

De novo transcriptome sequencing of *Rhododendron molle* and identification of genes involved in the biosynthesis of secondary metabolites

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Abstract

Background

Rhododendron molle (Ericaceae) is a traditional Chinese medicinal plant, its flower and root have been widely used to treat rheumatism and relieve pain for thousands of years in China. Chemical studies have revealed that *R. molle* contains abundant secondary metabolites such as terpenoids, flavonoids and lignans, some of which have exhibited various bioactivities including analgesia and hypotension. In despite of immense pharmaceutical importance, the mechanism underlying the biosynthesis of secondary metabolites remains unknown and the genomic information is unavailable.

Results

To gain molecular insight into the *R. molle*, we conducted deep transcriptome sequencing for *R. molle* flower and root using the Illumina HiSeq platform. In total, 100,603 unigenes were generated through de novo assembly with mean length of 778 bp, 57.1% of these unigenes were annotated in public databases and 17,906 of those unigenes showed significant match in the KEGG database. Unigenes involved in biosynthesis of secondary metabolites were annotated. Moreover, 3,376 transcription factors and 10,828 simple sequence repeats (SSRs) were also identified. Additionally, we further performed differential gene expression (DEG) analysis of the flower and root transcriptome libraries and identified numerous genes that were specifically expressed or up-regulated in flower.

Conclusions

To the best of our knowledge, this is the first time to generate and thoroughly analyze the transcriptome data of both *R. molle* flower and root. This study provided an important genetic resource which will shed light on elucidating various secondary metabolite biosynthetic pathways in *R. molle*, especially for those with medicinal value and allow for drug development in this herb.

Background

Rhododendron molle is a flowering perennial shrub indigenous to the south of the Yangtze River in China such as Guangdong and Guangxi Provinces. This plant grows mostly under shrubs or trees in the hilly area at the altitude of around 1,000 meters[1]. The medicinal properties of *R. molle* have been identified dating back to thousands of years ago. This toxic herb has been recorded and depicted in the earliest Chinese medicinal book *Shennong Bencao Jing* (Shennong's Classic of Materia Medica, compiled in the early period of the Eastern Han Dynasty dated from 25AD to 220AD). As one of the most famous traditional Chinese medicinal plant, *R. molle* has been extensively used as an anodyne and anesthetic since ancient times[2]. According to records in ancient medical books, both the dried flower and the root can be used as medicine to treat rheumatism, traumatic injury, migraine and neuropathic pain[1, 2]. In some rural areas, the herb was also be used as an insecticide[3]. Modern chemical studies have demonstrated that *R. molle* produces abundant secondary metabolites such as terpenoids, flavonoids,

and lignans [4-7]. Most of the compounds isolated from *R. molle* are terpenoids (e.g. grayanotoxin III, rhodojaponin III, both are tetracyclic diterpenoids) which account for the plant's various bioactivities including analgesia, antinociceptive effects [5], and hypotensive bioactivity [8]. In recent years, the RNA-Seq approach based on the NGS (next-generation sequencing) technology has been developed and widely used for fast and cost-effective transcriptome characterization of numerous vital medicinal plants like *Lilium regale* [9], *Glycyrrhiza uralensis* [10], *Eugenia uniflora* [11] and *Carthamus tinctorius* [12]. It also provides an effective way to accelerate discovering novel enzymes involved in the specific metabolic pathways [13, 14]. Recently, the transcriptome of *R. molle* flower was established [15], but it only provided focused on the mechanisms for flowering and the formation of flower color [15]. The genes involved in the secondary metabolic pathways remain unknown yet. In this study, deep *de novo* transcriptome sequencing for the flower and root of *R. molle* was performed using the Illumina HiSeq platform. A total of 100,603 unigenes with average lengths of 778 bp were obtained, among which 20,886 unigenes were over 1 kb in length. 57.1% of these unigenes were annotated based on sequence similarity searches and protein domain scanning against the publicly available databases. Moreover, bioinformatics analysis indicated that the genes encoding enzymes involved in the biosynthesis of the terpenoids backbone existed in the transcriptome of both *R. molle* flower and root. Total nine terpene synthases (TPS) were identified. In addition, the candidate genes putatively responsible for further backbone modifications were screened and a gene pool containing 61 cytochromes P450 (CYP) sequences was obtained. Putative members for biosynthesis of flavonoids and lignans were also identified in our transcriptome dataset. We profiled transcriptomes of flower and root separately and performed a comparative analysis, which provides a deeper insight into biosynthesis of secondary metabolites. Our transcriptome data provided a valuable resource for the discovery of functional genes involved in various metabolic pathways, especially for the putative genes related to the biosynthetic pathways of the medicinally important secondary metabolites and will pave the way towards enhanced biosynthesis of secondary metabolites with medicinal properties through synthetic biology approach.

Methods

1. Materials and RNA extraction

Rhododendron molle was collected from Guangxi Institute of Botany in Guilin, Guangxi Province of China and was identified by professor Guang-Zhao Li of Guangxi Institute of Botany. A voucher specimen (ID-24757) was deposited in the herbarium at the Department of Medicinal Plants, Institute of Materia Medica, Chinese Academy of Medical Sciences (CAMS). The plant was grown in the greenhouse at the Institute of Medicinal Plant Development, CAMS. The roots and flowers were harvested and washed with tap water and ultrapure water successively, dried on filter paper. The roots were chopped into small pieces. All samples were frozen immediately in liquid nitrogen and preserved at -80 °C before further processing. Total RNA was extracted from the root and flower using Trizol reagent and was treated with the RNase-free DNase I to eliminate genomic DNA. The quality and purity of the extracted RNA were assessed by spectrophotometer. The RNA integrity number (RIN) was checked by the Agilent Bioanalyzer 2100 system. Sample with RNA integrity number (RIN) value more than 8.0 was selected for further use.

2. cDNA library construction and sequencing

1 µg RNA sample was collected to construct the cDNA library using the NEBNext®Ultra™ RNA Library Prep Kit for Illumina®(NEB, USA) following manufacturer's instructions. Briefly, the mRNA molecules were purified with Magnetic Oligo (dT) beads, fragmented and subjected to cDNA synthesis, then cDNA library was generated through PCR. The quality of each sample library was assessed using the Agilent Bioanalyzer 2100 system. Ultimately the transcriptome library per tissue was sequenced by Illumina HiSeq 2000 platform (Biomarker Technologies Corporation, Beijing, China) and the paired-end reads were generated.

3. *De novo* transcriptome assembly and annotation

The raw reads obtained via the cDNA library sequencing were initially processed to produce clean reads by trimming the adapter and low quality reads. The clean reads were assembled using the Trinity software (version 2.5.1) to generate transcripts[16]. Then transcript analysis was performed to remove redundancies with TGICL software (version 2.1) and acquire unigenes without redundancy [17]. All assembled unigene sequences were subjected to similarity search against major public databases, including NCBI non-redundant protein (NR) (<https://www.ncbi.nlm.nih.gov/protein/>)[18], Swiss Prot database (<https://www.uniprot.org/uniprot/>)[19], Clusters of Orthologous Groups (COG) (<http://www.ncbi.nlm.nih.gov/COG/>)[20], eggNOG4(<http://eggnogdb.embl.de/>)[21], Pfam (<https://pfam.xfam.org/>)[22]. The blast algorithm was used to identify homologous sequences with a cut-off value less than 10^{-5} . The annotations of the best hits were recorded. Gene Ontology (GO) (<http://www.geneontology.org/>) was further used to categorize the function of the unigenes by Blast2GO software (version 2.5)with default parameters [23, 24]. The TransDecoder software (version 5.0.0) was used to predict the coding region sequence (CDS) and the corresponding amino acid sequence of unigenes, according to the alignment of amino acid sequence with protein domain sequence in the Pfam database.

4. Functional characterization using KEGG

All the assembled unigenes were mapped against the Kyoto Encyclopedia of Genes and Genome (KEGG) database (<http://www.genome.jp/kegg/>)[25] using the BLASTX with the threshold *E*-value of $<10^{-5}$. The KEGG orthology (KO) assignments were carried out via the KOBAS software (version 2.0) [26]with default parameters.

5. Differential expression analysis

Gene expression levels were calculated by the fragments FPKM[27] (per kilobase per million fragments mapped) approach using RSEM software(version 1.2.19)[28]. The EBSeq software (version 1.6.0) was used to carry out differential expression analysis of two samples. Pvalue was adjusted using qvalue[29], the threshold of $qvalue < 0.005$ and $\log_2(\text{fold change}) > 1$ was set as the two criteria for significantly differential expression.

6. Transcription factor analysis

To identify the transcription factor (TF) families, the assembled unigenes were inquired against the TF protein domains in the plant transcription factor database (PInTFDB) by BLASTX [30] (plant transcription factor database) by BLASTX with an *E*-value cutoff 1E^{-06} .

7. Identification of simple sequence repeats (SSRs)

For identification of SSR motifs, the unigenes generated from transcriptome sequences of both flower and root tissue were searched with MISA (Microsatellite searching Tool) (version 1.0). In this study, the microsatellites from mono-nucleotide to hexa-nucleotide were detected, and both the perfect (containing a single repeat motif) and compound repeats (containing two or more motifs separated by 100 base pairs) were identified.

8. Phylogenetic analyses

The phylogenetic tree was constructed using the amino acid sequences of TPSs and CYPs from the *R. molle* transcriptome, as well as the representative functionally characterized proteins from other plant species. Accession numbers of protein sequences derived from GenBank and swissprot were listed in additional file1: Table S1. Amino acid sequences were aligned in ClustalX 2.1. The alignment was manually refined, removed the terminal gaps. The TPS and CYP phylogenetic trees were constructed using the MEGA7 software [31] by the neighbor-joining (NJ) method. The significance level for the phylogenetic tree was assessed by bootstrap testing with 1,000 replicates.

9. Identification of genes related to terpenoids

Custom databases of TPSs and CYPs were established according to the publicly available protein sequences. The sequences retrieved from GenBank are given in additional file 2: Table S2. The tBLASTn program was conducted to mine the candidate genes from the generated assemblies, with the *E*-value threshold of 1.0×10^{-50} and the minimum read length of 500 base pairs. All the identified unigenes were validated by using BLAST search in NCBI database.

