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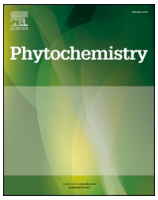

# Transcriptome analysis of *Paris polyphylla* var. *yunnanensis* illuminates the biosynthesis and accumulation of steroidal saponins in rhizomes and leaves

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ABSTRACT

*Paris polyphylla* var. *yunnanensis* can synthesize Paris saponins with multiple effective therapies, and its rhizome has become an indispensable ingredient in many patented drugs. However, how Paris saponin content changes in tissues at different stages and the molecular mechanisms underlying the production and accumulation of the bioactive compounds are unclear. This study aimed to uncover the mechanisms underlying the biosynthesis and accumulation by integrating transcriptome sequencing and phytochemical investigation of the leaves and rhizomes at different growth stages. Paris saponin content in leaves was lower during the fruiting stage than the vegetative stage, whereas the content in rhizomes increased during the fruiting stage. The candidate genes related to Paris saponin biosynthesis were determined by transcriptome analyses. Most biosynthetic genes were found to be abundantly expressed in the leaves during the vegetative stage in the light of expression profiles and functional enrichment results. The expression patterns of the differentially expressed genes related to the biosynthesis were positively correlated with the accumulation of saponins in tissues. These findings suggest that both leaves and rhizomes are capable of biosynthesizing Paris saponins, and that aerial plant parts can be used to extract them. The different patterns of biosynthesis and accumulation in the leaves and rhizomes were also determined here. This study will help improve our understanding of the mechanisms underlying the biosynthesis and accumulation of Paris saponins, and aid in the comprehensive development and utilization of this medicinal plant.

## 1. Introduction

*Paris polyphylla* var. *yunnanensis* (Franch.) Hand.-Mazz. (Melanthiaceae) is a perennial medicinal herb that is mainly distributed in Southwest China (Li, 2008; Chase et al., 2016). The dried rhizomes of *P. polyphylla* var. *yunnanensis* are widely used in Traditional Chinese Medicine, and its harvested rhizomes have become the indispensable component of more than 70 popular patented medicines (Qin et al., 2018). Paris saponins are the main bioactive ingredients, accounting for approximately 80% of the total number of active compounds. Paris saponins have remarkable efficacies when treating hemostasis and inflammation, and antitumor activities (Liu et al., 2012; Man et al., 2012). Although Paris saponins have important medicinal attributes, little is known about the biosynthesis and accumulation of the compounds in the different tissues of *P. polyphylla* var. *yunnanensis* at the functional genomic level.

Over the years, high throughput sequencing has answered many questions at level of the entire plant genome (Nützmann et al., 2016; Bevan et al., 2017; Giarola et al., 2017). In the absence of adequate genomic information, transcriptome analyses of non-model plants have elaborated our understanding of metabolic pathways and their regulation (Morozova et al., 2009). In view of the remarkable advances in

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transcriptomics, fruitful efforts to discover genes encoding enzymes related to the production of specialized metabolites in medicinal plants—e.g. *Panax* species—have been profoundly enhanced (Jayakodi et al., 2015; Xu et al., 2015; Tatsis et al., 2017; Zhang et al., 2017). Additionally, transcriptomes help delineate metabolic pathways and regulatory networks, and expose metabolic evolution in medicinal plants. Transcriptome profiling revealed that *Artemisia annua* evolved sophisticated transcriptional regulatory networks for artemisinin biosynthesis (Shen et al., 2018). *P. polyphylla* and *Trigonella foenum–graecum* recruit pairs of cytochromes P450s identified from transcriptomes, which catalyze 5,6-spiroketalization of cholesterol to produce diosgenin, with the evolutionary origins of which were traced to conserved phytohormone metabolism (Christ et al., 2019). *P. polyphylla* var. *yunnanensis* genome is estimated to be approximately 100 Gb, but this giant genome remains unreported (Pellicer et al., 2014). Previously, transcriptome sequencing of *P. polyphylla* was applied to predict the genes coding cytochromes P450 involved in biosynthesis of steroidal saponin, and the genes involved in germination (Liu et al., 2016; Yin et al., 2018; Christ et al., 2019; Yang et al., 2019; Liao et al., 2019; Zhang and Ling, 2020). However, the relationships between Paris saponins and their biosynthetic genes are unclear.

Steroidal saponins are important specialized metabolites, characterized by the structures containing a steroid aglycone and one or more sugar chains, which are sporadically distributed in monocots, e.g., some species of Liliaceae, Dioscoreaceae, Agave, and Palmae (Christ et al., 2019). Of the steroidal saponins, Paris saponins are specifically produced by *Paris* species. Steroidal saponins in plants are biosynthesized through the cytosolic mevalonate (MVA) pathway and the plastidial 2-C-methl-d-erythritol-4-phospate (MEP) pathway, leading to synthesizing squalene. Squalene is then converted into 2, 3-oxidosqualene under the catalysis of squalene epoxidase (SE), which undergoes cyclization by oxidosqualene cyclase (OSC). The cyclization products then undergo modifications (hydroxylation and glycosylation), ultimately yielding different types of steroidal saponin glycosides (Thimmappa et al., 2014; Upadhyay et al., 2018). Genes related to the biosynthesis of the saponin backbone have been recognized in resource plants like *Medicago truncatula*, *Asparagus racemosus*, *Allium fistulosum*, *T. foenum-graecum*, and *Trillium govanianum* (Carelli et al., 2011; Upadhyay et al., 2014; Ciura et al., 2017; Singh et al., 2017). However, the biosynthesis of Paris saponin backbone needs to be more clearly delineated.

