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Transcriptome profiling reveals metabolic alteration in *Andrographis paniculata* in response to continuous cropping

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ABSTRACT

Andrographis paniculata (Burm. f.) Nees is one of the most important economic crops for its various medicinal properties, which has been widely used as medicinal herb in several folk medicine systems all over the world. *A. paniculata* suffers from continuous cropping problem in cultivation; however, effects of continuous cropping on *A. paniculata* and the underlying mechanism at molecular level are rarely understood. In this study, Illumina paired-end RNA-seq was performed for *de novo* transcriptome construction of *A. paniculata* leaf with or without 2-years continuous cropping, to identify gene expression in response to continuous cropping stress. A total of 43,683 Unigenes were obtained by *de novo* assembly of 231.53 million paired-end clean reads. RNA-seq based gene expression profiling showed a total of 6193 Unigenes was significantly up-/down-regulated after continuous cropping. Integrating the GO and KEGG enrichment analysis of differentially expressed genes (DEGs) revealed that *A. paniculata* could alter enzyme genes expression to adjust the complicated metabolic pathways to tolerate the continuous cropping stress. Specially, accorded with the downtrend of active component contents in *A. paniculata*, majority of genes involved in the terpenoids biosynthesis, phenylpropanoid biosynthesis and flavonoids biosynthesis pathways were down-regulated, indicating that continuous cropping led to a declined synthesis of active ingredients through repressing the expression levels of genes involved in these metabolites' biosynthesis pathways. The transcriptome profiling will deepen the understanding of *A. paniculata* under continuous cropping stress at transcriptional level, and provide useful genomic resource for further interpretation on the regulation mechanism.

1. Introduction

Consecutively cultivated in the same field year after year will eventually lead to crop yield reduction, quality deterioration, poor growth status and disease aggravation, which is commonly known as continuous cropping problem or replanting problem. It is not only a common production problem in main economic crops, but also frequently happens in medicinal plants such as *Panax quinquefolius* (Dong et al., 2017), *Panax notoginseng* (Dong et al., 2016), *Rehmannia glutinosa* (Yang et al., 2013; Tian et al., 2017), *etc.* Continuous cropping problem is a complex environmental stress involving multiple factors, including the autotoxicity of root exudates, the rhizosphere microbial imbalance and physiological variation (Li et al., 2013; Wu et al., 2013). Consecutive monoculture research on *R. glutinosa* also suggested that, continuous cropping problem was a comprehensive stress, and the influence of continuous cropping practice on genes' expression profile of *R. glutinosa* was very similar to that of it when responding to salt and drought stresses (Tian et al., 2017).

Andrographis paniculata (Burm. f.) Nees in Acanthaceae family possesses various medicinal properties including antimicrobial and antioxidant, antimalaria, antiangiogenic, anti-inflammatory, anti-diabetic, *etc.* (Malahubban et al., 2013; Sheeja et al., 2007, 2008; Zein et al., 2013; Zhang and Tan, 2000). It is rich in diterpenoids and flavonoids, among which, diterpene lactones such as andrographolide, dehydroandrographolide, neoandrographolide and deoxyandrographolide mainly contribute to the pharmacological activities. This herb has been widely cultivated in the tropical area of Asia for its importance in medicinal industry. It was introduced into China in 1950s, the dried aerial part of *A. paniculata* (Andrographis herba, also called "*Chuanxinlian*"), has been widely used in traditional Chinese medical system for its efficacy of clearing heat and removing toxicity, cooling blood and detumescence. The raw herb is popularly used in numerous Chinese

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herbal compound prescriptions and Chinese patent medicine owing to its significant clinical efficacy, resulted in a vastly increasing demand of *A. paniculata*. In the commercially standardized cultivation, it was reported that *A. paniculata* suffers from replanting problem and farmers usually avoid the problem via crop rotation or leaving land unused. Either of these approaches declines the production of Andrographis herb. Studies have proved that the allelopathic effect of extracts from vegetative parts of *A. paniculata* inhibited the seed germination and seedling growth of its own and other plants (Li et al., 2014a; 2010). Diterpenoids and flavonoids with high richness in the plant were identified as powerful allelochemicals (Bais et al., 2004; Walker, 2003; Xuan et al., 2005). However, no research at molecular level on continuous cropping of *A. paniculata* has yet been reported.

For the advantages of efficient, cost-effective and no need of a reference genome, RNA-Seq coupled with digital gene expression (DGE) tag profiling have been widely used in characterizing non-model organisms. The transcriptome profiling of numerous plants under any given conditions have been dissected to identify differentially expressed genes (DEGs). Therefore, deciphering the transcriptome profiles of *A. paniculata* in response to continuous cropping stress is a vital step to understand the effects and mechanism of continuous cropping on biological functions and metabolic pathways at transcriptional level.

In this study, Illumina HiSeq™ 2000 platform was used to perform the large-scale transcriptome analysis of *A. paniculata* and, specifically, the transcriptome profile features of *A. paniculata* subjected to continuous cropping were emphatically investigated to elucidate changes of the biological processes and metabolic regulation occurred under such situation.

2. Materials and methods

2.1. Plant materials and continuous cropping treatment

A. paniculata seeds were germinated in sterilized soil and transplanted into earthen pots (25 cm height and internal diameter) at the three pair true leaves stage. Seedlings were randomly allocated into control group and continuous cropping experimental group, 50 seedlings in each group. In the control group (hereafter referred as AP0), seedlings were planted with a commercial horticultural soil without *A. paniculata* planting before (the first-year planting in this study); while in the experimental group (hereafter referred as AP2), seedlings were planted with the same kind of soil that had been continuously growing *A. paniculata* in the previous two years (two growth cycles, one cycle per year; the third-year planting in this study). Plants were grown in a plastic greenhouse during the months of July-September under natural temperature and light. Same cultivation conditions were applied. At the initial time of flowering (the end of September), leaf samples randomly collected from 10 plants in AP2 were mixed as one sample, as well as in AP0. After being quickly washed with sterile water, all samples were frozen immediately in liquid nitrogen for RNA extraction. Meanwhile, 10 individual plants randomly chosen from each group were collected for content determination of active compounds.

2.2. Extraction and HPLC analysis of andrographolide and dehydroandrographolide

Shade dried aboveground parts of *A. paniculata* were ground into 65 mesh powder as extraction samples. The extraction and content determination were conducted according to the Chinese Pharmacopoeia (2015 edition) with slight modifications, as described previously (Li et al., 2017). Ten *A. paniculata* plants randomly chosen from AP0 and AP2 respectively were determined in this experiment.

2.3. Extraction and determination of total flavonoids

Total flavonoids contents of crude extracts were determined by aluminium nitrate colorimetric method. In brief, 1 g of *A. paniculata* powder as described in *2.2* was precisely weighed and transferred to a conical flask. Petroleum ether (15 m L) was added to remove the lipids before extracting twice with 15 m L 60% (v:v) ethanol using an ultrasonic bath at a frequency of 33 kHz at 25℃ for 2 h. Extracts were combined and concentrated to 5 mL. The supernatant was diluted to 25 m L with 60% ethanol. One milliliter of crude ethanol extract was transferred to 10 m L colorimetric tube, 0.5 mL 5% $NaNO_2$ solution was added and stood for 6 min. After 6 min of incubation, 0.5 m L of 10% Al $(NO_3)_3$ solution was added and the mixture was allowed to stand for another 6 min. Then, 5 mL of 4% NaOH solution were added, and the final volume of the mixture was brought to 10 mL with 60% ethanol. After 15 min incubation, absorption of the mixture was determined at a wavelength of 500 nm. The content of total flavonoids was calculated by a standard curve using rutin in different concentration, and the result was expressed as mg rutin equivalent per dry weight of plant, and converted to percentage. Ten *A. paniculata* plants randomly chosen from AP0 and AP2 respectively were determined in this experiment.

2.4. RNA extraction, cDNA library construction and sequencing

Total RNA was isolated from continuous cropping samples (AP2) and control (AP0) using Trizol Kit (Promega, USA) following the manufacturer's instructions, and then the contaminant DNA was erased using RNase-free DNase I (Takara Bio, Japan). Integrity of RNA was checked by agarose gel electrophoresis and 2100 Bioanalyzer (Agilent Technologies, USA); and concentration of RNA was determined using a Nanodrop micro spectrophotometer (Thermo fisher, USA). Poly(A) mRNA was enriched by oligo dT magnetic beads (Qiagen); and the purified mRNA was fragmented by adding fragmentation buffer. First-strand cDNA was obtained by random hexamer-primed reverse transcription, and then RNase H and DNA polymerase I were used for the second-strand cDNA synthesis. Then, QIAquick PCR extraction kit (Qiagen, China) was used to purify the cDNA fragments. These purified fragments were then resolved in EB buffer for end reparation and A tailing, and ligated to sequencing adapters. After purification, the cDNA fragments were enriched by PCR to construct the final cDNA library. Finally, the established cDNA libraries were sequenced using Illumina HiSeq™ 2000 platform (Illumina Inc., USA) at Gene Denovo Co. Guangzhou, China.

2.5. Preprocessing, de novo assembly and functional annotation

Trimmomatic (Bolger et al., 2014) was used to generate clean reads by removing the adaptor sequences, reads with ambiguous 'N' bases and base quality less than Q30 from raw reads. Then, the clean reads were *de novo* assembled into contiguous sequences (contigs) using Trinity Program (Grabherr et al., 2011), and all obtained contigs were further assembled into Unigene, the constructed sequence that could not be extended on either end. For functional annotation, the resulted Unigenes were aligned against four public protein databases (Nr, COG, Swiss-Prot and KEGG) using BLASTx (Altschul et al., 1997) with a cut-off *E*-value of 10^{-5}. Gene ontology (GO) annotation and functional classification were analyzed using Blast2GO and WEGO software, respectively (Ye et al., 2006).

2.6. Differential gene expression analysis

The expression abundance of each Unigene was estimated using software package of RNA-seq by Expectation Maximization (RSEM) (Li and Dewey, 2011), the RPKM (Reads Per Kb per Million reads) method was applied to normalize the relative gene expression level. Unigenes with false discovery rate (FDR) ≤ 0.001 and $|\log_2\text{fold change}| \geq 1$ were defined as DEGs, which were identified across groups using the R package edgeR (Robinson et al., 2009).

2.7. Analysis of GO enrichment and KEGG pathway enrichment

Gene ontology (GO) enrichment analysis of the DEGs was implemented using Blast2GO with a corrected *P*-value ≤ 0.05. KEGG pathway enrichment analysis of the DEGs was performed using KOBAS with Q-value ≤ 0.05.

2.8. Validation of DEGs using quantitative real-time PCR (qRT-PCR)

For qRT-PCR, 1 μg total RNA of each sample for RNA-Seq was used as a template in 20 μL reaction using the SsoFast EvaGreen Supermix (Bio-Rad, USA). Quantitative RT-PCR analysis (with 3 biological replicates and 3 technical replicates for each sample) was performed using CFX96 system (Bio-Rad, USA). The *GAPDH* gene was used as endogenous reference for normalization of qRT-PCR CT values. The $2^{-\triangle\triangle CT}$ method was used for the relative expression level calculation. Primers used for qRT-PCR were presented in Supplementary Table S1.

3. Results

3.1. Contents of active compounds reduced in A.paniculata subjected to continuous cropping

In the current study, main active components of AP0 and AP2 plants were extracted, and the contents were determined using correspondent methods. As shown in Fig. 1, the contents of andrographolide, dehydroandrographolide and total flavonoids in AP2 were significantly lower than those in AP0 ($P < 0.01$).