10. Real-time PCR

RNA samples were isolated from the roots and flowers tissues. Reverse transcription was performed using the TransScript® One-Step gDNA Removal and cDNA Synthesis Super Mix kit (TransGene, Beijing, China) following manufacturer's instructions. The reaction was carried out at 42 °C for 15 min and 80 °C for 5 s. UltraSYBR Mixture (CWBIO, Beijing, China) and LightCycler480 II (Roche, Switzerland) Real-Time PCR System were used to conduct real-time quantification. The reaction mixture (20 µL) contained 10 µL of 2 × UltraSYBR Mixture, 0.5 µL of each forward and reverse primers, and 1 µL of template cDNA. PCR amplification was performed under the following conditions: 95 °C for 10 min and 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The gene-specific primers were designed using Primer 5.0 software and were listed in Additional file 3: Table S3. The GAPDH gene was used as an internal standard, Ct values were

determined based on three biological replicates of each sample and the relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [32].

Results

Transcriptome sequencing, and de novo assembly

Two cDNA libraries were constructed from the total RNA of *R. molle* flower and root, respectively. The libraries were sequenced using the Illumina HiSeq 2000 platform and approximately 26.58 Gb of clean data (89 million reads) were generated. The quality check showed that the base quality was above Q30 for 92% reads, and raw reads were trimmed prior to assembly. After trimming the adapter and low quality reads and removing those shorter than 50 bp, 47,559,180 and 41,387,924 high quality reads were obtained from the flowers and root libraries respectively for further analysis. Trinity software and TGI clustering tool (TGICL) were used for the *de novo* assembly and removed redundant clusters, a total of 100,603 unigenes were generated, with median length of 778 bp. The N50 length and average GC% were 1,384 bp and 47.7%, respectively. Among these unigenes, 40.88% (41,129) were longer than 500 bp, and 20.96% (20,886) were longer than 1000 bp, the length of most unigenes fell between 200 bp and 2000 bp, as shown in Additional file 4: Figure S1a. Additionally, a total of 20,213 coding sequences (CDSs) were predicted, with the average length of 515 bp, among which 41.1% (8,313) were longer than 200 bp in length (Additional file 4: Figure S1b). These results suggested the high-quality sequencing for subsequent functional annotations.

Functional annotation

For comprehensive annotation of assembled unigenes, sequence similarity search was performed against eight public databases. The result indicated that total 57,416 (57.1%) unigenes had significant matches in these public databases, while others were uninformative (e.g. “unknown” “unnamed” or “hypothetical protein”). Maximum annotation (56.2%) was resulted from NR database, while COG had the least number of annotated unigenes (16%). Additionally, 50.2%, 36.9%, 32.8%, 27%, 26.8% and 17.8% unigenes acquired significant hits in the eggNOG, GO (Gene Ontology), Pfam (Protein family), KOG (euKaryotic Orthologous Groups), swissprot and KEGG, respectively (Table 1). Moreover, the *E*-value and identity distribution were calculated to further analyze the BLAST results. Statistical analysis revealed that 45.71% of the mapped sequences displayed apparent homology ($< 1.0E^{-50}$), while the remaining unigenes had the *E*-value ranging from 1.0×10^{-50} to 1.0×10^{-11} (Additional file 5: Figure S2a). In addition, the identity distribution showed that the majority of the mapped unigenes (73.64%) exhibited a similarity of $> 60\%$, 47% unigenes showed similarity between 60–80%, while 13.83% unigenes showed similarity between 50–60%, only 13% unigenes were $< 50\%$ (Additional file 5: Figure S2b). The higher identity along with high quality *E*-value proved reliability of the *de novo* assembly generated in this study. According to Nr annotation result, 8.88% of the assembled unigenes had the highest homology with *Quercus Suber*, followed by *Vitis vinifera* with 5.95% resemblance and *Serendipita vermifera* with 4.82% resemblance (Additional file 5: Figure S2c). To facilitate the functional classification of the unigenes, GO annotation

was conducted, which provided the ontology of defined terms representing gene product properties. GO annotations were further classified into three major classes as biological process, cellular component and molecular functions, a total of 37,108 sequences were identified based on sequence homology and can be classified into 52 functional groups (Fig. 1). Cellular components category was divided into 15 classes, in which the predominant groups corresponded to the cell (16,736 unigenes, 45.10%) and cell part (16,728 unigenes, 45.07%) followed by membrane (14,451 unigene, 38.9 %), organelle (11,873 unigenes, 31.9%) and membrane part (11,098 unigenes, 29.9%). In the molecular function category, the top two groups were catalytic activity (18,589 unigenes, 50.09%) and binding (17,291 unigenes, 46.59%) which far outnumbered the unigenes corresponding to transporter activity (3,066 unigenes, 8.26%) and structural molecular activity (1,373 unigenes, 3.7 %). In the 22 groups of biological process, the most abundant unigenes belonged to metabolic processes (18,425 unigenes , 49.65 %), indicating rich secondary metabolites accumulated in *R.molle*, followed by those taking part in cellular processes (17,024 unigenes,48.65%) and single-organism process (12,502 unigenes, 32.73%).

Table 1 Annotation of unigenes against eight different databases

Annotated database	Annotated number	Percentage of annotated genes (%)
Nr	56,529	56.2%
eggNOG	50,523	50.2%
GO	37,108	36.9%
Pfam	32,970	36.9%
KOG	27,211	32.8%
Swissprot	26,983	26.8%
KEGG	17,906	17.8%
COG	16,102	16%
All annotated	57,416	57.1%

Pathway analysis by Kyoto Encyclopaedia of Genes and Genomes (KEGG).

Genes within the same pathway usually cooperate with each other to exercise their biological functions. Pathway-based analysis aid in understanding those functions and identification of unigenes involved in various biosynthetic pathways. In this study, KEGG pathway analysis was performed with the threshold *E*-value of $<10^{-5}$. A total of 17,906 (17.8%) unigenes were significantly matched into the KEGG database which were divided into five primary categories, including cellular process, environmental information processing, genetic information processing, metabolism , and organismal systems comprising 130 pathways (Fig. 2a). In our dataset, the highest numbers of unigenes were grouped into “carbohydrate metabolism(993 unigenes)” followed by “ribosome(881 unigenes)” and “biosynthesis of amino acid(703 unigenes) ”. We further explore the unigenes related to secondary metabolism, a total of 11 pathways including 437 unigenes were found to participate in “biosynthesis of other secondary metabolites”, among which the most unigenes were enriched in the phenylpropanoid biosynthetic pathway(232 unigenes)(Fig. 2b), followed by ‘flavonoid biosynthesis(53 unigenes) and ‘isoquinoline alkaloid

biosynthesis (50 unigenes). Furthermore, “metabolism of terpenoids and polyketides” subcategory contained 8 pathways including 253 unigenes, the cluster for ‘terpenoid backbone biosynthesis’ representing the largest group (102 unigenes), followed by carotenoid biosynthesis (49 unigenes) and diterpenoid biosynthesis (26 unigenes) (Fig. 2c).

Over view of differentially expressed genes

We performed DEGs analysis of the two transcriptome libraries to discover the unigenes with significant difference in expression. FPKM value was used to measure unigenes expression levels. The overall expression levels of flower unigenes were higher than root unigenes (Fig. 3a). Further analysis revealed that out of 100,603 unigenes generated from the combined assembly of both flower and root transcriptomes, 6,082 unigenes were differentially expressed in flower and root, including 1,120 up-regulated and 4,962 down-regulated unigenes in root vs flower (Fig. 3b). Furthermore, 507 unigenes were expressed uniquely in flower and 1,306 unigenes were expressed in both tissues, but at different levels. Hierarchical clustering of the 6,082 DEGs showed that the two tissues clustered relatively tight (Fig. 3c), indicating that some DEGs may involved in the same metabolic pathway. Out of 6,082 unigenes, 5,314 were annotated using different databases. For the GO enrichment analysis, the flower-specific up-regulated unigenes were assigned to several ontologies based on sequence homology, including 1,254 for cellular component, 716 for biological process, and 1,133 for molecular function. In the biological process category, the GO terms ‘metabolic process’ (GO:0008152) was most significantly enriched (304 unigenes), indicating the presence of vital metabolic activities in flowers (Fig. 4a). To further understand the involved metabolism pathways of DEGs, the KEGG enrichment analysis was performed. A total of 1,433 unigenes referring to 115 KEGG pathways were identified, the top three most abundant DEGs enrichment pathways were carbon metabolism (78 unigenes, 5.4%), starch and sucrose metabolism (66 unigenes, 4.6%), and biosynthesis of amino acids (61 unigenes, 4.3%), all of them were related to primary metabolism (Fig. 4b). Besides, DEGs were also enriched in biosynthetic pathways of secondary metabolites, a total of 222 DEGs involving 20 biosynthetic pathways were identified, among them 32 DEGs and 6 DEGs were clustered in flavonoid and phenylpropanoid biosynthetic pathways respectively. Moreover the “metabolism of terpenoids and polyketides” subcategory contained 8 pathways including 27 DEGs, and the highest numbers of DEGs (7) were clustered into terpenoid backbone biosynthesis. Furthermore, one DEG in monoterpene biosynthesis, four DEGs in sesquiterpene and triterpene biosynthesis, and six DEGs in diterpenoid biosynthesis (Additional file 6: Table S4) Further research on these genes can offer an improved understanding of terpenoid biosynthetic pathway.