There have been shortages in the supplies of *P. polyphylla* var. *yunnanensis* in recent years, partly because of its long growth cycles (ca. 7–8 years) and the sharp increase in the demand for rhizomes by the pharmaceutical industry (Qin et al., 2018). However, molecular analyses of the leaves as an alternative source of active ingredients have not been conducted. In addition, annual saponin accumulation in both leaves and rhizomes has not been reported. Thus, it is necessary to investigate changes in the active ingredients of tissues across development stages and the molecular mechanisms underlying these changes.

Here, leaves and rhizomes of 7-year-old *P. polyphylla* var. *yunnanensis* during the vegetative and fruiting stages were simultaneously subjected to high throughput transcriptome sequencing and phytochemical investigation. This enabled us to, (1) uncover the content changes of Paris saponins in the tissues during different development stages, (2) identify the differentially expressed genes (DEGs) related to this biosynthesis, and (3) determine the relationships between the changes in accumulation of the bioactive compounds and biosynthetic DEGs. This study sheds light on the mechanisms underlying the biosynthesis and accumulation of Paris saponins in *P. polyphylla* var. *yunnanensis*.

## 2. Results

### 2.1. Phytochemical analysis

The changes in steroidal saponin in different tissues of *P. polyphylla* var. *yunnanensis* (e.g., leaves and rhizomes) across the different development stages were not previously known. To characterize the active ingredients, quantitative analysis of Paris saponin in leaves and rhizomes during the vegetative stage (VL and VR, respectively) and the fruiting stage (FL and FR, respectively) was performed using high-performance liquid chromatography (HPLC). The total saponin content of the leaves was much higher than that of the rhizomes during the vegetative stage, whereas the opposite was found during the fruiting stage (Fig. 1a). Paris saponin I, Paris saponin II, Paris saponin VI, and Paris saponin VII are the official quality standards for Chinese Pharmacopoeia (Committee of National Pharmacopoeia, 2015). Three bioactive saponins including diosgenin-type saponins (Paris saponin I, II) and pennogenin-type saponins (Paris saponin VII) were detected, but Paris saponin VI was not. Furthermore, the proportion of Paris saponin II was the highest in all tissues during two stages. More types of saponin were discovered in the rhizomes during both vegetative and fruiting stages (Fig. 1b). These results illustrate the changes in saponin content in tissues during these two development stages.

### 2.2. Transcriptome sequencing and de novo assembly

Rhizomes and leaves of *P. polyphylla* var. *yunnanensis* from both development stages, were used simultaneously to generate libraries that were sequenced on an Illumina HiSeq 2500 platform with 2 × 150 bp paired-end reads; and each tissue was prepared using three replicates (Table S1). A total of 584, 977, 568 raw sequencing reads were generated from 12 libraries and a total of 581, 131, 084 clean reads were obtained after removing the adaptors, low-quality, and contaminated reads. In all the libraries, more than 95% of the sequences had a quality score greater than Q20, which indicated that the libraries had high-quality raw reads.

The sequencing depth achieved in this study (87 G) was better than those of previous transcriptome sequencing of *P. polyphylla* var. *yunnanensis* (Table S2). The clean bases of each sample sequenced here were guaranteed to be > 6 G. The clean reads from all tissues were assembled together with a contig N50 size of 952 bp. According to the assessment of the assembled transcriptome, 78.6% of the BUSCO groups were covered (Table S3). Similar to the huge genome, the repeats accounted for a large proportion of the transcriptome, and they were sometimes not sequenced owing to gene silencing and removal from experiments. However, these did not have a large impact on transcriptome sequencing. In short, the read quality and assessment of assembled results indicated that sequencing depth and transcriptomic data in this study were sound and effective.

A total of 85,677 unigenes were identified, with a median length of 668 bp (Table S4). The shortest and longest unigenes were 201 bp and 15,259 bp, respectively. And 35,442 (41.37%) unigenes were >500 bp and 15,447 (18.03%) unigenes were >1000 bp in length (Table S5). The gene expression profiles of all unigenes are provided in Table S6. All expressed unigenes, whose transcripts per kilobase million was greater than 0 (TPM > 0), were determined. The results revealed that 29,146, 23,661, 28,601, 225,86 unigenes were expressed in leaves and rhizomes during the vegetative and fruiting stages, respectively (Fig. 1c). A total of 16,280 shared unigenes were identified in all tissues, and over 4000 unigenes were particularly expressed in leaves during the two stages (Fig. S1). The overall expression levels of the leaf unigenes were higher than those of the rhizome unigenes (Fig. 1d).

### 2.3. Gene annotation and functional classification

To understand the functions of the assembled transcripts, *P. polyphylla* var. *yunnanensis* unigenes were annotated using BLAST searches against entries in the six public databases: Nr, GO, KEGG, Swiss-Prot, Pfam, and KOG (Table S7). The analyses showed that 33,408 (38.99%) unigenes had significant matches in the Nr database, 10,154 (11.85%) in GO, 9117 (10.64%) in KEGG, 17,018 (19.86%) in Swiss-