These results indicated that the accumulation of active compounds including andrographolide, dehydroandrographolide and total flavonoids in *A. paniculata* declined due to continuous cropping.

3.2. RNA sequencing, de novo assembly and annotation of A. paniculata transcriptome

To elucidate the mechanism of continuous cropping on *A. paniculata* at molecular level, the RNA-Seq high-throughput sequencing was employed to clarify the change of transcripts present in leaves of *A. paniculata* after treatment. The cDNA libraries of AP2 and AP0 were pooled together to represent the whole transcriptome of *A. paniculata*. A total of 231,536,164 clean reads were obtained with the Q20 and GC percentages of 96.95% and 49.32%, respectively (Supplementary Table S2). The clean reads were *de novo* assembled into 43,683 Unigenes with an average length of 1045 bp, an N50 of 1743 bp, and a GC percentage of 44.22% (Supplementary Table S3). The length distribution of *A. paniculata* Unigenes was shown in Supplementary Fig. S1. These obtained Unigenes would act as the reference transcripts of *A. paniculata*.

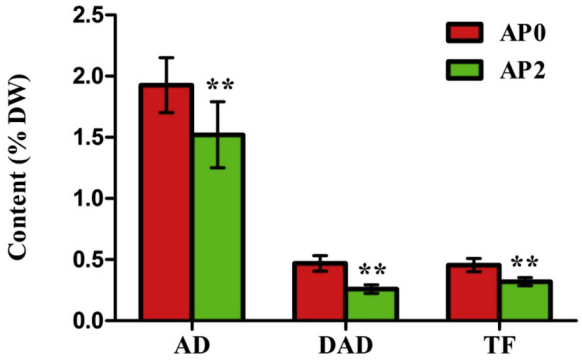

Fig. 1. Contents of active compounds in *Andrographis paniculata* continuous cropped for 0 (AP0) and 2 (AP2) years, respectively. The red color represents AP0, and green color represents AP2. DW, dry weight of *Andrographis paniculata*; AD, andrographolide; DAD, dehydroandrographolide; TF, total flavonoids. Values are mean ± SD of 10 biological replicates. The asterisks represented significant difference between AP0 and AP2, as indicated by the *t*-test (**$P < 0.01$).

As results of sequence homology searching against the public protein databases, a total of 27,418 (62.77%) assembled Unigenes had significant BLASTx hits in at least one of the four databases including Nr, COG, Swiss-Prot and KEGG (Supplementary Table S4). There were 5265 Unigenes had hits in all the four databases, 5991 were uniquely matched in Nr database and 79 found hits only in Swiss-Prot (Supplementary Fig. S2). There were also 12 and 5 Unigenes annotated solely by KEGG and COG, respectively. To study sequence homology between *A. paniculata* and other plant species, the species distribution of Unigene datasets were analyzed by aligning sequences against the Nr database, and the best match from each sequence was shown in Supplementary Fig. S3. The Unigenes showed significant similarity to those from *Solanum lycopersicum* (15.14% of the total Unigenes), *Theobroma cacao* (9.33%), *Vitis vinifera* (8.59%), *Cucumis sativus* (3.13%) and *Arabidopsis thaliana* (2.65%) at different levels (Supplementary Fig. S3).

For the prediction and classification of possible functions, all Unigenes were aligned to the COG database. A total of 10,584 (24.23%) Unigenes were grouped into 24 COG classifications. The top three categories were “General function prediction only” (3,107, 29.35%), “Transcription” (1,639, 15.48%) and “Replication, recombination and repair” (1,476, 13.95%). In the meantime, 586 (5.54%) Unigenes were assigned to the cluster “Secondary metabolites biosynthesis, transport and catabolism” (Supplementary Fig. S4).

Gene ontology (GO) analysis was carried out to categorize the possible functions of all Unigenes. A total of 23,726 Unigenes were categorized into “biological process” with 23 GO terms, 22,596 Unigenes were classified into “cellular component” with 11 GO terms and 12,884 Unigenes were related to “molecular function” with 10 GO terms (Supplementary Fig. S5).

For biochemical pathways prediction in the KEGG database, 8024 Unigenes were mapped into 122 KEGG pathways. Among the 122 pathways, 2043 Unigenes were matched to “Metabolic pathways” (pathway ID Ko01100), followed by “Biosynthesis of the secondary metabolites” (pathway ID Ko01110, 1063 Unigenes), “Ribosome” (pathway ID Ko03010, 516 Unigenes), “Plant hormone signal transduction” (pathway ID Ko04075, 283 Unigenes) and “Protein processing in endoplasmic reticulum” (pathway ID Ko04141, 232 Unigenes). There were 132 (1.65%) Unigenes mapped into “Phenylpropanoid biosynthesis” (pathway ID Ko00940), 74 (0.92%) Unigenes mapped to “Terpenoid backbone biosynthesis” (pathway ID Ko00900), 26 (0.32%) mapped to “Diterpenoid biosynthesis” (pathway ID Ko00904), and 32 (0.4%) mapped to “Flavonoid biosynthesis” (pathway ID Ko00941).

3.3. Differentially expressed genes in response to continuous cropping

A total of 6193 DEGs were found with FDR ≤ 0.001 and $|\log_2\text{Ratio (AP2/AP0)}| \geq 1$ in response to continuous cropping (Supplementary data: sheet 1), of which, 1852 were up-regulated and 4341 were down-regulated (Fig. 2), indicating more suppression than activation of genes under continuous cropping. In AP0, 103 undetectable (RPKM = 0.001) DEGs were uniquely expressed in AP2, showing an induction of these genes by continuous cropping; 462 DEGs were totally suppressed (RPKM = 0.001) in AP2 among the down-regulated DEGs.

In the 103 DEGs induced by continuous cropping, 51 DEGs had function annotation and only 14 of them were classified in specific KEGG pathway(s). Five of the 14 DEGs including 60S ribosomal protein, 60S ribosomal protein L37a, 60S ribosomal protein L1Ae, ribosomal protein L27 and ribosomal protein 30 40S small ribosomal subunit were mapped to Ribosome pathway (ko03010). Four DEGs including fructose-bisphosphate aldolase, formate dehydrogenase, cytochrome c oxidase subunit 1, and cytochrome c oxidase subunit 3 were associated with Metabolic pathway (ko01100). Some interesting genes were also spotted such as manganese superoxide dismutase, peroxidase 3, heat

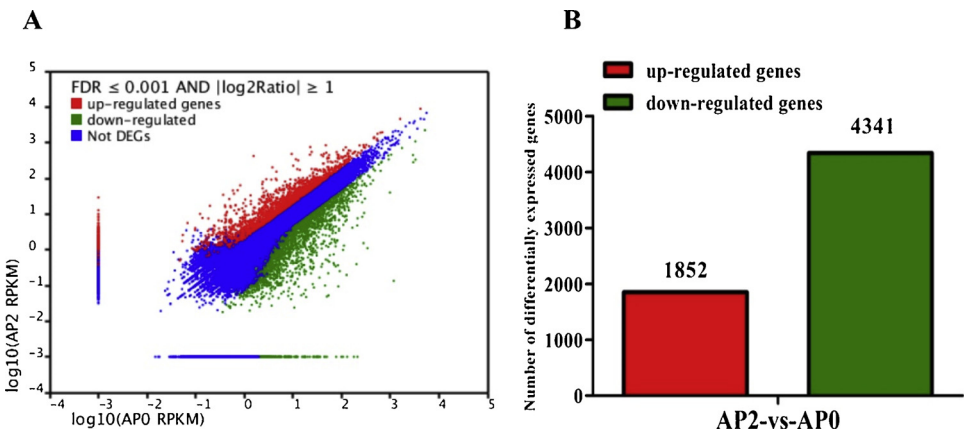

Fig. 2. Differentially expressed genes (DEGs) in *Andrographis paniculata* under continuous cropping stress. (A), Scatter plot of DEGs (FDR ≤ 0.001 and $|\log_2\text{Ratio(AP2/AP0)}| \geq 1$) illustrating the full set of genes in samples. Red dots are up-regulated genes, blue dots are non-DEGs, and green dots are down-regulated genes. (B), The number of DEGs identified in each library contrast by applying a threshold of the ratio change ≥ 2 and a q-value of < 0.05. The red and green columns represent genes up- and down-regulated by continuous cropping stress, respectively.

shock protein 70-2, transcription factor MYB1, R2R3-MYB protein, E3 ubiquitin-protein ligase ATL42, etc., which played vital role in response to biotic and abiotic stress in the annotated Unigenes. Many enzyme genes involved in various biological processes, including nitrate reductase, transposase, peptidylprolyl isomerase, pectate lyase, alcohol dehydrogenase ($NADP^+$) and NADP dependent sorbitol 6-phosphate dehydrogenase (Supplementary data: sheet 2) were also up-regulated. These uniquely up-regulated genes might exert positive response to the continuous cropping.

Within 462 DEGs that suppressed in AP2, 326 were annotated and 57 of them were classified into specific KEGG pathways. These 57 DEGs distributed in various biological processes related to plant growth, development, metabolic and stress response of *A. paniculata*. Among them, enzyme genes such as geraniol synthase, dehydrodolichyl diphosphate synthase, flavonoid 3′-hydroxylase, flavonol synthase and squalene monooxygenase were involved in the biosynthesis of secondary metabolites. Some genes like peroxidase, glutathione S-transferase, heat shock protein, *etc*., were related to stress response (Supplementary data: sheet 3).

3.4. GO enrichment analysis of DEGs

A total of 6193 DEGs were separated into three main categories including biological processes, molecular functions, and cellular components. GO classification of up- and down-regulated DEGs were shown in Supplementary Fig. S6. There were 1350, 1260, and 711 DEGs up-regulated, as well as 3020, 2506 and 1609 DEGs down-regulated in the three main categories, respectively. These results also suggested that more genes were down-regulated in *A. paniculata* during the continuous cropping process. With a corrected *P* value ≤ 0.05, the top 24 most enriched functional groups were screened out (Supplementary Fig. S7); 5, 9 and 11 functional groups were significantly enriched within the biological process category, molecular function category and cellular component category, respectively. In biological process category, Cellular aromatic compound metabolic process had the maximum number of DEGs, followed by Secondary metabolic process, Phenylpropanoid metabolic process, Aromatic compound biosynthesis process and Phenylpropanoid biosynthetic process. These indicated that the biosynthesis and metabolic of Phenylpropanoid and aromatic compounds were significantly influenced by continuous cropping.

3.5. KEGG pathway enrichment analysis of DEGs

To further understand the function of DEGs in response to continuous cropping, the DEGs were mapped to the KEGG database, 1266 out of 6193 DEGs were assigned to 113 KEGG pathways. With a Q-value ≤ 0.05, a total of 20 pathways were significantly affected by continuous cropping (Supplementary Fig. S8). The metabolic pathways (421 DEGs, 33.25%), and biosynthesis of secondary metabolites (226, 17.85%) were the two most significantly enriched KEGG pathways with a majority number of DEGs. Some primary metabolic pathways that were essential for plant growth and development were also significantly enriched, including pentose and glucuronate interconversions, starch and sucrose metabolism, glycerophospholipid metabolism, carbon fixation in photosynthetic organisms, arachidonic acid metabolism, linoleic acid metabolism, fatty acid biosynthesis, DNA replication and photosynthesis. Besides, some important protective activities pathways including biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, phenylalanine metabolism, flavonoid biosynthesis, terpenoid backbone biosynthesis, Stilbenoid, diarylheptanoid and gingerol biosynthesis, Zeatin biosynthesis and Carotenoid biosynthesis, which played important role in plant stress responses. These results indicated that continuous cropping had extensively affected the primary and secondary metabolism processes of *A. paniculata*, and *A. paniculata* adapted to the complicated stress by regulating expression of genes involved in these metabolic pathways.