Analysis of the secondary metabolic pathways

Identification of genes involved in terpenoid biosynthesis

Terpenoids are the major secondary metabolites accumulated in *R. molle*, which have been proved to possess many pharmacological activities. The biosynthesis process of terpenoids can be divided into two stages, namely, the synthesis of terpenoid backbone and specific terpene formation and modification. The terpenoid backbone is derived from the universal C-5 building blocks, dimethylallyl

diphosphate(DMAPP) and isopentenyl diphosphate (IPP)[33, 34]. In plants both the cytoplasmic mevalonate(MVA) and the plastidic methylerythritol phosphate(MEP) pathway contribute to supplying DMAPP and IPP with cross flow[35, 36], then the sequential condensation of DMAPP with IPP catalyzed by isoprenyl diphosphate synthases lead to the formation of the starting precursors of different classes of terpenes, geranyl diphosphate(GPP,C-10) for monoterpenes, farnesyl diphosphate(FPP, C-15) for sesquiterpene and geranylgeranyl diphosphate(GGPP, C-20) for diterpenes[33].(Fig. 5).

Based on the KEGG pathway assignment, a total of 102 unigenes for 17 key enzymes related to the biosynthesis of terpenoid backbone were annotated, accounting for 0.57% of all the assembled unigenes with pathway annotation. These unigenes were mainly distributed in the MVA(46 unigenes, 6 enzymes) and MEP(17 unigenes, 6 enzymes) pathways, which may participate in the biosynthesis of IPP, the common building block of terpenoids. Moreover, several genes(28 unigenes, 3 enzymes) were distributed in the downstream. In most cases, more than one unigenes was annotated as the same enzyme, suggesting that these unigenes might represent different members of the same gene family or the different fragments of a single transcript, or both. Corresponding unigenes were listed in Table 2. Among them, seven DEGs were discovered, three up-regulated unigenes were related to MEP pathway, including one for DXS, one for 2-C-Methyl-D-erythritol 2,4 cyclodiphosphate(MDS) and one for isoprene synthase. Besides, we also found three down-regulated unigenes which were involved in MVA pathway, including two for HMGS, one for PMK(Table 2). These results indicated that the MEP pathway was mainly responsible for synthesizing terpenoids in flowers.

Table 2 Unigenes involved in the terpenoid backbone biosynthesis in *R.molle*

Enzyme name	EC number	Unigene number	DEGs number
AACT	2.3.1.9	18	*
HMGS	2.3.3.10	10	2
HMGR	1.1.1.34	12	*
MK	2.7.1.36	1	*
PMK	2.7.4.2	2	1
MVD	4.1.1.33	3	*
DXS	2.2.1.7	3	1
DXR	1.1.1.267	1	*
CMS	2.7.7.60	1	*
MCS	4.6.1.12	1	1
HDS	1.17.7.1	2	*
HDR	1.17.7.2	2	*
IPPI	5.3.3.2	4	*
GPPS	2.5.1.1	12	*
FPPS	2.5.1.10	10	*
GGPS	2.5.1.1	6	*

* DEGs were not found AACT: acetyl-CoA acetyltransferase HMGS:hydroxymethylglutaryl-CoA synthase HMGR: hydroxymethylglutaryl-CoA reductase MK: mevalonate kinase PMK: phosphomevalonate kinase MVD: mevalonate diphosphate decarboxylase DXS: 1-deoxy-D-xylulose-5-

phosphate synthase DXR: 1-deoxy-D-xylulose-5-phosphate reductoisomerase CMS: 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase MCS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase HDS: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase IPPI: isopentenyl diphosphate isomerase GPPS: Geranyl diphosphate synthase FPPS:Farnesyl diphosphate synthase GGPS: Geranylgeranyl diphosphate synthase

The biosynthesis of specific terpenes starts from the common precursors GPP(10- carbon), FPP(15- carbon) and GGPP(20-carbon), these isoprenoid diphosphates are converted to distinct terpene scaffolds by terpene synthases(TPSs)[33]. The products of TPSs can undergo modifications primarily through the activity of cytochromes P450(CYP) enzymes and various types of transferases(e.g. acyl-, aryl-, methyl-, or glycosyltransferases). Particularly, CYPs can greatly diversify the terpenoid structure. To identify TPS and CYP candidates, the custom databases were built based on the publicly available protein sequences, which represented the least populous sequence sets without redundancy. A panel of nine terpene synthases was identified from the transcriptome data according to the sequence homology to the NCBI NR database(Additional file 7: Table S5), in which three unigenes were annotated as linalool synthase involved in monoterpene biosynthesis, and one unigenes as germacrene D synthase. Additionally, two copies of copalyl diphosphate synthase and one copy of ent-kaurene synthase were also identified, and details were shown in Table 3. These enzymes can be grouped into four families according to phylogenetic relationships(Fig. 6), six out of these TPS candidates (RmTPS1-5, RmTPS6) belonged to the TPS-a family, and RmTPS8 was classified into the TPS-e/f family. All the above-mentioned TPSs possessed the features of a class I terpene synthase, and only two class II terpene synthases were discovered in our dataset, which were RmTPS6 and RmTPS7 belonging to the TPS-c family.

Table 3 Terpene synthase candidate genes of *R. molle*

Terpene synthase	Gene	Unigene ID	Annotation
Mono-	RmTPS3	c135826	linalool synthase
	RmTPS4	c61279	linalool synthase
	RmTPS5	c97331	linalool synthase
Sesqui-	RmTPS9	c71656	germacrene D synthase
Di	RmTPS6	c95076	copalyl diphosphate synthase
	RmTPS7	c86861	copalyl diphosphate synthase
	RmTPS8	c92044	ent-kaurene synthase
*	RmTPS1	c79171	putative terpene synthase 9 [<i>Quercus suber</i>]
*	RmTPS2	c91666	putative terpene synthase2 [<i>Camellia sinensis</i>]

Cytochrome P450 monooxygenases (CYP) represent the largest superfamily of enzymes (around 1% of the sequenced plant genomes) in plant, but only few CYPs involved in terpenoid metabolism have been characterized, making it challenging to identify CYPs in the specialized terpenoids biosynthetic pathway of *R. molle*. In this study, the *R. molle* flower and root transcriptomes were mined against a P450-specific protein database, and a total of 61 candidates were identified(Additional file 7: Table S5).Phylogenetic analysis classified these CYPs candidates into 4 clans, members of the CYP71 clan were the most represented(Fig. 7), this clan harbors the most of CYP families involved in plant secondary metabolism[37, 38]. Terpenoid metabolism in plants is dominated by a few CYP families, among which

the CYP71 and CYP76 families are major contributor[38, 39]. We used reported proteins from these two CYP families as probes to investigate our transcriptomes and a total of nine CYP enzymes from CYP71 and CYP76 families were identified(Fig. 8). To further screen the highly probable CYP candidates, the gene expression levels were assessed based on the FPKM values. The results indicated that the majority of the genes were expressed in both of the two tissues but showed different expression levels. Specifically, Rm89174 was significantly up-regulated in the flower tissue, while Rm66646 and Rm92121 were highly expressed in the root tissue of *R. molle*(Fig. 9a). The unigenes expression levels detected in FPKM analysis were further verified through qRT-PCR analysis(Fig. 9b). Generally, expression level measured by qRT-PCR was consistent with RNA-Seq data. The CYP unigenes showed accordant expressions in both qRT-PCR and FPKM analysis, confirming the reliability of the sequencing results. These results provide a reference for future functional characterization of TPS and CYP candidates involved in terpenoids biosynthesis in *R. molle*. Nevertheless, further research is warranted to uncover the true functions of these unigenes.

Lignan biosynthetic genes

Phenylpropanoids are derived from phenylalanine and comprise a large group of plant natural products with extensive bioactivities, such as hepatoprotection and antioxidation. These compounds contribute to all aspects of plant responses towards biotic and abiotic stimuli[40]. The general phenylpropanoid metabolism generates enormous secondary metabolites based on the few intermediates of the shikimate pathway. The biosynthetic pathway starts with the formation of cinnamic acid from phenylalanine, which results in the formation of cinnamoyl-CoA and p-coumaroyl-CoA. These CoA-activated compounds are the precursor for synthesizing lignans, flavonoids, flavonols as well as numerous other secondary metabolites (Fig.10). In the present study, we performed KEGG analysis on both *R. molle* flowers and roots transcriptome and the results revealed a total of 232 unigenes were involved in the phenylpropanoid biosynthetic pathway. Ten unigenes were annotated for coding phenylalanine ammonia-lyase (PAL) and three unigenes were annotated to code the trans-cinnamate 4-monooxygenase. Both of the two enzymes play a significant role in the formation of important intermediate cinamic acid. Besides, 18 unigenes were annotated as 4-coumarate–CoA ligase (4CL) and 6 unigenes were annotated to code cinnamoyl-CoA reductase (CCR). Moreover, enzymes at branching points were also identified, and the representative enzymes are listed in Table 4.

Table 4 Representative enzymes in phenylpropanoid biosynthetic pathway in *R. molle*

Enzyme name	Annotation	EC number	Unigene number
PAL	phenylalanine ammonia lyase	4.3.1.24	10
4CL	4-coumarate--CoA ligase	6.2.1.12	18
CCR	cinnamoyl-CoA reductase	1.2.1.44	6
CYP73A	Trans-cinnamate 4-monooxygenase	1.14.14.91	3
HCT	shikimate O-hydroxycinnamoyl transferase	2.3.1.133	10
CYP98A	5-O-(4-coumaroyl)-D-quinic acid 3-O-methyltransferase	3'- 1.14.14.96	4
CYP84A	ferulate-5-hydroxylase	1.14--	4
COMT	caffeic acid 3-O-methyltransferase	2.1.1.68	12

Identification of genes related to flavonoid biosynthesis

Flavonoids are important polyphenolic plant secondary metabolites that can be categorized into flavones, flavonols, flavanone, isoflavones, catechins and chalcones [41]. Appropriate intake of flavonoids can reduce the incidence of cancer, cardiovascular disease, lipid peroxidation and osteoporosis[42]. Considering the diverse beneficial effects of flavonoids, this study explored the unigenes related to flavonoid biosynthesis in the transcriptomes of *R. molle* flower and root. Coumaroyl-CoA and malonyl-CoA are the common precursors for the biosynthesis of flavonoids, which are derived from phenylpropanoid pathway and carbohydrate metabolism, respectively. The biosynthesis of flavonoids is initiated by chalcone synthase(CHS), which generates chalcone as the important intermediate and the pathway proceeds with several enzymatic steps for forming other classes of flavonoids, like flavanones and dihydroflavonols. In addition, the side branches of the flavonoid pathway lead to synthesis of other flavonoid classes including isoflavones, flavones, and flavonols(Fig. 11). A total of 53 unigenes related to flavonoid biosynthetic pathway were annotated. Starting from the initial committed enzymes for the biosynthesis of flavonoids, chalcone synthase(CHS), chalcone isomerase(CHI) and flavanone hydroxylase(F3H) were identified, all of which continuously catalyzed p-coumaroyl-CoA and malonyl-CoA into the important intermediate dihydrokaempferol. Additionally, the flavonoid-3 β -hydroxyase and flavonoid-3 β ,5 β -hydroxyase, which are essential for converting dihydrokaempferol into dihydroquercetin and dihydromyricetin were also identified. The main enzymes involved in flavonoid biosynthesis are listed in Table 5.