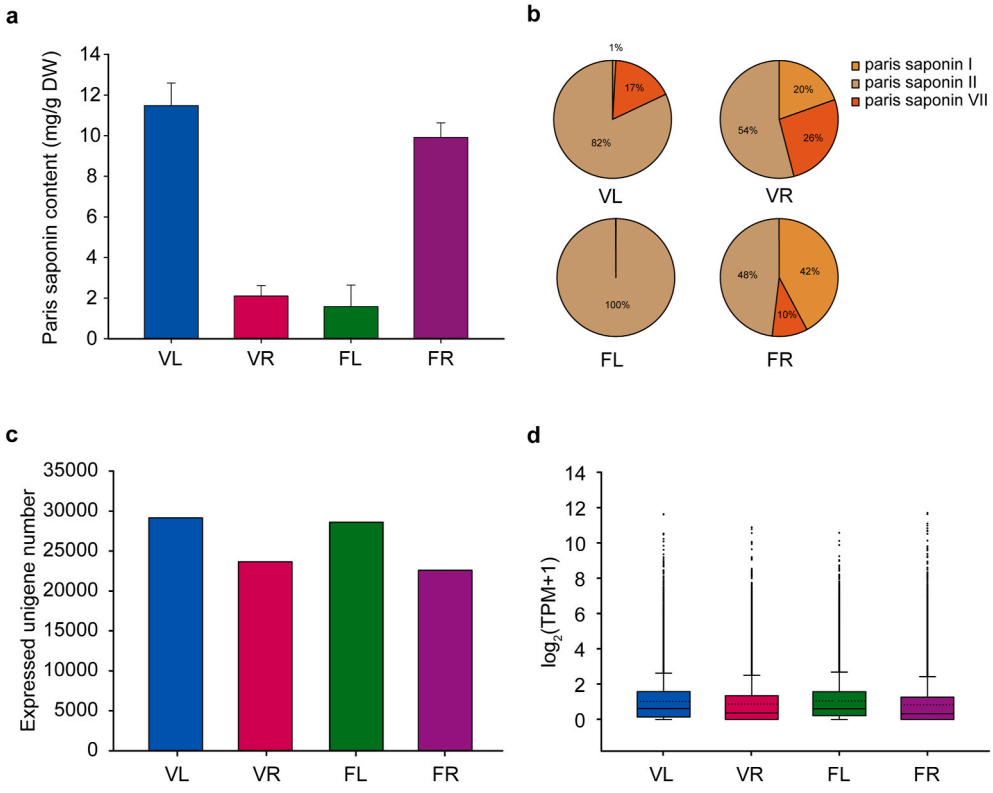


**Fig. 1.** The bioactive compound content and transcriptome characters. (a) Total content of three typical types of Paris saponins in leaves and rhizomes during the vegetative and fruiting stages. VL: leaves at vegetative stage, VR: rhizomes at vegetative stage, FL: leaves at fruiting stage, and FR: rhizomes at fruiting stage. (b) Proportion of three types of Paris saponins in leaves and rhizomes. (c) Distribution of the expressed unigenes in tissues during the two stages ($\log_2$ (TPM+1) > 0). (d) Boxplot of unigene expression profiles.

Prot, 16,395 (19.16%) in Pfam, and 21,576 (25.18%) in KOG. In total, 34,020 (39.71%) unigenes were successfully annotated in at least one of the six databases, and 4171 (4.87%) unigenes were annotated in all the databases. There were 51,657 unigenes with no homologous entries in the six public databases, of which, 48,376 were thought to be noncoding RNAs, as determined by noncoding RNA identification. No species in Liliales (the order of *P. polyphylla* var. *yunnanensis*) have had its genome sequenced, so it is understandable that some unigenes from *P. polyphylla* var. *yunnanensis* have not yet been annotated. These unannotated genes may be involved in unknown functions and further investigations are required to determine their functions.

Unigenes related to "catalytic activity" and "binding" from the molecular function category, and "metabolic process" and "cellular process" from the biological process category accounted for much higher proportions of the GO annotation results than unigenes related to other GO subcategories (Fig. S2). To discover the most important biological pathways, unigenes annotated in the KEGG database were classified into five classes. There were large groups of unigenes related to "Biosynthesis of other secondary metabolites", and "Metabolism of terpenoids and polyketides" (Fig. S3). KOG classification showed that "General functional prediction only" was the largest group, followed by "Post-translational modification", "Protein turnover", "Chaperones", and "Signal transduction mechanisms". Notably, "Secondary metabolites biosynthesis, transport and catabolism" was also a large group, including 877 unigenes (Fig. S4). The putative genes related to the biosynthesis of the saponin backbone were also determined. Fifty-three candidate genes involved in steps before squalene synthesis and 34 candidate genes that participated in downstream of the saponin backbone biosynthesis were identified based on the KEGG pathway assignments (Fig. 2).

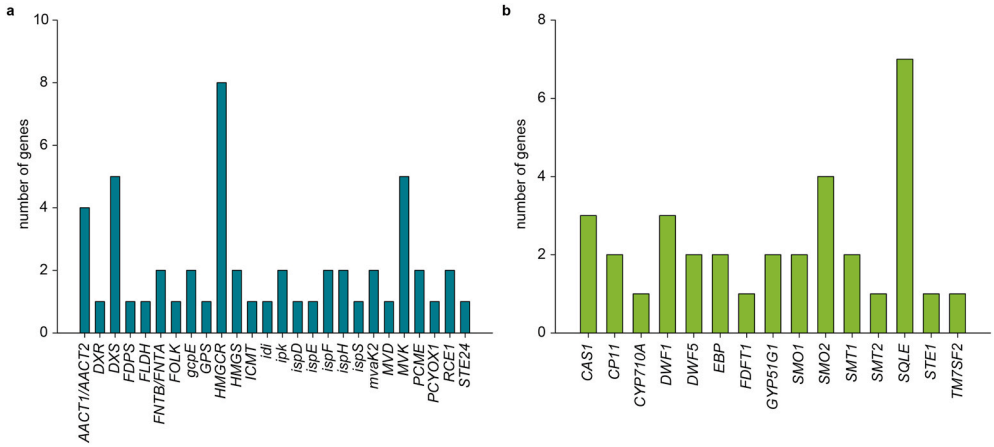


**Fig. 2.** Genes involved in Paris saponin biosynthesis. (a) Genes participated in the MVA and MEP pathways. (b) Genes participated in the downstream of saponin backbone biosynthesis.