3.6. Analysis of SSRs information

A total of 43,683 Unigenes in *A. paniculata* transcriptome were used to explore SSRs. As previously reported, 14,135 SSR loci were found in the transcriptome of *A. paniculata*, which distributed in 9973 Unigenes with a distribution frequency of 32.36%. Di-nucleotide and Tri-nucleotide repeat were the main types, accounted for 75.54% of all SSRs. AT/AT and CCG/CGG were the predominant repeat types of Di-nucleotide and Tri-nucleotide, respectively (Li et al., 2018). In the transcriptome profiling, a total of 2031 Unigenes containing SSR loci were differentially expressed after continuous cropping, accounted for 32.80% of DEGs. Of which, 1393 (68.59%) were downregulated while 638 (31.41%) were upregulated, indicating that more Unigene containing SSR loci were repressed under continuous cropping (Supplementary data: sheet 4).

3.7. Validation of DEGs by qRT-PCR

The RNA-seq results were verified by qRT-PCR analysis of 8 genes randomly selected from the DEGs, including Unigene0023529 (cytochrome P450), Unigene0003869 (glycine-rich RNA-binding protein 2), Unigene0007773 (aluminium induced protein), Unigene0041010 (flavonol 3-O-methyltransferase), Unigene0014861 (cysteine protease), Unigene0016422 (flavonoid 3′-hydroxylase), Unigene0014860 (vacuolar-processing enzyme) and Unigene0013708 (*ent*-copalyl diphosphate synthase). Significant expression differences for these 8 genes agreed with those detected by RNA-Seq with similar expression patterns, although the fold changes were not the same (Fig. 3).

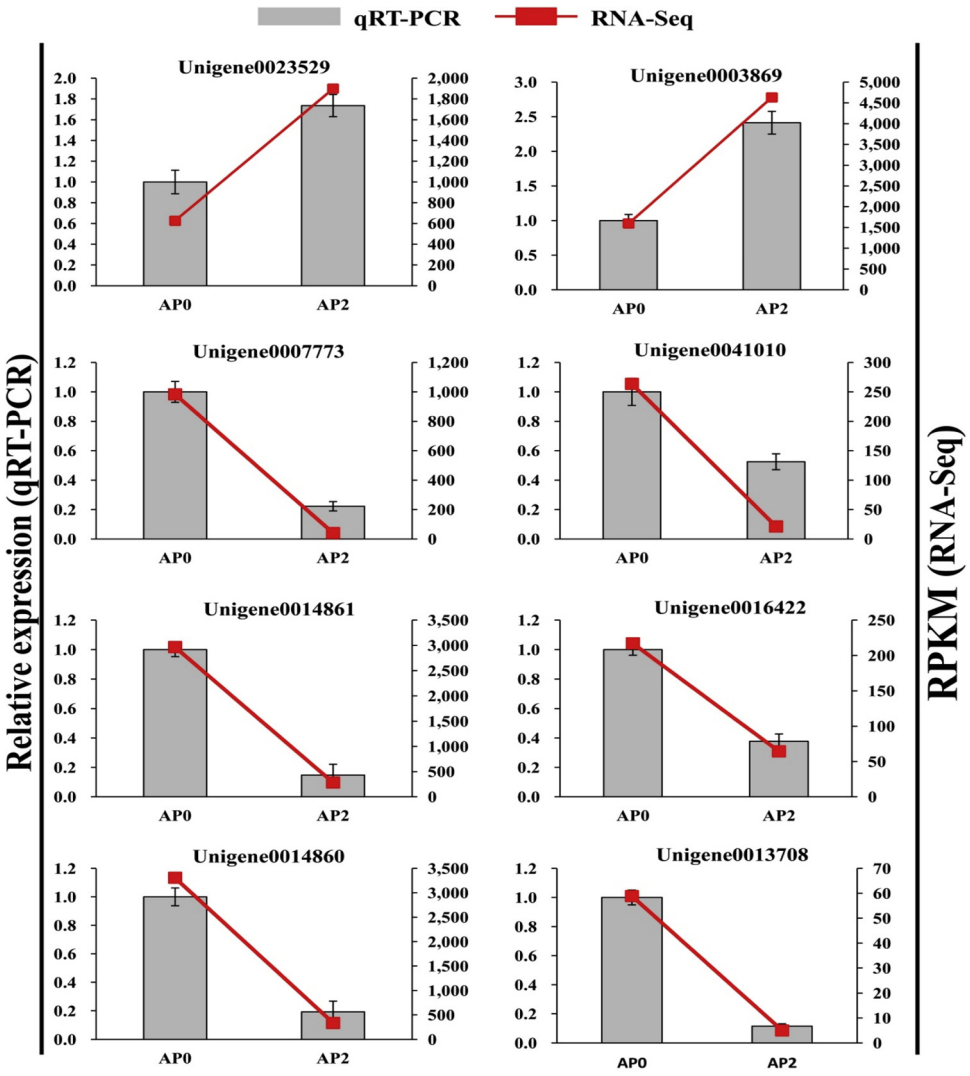

Fig. 3. Validation of expression patterns of 8 DEGs between AP0 and AP2 by qRT-PCR. The relative expression levels of eight DEGs were calculated according to the $2^{-\triangle\triangle CT}$ method using the *Andrographis paniculata GAPDH* as internal reference gene. Error bars represent standard deviations. The relative expression level of qRT-PCR is indicated on the left y-axis and the normalized expression level (RPKM) of RNA sequencing is indicated on the right y-axis.

4. Discussion

For its value as Chinese material medica, *A. paniculata* has become an important economic crop with an increasing market. In China, Andrographis herba is mainly produced by domestic growing. The quality and production of this herb in cultivation are unstable, that not only due to the influences of environmental conditions, uneven harvesting time and genetic variation (Bhan et al., 2006), but also the continuous cropping problem. Therefore, a detailed research on the response mechanism of *A. paniculata* under continuous cropping is urgently needed. In this study, the transcriptomes of *A. paniculata* continuously cropped for 0 year and 2 years were compared, to characterize *A. paniculata* under continuous cropping at molecular level. Many genes involved in important biological pathways were down- or up-regulated under continuous cropping, which might be self-adjustment of *A. paniculata* to fit the stress. Particularly, numerous DEGs were involved in secondary metabolic pathways that essential for plant stress response. In the present experiment, the contents of andrographolide, dehydroandrographolide and total flavonoids in *A. paniculata* significantly decreased after continuous cropping, which might have connection with the related genes' expression. Therefore, attention was drawn to the pathways related to the synthesis of active compounds in *A. paniculata*, including terpenoids biosynthesis, phenylpropanoid biosynthesis and flavonoids biosynthesis pathways. The expression profiles of DEGs involved in these pathways are emphatically discussed below.

4.1. Continuous cropping altered the expression of genes involved in terpenoid backbone biosynthesis and diterpenoid biosynthesis

Terpenoids are the largest group of natural products that extensively used for their aromatic qualities and effects on curing diseases. Terpenoids play important roles in almost all basic plant processes, including growth, development, reproduction, biotic and abiotic stress defense and signal transduction (Loreto et al., 2014; Soto et al., 2011; Wink, 2010). In plant, all terpenoids are derived from the C5 unit isopentenyl diphosphate (IPP), which can be synthesized via mevalonate (MVA) pathway in the cytoplasm and the methylerythritol phosphate (MEP) pathway in plastids (Nagegowda, 2010).

The MVA pathway in plants starts with acetyl-CoA and finally are converted into isopentenyl diphosphate (IPP), which are orderly catalyzed by six enzymes including acetyl-CoA acyltransferase (AACT, EC 2.3.1.9), 3-hydroxy-3-methylglutaryl-CoA (HMGS, EC 2.3.3.10), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34), mevalonate kinase (MVK, EC 2.7.1.36), phosphomevalonate kinase (PMK, EC 2.7.4.2) and mevalonate 5-dinhophate decarboxylase (MVD, EC