Table 5 Unigenes involved in the flavonoid biosynthetic pathway in *R. molle*

Enzyme name	Annotation	EC number	Unigene number
CHS	chalcone synthase	2.3.1.74	6
CHI	chalcone isomerase	5.5.1.6	3
F3H	flavanone hydroxylase	1.14.11.9	7
F3'H	flavonoid-3'-hydroxylase	2.7.1.36	2
F3'5'H	flavonoid-3'5'-hydroxylase	1.14.14.81	3
DFR	dihydroflavonol-4-reductase	1.1.1.219	3
ANS	anthocyanidin synthase	1.14.20.4	7
FLS	flavonol synthase	1.14.20.6	2

Identification of transcription factors

In plant, transcription factors (TFs) often play a key role in regulating gene expression at the transcriptional level, which can also affect the metabolic flux by interacting with the promoter regions of gene. Based on our Blast X search against the known Plant Transcription Factor database, 3376 putative *R. molle* transcription factor distributed in at least 49 TF families were identified, which represented 3.35% of the total assembled unigenes (Fig. 12). Among them, C2H2 was the most abundant TF family (329 unigenes, 9.7%), followed by zn-clus (179 unigenes, 5.3%), and bZIP (105 unigenes, 3.1%). C2H2 family members are crucial to plant developmental processes including floral organogenesis, initiation of leaves and lateral shoots and seed development [43]. bZIP regulates processes including pathogen defense and stress signaling [44]. The present study also identified 59 and 97 unigenes encoding for MYB and MYB related TFs, respectively. MYB TFs regulate the phenylpropanoid biosynthesis in several plant species and mostly include the R2R3-MYB TFs, which have also been shown to regulate the main branch viz. flavonoid metabolic pathway in phenylpropanoid biosynthesis in several plants including *Arabidopsis thaliana* [45], *Prunu spersica* [46] and *Epimedium sagittatum* [46]. In addition, 89, 83 and 51 unigenes were also found to be related to bHLH, AP2-ERF and WRKY, respectively. These TFs have various roles throughout the whole life cycle of plant, from the regulation of several developmental processes to the response to environmental stress [47-49]. Moreover, they are especially important to secondary metabolism in plants. In addition, bHLH TFs also regulate the flavonoid biosynthetic pathway in plants [50, 51]. The AP2/ERF TF family members modulate the biosynthetic genes for terpenoid indole alkaloids in *Catharanthus roseus* [52]. Further investigation on these TFs may provide a clear profile on the regulatory network for the biosynthesis of secondary metabolites in *R. molle*.

Identification of SSRs

Simple sequence repeats (SSRs) also termed as microsatellites, are tandemly repeated short DNA motifs which are ranging from one to six base pairs, they are extensively distributed in eukaryotes including plants, animals and fungi as well as in some prokaryotes [53]. SSRs are generally associated with phenotypic variations which have become the most extensively utilized informative molecular markers that favor for a variety of applications, including the genetic breeding of plants, gene mapping and genetic marker-assisted selection [54]. To identify SSRs, all the assembled unigenes of both *R. molle*

flower and root were analyzed using MISA. Overall 10,828 SSRs were identified from 7,799 unigene, in which the most abundant SSRs were mono-nucleotide repeat motif(4,868, 44.95%), followed by di-nucleotide(3,872, 35.75%) and tri-nucleotide(1,292, 11.93%), while hexa-nucleotide SSRs(6, 0.055%) had the lowest abundance. Additionally, there were 66(6.1%) tetra-nucleotide and 14(0.13%) penta-nucleotide SSR.(Additional files 8: Figure S3). In particular, several SSR motifs were linked with the unigenes encoding enzyme(eg. HMGR, DXS) involved in terpenoid biosynthesis(Table 6).These unique markers may represent a valuable genetic resource for future genetic diversity analysis in *R. molle* and the related species.

Table 6 SSR motifs in unigenes related to terpenoid biosynthesis

Enzyme name	Unigene ID	Number of SSRs	SSR motif	Number of repeats
HMGR	c88044	4	AAC	6
			T	13
			GA	7
			AAC	5
MCT	c86870	1	TC	7
GGPS	c83679	1	*	10
DXS	c83170	1	1.14.20.4	7
	c84061	1	1.14.20.6	2

* represent compound repeat type. SSR motif: (T)GATCAGCAGAAAGATGAGG ACTTTGATTCATGGTACTGTAACAGCATCTGACGTTTTGCAGG(A)

Discussion

R. molle is a well known traditional Chinese medicinal plant, which has been extensively investigated on the natural plant over the past few years [3-5]. It contains abundant secondary metabolites including terpenoids, flavonoids, and lignans, which account for the broad bioactivities, such as analgesic effect, antioxidation and sodium channel modulation[5, 55]. However, despite the important medicinal value of *R. molle*, the molecular mechanism and putative genes involved in secondary metabolic biosynthetic pathway remain unclear so far. With the advent of the next generation sequencing (NGS) technology, the RNA-Seq approach has been extensively used to elucidate the biosynthetic pathways of natural products through gene discovery[56-58]. In this study, we performed high-throughput transcriptome sequencing for root and flower of *R. molle*. In total, 100,603 unigenes were assembled with the average length of 778 bp and N50 1,384 bp, which indicated the assembly integrity was qualified and could be used for subsequent analysis. Among them, 57,416 unigenes were annotated in the public databases. However, 42.9% unigenes remained unannotated, which suggested the publicly available plant transcriptome and genome data are insufficient due to few related species of *R. molle* were sequenced, these unigenes may be related to the biosynthesis of specific secondary metabolites produced in *R. molle*, which deserves our attention and further study. In addition, there were 20,213 predicted CDSs, accounting for 20.1% of the

total unigenes, which are beneficial for further analysis on unigene function at the protein level and provide information for studying the crucial genes.

Furthermore, the Gene Ontology assignment program was employed for functional categorization of the annotated unigenes, and a total of 37,108 unigenes obtained GO terms. In many cases, multiple terms were assigned to the same transcript, which resulted in 25,634 assignments to cellular components, 27,218 to biological processes, and 29,126 to molecular functions. The rest of the unigenes remained unannotated, which may be due to relatively short sequences that were unable to cover the conserved protein domains. Within the molecular function category, the vast majority of unigenes were related to 'metabolic process', which was in accordance with the abundant secondary metabolites produced in *R. molle*. Through mapping unigenes onto the KEGG pathways, 232 unigenes were discovered to be enriched in phenylpropanoids biosynthetic pathway, which represented the largest cluster of all secondary metabolic pathways, and this result indicated that the biosynthesis process of phenylpropanoids serves as a reservoir to provide intermediates for the biosynthesis of other diverse secondary metabolites in *R. molle*. Our study also found 53 unigenes related to flavonoid biosynthesis. Moreover, numerous unigenes were identified to be involved in terpenoid backbone biosynthesis, including AACT, HMGR, DXR, MCS, IPPI, FPPS, and GPPS. Further characterization of these unigenes will improve our understanding of the molecular mechanisms underlying terpenoid biosynthesis. TPS is suggested to be the first committed enzyme in the biological process of terpenoids biosynthesis, which can initially cyclize the common isoprenyl diphosphate precursors (GPP, C-10 FPP, C-15 and GGPP C-20) to form terpene scaffolds. We mined the sequenced transcriptome of *R. molle* flower and root and a small TPS gene family was identified including three monoterpene synthases annotated as linalool synthase and three diterpene synthases (diTPSs). Out of the three diTPS, two unigenes were annotated as copalyl diphosphate synthase and one unigene as kaurene synthase, while only one sesquiterpene synthase was identified and annotated as the germacrene D synthase. The structural complexity of terpenoids indicates that most of them carry a high oxygenation degree, which is quite possible to be catalyzed by CYPs. To explore the molecular underpinnings of terpene backbone modification, 61 unigenes putatively encoding CYPs were identified from the transcriptome, among which four and five unigenes belonged to the CYP71 and CYP76 families, respectively. These two families have been considered as the main driving force of terpenoids diversity in plants. However, sequence similarity cannot guarantee specific functions due to the functional plasticity within the TPS and CYP families. Therefore biochemical approaches are still necessary for the accurate functional annotation of these TPS and CYP candidates. In addition, our study examined the differentially expressed unigenes between the flower and root based on FPKM values. The results suggested that several unigenes were uniquely expressed in either the flower or root tissues and many unigenes were expressed at different levels. Further study on these DEGs combined with metabolomes will enable us to more clearly understand the biosynthetic process of secondary metabolites. Besides, transcriptomes also serve as invaluable resource for discovery of SSRs. In this study, 10,828 SSRs were identified which will be useful for cultivar fingerprinting, selection of desirable genotypes in *R. molle* breeding and facilitate genetic manipulations in *R. molle*. TFs affect the metabolic flux through regulating gene expression. In this work, a total of 3376 TFs were identified,

including bHLH (89), AP2/ERF (83), MYB (59), MYB related (97) and WRKY (51) families. The number of TFs in *R. molle* is slightly higher than that of the well known diterpenoid-producing medicinal plant, *S. miltiorrhiza* (1948 TFs), and than that of the model plant *A. thaliana* (2357 TFs), indicating that there are complex metabolic regulation networks in *R. molle*. These TFs may play significant roles in regulating biosynthesis of secondary metabolite. In *Artemisia annua*, the biosynthesis of artemisinin has been effectively regulated by the bHLH transcription factor AabHLH1[59], in *Catharanthus roseus*, the AP2/ERF proteins of ORCA2 and ORCA3 regulate terpenoid indole alkaloid metabolism through binding to the promoter of the strictosidine synthase (STR)[52]. It is of substantial significance to use the genetic engineering methods to control TFs for regulating terpenoid biosynthesis in *R. molle*. Further investigate on these TFs will be helpful for manipulating the metabolic pathways and ultimately increasing the yield of secondary metabolites with medicinal value in *R. molle*.

