cm), polysaccharide content (%) and yield (kg/m^2).

and yield also decreased (Figure 1B and E). Thus, the growth status, output and quality of CC *P. odoratum* were significantly reduced.

De novo transcriptome assembly and functional annotation

Due to a lack of reference genome sequences, the sequencing data from two tissues of *P. odoratum* under FC and CC were used to generate a *de novo* assembly. After removing low-quality sequences, the further assembly was performed using the Trinity and generated 842,213 transcripts and 510,970 unigenes. The N50 lengths of the transcripts and unigenes were 931 bp and 1,167 bp, respectively. The unigenes have a length distribution from 201 to 1, 5991 bp with an average length of 859 bp.

Identification of DEGs during the replanting of P. odoratum

We constructed four RNA-seq libraries from FCL, FCR, CCL and CCR to identify CMP-associated genes. A total of 7,932 and 5,866 DEGs were up-regulated and down-regulated in CCL vs FCL, respectively; while 4,843 up-regulated and 10,945 down-regulated genes were identified in CCR vs FCR. There were 1,093 and 476 DEGs shared in the comparisons of CCL vs FCL and CCR vs FCR. GO enrichment analysis showed that the DEGs were involved in metabolic process, protein phosphorylation and oxidation-reduction. The pathway analysis revealed that the DEGs of CCL vs FCL were mainly related to stilbenoid, brassinosteroid and phenylpropanoid biosynthesis and phenylalanine metabolism (Figure 2A). Similarly, among the DEGs of CCR vs FCR (Figure 2B, C), 35 DEGs participated in the phenylalanine biosynthesis, 47 for terpenoid backbone biosynthesis, and 23 in the flavonoid biosynthesis and redox reaction.

Identification and functional analysis of replanting-responsive miRNAs

In total, 284 and 81 were considered as known and novel miRNAs, respectively, and 184 miRNAs were identified responsive to CMP. Then 2,262 targets for known miRNAs and 823 target genes for novel miRNA were identified. GO and KEGG pathway analysis suggested that the target genes were mainly involved in starch and sucrose, glycerophospholipid, and galactose metabolism, RNA degradation, nucleotide excision repair and circadian rhythm. Among all target genes, there were 10 target DEGs for 7 differentially expressed miRNAs such as *miR164* and *miR398* in CCL vs FCL, and 28 target DEGs for 11 differentially expressed miRNAs including *miR156* and *miR319* in CCR vs FCR (Table 1).

Validation of miRNAs and the expression of their targets by qRT-PCR

It was confirmed that *miR164*, *miR167* and *miR398* were down-regulated in the leaves, which is coincident with the sequencing data (Figure 3A). Meanwhile, their targets *NAC22*, *ARF17* and *CCS* were up-regulated in the leaves as expected (Figure 3B). In roots, the expression level of *miR156*, *miR160* and *miR319* was higher in the CC plants than that in FC plants (Figure 3C), and accordingly, the expression level of their targets *SPL7*, *ARF18* and *TCP2* was down-regulated in the CC plants (Figure 3D). The results suggested that the high-throughput sequencing data were reliable.

Discussion

The DEGs related to secondary metabolism play important roles in CMP

The formation of CMP has been reported to be in-

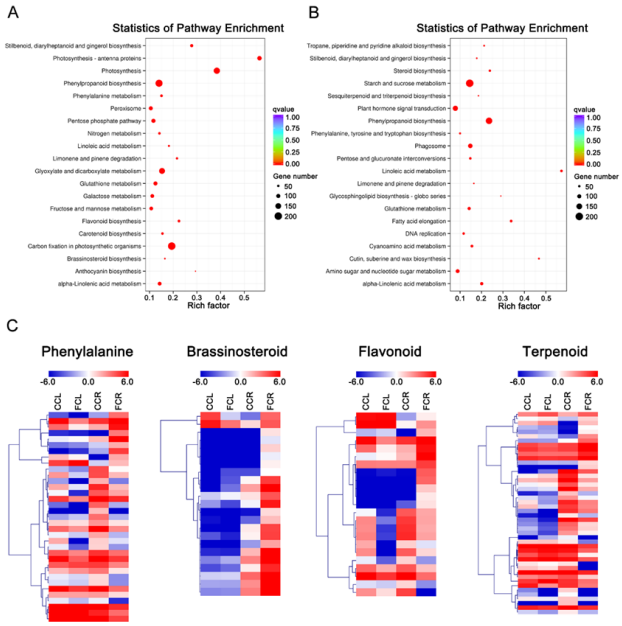

Figure 2. KEGG pathway enrichment of the DEGs. **A:** KEGG pathway enrichment of the DEGs in FCL vs CCL. **B:** KEGG pathway enrichment of the DEGs in FCR vs CCR. The red circle represents the significantly enriched pathway, and the more DEGs involved, the larger the circular. **C:** Heatmaps of the DEGs in the significant KEGG pathways. Red color indicates up-regulation and blue color represents down-regulation.