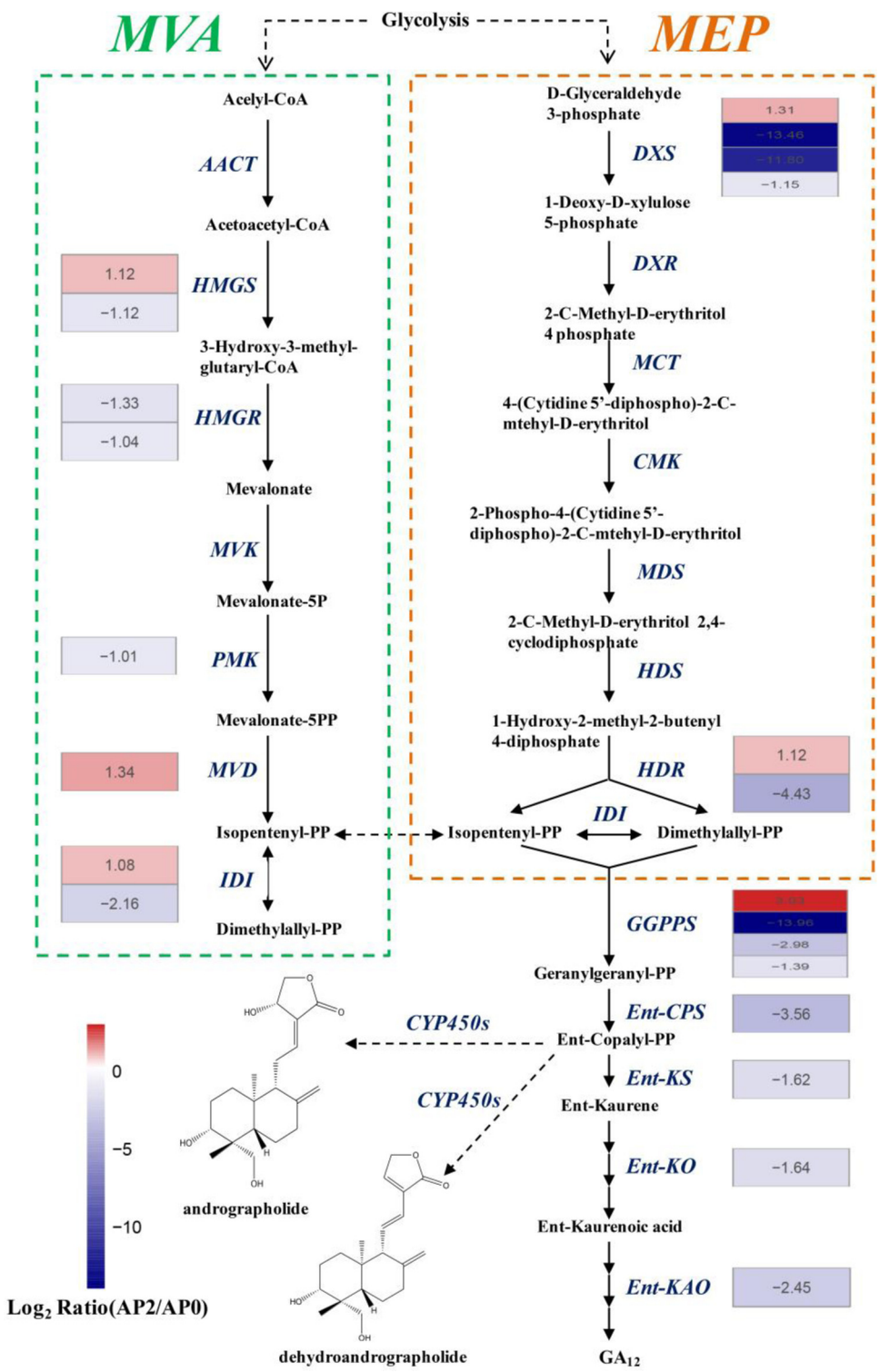

Fig. 4. DEGs involved in terpenoid backbone and diterpenoid biosynthesis pathway in transcriptome profile of *Andrographis paniculata* under continuous cropping. The number in the heat map represents the value of $\log_2$Ratio (AP2/AP0), $\log_2$Ratio(AP2/AP0) ≥ 1 (upregulation) or $\log_2$Ratio(AP2/AP0) ≤ −1 (downregulation) means DEGs. The red color in the heat map means upregulated expression, while blue color means downregulated expression. AACT, acetyl-CoA acyltransferase; HMGS, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; MVD, mevalonate 5-dinhophate decarboxylase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, MEP cytidyltransferase; CMK, 4-(Cytidine5-diphospho)-2-C-methylerythritol kinase; MDS, 2-C-Methy-D-erythritol2,4-cyclodiphosphate synthase; HDS, hydroxymethylbutenyl 4-diphosphatesynthase; HDR, 4-hydroxy-3-methylbut-2-enyldiphosphatereductase; IDI, isopentenyl diphosphate isomerase; GGPPS, geranylgeranyl diphosphate synthase; *ent*-CPS, *ent*-copalyl diphosphate synthase; *ent*-KS, *ent*-kaurene synthase; *ent*-KO, *ent*-kaurene oxidase; *ent*-KAO, *ent*-kaurenoic acid oxidase.

4.1.1.33). Sesquiterpenes and triterpenoids are usually synthesized by the MVA pathway. In *A. paniculata* transcriptome, 13 Unigenes were identified to be involved in MVA pathway (Supplementary Fig. S9), including 2 AACT genes, 2 HMGS genes, 5 HMGR genes, 1 MVK gene, 2 PMK genes and 1 MVD gene. Among them, 1 HMGS gene and the MVD gene were up-regulated, while 1 HMGS gene, 2 HMGR genes and 1 PMK gene were down-regulated in response to continuous cropping stress (Fig. 4; Supplementary data: sheet 5). After HMGS catalyzes the conversion of Acetoacetyl-CoA to 3-Hydroxy-3-methylglutaryl-CoA, the 3-Hydroxy-3-methylglutaryl-CoA is then converted into mevalonate by HMGR, which is the rate-limiting step in MVA pathway. Many studies have demonstrated the important regulatory role of HMGR in the biosynthesis of phytosterols, triterpenoids and sesquiterpenoid phytoalexins, although flux control often involves additional downstream enzymes (Tholl, 2015). Mevalonate-5-diphosphate produced by MVK is finally converted into IPP by PMK and MVD. Therefore, differential expression of HMGS and HMGR might affect the biosynthesis of 3-Hydroxy-3-methylglutaryl-CoA as well as mevalonate, and mevalonate-5-diphosphate and IPP contents could also be influenced by differential expression of PMK and MVD.

The MEP pathway consists of seven enzymatic steps orderly catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS, EC 2.2.1.7), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR, EC 1.1.1.267), MEP cytidyltransferase (MCT, EC 2.7.7.60), 4-(Cytidine 5-diphospho)-2-C-methylerythritol kinase (CMK, EC 2.7.1.148), 2-C-Methy-D-erythritol 2,4-cyclodiphosphate synthase (MDS, EC 4.6.1.12), hydroxymethylbutenyl 4-diphosphatesynthase (HDS, EC 1.17.7.1) and 4-hydroxy-3-methylbut-2-enyldiphosphatereductase (HDR, EC 1.17.1.2). The MEP pathway is essential for the biosynthesis of monoterpenes and diterpenes. In *A. paniculata* transcriptome, 18 Unigenes encoding these seven enzymes were found in this pathway (Supplementary Fig. S9) included 7 DXS genes, 2 DXR genes, 1 MCT gene, 1 CMK gene, 1 MDS gene, 2 HDS genes and 2 HDR genes. One in each of these DXS and HDR genes was up-regulated, while other 3 of DXS and 1 HDR genes were down-regulated in AP2 (Fig. 4; Supplementary data: sheet 5). The DXSs carry out the first enzymatic reaction in MEP pathway, converting the pyruvate and D-Glyceraldehyde 3-phosphate into 1-Deoxy-D-xylulose-5-phosphate. Many studies have confirmed the vital regulatory and rate-limiting functions of DXS in the biosynthesis of plastidial isoprenoids (Vranova et al., 2013). Overexpression or suppression of DXS would increase or decrease the levels of specific isoprenoid final products in Arabidopsis (Estévez et al., 2001), tomato (Enfissi et al., 2005) and potato (Morris, 2006). To date, DXS represents a vital target for manipulating the isoprenoid biosynthesis. On the other hand, HDRs catalyze the last reaction in MEP pathway and consequently HMBPP is converted to a mixture of IPP and dimethylallyl diphosphate (DMAPP) with a ratio of 5:1- 6:1 (Rohdich et al., 2000; Tritsch et al., 2010). Reportedly, HDRs also play important part in controlling the production of MEP-derived precursors (Botella-Pavía et al., 2004), therefore, in this study the downregulation of HDR gene might result in low contents of IPP and DMAPP. The DXS and HDR have also been proved to be the key enzymes that control flux in the MEP pathway (Rodríguez-Concepción, 2006).

The common precursor IPP can be converted to DMAPP by isopentenyl diphosphate isomerase (IDI, EC 5.3.3.2). Subsequently, IPP and DMAPP condensed to form geranyldiphosphate (GPP), diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) by a series of isoprenyl diphosphate synthases (IDSs), including geranyl diphosphatesynthase (GPPS, EC 2.5.1.1), farnesyl diphosphate synthase (FPPS, EC 2.5.1.10) and geranylgeranyl diphosphate synthase (GGPPS, EC 2.5.1.29). It has been reported that IDSs involved in terpenoid biosynthesis were encoded by small gene families with at least two members (Ma et al., 2012). In the present work, 3 IDI genes, 16 GPPS genes, 15 FPPS genes and 8 GGPPS genes were annotated in *A. paniculata* transcriptome (Supplementary Fig. S9). One in each of the IDI and GGPPS genes were up-regulated, while 1 IDI and 3 GGPPS genes were down-regulated after continuous cropping (Fig. 4; Supplementary data: sheet 5). The suppressed expression of GGPPS might reduce the synthesis of GGPP, the precursor of diterpenoid, and consequently down-regulated the diterpenoid synthesis.

As mentioned above, IPP and DMAPP are synthesized through the MVA and MEP pathways, 3 IPP and 1 DMAPP are head-to-tail assembled into GGPP catalyzed by GGPPS. The GGPP is then catalyzed by terpene synthases (TPSs) and various modifying enzymes, finally produce all kinds of downstream diterpenoid compounds, such as carotenoids, chlorophylls, gibberellins and specialized diterpenoids including taxol, tanshinones and andrographolide. In this study, almost all genes encoding enzymes involving diterpenoid biosynthesis were found in *A. paniculata* transcriptome (Supplementary Fig. S10), including 4 *ent*-copalyl diphosphate synthase (*ent*-CPS, EC 5.5.1.13) genes, 4 *ent*-kaurene synthase (*ent*-KS, EC 4.2.3.19) genes, 1 *ent*-kaurene oxidase (*ent*-KO, EC 1.14.13.78) gene, 3 *ent*-kaurenoic acid oxidase (*ent*-KAO, EC 1.14.13.79) genes, 5 gibberellin-44 dioxygenase (GA44ox, EC 1.14.11.12) genes, 4 gibberellin 2β-dioxygenase (GA2ox, EC 1.14.11.13) genes, 1 gibberellin 3β-dioxygenase (GA3ox, EC 1.14.11.15) gene, 1 *syn*-copalyl-diphosphate synthase (EC 5.5.1.14) gene and 3 casbene synthase (EC 4.2.38) genes. The *ent*-KO and one in each of the *ent*-CPS, *ent*-KS, *ent*-KAO genes were down-regulated in AP2 (Fig. 4; Supplementary data: sheet 5). Previous researches have demonstrated that *A. paniculata* is rich in labdane-related diterpenoids (LRD) such as andrographolide and dehydroandrographolide. The bicyclic labdane, core structure of all LRD, is derived from copalyl diphosphate (CPP), which is cyclized from GGPP through CPP synthase (CPS). Three kinds of CPSs are involved in the biosynthesis of LRD, and produce three types of CPP with different stereochemical configurations including *ent*-, *syn*- and normal, respectively. Based on the chemical structure, andrographolide and dehydroandrographolide should be initiated from the cyclization of GGPP to form *ent*-CPP catalyzed by *ent*-CPS. *Ent*-CPP is the direct precursor of andrographolides and gibberellins in *A. paniculata*, as well as of phytoalexins in rice and maize, and the steviol glycoside in *Stevia rebaudiana* (Harris et al., 2005; Prisic, 2004; Yoneda et al., 2017). As the key enzyme in the *ent*-CPP formation, expression level of *ent-CPS* was consistent with andrographolide accumulation in *A. paniculata* induced by Methyl jasmonates (Shen et al., 2016a). Furthermore, virus-induced gene silencing (VIGS) of the *ent*-CPS in *A. paniculata* resulted in a significant decreased of andrographolide accumulation (Shen et al., 2016b). Downregulation of *ent*-CPS will directly decrease the biosynthesis of *ent*-CPP, result in a declined accumulation of andrographolide and dehydroandrographolide. After the formation of *ent*-CPP catalyzed by *ent*-CPS, *ent*-KS converts *ent*-CPP to *ent*-kaurene, which will be subsequently converted into *ent*-kaurenoic acid catalyzed by *ent*-KO through a three-stage oxidation reaction. The next step is the *ent*-KAO catalyzation of a three-step oxidation reaction and oxidative extrusion of an endocyclic ring carbon from *ent*-kaurenoic acid via *ent*-7-hydroxy-kaurenoic acid to GA_{12}, the common precursor for all GAs in higher plants. The resulted product will then be further converted into bioactive GAs by several oxidation steps (Salazar-Cerezo et al., 2018). The important roles of *ent*-KS, *ent*-KO and *ent*-KAO in GA biosynthesis have been demonstrated in various plant species including Arabidopsis, rice, *Salvia miltiorrhiza*, *Scoparia dulcis*, *etc*. (Helliwell et al., 2001a, 2001b; Sakamoto et al., 2003; Su et al., 2016; Yamamura et al., 2018). Therefore, the down-regulation of these genes would affect the biosynthesis of downstream GAs.