Conclusions

In this study, we presented the *de novo* transcriptome sequencing of *R. molle* flower and root. A total of 100,603 unigenes were generated and 57,416 unigenes were annotated by public databases. 17,906 unigenes were matched with the KEGG database, which were involved in 133 different pathways of plant metabolism. Out of 17,906 unigenes, 102 unigenes were involved in terpenoid backbone biosynthesis. Moreover, 9 TPSs were identified through BLAST similarity search. 61 CYP enzymes were also discovered, among them 9 CYPs were from CYP71 and CYP76 families. The transcriptome information presented in our study also revealed that various genes involved in the biosynthetic pathways of lignans, flavonoids, and other secondary metabolites. Additionally, our study identified several transcription factors related to the biosynthesis of secondary metabolites and 10,828 SSRs were also discovered from our transcriptomic database, which are potential for genetic manipulations in *R. molle*. Taken together, the transcriptome data generated in our study, will allow for discovering novel genes involved in specific secondary metabolic pathways, and also provide basis for improving the yields of valuable metabolites in plants or in microbial hosts by metabolic engineering. Moreover, it is also highly valuable to pave the way for functional and comparative genomic studies of this promising medicinal plant in future.

Additional Files

Additional files 1: Table S1. Accession numbers of protein sequences derived from GenBank and swissprot used for the phylogenetic analysis. (XLSX 15 kb)

Additional files 2: Table S2. TPSs and CYPs sequences derived from GenBank used for building custom databases. (XLSX 92 kb)

Additional files 3: Table S3. Primers used for qPCR. (XLSX 11 kb)

Additional files 4: Figure S1. (a) Length distribution of assembled unigenes. (b) Length distribution of predict CDSs. (TIF 429 kb)

Additional files 5: Figure S2. Characteristic of homology search of assembled unigenes against NR database. (a) E-value distribution of top Blast hits. (b) Similarity distribution of unigenes. (c) Species distribution of Blast hits. (TIF 362 kb)

Additional files 6: Table S4. DEGs involved in biosynthetic pathways of secondary metabolites. (XLSX 16kb)

Additional files 7: Table S5. RNA-Seq data of identified TPSs and CYPs candidates in *R. molle*. (XLSX 65kb)

Additional files 8: Figure S3. SSR Density Distribution Map. The X-axis is SSR type, the Y-axis is the number of SSRs per Mb sequence. SSR type (p1: mono-nucleotide repeat motif p2: di-nucleotide repeat motif p3: tri-nucleotide repeat motif p4: tetra-nucleotide repeat motif p5: penta-nucleotide repeat motif p6: hexa-nucleotide repeat motif c: complex repeat motif c*: two SSR sequences with repetitive common parts. (TIF 83 kb)

Abbreviations

AACT: acetyl-CoA acetyltransferase CCR: cinnamoyl-CoA reductase CDS : coding sequence CHI: chalcone isomerase CHS: chalcone synthase CL: 4-coumarate-CoA ligase CMS: 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase COG: Clusters of Orthologous Groups CYP: Cytochromes P450 DEGs: Different expressed genes; DMAPP: Dimethylallyl pyrophosphate DXR: 1-deoxy-D-xylulose-5-phosphate reductoisomerase DXS: 1-deoxy-D-xylulose-5-phosphate synthase

FPKM: Fragments per kilobase of transcript per million mapped reads

FPP: Farnesyl diphosphate FPPS: Farnesyl diphosphate synthase

GGPP: Geranylgeranyl diphosphate GGPPS: Geranylgeranyl diphosphate synthase GO: Gene Ontology GPP: geranyl diphosphate

HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase

HDS: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase

HMGR: Hydroxymethylglutaryl-CoA reductase HMGS: Hydroxymethylglutaryl-CoA synthase IPP: Isopentenyl diphosphate IPPI: isopentenyl diphosphate isomerase KEGG: Kyoto Encyclopedia of Genes and Genomes; KOG: EuKaryotic Ortholog Groups; MCS: 2,4-cyclodiphosphate synthase ; MEP: Methylerythritol phosphate ; MK: mevalonate kinase; MVA: Mevalonic acid; NJ: Neighbor-Joining;

NGS: next generation sequencing Nr: NCBI Non-redundant Protein; PAL: Phenylalanine ammonia lyase ; Pfam(Protein family); PlnTFDB: Plant transcription factor database ; PMK: Phosphomevalonate kinase, TFs: Transcription factors; TPS: Terpene synthases; SSR: Simple sequence repeats

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

Raw data of all sample-sequencing results are available under NCBI BioProject database under accession number PRJNA565490. All the supporting data are included as additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

GL Z designed and conducted this study and draft the manuscript. P Z conceived, designed and supervised this study and revised the manuscript. All authors approved the final manuscript.