### 2.4. Analyses of DEGs

A total of 1,118, 1,402, 1,247, and 35 genes were found to be differentially expressed in the VL vs. VR, FL vs. FR, FL vs. VL, and FR vs. VR paired groups, respectively. The first three groups had more specific DEGs than did the rhizome paired comparison (Fig. 3a and Fig. S5). Comparisons between VL and VR, FL and FR, FL and VL, VR and FR, revealed 695, 923, 599, and 21 up-regulated genes in VL, FL, FL, and VR, respectively (Fig. 3b). No common DEGs were found in these paired comparisons. There were more up-regulated genes than down-regulated ones in leaves during the same development stage. Additionally, hierarchical clustering of the expression profiles of all DEGs indicated that gene expression patterns as a whole were similar in a particular tissue across the development stages, but these patterns differed between leaves and rhizomes, implying that the biological processes occurring in a particular tissue were similar across different development stages (Fig. 4a). GO enrichment analysis was implemented to reveal the possible functions of the DEGs. The top 15 significantly enriched GO terms in each of the three main GO categories are presented in Fig. 4b. The DEGs were mainly found to be involved in primary metabolic processes.

All DEGs were then subjected to a KEGG pathway enrichment analysis to characterize the complex biological behaviors of the transcriptome and further explore the potential biological functions of DEGs. DEGs were assigned to a total of 158 KEGG pathways. The 17 enriched pathways identified are presented in Fig. 5. The enrichment pathways of DEGs reflected the preferential biological functions of tissues. More genes were overexpressed in the leaves than in the rhizomes with respect to "Photosynthesis", "Carbon metabolism", and "Porphyrin and chlorophyll metabolism", indicating that leaves play a vital role in providing materials and energy for *P. polyphylla* var. *yunnanensis* growth and development. Photosynthetic genes were significantly enriched in leaves during vegetative stage according to enrichment results of DEGs from leaves at two stages, implying that photosynthesis sharply declines during the fruiting stage. Similar to the enrichment results for leaves, the DEGs of rhizomes during both vegetative and fruiting stages shared similar enrichment patterns. The genes involved in "MAPK signaling pathway", "Phenylpropanoid biosynthesis", "Stilbenoid, diarylheptanoid and gingerol biosynthesis", "Amino sugar and nucleotide sugar metabolism", and "Flavonoid biosynthesis" were significantly enriched. Noteworthy is that the pathways related to bioactive metabolisms were the main subjects of focus for medicinal plants. The saponin biosynthesis-related genes were clearly enriched in leaves during the vegetative growth stage.

### 2.5. Paris saponin biosynthesis and accumulation

The genes of saponin backbone biosynthesis differentially expressed in leaves and rhizomes during both development stages were determined (Fig. 6). The expression of genes encoding the same catalytic enzymes showed clear differences between the tissue types, such as the genes encoding farnesyl diphosphate synthase (FDPS)/geranylgeranyl diphosphate synthase (GGPS), and squalene monooxygenase (SQLE). Specifically, the expression of most genes was the highest in leaves during the vegetative stage. According to Pearson correlations, saponin content changes were positively correlated with the expression levels of most biosynthetic DEGs ($r > 0.6$) (Fig. S6). Moreover, MVA and MEP pathways were identified as the early biosynthetic steps of steroidal saponins, suggesting that both of them played roles in saponin biosynthesis. Although the expression of DEGs in the MEP pathway was promiscuous, DEG expression in the MVA pathway appeared to be correlated with changes in accumulated Paris saponins. These results indicated that the MVA pathway was dominant and that the leaf was an important organ for steroid synthesis.

The DEGs provided crucial information for further investigation into the molecular mechanisms underlying Paris saponin biosynthesis and accumulation. Higher contents of Paris saponin were detected in leaves during the vegetative stage and the rhizomes during the fruiting stage, while the more abundant types of steroid saponins were detected in the rhizomes during both development stages. Rhizomes also appeared to be capable of synthesizing steroidal saponin like saponin I and become the final storage organ for the bioactive compounds. Leaves of *P. polyphylla* var. *yunnanensis* during the vegetative stage, which biosynthesized high amounts of Paris saponin II and VII, could thus be considered potential raw materials for screening the active medicinal compounds. Together, the characterization of the saponin metabolic branches and its biosynthesis and accumulation are critical steps to help further understanding the biosynthetic mechanisms regulating the medicinal compounds of *P. polyphylla* var. *yunnanensis*.

### 2.6. Validation of gene expression profiles by qRT-PCR

To verify RNA sequencing results, eight DEGs from the steroid saponin biosynthetic pathway were selected for relative quantitative analysis. The primers used in qRT-PCR are listed in Table S8. The relative expressions of the eight genes noted in leaves during vegetative stage were significantly higher than those in rhizomes during this growth stage. The relative expression of these genes in leaves during the vegetative stage was also significantly higher than those in leaves during fruiting stage (Fig. 7). These findings are consistent with the results of the transcriptomic analysis.

## 3. Discussion

### 3.1. Saponin production and changes in saponin content

DEGs involved in Paris saponin biosynthesis and HPLC-determined contents of Paris saponin reflected different roles that leaves and rhizomes played in the biosynthesis and accumulation, as well as their defense responses to the various environmental cues. Plants often