Extensive works have confirmed that, alteration in metabolic flux by overexpression or suppression of genes in the core terpenoid biosynthesis pathways will promote pathway feedback or feedforward signals that modify the expression of up- or downstream genes, and finally affect the synthesis of targeted terpenoids (Tholl, 2015). Overexpressing any of the *SmHMGR*, *SmDXS2*, *SmGGPPS*, *SmHMGR-SmGGPPS*, *SmHMGR-SmDXR* or *SmGPPS-SmDXS2* in tanshinone biosynthetic pathway, could increase the tanshinone accumulation; while suppressed expression of *SmIPI* and *SmCPS* caused decline of tanshinone (Shi et al., 2016, 2014; Zhang et al., 2015b; Cheng et al., 2014; Kai et al., 2011; Liao et al., 2009). The similar response was also observed in artemisinin that, overexpression of *AaHMGR*, *AaFPS*, *AaHDR1* and *AaDXR* led to enhanced artemisinin biosynthesis, while suppression of *AaHDR1* resulted in decreased artemisinin content (Ma et al., 2017; Xiang et al., 2012; Aquil et al., 2009; Chen et al., 2000). Correlation between the accumulation of andrographolide and expression levels of genes including HMGS, HMGR, DXS, DXR and GGPS were also confirmed (Sharma et al., 2015). The present gene expression profiles showed that, expression levels of most genes participated in terpenoid biosynthesis were downregulated under continuous cropping, which accorded with the decreasing tendency of andrographolide and dehydroandrographolide contents (Fig. 1). In line with these observations, it was apparent that continuous cropping practice, a comprehensive environmental stress, could repress the expression of genes involved in terpenoid biosynthesis pathway and finally resulted in less synthesis of andrographolide and dehydroandrographolide in *A. paniculata*. However, the specific regulation mechanisms still need further investigation. In addition, it was reported that terpenoid precursor biosynthesis was controlled at both transcriptional and posttranscriptional levels

(Tholl, 2015; Vranova et al., 2013). In our study, *A. paniculata* might response to the complex stress of continuous cropping through regulating the expression of genes involved in the terpenoid biosynthesis pathway at transcriptional level.

4.2. Genes involved in phenylpropanoid biosynthesis pathway were suppressed under continuous cropping

In plant, the phenylpropanoid pathway serves as a rich source of metabolites, being required for biosynthesis of lignin, flavonoids, coumarins and hydroxycinnamic acids (Fraser and Chapple, 2011). Phenylpropanoids play important roles in plant defense, structural support, and survival (Vogt, 2010). Phenylpropanoid biosynthesis starts with the formation of the aromatic amino acid phenylalanine, and then phenylalanine converts to cinnamic acid catalyzed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.24). Subsequently, cinnamate 4-hydroxylase (C4H, EC 1.14.13.11) and 4-coumarate-CoA ligase (4CL, EC 6.2.1.12) catalyze the conversion of cinnamic acid to p-coumaroyl-CoA, which is the precursor for many phenylpropanoid products including lignin, anthocyanins, flavonols and isoflavonoids (Vogt, 2010). As the first and committed enzyme in the phenylpropanoid pathway, PAL activity determined the overall flux through the phenylpropanoid pathway and the rate of downstream products (Bate et al., 1994). Plants also respond to various stresses by regulating the PAL activity and phenylpropanoid accumulation (Dixon and Paiva, 1995). Inhibition of the activity of PAL with competitive PAL inhibitor AIP led to a reduced biosynthesis of subsequent phenolic compounds, and consequently reduced the oxidative browning in *A. annua* callus culture (Jones and Saxena, 2013). Being positioned at the second step of the phenylpropanoid pathway, C4H catalyzes conversion of trans-cinnamic acid to p-coumaric acid. It also controls the carbon fluxes, and the expression level of C4H gene would affect the biosynthesis of phenolics content and the stress response in plant (Cheng et al., 2018; Wang et al., 2017; Xia et al., 2017). As the third and key branch point enzyme of the general phenylpropanoid pathway, 4CL contributes in channelizing overall flux of the hydroxycinnamic acids into subsequent biosynthetic pathways (Rastogi et al., 2013). Characterization of 4CLs from various plants have suggested that a single 4CL was required for the lignin and flavonoid biosynthesis, alteration in expression level of 4CL would influence the biosynthesis of downstream products (Li et al., 2014b; Rao et al., 2015). In *A. paniculata* transcriptome, a total of 132 Unigenes were mapped to the phenylpropanoid biosynthesis pathway, of which, 35 genes encoding 12 enzymes were found differentially expressed under continuous cropping (Fig. 5; Supplementary data: sheet 6). Among them, 30 DEGs were down-regulated, including 1 PAL gene, 4 C4H genes, 3 4CL genes, 1 cinnamoyl-CoA reductase (CCR, EC 1.2.1.44) gene, 2 β-glucoside (EC 3.2.1.21) genes, 1 HST (shikimate O-hydroxycinnamoyltransferase, EC 2.3.1.133) gene, 10 peroxidase (EC 1.11.1.7) genes, 1 caffeic acid 3-O-methyltransferase (COMT, EC 2.1.1.68) gene, 1 F5H gene, 2 coniferyl-aldehyde dehydrogenase (REF1, EC 1.2.1.68) genes, 1 C3H (p-coumarate 3-hydroxylase, EC 1.14.13.-) gene and 3 caffeoyl-CoA O-methyltransferase (CCoAOMT, EC 2.1.1.104) genes. In addition, 5 DEGs were up-regulated, including 1 PAL gene, one 4CL gene, 1 β-glucoside gene and 2 peroxidase genes. Being the key and rate-limiting enzymes involved in phenylpropanoid biosynthesis pathway (Vogt, 2010), the suppression of PAL, C4H and 4CL genes might directly lead to decline of the cinnamic acid and p-coumaroyl-CoA synthesis, and then affect the downstream product synthesis. Taken together, these results indicated that genes involved in phenylpropanoid biosynthesis pathway were suppressed under continuous cropping, which would also affect the downstream flavonoids synthesis.

4.3. Genes involved in flavonoids biosynthesis were down-regulated by continuous cropping

Flavonoids are the most common compounds in the plant kingdom and have diverse functions in plants, including stress defense, protection against ultraviolet radiation and phytopathogens, auxin transport inhibition, allelopathy, and flower coloring (Buer et al., 2010; Falcone Ferreyra et al., 2012). Flavonoids originate from the phenylpropanoid pathway; phenylalanine is transformed into 4-coumaroyl-CoA orderly catalyzed by PAL, C4H and 4CL, which eventually enters the flavonoid biosynthesis pathway. Chalcone synthase (CHS, EC 2.3.1.74) is the first enzyme specifically for the flavonoid pathway, produces chalcones from which all flavonoids derive from. Subsequently, chalcone isomerase (CHI, EC 5.5.1.6) catalyzes the isomerization of chalcones into naringenin, which then can be converted to eriodictyol and dihydrotricetin by flavonoid 3′-hydroxylase (F3′H, EC 1.14.13.21) and flavonoid 3′,5′-hydroxylase (F3′5′H), respectively. The flavone synthase (FNS, EC 1.14.11.22) catalyzes the conversion of flavanones to flavones which are subsequently converted into dihydroflavonols catalyzed by flavanone 3-hydroxylase (F3H, EC 1.14.11.9). Then, dihydroflavonols can be converted into flavonols and leucoanthocyanidin catalyzed by flavonol synthase (FLS, EC 1.14.11.23) and dihydroflavonol 4-reductase (DFR, EC 1.1.1.219), respectively. Leucoanthocyanidin can be converted into anthocyanidins by the action of anthocyanidin synthase (ANS, EC 1.14.11.19). In *A. paniculata* transcriptome, 32 Unigenes were mapped to the flavonoid biosynthesis pathway, of which, 18 DEGs encoding 9 enzymes were found (Fig. 6; Supplementary data: sheet 7). After continuous cropping, except one of the F3′H genes was up-regulated, the remained 17 DEGs including 4 C4H genes, 2 CHS genes, 1 CHI gene, 3 F3′H genes, 1 FLS gene, 1 ANS gene, 3 CCoAOMT genes, 1 C3H gene and 1 HST gene, were all down-regulated. Similar gene expression pattern was also found on the *R. glutinosa* under consecutive monoculture (Tian et al., 2017). As mentioned above, the three core enzymes in the general phenylpropanoid biosynthesis pathway, PAL, C4H and 4CL, played vital roles in regulating the downstream flavonoids biosynthesis. Expression of CsPAL and CsC4H in tea had a positive correlation with the catechin contents, suggested a critical role of PAL and C4H in catechin biosynthesis and a crosstalk between the phenylpropanoid and flavonoids biosynthesis pathways (Singh et al., 2009). In leaves of three crabapple cultivars (Malus spp.), expression levels of PAL, C4H and 4CL were consistent with the content of flavonoids, especially flavonols (Zhang et al., 2015a). Similar results also found in *Arnebia euchroma* and *Selaginella bryopteris* (Singh et al., 2010, 2018). Therefore, downregulated C4H might lead to less synthesis of chalcones. CHS is the first enzyme specific for the flavonoid pathway, whose activity will directly impact on the flavonoid biosynthesis. RNAi of CHS in tomato plants resulted in a reduction of total flavonoid levels compared with the corresponding wild type (Schijlen et al., 2007). The expression pattern of PAL, CHS and ANS were also reported along with the correspondingly varied contents of total flavonoids and anthocyanins in developing olive fruit (Martinelli and Tonutti, 2012). Also, 3 UDP-glycosyltransferase 73C (UGT73C, EC 2.4.1.-) genes, 1 flavonol 3-O-methyltransferase (EC 2.1.1.76) gene and 1 flavonoid 3-O-glucosyltransferase (EC 2.4.1.91) gene were found differentially expressed in the flavone and flavonol biosynthesis pathway (Supplementary Fig. S11; Supplementary data: sheet 7). Among these DEGs, 2 of the UGT73C were up-regulated, and the remaining genes were down-regulated. UDP-glycosyltransferases encoding enzymes catalyze the glycosylation reaction of various flavonoids. Flavonols commonly occur in plant as glycosides with greatly improved solubility of the parent flavonol structures and better transport into the vacuole, as well as protecting their reactive groups from oxidation by free radicals (Rouhier et al., 2008; Vogt and Jones, 2000). Thus, decreased expression of the above enzyme genes involved in flavonoids biosynthesis pathway might result in down-regulated biosynthesis of their downstream products and finally decrease the flavonoids contents. The