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Figures

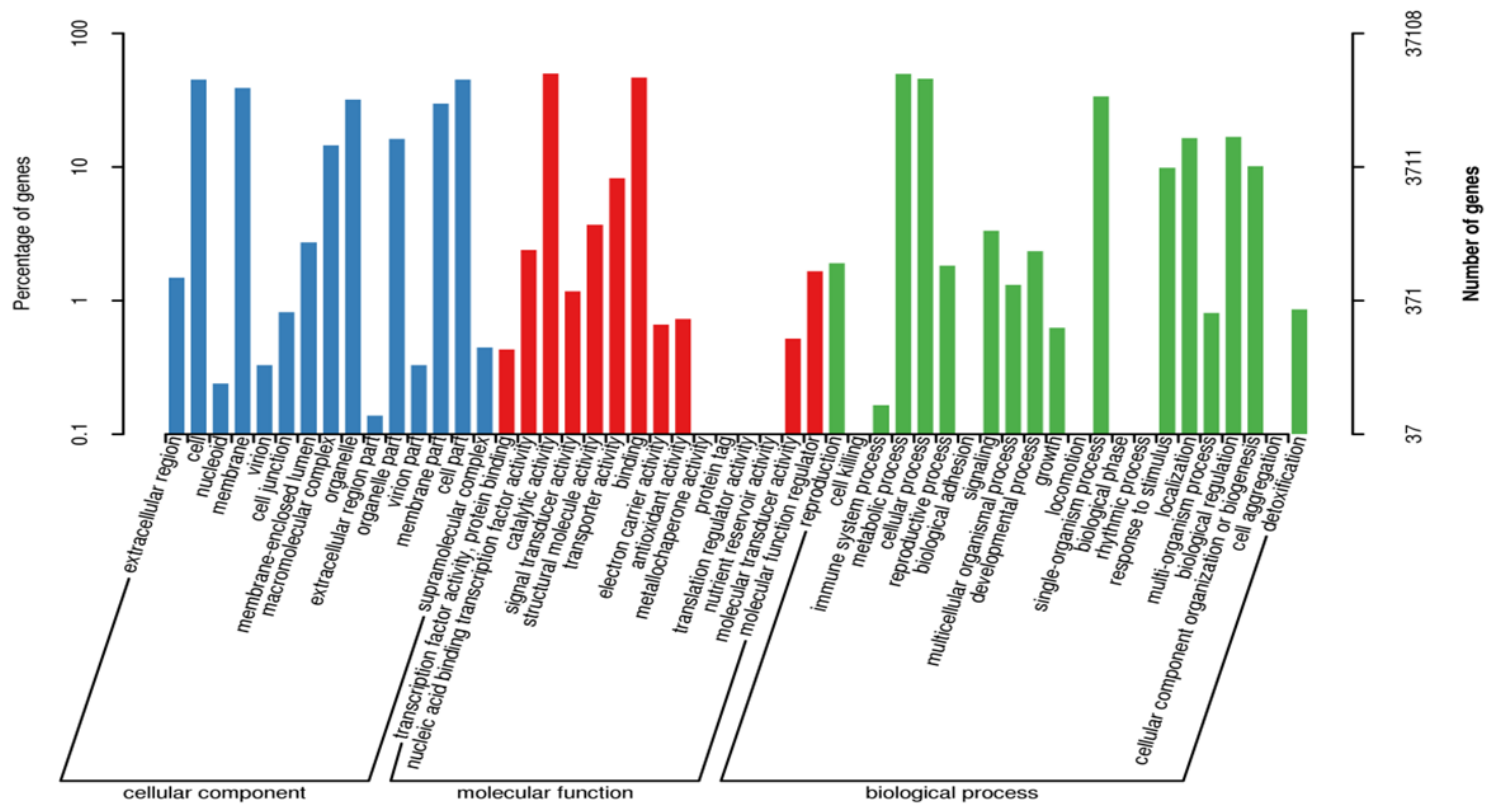


Figure 1

GO functional classifications of *R. molle* unigenes

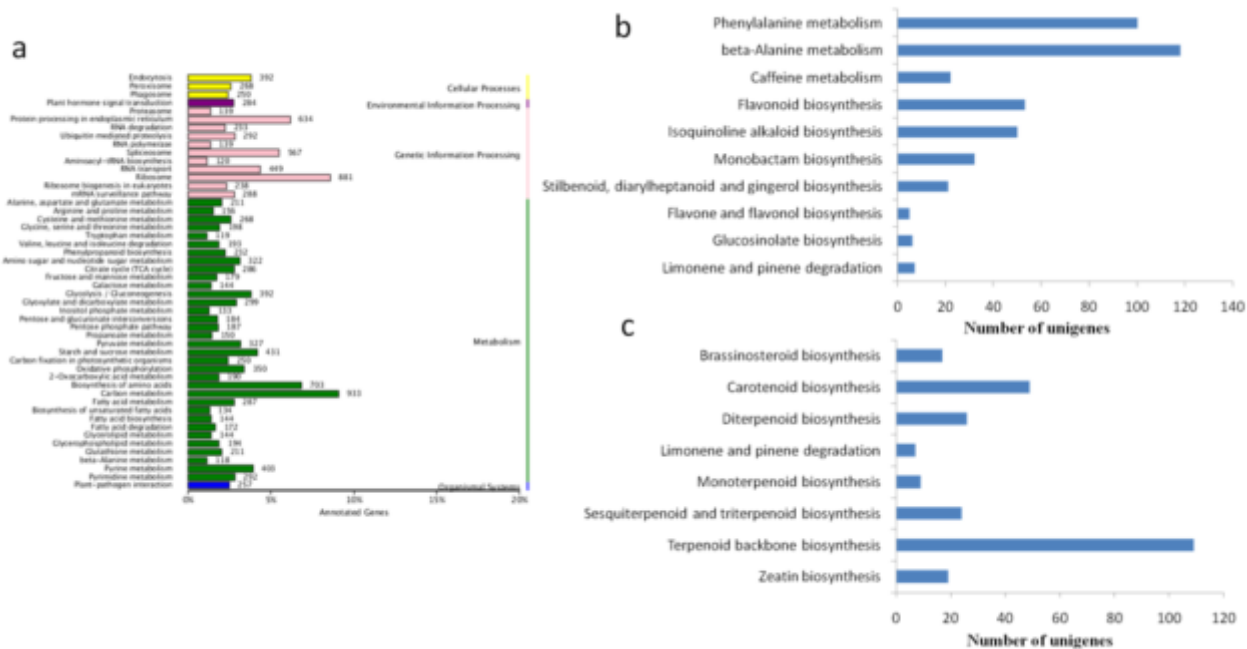


Figure 2

KEGG annotation of *R. molle* unigenes. a KEGG functional classifications of assembled unigenes. b Classifications of the subcategory "biosynthesis of other secondary metabolites". c Classifications of the

subcategory “metabolism of terpenoids and polyketides”.

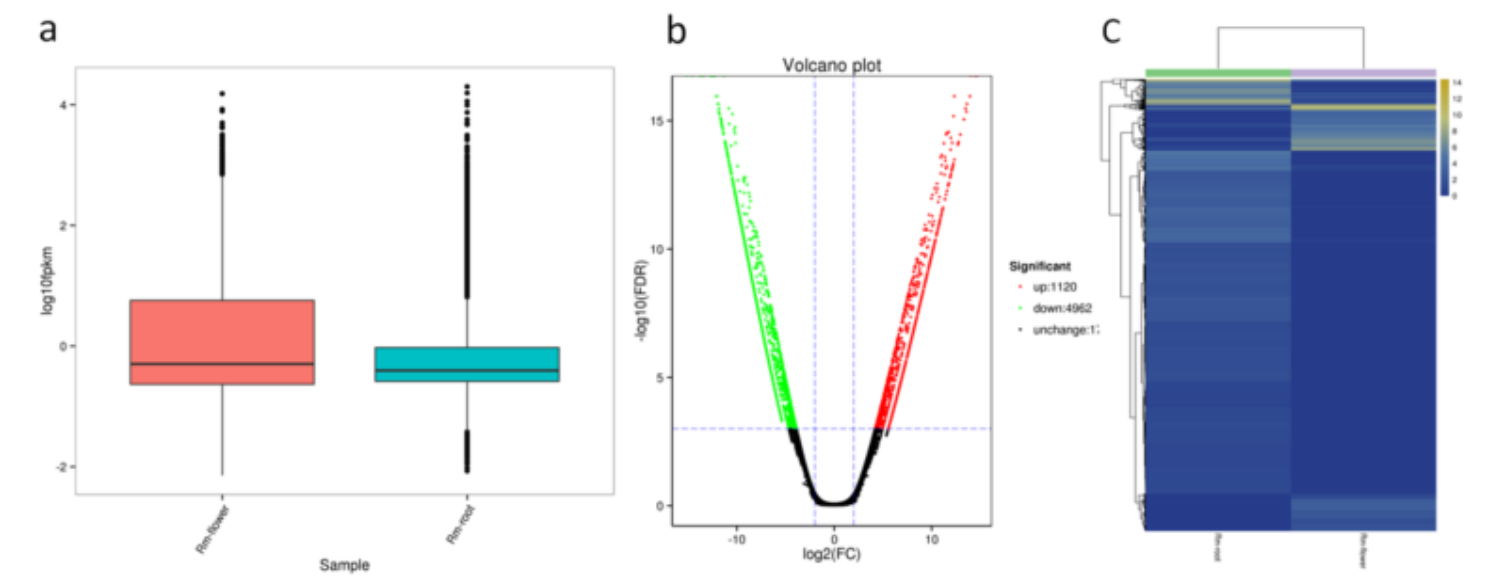


Figure 3

Overview of expression profiles in *R. molle* flower and root. a Boxplot of unigenes expressed in the two tissues. b The volcano plots of differentially expressed genes between flower and root. (C) Heatmap of unigenes co-expressed in the two tissues. The intensity of the colour scheme is scaled to the log2 (FPKM) expression values, and yellow and blue represent high and low expression levels, respectively.

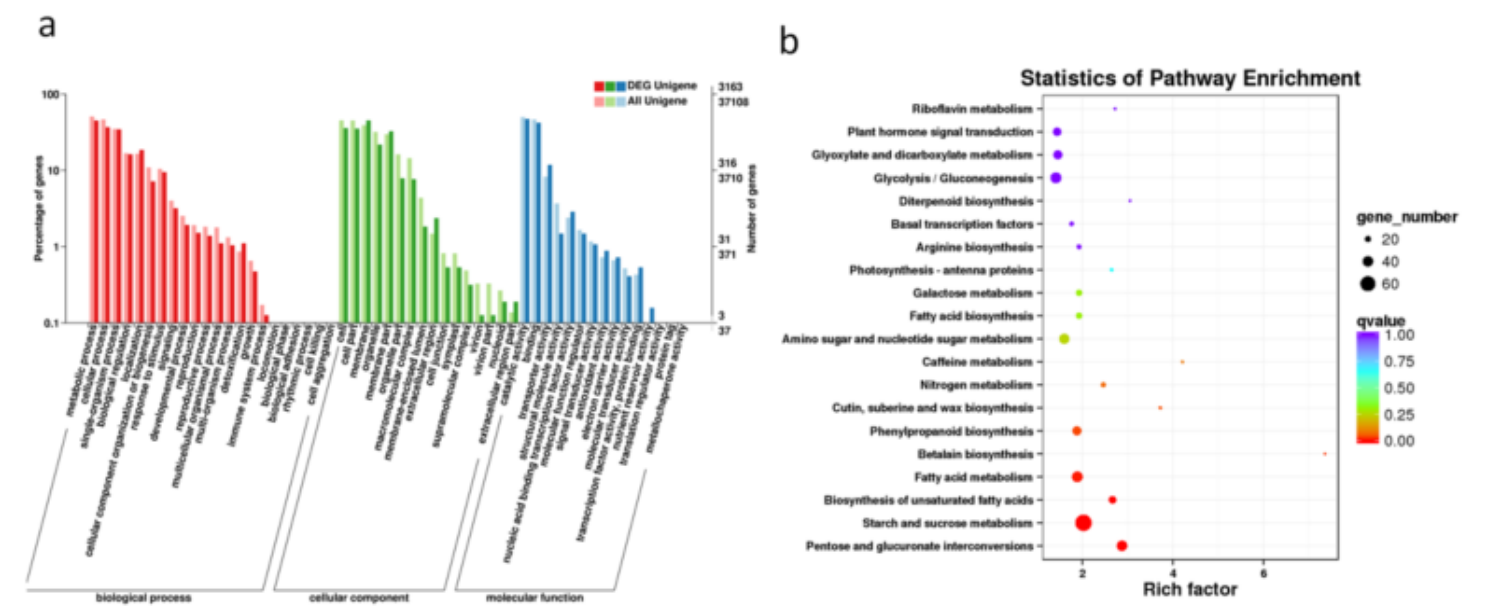


Figure 4

GO and pathway enrichment analysis of DEGs. a The gene enrichment of each secondary