Table 1. Some miRNAs regulated differentially expressed transcription factors (TFs) during the replanting of *P. odoratum*.

miRNA	DEGs (TFs)	Annotation	CCL	FCL	CCR	FCR
CCL vs FCL						
pod-miR160b	Cluster-60288.240563	MADS	1.54	0.10	0.00	0.04
pod-miR164a	Cluster-60288.119908	NAC21	3.71	0.16	0.19	0.93
CCR vs FCR						
pod-miR156a	Cluster-60288.418576	SPL7	0.00	0.03	0.19	2.87
pod-miR156a	Cluster-60288.418577	SPL7	0.00	0.00	0.26	3.12
pod-miR160	Cluster-60288.106698	ARF18	1.62	2.31	0.98	4.74
pod-miR319	Cluster-60288.259333	PCF6	7.52	6.50	0.42	2.13

DEGs: differentially expressed genes.

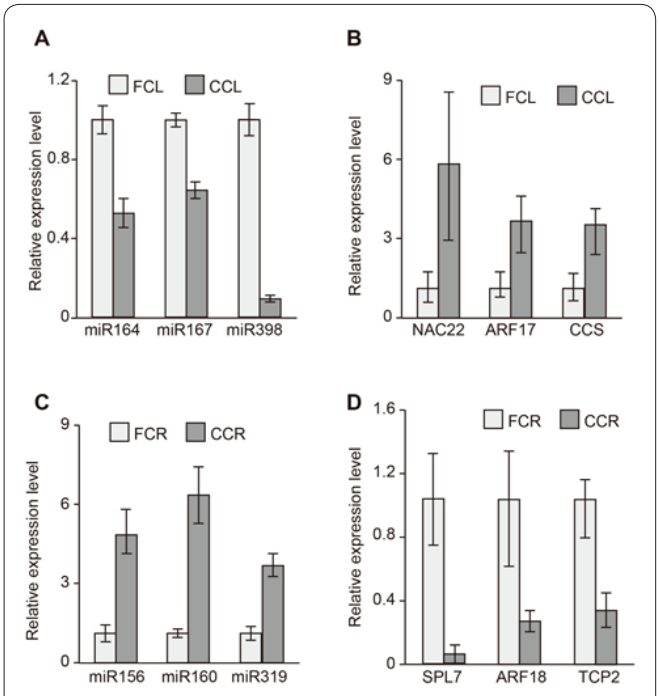

Figure 3. Expression confirmation of miRNAs and their targets by qRT-PCR. Three independent experiments were performed. **A, B:** The expression level of miRNAs and target genes in FCL and CCL. **C, D:** The expression level of miRNAs and target genes in FCR and CCR. Error bars represent S.D. (*n*=3).

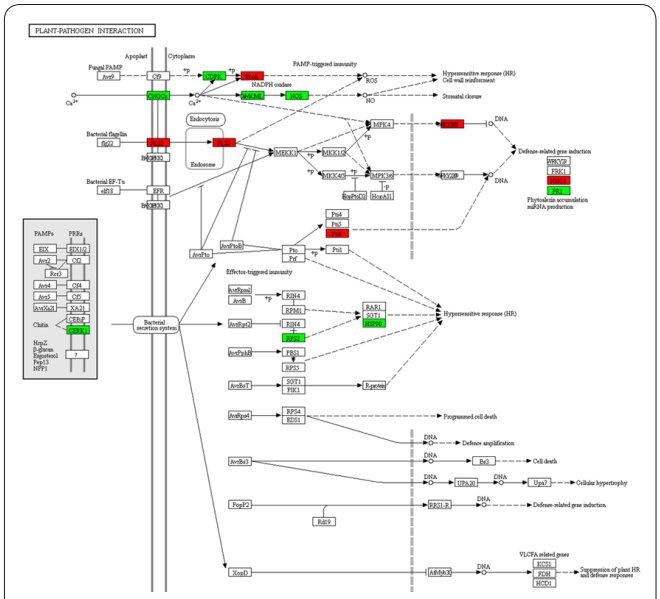

Figure 4. Plant-pathogen interaction enrichment of the DEGs between CCR and FCR. All DEGs between CCR and FCR were mapped to the KEGG database. Green, down-regulated, Red, up-regulation.

volved in the rhizosphere micro-ecosystem among the root exudates, plants and rhizosphere microbes (10). The ongoing development of high-throughput sequencing technologies has been applied to decode replant-stress regulation in many Chinese medicine plants (9-12). In the study, the pathway analysis showed that lots of DEGs were involved in metabolic processes, including terpenoids biosynthesis, phenylalanine metabolism, and brassinosteroid biosynthesis. Terpenoid and phenylalanine biosynthesis plays an important role in the stress defense (10), indicating their important roles in response to CMP of *P. odoratum*. A lot of genes associated with flavonoids metabolism may participate in plant root-microbe interaction (34). Previous studies have been reported that secondary metabolites like flavonoids, phenolics and terpenoid widely mediated the communication between inter- and intra-specific roots (4). Therefore, these DEGs related to the secondary metabolism were involved in CMP of *P. odoratum*.