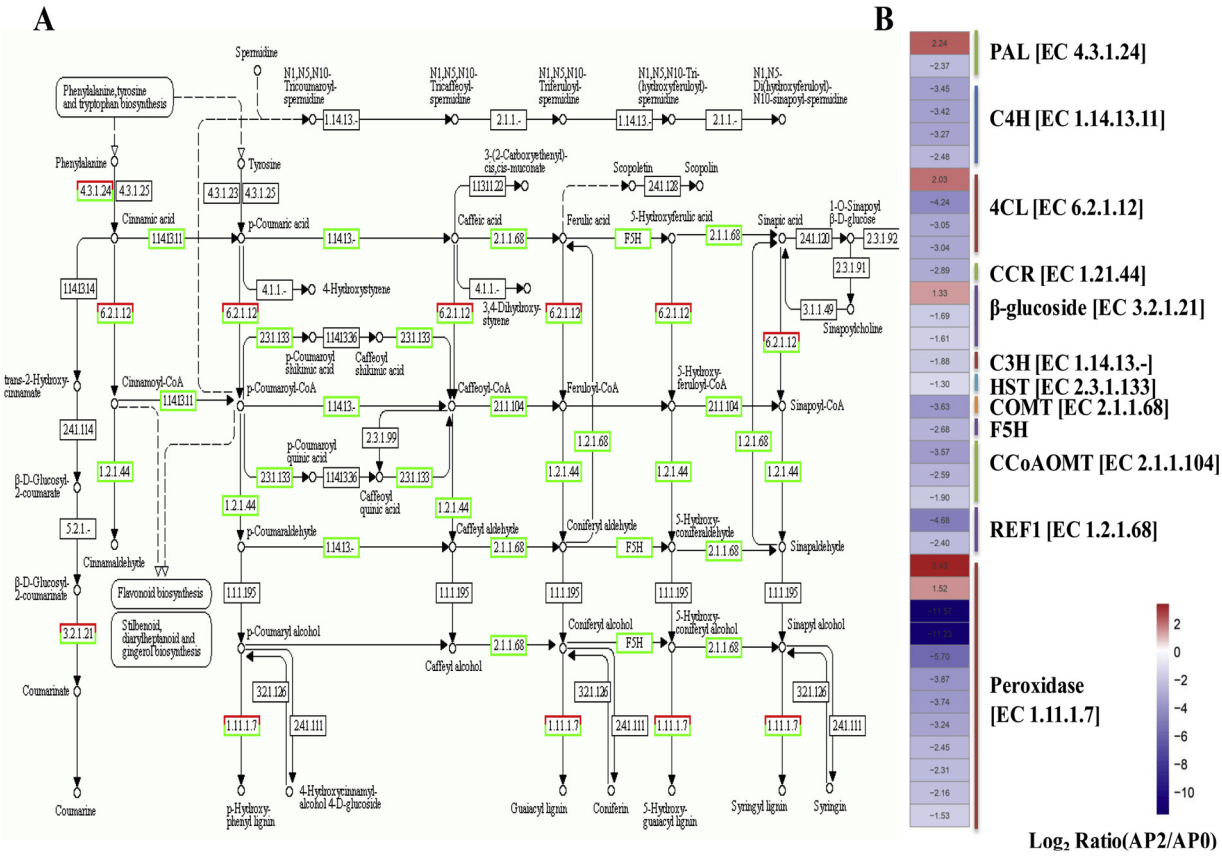

Fig. 5. (A) DEGs involved in phenylpropanoid biosynthesis pathway in transcriptome profile of *Andrographis paniculata* under continuous cropping. Red boxes indicate DEGs up-regulated and green boxes mean DEGs down-regulated under continuous cropping. (B) The heat map showing an expression of genes involved in phenylpropanoid biosynthesis pathway based on $\log_2$Ratio(AP2/AP0). The number in the heat map represents the value of $\log_2$Ratio(AP2/AP0), $\log_2$Ratio(AP2/AP0) ≥ 1 (upregulation) or $\log_2$Ratio(AP2/AP0) ≤ -1 (downregulation) means DEGs. The red color in the heat map means upregulated expression, while blue color means downregulated expression. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; C3H, p-coumarate 3-hydrolase; 4CL, 4-hydroxy cinnamoyl COA ligase; HST, shikimate O-hydroxycinnamoyltransferase; COMT, caffeic acid 3-O-methyltransferase; CCR, cinnamoyl-CoA reductase; CCoAOMT, caffeoyl-CoA O-methyltransferase; REF1, coniferyl-aldehyde dehydrogenase.

significant decrease content of total flavonoids in AP2 (Fig. 1), indicated that continuous cropping down-regulated the expression of genes involved in flavonoids biosynthesis and led to declined accumulation of total flavonoids.

4.4. The complexity of continuous cropping stress on A. paniculata

As our results shown, most genes involved in biosynthesis of terpenoids, phenylpropanoid and flavonoids in *A. paniculata* were down-regulated after continuous cropping, which was consistent with the genes' expression profile of *R. glutinosa* under continuous cropping, salt, and drought stresses (Tian et al., 2017). In general, secondary metabolites syntheses were considered increased under stress condition. However, in this study, the active ingredients of *A. paniculata* were significantly decreased after continuous cropping, such result was also observed in medicinal plants such as *R. glutinosa* (Wu et al., 2015), *Pseudostellariae heterophylla* (Chen et al., 2017) and *S. miltiorrhiza* (Liu et al., 2013). The formation and aggravation of continuous cropping problem in medicinal plants are extremely complicated that involving the complex rhizosphere microecosystems including root exudates, plants, and rhizosphere microbes. Secondary metabolites such as flavonoids, phenolics and terpenoids have been widely accepted as mediators that act between inter- and intraspecific root (Bais et al., 2004; Weston et al., 2012). Previous studies also indicated that the flavonoids could be secreted into rhizosphere passively by decomposing the root cap and border cells to respond to elicitors (Cesco et al., 2010). A significant increase of MDA content after continuous cropping was also found in *A. paniculata* (Li et al., 2017). Therefore, continuous cropping might disorganize the structure stability of membrane and metabolism in *A. paniculata*, which would promote the release of the allelopathic chemicals like terpenoids and flavonoids, thus decrease the accumulation of them. Moreover, the flowering and primary fruit stages of *A. paniculata* were both brought forward after continuous cropping (Li et al., 2017). It is known that flowering is the key point of the conversion from vegetative period to reproductive stage in plant. When facing the environmental stress, earlier conversion from vegetative period to reproductive stage were found in some plants. The accumulation of active ingredients in *A. paniculata* increased with the length of the vegetative period, and quickly increased before the initial time of flowering and reached the highest level in initial time of flowering (Chen et al., 2014). Therefore, the decline of active ingredients in *A. paniculata* after continuous cropping might be partially attributed to the shortened vegetative period as well as the accumulation time of active ingredients. At molecular level, all these changes induced by continuous cropping might depend on altered expression of related genes in *A. paniculata*, and further experimental studies are needed to illuminate the underlying mechanism.

It was shown that the genes' expression profile of *R. glutinosa* under continuous cropping was very similar to the profiles of its responses to salt and drought stresses (Tian et al., 2017). In current study, a significant influence of continuous cropping on genes' expression of *A. paniculata* was also revealed. Functional analysis indicated that these identified DEGs were involved in a wide range of biological processes in *A. paniculata*, of which, most of DEGs were also reported in other plant

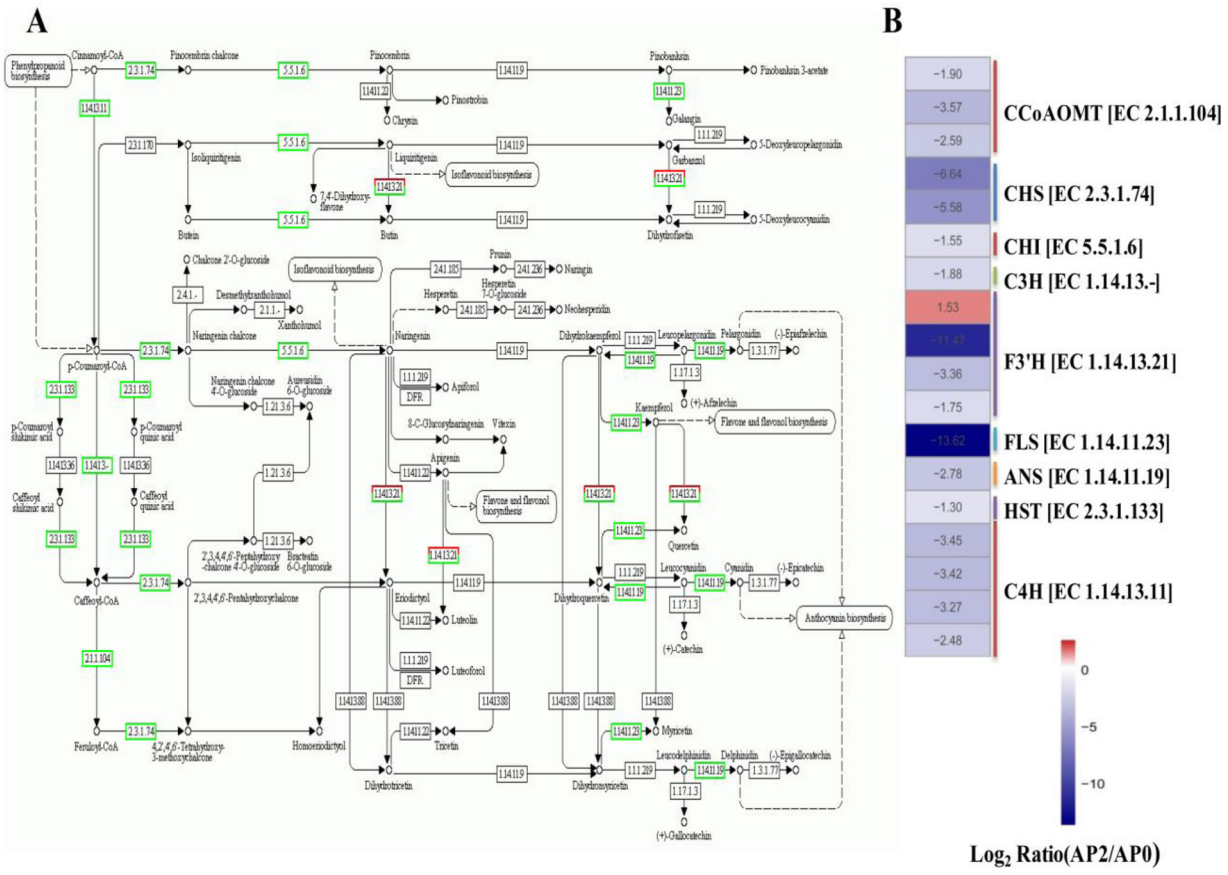

Fig. 6. (A) DEGs involved in flavonoid biosynthesis pathway in transcriptome profile of *Andrographis paniculata* under continuous cropping. Red boxes indicate DEGs up-regulated and green boxes mean DEGs down-regulated under continuous cropping. (B) The heat map showing an expression of genes involved in flavonoid biosynthesis pathway based on $\log_2$Ratio(AP2/AP0). The number in the heat map represents the value of $\log_2$Ratio(AP2/AP0), $\log_2$Ratio(AP2/AP0) ≥ 1 (upregulation) or $\log_2$Ratio(AP2/AP0) ≤ -1 (downregulation) means DEGs. The red color in the heat map means upregulated expression, while blue color means downregulated expression. C4H, cinnamate 4-hydroxylase; CHS, Chalcone synthase; CHI, chalcone isomerase; C3H, p-coumarate 3-hydrolase; HST, shikimate O-hydroxycinnamoyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; FLS, flavonol synthase; flavonoid 3′-hydroxylase; ANS, anthocyanidin synthase.

species under other abiotic stresses. However, to date, data is lacking on the gene expression of *A. paniculata* under abiotic stress, such as salt, drought, heat, and cold *etc*. A proteomic analysis of *A. paniculata* leaf identified three known proteins that induced by salt stress, including ribulose-1,5-bisphosphate carboxylase/oxygenase, superoxide dismutase (Cu-Zn) and Os04g0416400 protein (fragment) (Talei et al., 2014). Two DEGs encoding ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (Unigene0016160) and manganese superoxide dismutase (Unigene0004508), respectively, were found upregulated by continuous cropping in present study. Characterization of novel genes uniquely response to continuous cropping stress of *A. paniculata* awaits further studies on gene expression profiles of *A. paniculata* responses to other stresses.