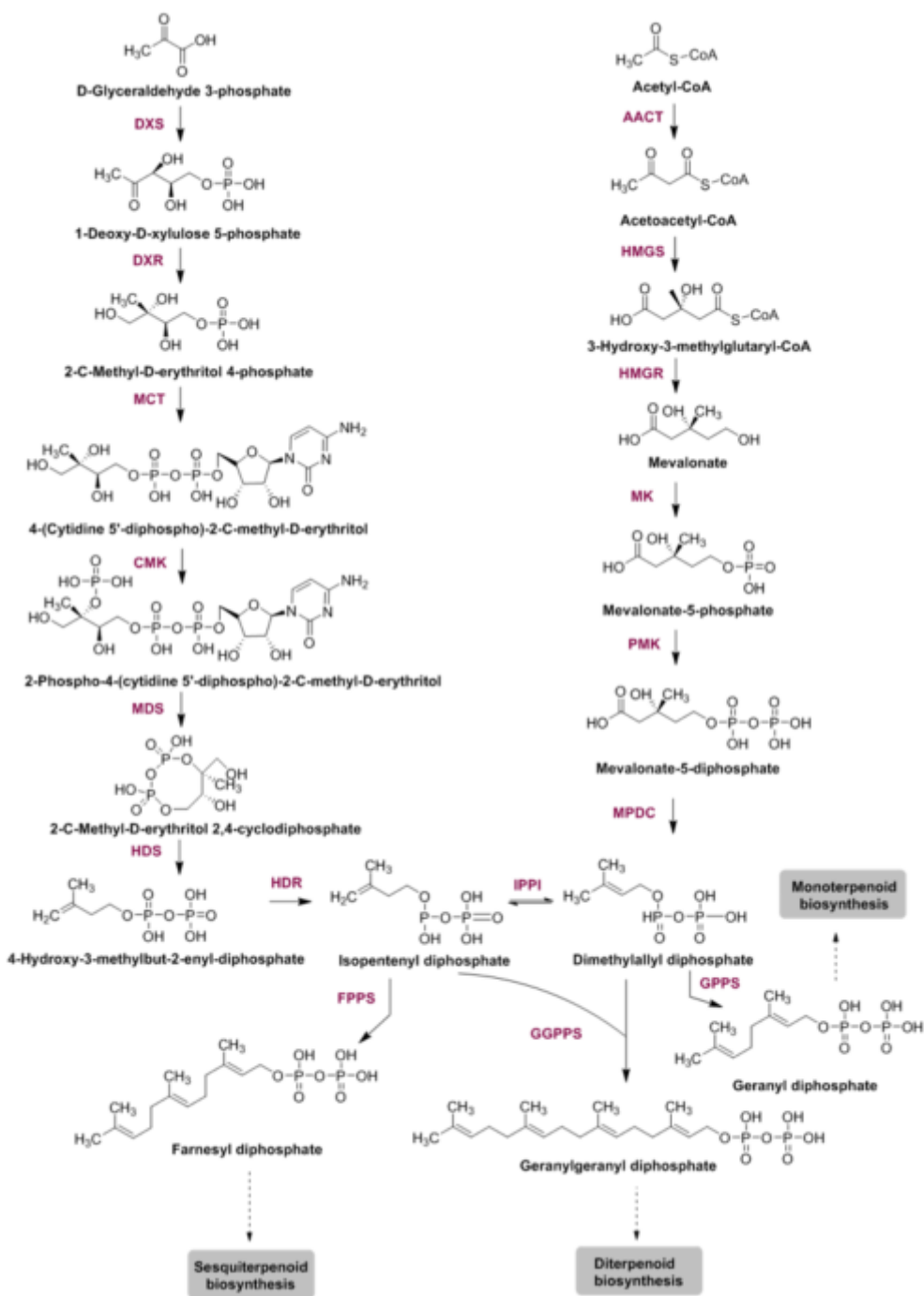


Figure 5

The terpenoid biosynthetic pathway in *R. molle*

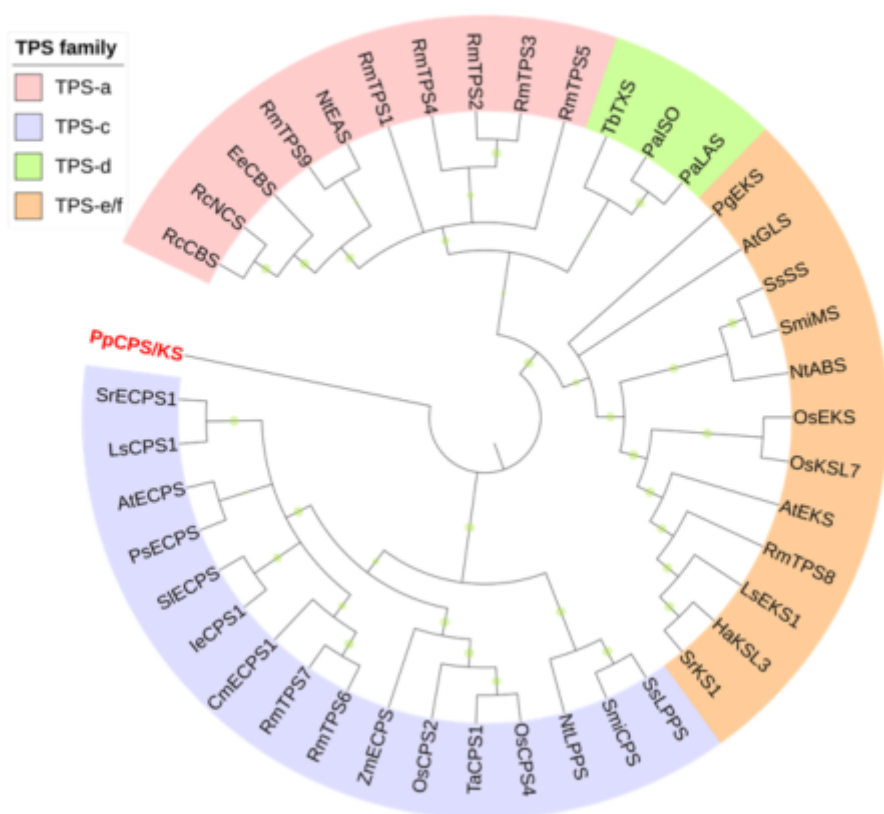


Figure 6

The phylogenetic tree of terpene synthases from *R. molle*. Neighbor joining tree. *P. patens* copalyl diphosphate synthase/kaurene synthase (PpCPS/KS) was used as the tree root.