The miRNAs-regulated genes contribute to relieving the CMP of P. odoratum

We found that *miR156* and *miR319* were up-regulated only in the roots of the plant with CC. However, *miR164*, *miR167*, and *miR398* were down-regulated only in leaves. Previous studies have reported that the *SPL7* gene targeted by *miR156* promotes the shift to the reproductive growth of *Arabidopsis* (35). The recent study revealed that *P. euphratica* may increase taproot elongation and reduce lateral root number to adapt to drought and salt stresses via *peu-miR164*-regulated *PeNAC70* expression patterns (36). Based on the functional analysis of *miR156*, a simple model of *miR156-SPL9-DFR* (DIHYDROFLAVONOL-4-REDUCTASE) pathway was postulated (37). The transgenic study revealed that the plants with overexpression-*miR156* exhibited greater susceptibility to *Pseudomonas syringae pv.* Tomato DC3000 infection through repressing the *SPL* gene, regulating reactive oxygen species (ROS) accumulation (38). In addition, both of *miR164* and *miR156* were also identified as negative regulators in response to the replanting disease of *R. glutinosa* (12). In our study, *miR164* was repressed, and its target gene *NAC22* was up-regulated in CCL. And in CCR, *miR156* was induced, and its target gene *SPL7* was down-regulated (Figure 3). Therefore, the data indicated the positive roles of these miRNAs and their target genes in response to CMP of *P. odoratum*.

In *Arabidopsis*, *miR398*-directly cleaves its target CCS (copper chaperone for CSD1 and CSD2) mRNAs, which may be involved in oxidative stress tolerance (39). According to the transcriptome data, thousands of genes related to redox were differentially expressed between CCL and FCL. The *miR398* was up-regulated in CCL and negatively regulated *CSD1* and *CSD2* (Figure 3A and B). Another stress-responsive miRNA, *miR319*, has been uncovered to be involved in repression of cell proliferation and leaf development (40). In the present study, *miR319* was slightly up-regulated and its target gene *TCP2* was down-regulated in the CCR vs FCR (Figure 3C and D). The present results indicated that consecutive cropping may influence the ROS level in plants, and further repress plant growth (Figure 1).

Plant-pathogen interaction exhibited conserved roles in CMP of P. odoratum

To further our understanding of how the consecutive cropping affects *P. odoratum* growth, the KEGG analysis of all DEGs between CCR and FCR uncovered the profile related to plant-pathogen interaction (Figure 4). The recent studies showed that cyclic nucleotide-gated channels (CNGCs) play important roles in sensing Ca^{2+} in diverse signaling pathways via forming important interactions with Ca^{2+} sensor calmodulin (41), and silencing of CNGC genes in tomato might be involved in disease resistance (42). The downstream events re-

quire the cross-talk between calmodulin and nitric oxide (NO) to induce antioxidant defense and stomatal closure to reduce transpiration water loss in plants under various stress conditions (43, 44). Our data showed that the CNGC gene (KO: K05391) was down-regulated in *P. odoratum* with consecutive cropping, which may lead to the enhancement of disease resistance of *P. odoratum*. However, the other Ca^{2+}-signaling genes including CaLM and CML (K02183), and nitric oxide synthase (NOS, K13427) exhibited down-regulated in CCR compared with FCC, which may suggest the serious damage to *P. odoratum* roots under consecutive cropping, and the detailed mechanism remains clarified.

It is well-known that ROS play central roles in plant response to adverse stresses (45). As shown in Figure 4, Rboh proteins play as the predominant players for apoplastic ROS production, and bacterial flagellin (flg22) receptor FLS2 also contributes to the ROS burst, which results in a hypersensitive response (46). At the same time, the lock-out of *WRKY33* in *Arabidopsis* showed higher susceptibility to pathogens via increasing expression of PR genes (47), and the study revealed the conserved roles of WRKY33 proteins in plant stress responses (48). The previous study uncovered that *Arabidopsis* glycerol kinase (glpk) seedling mutants promote resistance to abiotic stresses through the accumulation of glycerol (49, 50). In the present study, the genes encoding Rboh (K13447), FLS2 (K13420), WRKY33 (K13424) were up-regulated in CCR compared with FCR, indicating that there are continuous stresses to *P. odoratum* roots with higher ROS level. Accordingly, the *PR1* gene (K13449) was down-regulated, and *NHO1* gene (glpk, K00864) was up-regulated in CCR, resulting in susceptibility to pathogens and lower level of glycerol in CCR, respectively, both of which suggest the lethal damage of consecutive cropping to *P. odoratum* roots and growth (Figure 1).

In the present study, the integrated transcriptome and miRNA expression profiles analysis of roots and leaves from *P. odoratum* with FC and CC was performed and discovered that the DEGs and differentially expressed miRNAs involved in terpenoids, phenylpropanoid and brassinosteroid biosynthesis may be related to CMP. The KEGG analysis revealed the conserved roles of plant-pathogen interaction in CMP of *P. odoratum*. Overall, the study could provide a foundation for further functional analysis of genes and miRNAs for the improvement of CMP in *P. odoratum*.

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