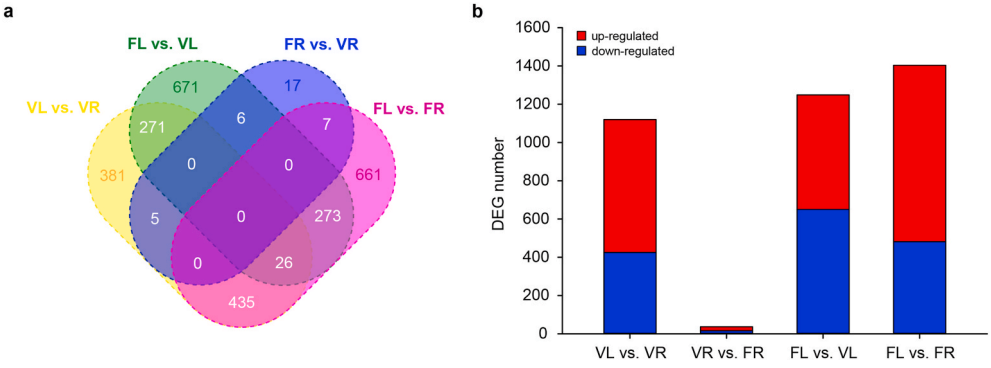


**Fig. 3.** DEG statistics. (a) Venn diagram of DEGs from the four paired comparisons. (b) The number of up-down regulated DEGs of the four paired comparisons.

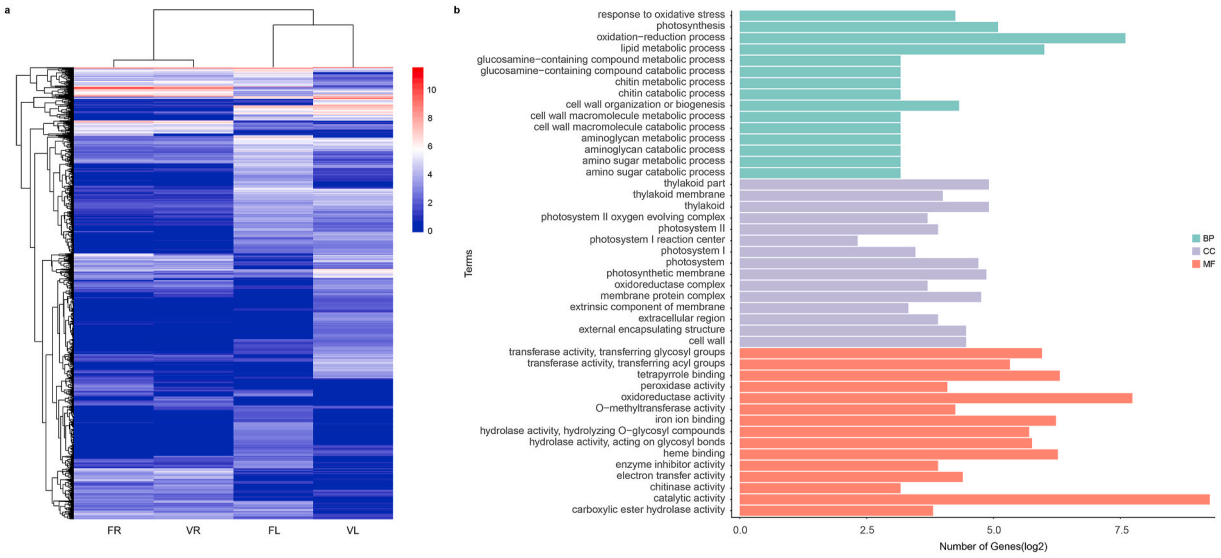


**Fig. 4.** An overview of DEG expression patterns and GO enrichments. (a) Heatmap of expression values for all DEGs. (b) GO enrichments of DEGs, with displaying the top fifteen subcategories for each category.

synthesize specialized metabolites in response to environmental factors, and the biosynthesis of defensive compounds can be stimulated by herbivore damage and microbial infections (Yang et al., 2012). Saponins possess a broad spectrum of biological properties such as antifungal, insecticidal, anti-herbivore, phytotoxic, and allelopathic effects (Waller and Yamasaki, 2013). These properties explain why saponins are synthesized constitutively in some plant species and participate in plant defense mechanisms (Osbourn, 1996; Christ et al., 2019).

In our study, total Paris saponin content in leaves during the vegetative stage was much higher than that in the leaves during the fruiting stage, whereas its content in rhizomes during the vegetative stage was lower than that during the fruiting stage. KEGG enrichment results demonstrated that genes involved in the biosynthesis were highly expressed in young leaves, indicating that Paris saponins were mainly synthesized in the leaves during vegetative stage. Plant organs with high fitness values or are under a high risk of attack may be best protected by constitutive defenses, whereas others may be defended better by induced responses (Wittstock and Gershenzon, 2002). Young leaves synthesize saponins as a coping mechanism against ecological challenges to biotic stress, including herbivore attacks and fungal infections. Rhizomes may identify shoot-root defense signals and trigger the transport of Paris saponins to the aboveground parts, augmenting its concentrations in the leaves and increasing resistance (Erb et al., 2009; De Coninck et al., 2015). Consequently, Paris saponin content in rhizomes was lower during the vegetative stage than the fruiting stage. In terms of plant growth and development, Paris saponin levels increased in rhizomes during particularly the fruiting stage via progressively accumulating these saponins originating produced locally and from leaves. This finding is in accordance with that for *P. notoginseng*: ginsenosides are synthesized in plant shoots as well as the roots, but accumulate quickly in the roots after flowering in *P. notoginseng* (Zhang et al., 2017). Rhizomes with many defense compounds protect themselves and prepare for the following year.

In addition, *P. polyphylla* var. *yunnanensis* preferred the MVA pathway for Paris saponin biosynthesis over the MEP pathway as determined using the gene expression analysis; this result is consistent with triterpenes biosynthesis in plants (Thimmappa et al., 2014). At the same time, it was easier to detect Paris saponin I in rhizomes than leaves. Previous studies have demonstrated that more types of saponins were reported in rhizomes than in other tissues (Upadhyay et al., 2018). These investigations indicated that rhizomes may be able to diversify defense compounds other than the biosynthesis and accumulation. Furthermore, saponin biosynthesis in rhizomes can be affected by plant endophytes. Some endophytic fungi as elicitors can also participated in saponin biosynthesis and increase saponin production in rhizomes (Yang et al., 2015; Li et al., 2016; Gai et al., 2017). These findings indicated that the biosynthesis and accumulation of Paris saponin is an intricate process that is affected by plant growth and development, microorganism interventions, defense responses, and even adaptions to ecological niches.