5. Conclusions

This study provides the first large-scale transcriptome data set from *A. paniculata* subjected to continuous cropping stress. More than 231 million clean reads were generated and assembled into 43,683 Unigenes. 6193 DEGs were identified and most of them were down-regulated by continuous cropping. Functional annotation of DEGs based on GO and KEGG showed that large part of down-regulated DEGs involved in plant metabolic processes, particularly the secondary metabolic pathways. Furthermore, biosynthesis pathways of active ingredients in *A. paniculata* were mainly emphasized, including terpenoid backbone biosynthesis, diterpenoid biosynthesis, phenylpropanoid biosynthesis and flavonoids biosynthesis, and found that DEGs involved in these pathways were suppressed under continuous cropping. Positive correlation between the gene expression and the active ingredients contents was found that contents of andrographolide, dehydroandrographolide and total flavonoids were significantly declined in *A. paniculata* subjected to continuous cropping. These results suggested that continuous cropping repressed the gene expression in these pathways and finally decreased the biosynthesis of target products, although the underlying regulation mechanism remains unclear. The current study enhances the understanding of the metabolic regulation at transcriptional level of *A. paniculata* in response to continuous cropping stress, and provides an extensive sequence resource for further studies of the specific continuous cropping mechanism at molecular level in *A. paniculata*.

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Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2019.05.067.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Aquil, S., Husaini, A.M., Abdin, M.Z., Rather, G.M., 2009. Overexpression of the HMG-CoA reductase gene leads to enhanced artemisinin biosynthesis in transgenic *Artemisia annua* plants. Planta Med. 75, 1453–1458.

Bais, H.P., Park, S., Weir, T.L., Callaway, R.M., Vivanco, J.M., 2004. How plants communicate using the underground information superhighway. Trends Plant Sci. 9, 26–32.

Bate, N.J., Orr, J., Ni, W., Meromi, A., Nadler-Hassar, T., Doerner, P.W., Dixon, R.A., Lamb, C.J., Elkind, Y., 1994. Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proc. Natl. Acad. Sci. U. S. A. 91, 7608–7612.

Bhan, M.K., Dhar, A.K., Khan, S., Lattoo, S.K., Gupta, K.K., Choudhary, D.K., 2006. Screening and optimization of *Andrographis paniculata* (Burm.f.) Nees for total andrographolide content, yield and its components. Sci. Hortic.-Amsterdam 107, 386–391.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120.

Botella-Pavía, P., Besumbes, Ó., Phillips, M.A., Carretero-Paulet, L., Boronat, A., Rodríguez-Concepción, M., 2004. Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. Plant J. 40, 188–199.

Buer, C.S., Imin, N., Djordjevic, M.A., 2010. Flavonoids: new roles for old molecules. J. Integr. Plant Biol. 52, 98–111.

Cesco, S., Neumann, G., Tomasi, N., Pinton, R., Weisskopf, L., 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil 329, 1–25.

Chen, D., Ye, H., Li, G., 2000. Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci. 155, 179–185.

Chen, J., Wu, L., Xiao, Z., Wu, Y., Wu, H., Qin, X., Wang, J., Wei, X., Khan, M.U., Lin, S., Lin, W., 2017. Assessment of the diversity of Pseudomonas spp. and fusarium spp. In radix pseudostellariae rhizosphere under monoculture by combining DGGE and quantitative PCR. Front. Microbiol. 8, 1748.

Chen, J., Gu, W., Duan, J., Su, S., Shao, J., Sun, H., Geng, C., 2014. Study on active components of *Andrographis paniculata* at various growth periods and differential expression of key enzyme gene. Chinese Traditional and Herbal Drugs 45, 3149–3152.

Cheng, Q., Su, P., Hu, Y., He, Y., Gao, W., Huang, L., 2014. RNA interference-mediated repression of SmCPS (copalyldiphosphate synthase) expression in hairy roots of *Salvia miltiorrhiza* causes a decrease of tanshinones and sheds light on the functional role of SmCPS. Biotechnol. Lett. 36, 363–369.

Cheng, S., Yan, J., Meng, X., Zhang, W., Liao, Y., Ye, J., Xu, F., 2018. Characterization and expression patterns of a cinnamate-4-hydroxylase gene involved in lignin biosynthesis and in response to various stresses and hormonal treatments in Ginkgo biloba. Acta Physiol. Plant. 40, 7.

Dixon, R.A., Paiva, N.L., 1995. Stress-induced phenylpropanoid metabolism. Plant Cell 7, 1085–1097.

Dong, L., Xu, J., Feng, G., Li, X., Chen, S., 2016. Soil bacterial and fungal community dynamics in relation to *Panax notoginseng* death rate in a continuous cropping system. Sci. Rep. 6, 31862.

Dong, L., Xu, J., Zhang, L., Yang, J., Liao, B., Li, X., Chen, S., 2017. High-throughput sequencing technology reveals that continuous cropping of American ginseng results in changes in the microbial community in arable soil. Chin. Med. 12, 18.

Enfissi, E.M.A., Fraser, P.D., Lois, L., Boronat, A., Schuch, W., Bramley, P.M., 2005. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol. J. 3, 17–27.

Estévez, J.M., Cantero, A., Reindl, A., Reichler, S., León, P., 2001. 1-deoxy-d-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J. Biol. Chem. 276, 22901–22909.

Falcone Ferreyra, M.L., Rius, S.P., Casati, P., 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front. Plant Sci. 3, 222.

Fraser, C.M., Chapple, C., 2011. The phenylpropanoid pathway in Arabidopsis. Arabidopsis Book 9, e0152.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 29, 644–652.

Harris, L.J., Saparno, A., Johnston, A., Prisic, S., Xu, M., Allard, S., Kathiresan, A., Ouellet, T., Peters, R.J., 2005. The Maize An2 gene is induced by Fusarium attack and encodes an ent-Copalyl diphosphate synthase. Plant Mol. Biol. 59, 881–894.

Helliwell, C.A., Chandler, P.M., Poole, A., Dennis, E.S., Peacock, W.J., 2001a. The CYP88A cytochrome P450, ent-kaurenoic acid oxidase, catalyzes three steps of the gibberellin biosynthesis pathway. Proc. Natl. Acad. Sci. U. S. A. 98, 2065–2070.

Helliwell, C.A., Sullivan, J.A., Mould, R.M., Gray, J.C., Peacock, W.J., Dennis, E.S., 2001b. A plastid envelope location of Arabidopsis ent-kaurene oxidase links the plastid and endoplasmic reticulum steps of the gibberellin biosynthesis pathway. Plant J. 28, 201–208.

Jones, A.M.P., Saxena, P.K., 2013. Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: a novel approach to reduce oxidative browning in plant tissue culture. PLoS One 8, e76802.

Kai, G., Xu, H., Zhou, C., Liao, P., Xiao, J., Luo, X., You, L., Zhang, L., 2011. Metabolic engineering tanshinone biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures. Metab. Eng. 13, 319–327.

Li, B., Dewey, C.N., 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12, 323.

Li, J., Chen, X., Tang, X., Zeng, X., Zhuo, Y., He, R., Zhan, R., 2017. Effects of continuous cropping on *Andrographis paniculata* growth and herb quality. Traditional Chinese Drug Research and Clinical Pharmacology 28, 797–801.

Li, J., Chen, X., Tang, X., He, R., Zhan, R., 2018. SSR loci information analysis in transcriptome of *Andrographis paniculata*. China J. Chinese Matera Med. 43, 2503–2508.

Li, M.J., Yang, Y.H., Chen, X.J., Wang, F.Q., Lin, W.X., Yi, Y.J., Zeng, L., Yang, S.Y., Zhang, Z.Y., 2013. Transcriptome/degradome-wide identification of *R. glutinosa* miRNAs and their targets: the role of miRNA activity in the replanting disease. PLoS One 8, e68531.

Li, M., Zhou, X., Lu, Z., 2010. Allelopathy of *Andrographis paniculata* vegetative. Journal of Chinese Medicinal Materials 33, 1829–1833.

Li, Y., Li, M., Tang, K., Zhao, P., Dong, S., Huang, J., Li, L., Pang, D., 2014a. Study on autotoxicity of *Andrographis paniculata* (Burm. F.) Nees vegetative. Northern Horticulture 12, 157–160.

Li, Z.B., Li, C.F., Li, J., Zhang, Y.S., 2014b. Molecular cloning and functional characterization of two divergent 4-coumarate: coenzyme A ligases from Kudzu (*Pueraria lobata*). Biol. Pharm. Bull. 37, 113–122.

Liao, P., Zhou, W., Zhang, L., Wang, J., Yan, X., Zhang, Y., Zhang, R., Li, L., Zhou, G., Kai, G., 2009. Molecular cloning, characterization and expression analysis of a new gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from Salvia miltiorrhiza. Acta Physiol. Plant. 31, 565–572.

Liu, W., Zhang, L., Zhang, Y., Zhou, J., Sun, Q., Wang, X., Li, F., 2013. Influence of continuous cropping years on yield and active compounds in *Salvia miltiorrhiza* f. *alba*. China J. Chinese Matera Med. 38, 4252–4256.

Loreto, F., Dicke, M., Schnitzler, J., Turlings, T.C.J., 2014. Plant volatiles and the environment. Plant Cell Environ. 37, 1905–1908.

Ma, D., Li, G., Zhu, Y., Xie, D., 2017. Overexpression and suppression of *Artemisia annua* 4-hydroxy-3-methylbut-2-enyl diphosphate reductase 1 gene (AaHDR1) differentially regulate artemisinin and terpenoid biosynthesis. Front. Plant Sci. 8, 77.

Ma, Y., Yuan, L., Wu, B., Li, X.E., Chen, S., Lu, S., 2012. Genome-wide identification and characterization of novel genes involved in terpenoid biosynthesis in *Salvia miltiorrhiza*. J. Exp. Bot. 63, 2809–2823.

Malahubban, M., Alimon, A.R., Sazili, A.Q., Fakurazi, S., Zakry, F.A., 2013. Phytochemical analysis of *Andrographis paniculata* and *Orthosiphon stamineus* leaf extracts for their antibacterial and antioxidant potential. Trop. Biomed. 30, 467–480.

Martinelli, F., Tonutti, P., 2012. Flavonoid metabolism and gene expression in developing olive (*Olea europaea* L.) fruit. Plant Biosyst. 146, 164–170.

Morris, W.L., 2006. Overexpression of a bacterial 1-deoxy-D-xylulose 5-phosphate synthase gene in potato tubers perturbs the isoprenoid metabolic network: implications for the control of the tuber life cycle. J. Exp. Bot. 57, 3007–3018.

Nagegowda, D.A., 2010. Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation. FEBS Lett. 584, 2965–2973.

Prisic, S., 2004. Rice contains two disparate ent-copalyl diphosphate synthases with distinct metabolic functions. Plant Pysiology 136, 4228–4236.

Rao, G., Pan, X., Xu, F., Zhang, Y., Cao, S., Jiang, X., Lu, H., 2015. Divergent and overlapping function of five 4-coumarate/coenzyme A ligases from *Populus tomentosa*. Plant Mol. Biol. Rep. 33, 841–854.

Rastogi, S., Kumar, R., Chanotiya, C.S., Shanker, K., Gupta, M.M., Nagegowda, D.A., Shasany, A.K., 2013. 4-Coumarate: CoA ligase partitions metabolites for eugenol biosynthesis. Plant Cell Physiol. 54, 1238–1252.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2009. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–140.

Rodríguez-Concepción, M., 2006. Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. Phytochem. Rev. 5, 1–15.

Rohdich, F., Wungsintaweekul, J., Eisenreich, W., Richter, G., Schuhr, C.A., Hecht, S., Zenk, M.H., Bacher, A., 2000. Biosynthesis of terpenoids: 4-Diphosphocytidyl-2C-methyl-D-erythritol synthase of *Arabidopsis thaliana*. PNAS 97, 6451–6456.