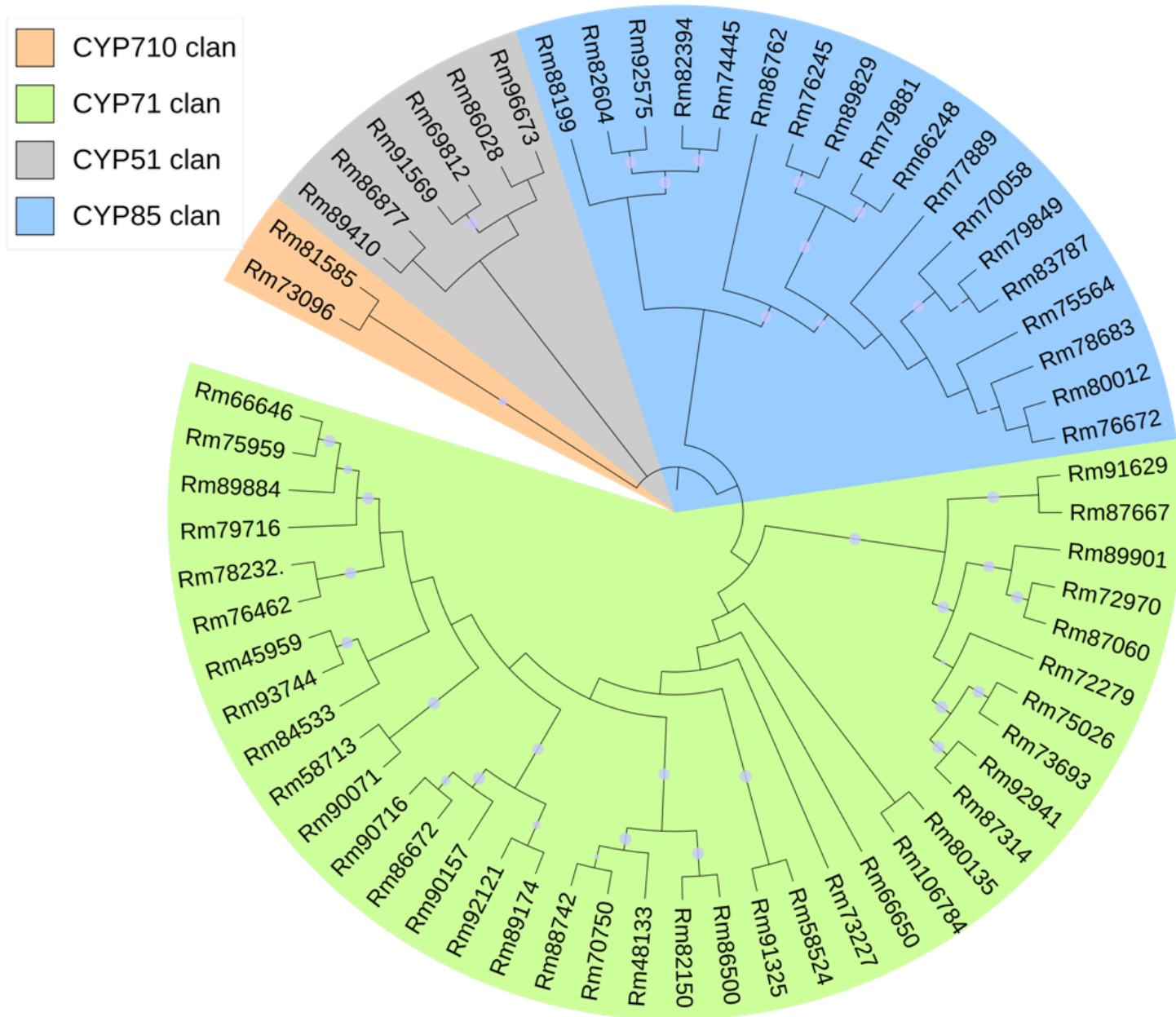


Figure 7

Phylogenetic analysis of CYPs from *R. molle*

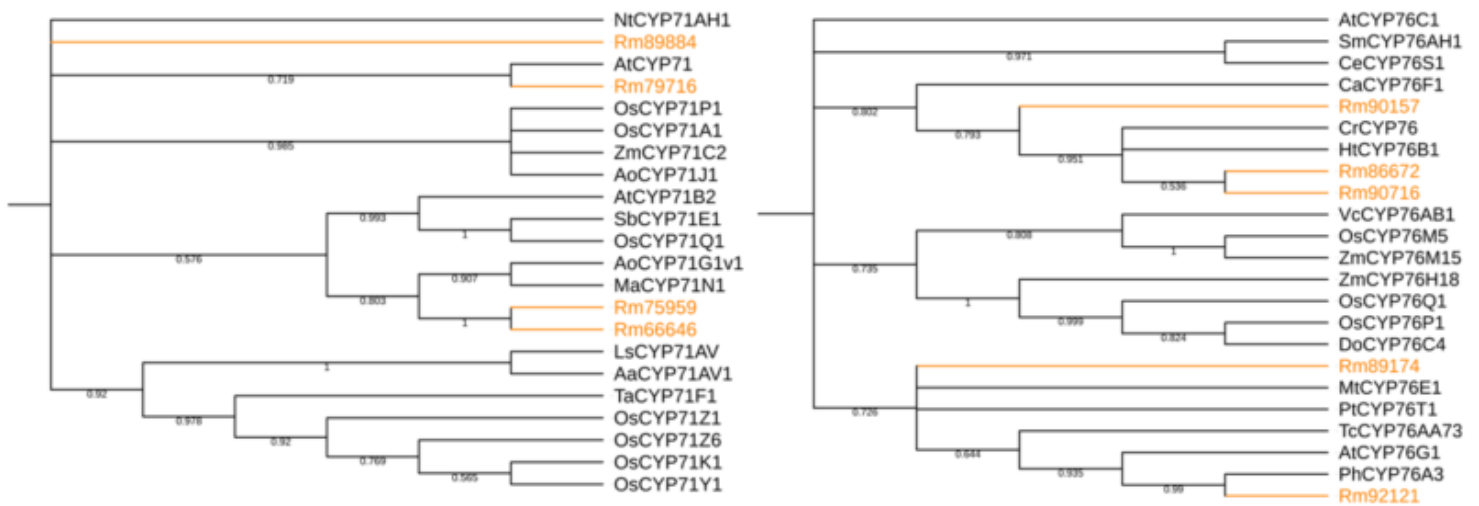


Figure 8

The phylogenetic analysis of CYP71 and CYP76 families in *R. molle*

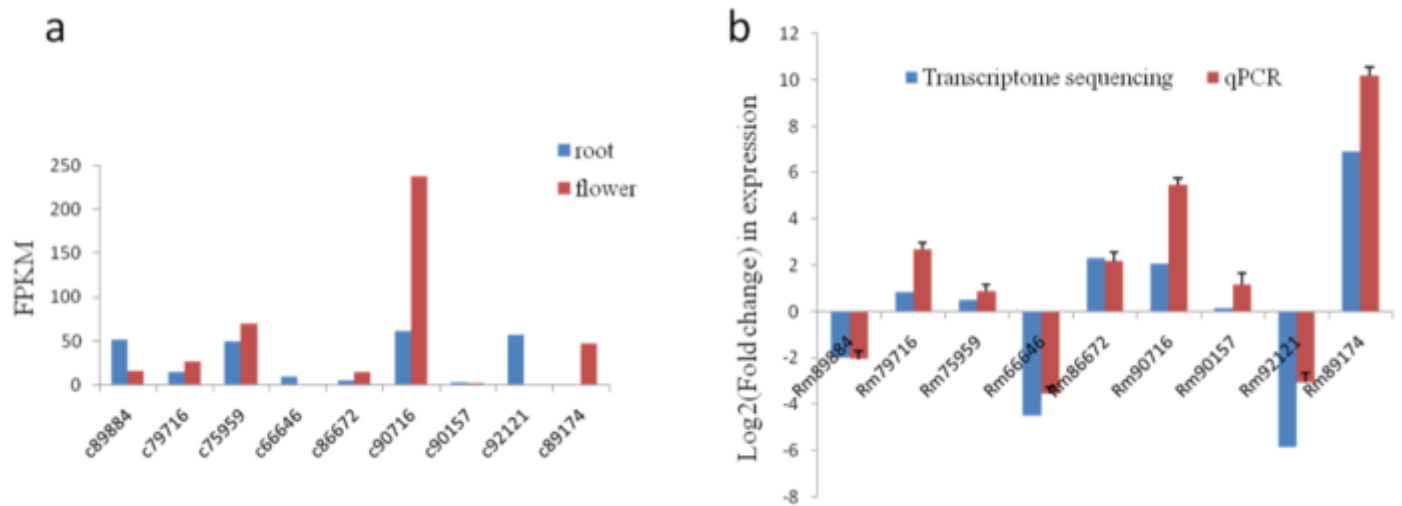


Figure 9

Relative expression of CYP71 and CYP76 families candidates in *R. molle*. a Expression profile of CYP candidates based on FPKM. b Gene expression levels of CYP candidates verified by qRT-PCR.

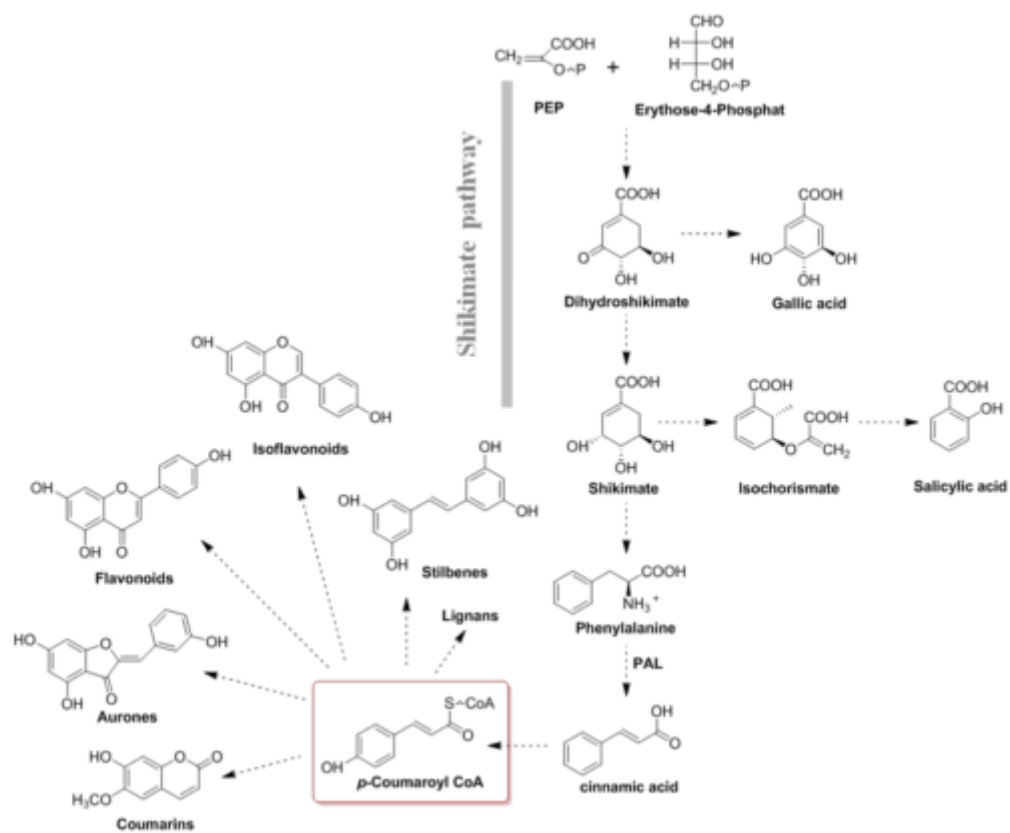


Figure 10

Phenylpropanoid Pathway and diversification of Phenylpropanoids in *R. molle*

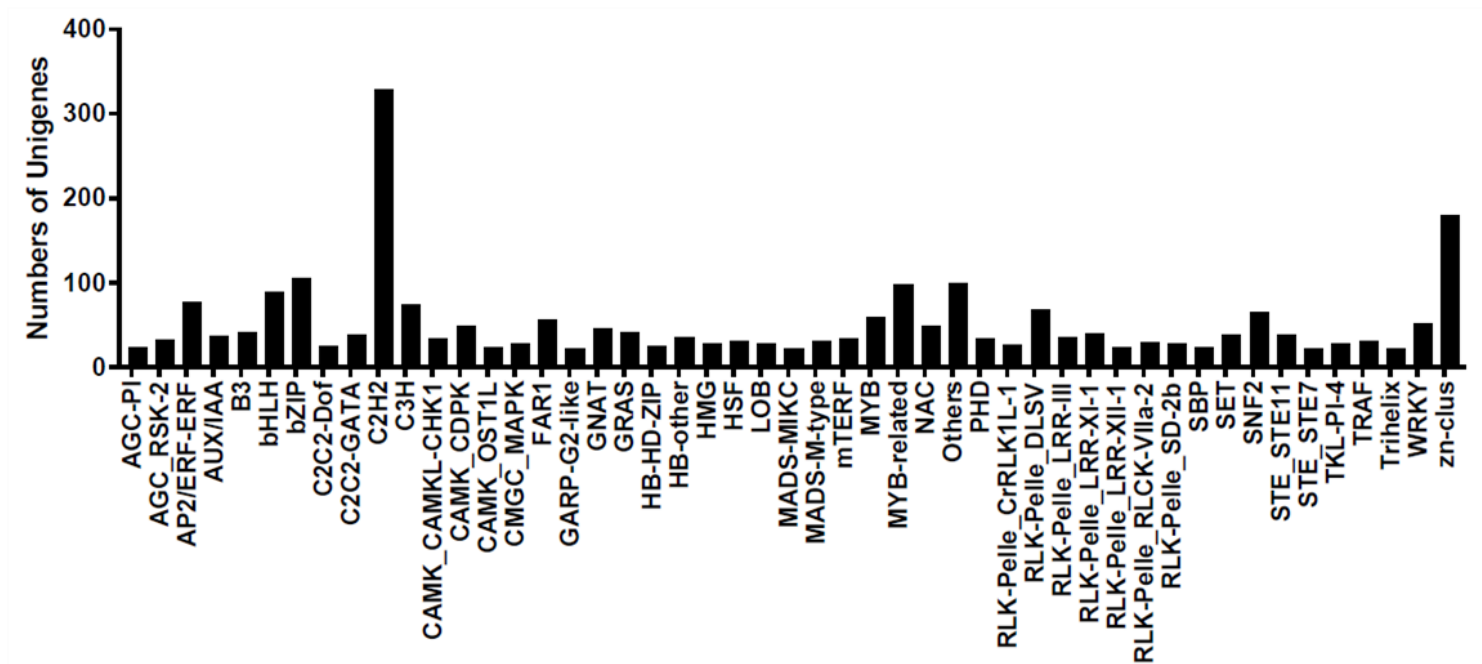


Figure 12

TF family classification of *R. molle* unigenes

Supplementary Files

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- [Additionalfiles8.tif](#)
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- [Additionalfiles4.tif](#)
- [Additionalfiles5.tif](#)