### 3.2. *Alternative sustainable sources and harvest times*

The wild resources available for *Paris* spp. are being threatened by extensive exploitation, and rapid habitat loss (Gao et al., 2018). Although *P. polyphylla* var. *yunnanensis* has been widely planted in Southwest China, not enough of its rhizomes can be produced to satisfy

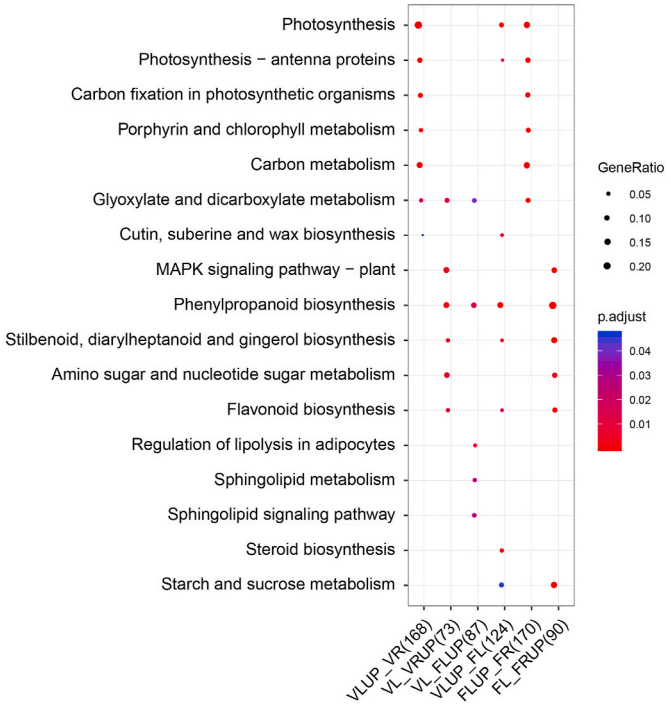


**Fig. 5.** KEGG enrichments of DEGs.

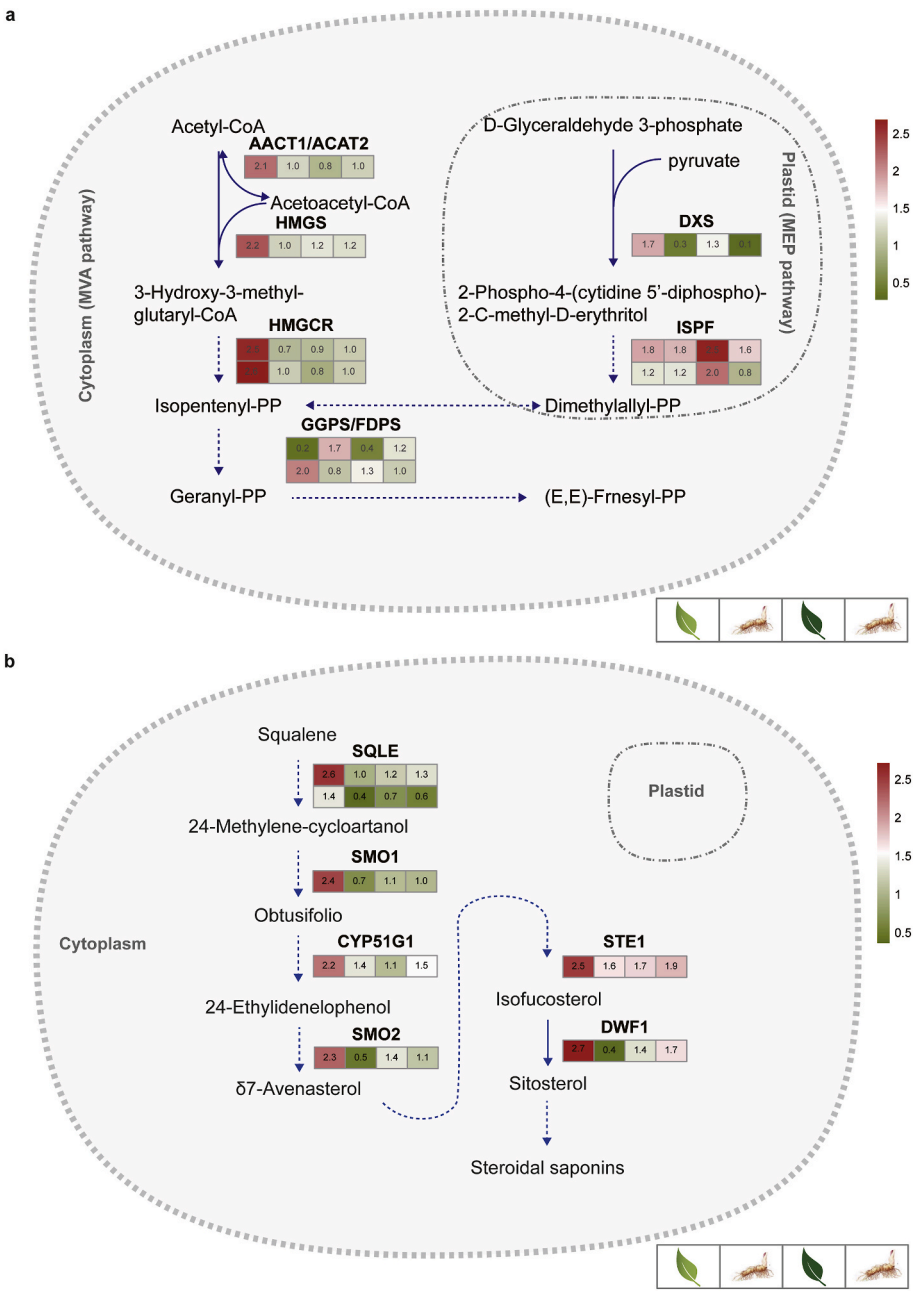


**Fig. 6.** Overview of DEGs related to Paris saponin biosynthesis pathway. (a) DEGs encoding enzymes involved in MVA and MEP pathways. (b) DEGs encoding enzymes involved in the downstream of the saponin backbone biosynthesis.