Rouhier, N., Lemaire, S.D., Jacquot, J., 2008. The role of glutathione in photosynthetic organisms: emerging functions for glutaredoxins and glutathionylation. Annu. Rev. Plant Biol. 59, 143–166.

Sakamoto, T., Morinaka, Y., Ishiyama, K., Kobayashi, M., Itoh, H., Kayano, T., Iwahori, S., Matsuoka, M., Tanaka, H., 2003. Genetic manipulation of gibberellin metabolism in transgenic rice. Nat. Biotechnol. 21, 909–913.

Salazar-Cerezo, S., Martínez-Montiel, N., García-Sánchez, J., Pérez-y-Terrón, R., Martínez-Contreras, R.D., 2018. Gibberellin biosynthesis and metabolism: a convergent route for plants, fungi and bacteria. Microbiol. Res. 208, 85–98.

Schijlen, E.G.W.M., de Vos, C.H.R., Martens, S., Jonker, H.H., Rosin, F.M., Molthoff, J.W., Tikunov, Y.M., Angenent, G.C., van Tunen, A.J., Bovy, A.G., 2007. RNA interference

silencing of chalcone synthase, the first step in the flavonoid biosynthesis pathway, leads to parthenocarpic tomato fruits. Plant Physiol. 144, 1520–1530.
Sharma, S.N., Jha, Z., Sinha, R.K., Geda, A.K., 2015. Jasmonate-induced biosynthesis of andrographolide in *Andrographis paniculata*. Physiol Plantarum. 153, 221–229.
Sheeja, K., Guruvayoorappan, C., Kuttan, G., 2007. Antiangiogenic activity of *Andrographis paniculata* extract and andrographolide. Int. Immunopharmacol. 7, 211–221.
Sheeja, K., Shihab, P.K., Kuttan, G., 2008. Antioxidant and anti-inflammatory activities of the plant *Andrographis paniculata* Nees. Immunopharm. Immunot. 28, 129–140.
Shen, Q., Li, L., Jiang, Y., Wang, Q., 2016a. Functional characterization of ent-copalyl diphosphate synthase from *Andrographis paniculata* with putative involvement in andrographolides biosynthesis. Biotechnol. Lett. 38, 131–137.
Shen, Q., Liu, Q., Li, C., Fu, Y., Wang, Q., 2016b. Functional characterization of ApCPS involved in Andrographolides biosynthesis by Virus-induced gene silencing. Acta Botanica Boreali-Occidentalia Sinica 36, 17–22.
Shi, M., Luo, X., Ju, G., Li, L., Huang, S., Zhang, T., Wang, H., Kai, G., 2016. Enhanced diterpene tanshinone accumulation and bioactivity of transgenic *Salvia miltiorrhiza* hairy roots by pathway engineering. J. Agr. Food Chem. 64, 2523–2530.
Shi, M., Luo, X., Ju, G., Yu, X., Hao, X., Huang, Q., Xiao, J., Cui, L., Kai, G., 2014. Increased accumulation of the cardio-cerebrovascular disease treatment drug tanshinone in *Salvia miltiorrhiza* hairy roots by the enzymes 3-hydroxy-3-methylglutaryl CoA reductase and 1-deoxy-d-xylulose 5-phosphate reductoisomerase. Funct. Integr. Genomic. 14, 603–615.
Singh, K., Kumar, S., Rani, A., Gulati, A., Ahuja, P.S., 2009. Phenylalanine ammonia-lyase (PAL) and cinnamate 4-hydroxylase (C4H) and catechins (flavan-3-ols) accumulation in tea. Funct. Integr. Genomic. 9, 125–134.
Singh, R.S., Gara, R.K., Bhardwaj, P.K., Kaachra, A., Malik, S., Kumar, R., Sharma, M., Ahuja, P.S., Kumar, S., 2010. Expression of 3-hydroxy-3-methylglutaryl-CoA reductase, p-hydroxybenzoate-m-geranyltransferase and genes of phenylpropanoid pathway exhibits positive correlation with shikonins content in arnebia [*Arnebia euchroma* (Royle) Johnston]. BMC Mol. Biol. 11, 88.
Singh, R.S., Kesari, R., Kumar, U., Jha, V.K., Kumar, A., Kumar, T., Pal, A.K., Singh, P.K., 2018. Candidate genes of flavonoid biosynthesis in *Selaginella bryopteris* (L.) Baker identified by RNA-Seq. Funct. Integr. Genomic. 18, 505–517.
Soto, G., Stritzler, M., Lisi, C., Alleva, K., Pagano, M.E., Ardila, F., Mozzicafreddo, M., Cuccioloni, M., Angeletti, M., Ayub, N.D., 2011. Acetoacetyl-CoA thiolase regulates the mevalonate pathway during abiotic stress adaptation. J. Exp. Bot. 62, 5699–5711.
Su, P., Tong, Y., Cheng, Q., Hu, Y., Zhang, M., Yang, J., Teng, Z., Gao, W., Huang, L., 2016. Functional characterization of ent-copalyl diphosphate synthase, kaurene synthase and kaurene oxidase in the *Salvia miltiorrhiza* gibberellin biosynthetic pathway. Sci. Rep. 6, 23057.
Talei, D., Valdiani, A., Rafii, M.Y., Maziah, M., 2014. Proteomic analysis of the salt-responsive leaf and root proteins in the anticancer plant *Andrographis paniculata* Nees. PLoS One 9, e112907.
Tholl, D., 2015. Biosynthesis and biological functions of terpenoids in plants. Adv. Biochem. Eng. Biotechnol. 148, 63–106.
Tian, Y., Feng, F., Zhang, B., Li, M., Wang, F., Gu, L., Chen, A., Li, Z., Shan, W., Wang, X., Chen, X., Zhang, Z., 2017. Transcriptome analysis reveals metabolic alteration due to consecutive monoculture and abiotic stress stimuli in *Rehamannia glutinosa* Libosch. Plant Cell Rep. 36, 859–875.
Tritsch, D., Hemmerlin, A., Bach, T.J., Rohmer, M., 2010. Plant isoprenoid biosynthesis via the MEP pathway: In vivo IPP/DMAPP ratio produced by (E)-4hydroxy-3-methylbut-2-enyl diphosphate reductase in tobacco BY-2 cell cultures. FEBS Lett. 584, 129–134.
Vogt, T., 2010. Phenylpropanoid biosynthesis. Mol. Plant 3, 2–20.
Vogt, T., Jones, P., 2000. Glycosyltransferases in Plant Natural Product Synthesis: Characterization of a Supergene Family, vol. 5. Elsevier Ltd, England, pp. 380–386.
Vranova, E., Coman, D., Gruissem, W., 2013. Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu. Rev. Plant Biol. 64, 665–700.
Walker, T.S., 2003. Root exudation and rhizosphere biology. Plant Physiol. 132, 44–51.
Wang, A., Zhu, M., Luo, Y., Liu, Y., Li, R., Kou, M., Wang, X., Zhang, Y., Meng, X., Zheng, Y., Ma, D., 2017. A sweet potato cinnamate 4-hydroxylase gene, IbC4H, increases phenolics content and enhances drought tolerance in tobacco. Acta Physiol. Plant. 39, 276.
Weston, L.A., Ryan, P.R., Watt, M., 2012. Mechanisms for cellular transport and release of allelochemicals from plant roots into the rhizosphere. J. Exp. Bot. 63, 3445–3454.
Wink, M., 2010. Biochemistry of Terpenoids: Monoterpenes, Sesquiterpenes and Diterpenes. Wiley-Blackwell, Oxford, UK, pp. 258–303.
Wu, L., Li, Z., Li, J., Khan, M.A., Huang, W., Zhang, Z., Lin, W., 2013. Assessment of shifts in microbial community structure and catabolic diversity in response to *Rehmannia glutinosa* monoculture. Agric., Ecosyst. Environ., Appl. Soil Ecol. 67, 1–9.
Wu, L., Wang, J., Huang, W., Wu, H., Chen, J., Yang, Y., Zhang, Z., Lin, W., 2015. Plant-microbe rhizosphere interactions mediated by *Rehmannia glutinosa* root exudates under consecutive monoculture. Sci. Rep. 5, 15871.
Xia, J., Liu, Y., Yao, S., Li, M., Zhu, M., Huang, K., Gao, L., Xia, T., 2017. Characterization and expression profiling of *Camellia sinensis* cinnamate 4-hydroxylase genes in phenylpropanoid pathways. GenesBasel 8, 193.
Xiang, L., Zeng, L., Yuan, Y., Chen, M., Wang, F., Liu, X., Zeng, L., Lan, X., Liao, Z., 2012. Enhancement of artemisinin biosynthesis by overexpressing dxr, cyp71av1 and cpr in the plants of *Artemisia annua* L. Plant Omics Journal 5, 503–507.
Xuan, T.D., Shinkichi, T., Khanh, T.D., Chung, I.M., 2005. Biological control of weeds and plant pathogens in paddy rice by exploiting plant allelopathy: an overview. Crop Prot. 24, 197–206.
Yamamura, Y., Taguchi, Y., Ichitani, K., Umebara, I., Ohshita, A., Kurosaki, F., Lee, J., 2018. Characterization of ent-kaurene synthase and kaurene oxidase involved in gibberellin biosynthesis from *Scoparia dulcis*. J. Nat Med-Tokyo 72, 456–463.
Yang, Y.H., Zhang, Z.Y., Fan, H.M., Zhao, Y.D., Li, M.J., Li, J., Chen, J.Y., Lin, W.X., Chen, X.J., 2013. Construction and analysis of different expression cDNA libraries in *Rehmannia glutinosa* plants subjected to continuous cropping. Acta Physiol. Plant. 35, 645–655.
Ye, J., Fang, L., Zheng, H., Zhang, Y., Chen, J., Zhang, Z., Wang, J., Li, S., Li, R., Bolund, L., Wang, J., 2006. WEGO: a web tool for plotting GO annotations. Nucleic Acids Res. 34, W293–W297.
Yoneda, Y., Nakashima, H., Miyasaka, J., Ohdoi, K., Shimizu, H., 2017. Impact of blue, red, and far-red light treatments on gene expression and steviol glycoside accumulation in *Stevia rebaudiana*. Phytochemistry 137, 57–65.
Zein, U., Fitri, L.E., Saragih, A., 2013. Comparative study of antimalarial effect of sambiloto (*Andrographis paniculata*) extract, chloroquine and artemisinin and their combination against *Plasmodium falciparum* in-vitro. Acta Med. Indones. 45, 38.
Zhang, J., Song, T.T., Meng, X.N., Han, Z.Y., Yao, Y.C., 2015a. Early phenylpropanoid biosynthetic pathway genes are responsible for flavonoid accumulation in the leaves of three crabapple (Malus spp.) cultivars. J. Hortic. Sci. Biotech. 90, 489–502.
Zhang, X.F., Tan, B.K., 2000. Anti-diabetic property of ethanolic extract of *Andrographis paniculata* in streptozotocin-diabetic rats. Acta Pharmacol. Sin. 21, 1157–1164.
Zhang, X., Guan, H., Dai, Z., Guo, J., Shen, Y., Cui, G., Gao, W., Huang, L., 2015b. Functional analysis of the isopentenyl diphosphate isomerase of *Salvia miltiorrhiza* via color complementation and RNA interference. Molecules 20, 20206–20218.