the needs of the medicinal plant markets (Chinese Herbal Medicine Network of Heaven and Earth, 2016). However, over 500 tons of the renewable aerial parts of *P. polyphylla* var. *yunnanensis* are discarded as waste materials each year (Qin et al., 2018). Consequently, alternative resources of *P. polyphylla* rhizomes should be identified, and efforts should be made to utilize this herb more comprehensively. The leaf, as indispensable aerial plant parts, was proven to be capable of producing Paris saponins. Paris saponin II could be acquired from the leaves during the fruiting stage, according to the phytochemical results in this study. The leaves of *P. polyphylla* var. *yunnanensis* may thus be of pharmacological importance (Liang et al., 2019). They will consequently be profitable as an alternative source of steroid materials to increase saponin yields and better utilize *Paris* resources. Our study provides methods for the rationally utilizing aerial plant parts (leaves) of *P. polyphylla* var. *yunnanensis*.

This study found dynamic changes in the saponin content across different development stages of *P. polyphylla* var. *yunnanensis*. A recent investigation reported the effects of cultivation years on saponin content in the rhizomes of *P. polyphylla* var. *yunnanensis* and proposed optimal harvest times to be the 7th or 8th year of growth (Wang and Li, 2018). Some medicinal plants such as *Lonicera japonica*, and *Chamaenerion angustifolium* have been explored to optimize the harvest times according to the time-course accumulation profiles of the main bioactive compounds (Kong et al., 2017; Agnieszka et al., 2018). Variations in Paris saponin concentrations across development stages inspired us to reconsider the best time to harvest *P. polyphylla* var. *yunnanensis*. The plants are usually harvested during the senescence stage under normal agricultural practices, i.e., the aerial parts are withered (Qin et al., 2018). The fruiting stage could be considered as another appropriate harvesting time base on the active compounds in the rhizomes and leaves. The expression of genes involved in saponin biosynthesis is governed by the development stage. Additionally, it was reported that

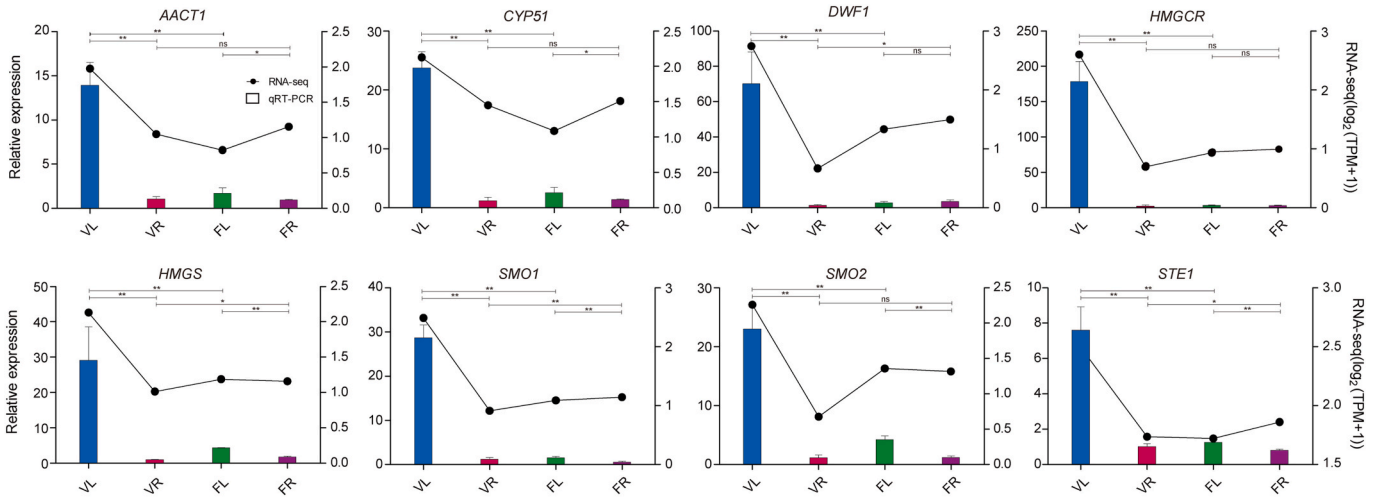


**Fig. 7.** QRT-PCR validation of RNA-Seq data. Expression profiles of eight selected genes were determined by transcriptome and qRT-PCR data. The left vertical axis represents the relative expression of the gene based on qRT-PCR. The right vertical axis represents the expression level of the gene based on transcriptome sequencing. The asterisk above the bar chart denotes statistical significance based on the qRT-PCR data (* denotes $P$ value $< 0.05$, ** denotes $P$ value $< 0.01$, ns denotes $P$ value $> 0.05$).

when plants reach the end of their fruiting stage and approach senescence, their leaves gradually lose their metabolic and developmental potential they possessed during younger growth stages (Kim et al., 2016). The underground buds develop enough before the senescence stage as they prepare for frondescence in the next year. Thus, variations in bioactive compounds in the tissues are associated with development stages as well as with cultivation periods. However, more phytochemical evidence is required to improve agricultural utilization of *P. polyphylla* var. *yunnanensis*.

In short, the findings reveal that both leaves and rhizomes are important organs for biosynthesizing Paris saponins, and that aerial plant parts can be used to extract them. The patterns of biosynthesis and accumulation in the leaves and rhizomes are different. This study provides insight into the underlying mechanisms responsible for the biosynthesis and accumulation of Paris saponins, and helps comprehensively utilize the medicinal plant.

## 4. Experimental

### 4.1. Sampling of plant materials

*P. polyphylla* var. *yunnanensis* were cultivated under the same conditions including light, temperature, soil, water supply, etc. in the green house of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (Kunming). Fresh leaves and rhizomes were sampled from the healthy 7-year-old plants at vegetative stage and fruiting stage. The tissues at two stages had three replicates, and each tissue was comprised of three different varieties with similar size.

### 4.2. Effective ingredient determination

The fresh leaves and rhizomes were dried to achieve constant weight in the oven at 40 °C and the dried tissue samples were grounded into powder, respectively. An aliquot (0.5 g) of the powder was accurately weighted and exhaustively mixed with 70% EtOH (25 mL). The mixture was heated under reflux 30 min and added 70% EtOH for the weight loss after cool down, and the supernatant was then filtered to get sample solution after centrifugation. Quantitative analysis of typical Paris saponin I, Paris saponin II and Paris saponin VI, Paris saponin VII were carried out on an Agilent HPLC 1260 series system (Agilent, USA). The chromatographic separation was performed at temperature 30 °C using acetonitrile (A) and water (B) under gradient conditions (0–40 min, linear gradient 30–60% A, linear gradient 70–40% B; 40–50 min, linear gradient 60–30% A, linear gradient 40–70% B) as the mobile phase at a flow rate of 1.0 mL/min (Man et al., 2010).

### 4.3. RNA extraction and sequencing data

Total RNA was extracted from the leaves and rhizomes for each replicate, treated with RNAse-Free DNAse and purified using RNasy (QIAGEN, Hilden, Germany). The quality and quantity of RNA was determined by agarose gel electrophoresis, NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, MA, USA) and Aglient 2100 Bioanalyzer (Agilent Technologies, CA, USA). RNA samples were sequenced on an Illumina HiSeq 2500 platform with 2 × 150 bp paired-end reads at Vazyme Biotech Co., Ltd (Nanjing, China). The quality of the paired-end raw reads generated by Illumina was checked using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The adapters used in sequencing process, low quality sequences with a Phred quality score Q < 20 and ambiguous sequence with N were removed. The clean reads were registered in the NCBI SRA database with the accession number PRJNA630028.

### 4.4. De novo transcriptome assembly and annotation

In order to obtain high-quality data, the clean reads were preprocessed and trimmed using Trimmomatic (Bolger et al., 2014). The high quality clean reads were assembled using Trinity v2.5.1 with min_kmer_cov set to 15 (Haas et al., 2013). The accuracy and completeness of assembled transcriptome was evaluated using BUSCO v3.0.2 (Simao et al., 2015). The longest isoform per each gene was selected to generate unigene set. To find the putative functions, homology searching was performed against databases Nr and KOG using BLAST with an E-value cutoff of $10^{-5}$. Subsequently, assembled unigenes were functionally categorized by GO, Pfam, and Swiss-Prot using Trinotate pipeline (http://trinotate.github.io/). Finally, the unigenes were mapped to KEGG pathways using KAAS (http://www.genome.jp/tools/kaas/). If unigenes did not annotated by any above databases, they were subjected to noncoding RNA prediction using both CNCI and CPC2 (Sun et al., 2013; Kang et al., 2017).

### 4.5. Differential expression gene analysis

The expression levels were calculated and normalized as TPM by RSEM and TMM methods (Li et al., 2009; Li and Dewey, 2011).

Differential expression transcripts were identified using DESeq2 package (Love et al., 2014). Raw *p*-value was adjusted for multiple comparisons by Benjamin-Hochberg procedure for controlling the false discovery rate (FDR $\leq$0.05) (Eddy, 1998). Adjusted P-value $< 0.001$ and $|\log_2 \text{(fold change)}| \geq 2$ was set as the threshold for significantly differential expression.

### 4.6. GO and KEGG enrichment analysis

GO and KEGG enrichment analyses provide all GO terms or KEGG pathways significantly enriched in DEGs in comparison to the transcriptome background. GO enrichment was performed using GOseq R package (Young et al., 2010). The *P*-values were adjusted using Benjamini-Hochberg's method (Benjamini and Hochberg, 1995). We considered a corrected *P* value $< 0.05$ as a significantly enriched GO term. The KEGG enrichment analysis was performed using the clusterProfiler R package (Yu et al., 2012), applying a hypergeometric test with FDR correction. Adjusted *P* value $< 0.05$ was used as a threshold to determine significant enrichment of the gene sets.

### 4.7. Quantitative real time PCR verification analysis

Eight random DEGs related to the biosynthesis were validated by qRT-PCR with three replicates to verify the RNA-Seq data. Total RNA was extracted as above. The first-strand cDNA was synthesized from 1 μg of total RNA by using the RvertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, MA, USA) according to the manual. The qRT-PCR primers were designed for the eight genes using Primer Premier v5.0. QRT-PCR was performed using SYBR Premix with following conditions: 95 °C for 3 min; 40 cycles of 94 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s; final at 72 °C for 5 min. All of the PCR reactions were conducted in triplicated and the average expression values were calculated. The target genes were normalized to the housekeeping gene (*Actin*) shown as $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak, 2008). T-test was used to test mean differences in four paired comparisons of tissues.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112460.

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