

Phytochemical and comparative transcriptome analysis reveals different regulatory mechanisms in terpenoids pathways between *Matricaria recutita* L. and *Chamaemelum nobile* L

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Abstract

Background *Matricaria recutita* (German chamomile) and *Chamaemelum nobile* (Roman chamomile) belong to the botanical family Asteraceae. These two herbs are not only morphologically distinguishable, but their secondary metabolites – especially the essential oils in flowers are also different, especially the terpenoids. The aim of this project was to preliminarily reveal differential regulatory mechanisms in terpenoids biosynthetic pathway between German and Roman chamomile by performing comparative transcriptomic and metabolomics analyses. **Results** We determined the content of essential oil in disk florets and ray florets in these two chamomiles, and found that the terpenoids content of German chamomile is greater than that of Roman chamomile. In addition, comparisons between German and Roman chamomile were studied by RNA-Seq, which showed that 54% of genes shared >75% sequence identity between the two species. In particular, more highly expressed DEGs (differentially expressed genes) and TFs (transcription factors), different regulation of CYPs (cytochrome P450 enzymes) and rapid evolution of the downstream enzymes in terpenoids biosynthetic pathways in German chamomile, and maybe the reasons result to differences in the types and levels of terpenoids compounds in these two chamomiles. In addition, the phylogenetic tree of single copy genes showed that German chamomile and Roman chamomile had a high identity to *C. nankingense*. **Conclusion** This work provided first insights into the terpenoids biosynthesis of chamomiles, and the candidate unigenes related to terpenoids biosynthesis can serve as important information for molecular breeding approaches to modulate the essential oil composition.

Background

German chamomile and Roman chamomile are the two most popular chamomile species in the Asteraceae, and the chemical compositions differ between the two species. The main characteristic constituents of chamomile are the essential oils in the flowers. Chamomile flowers are organized into flower heads that consist of a ring of male sterile outer ray florets (RF) and inner hermaphroditic disk florets (DF).

German chamomile is an annual herb native to Europe that has been known to humans as a medicinal herb and valued for thousands of years [1] for the characteristics of its aromatic oils. The flowers of German chamomile contain 0.2 to 1.9% essential oils [2] that consist mainly of terpenoids. As a traditional herbal medicine, German chamomile is widely used for the treatment of influenza, rheumatism pain, muscle spasm, gastrointestinal disorders, menstrual cramps, hemorrhoids, skin inflammation, and mucosal ulceration [3]. In addition, German chamomile has a mild calming effect, and can be used to reduce anxiety, treat convulsions, and as a sleep aid. Furthermore, chamomile is consumed as a popular herbal tea, and chamomile tea bags [4], which contain powdered chamomile flowers, are readily available on the market. In contrast to German chamomile, Roman chamomile is a perennial herb. The essential oil, which is present in the dry flowers of Roman chamomile at 0.3-1.5%, consists mainly of esters and a small amount sesquiterpenes such as angelic acid, angelic acid butyl ester, and chamazulene [5, 6]. While

the essential oil is used mainly in cosmetics and perfumes, the primary medicinal uses are as a sedative, anxiolytic, and antispasmodic. The oil is also used to treat mild skin irritation and inflammation[7] [8, 9].

Terpenoids represent the largest class of floral volatiles and include such well known and widely distributed constituents of floral scents as the monoterpenes linalool, limonene, myrcene, ocimene, and geraniol, and the sesquiterpenes farnesene, nerolidol, caryophyllene, and germacrene. Generally, there are two well-established pathways generating IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl diphosphate) in plants: one is the mevalonate (MVA) pathway and the other is the methylerythritol phosphate (MEP) pathway [10]. In recent studies, monoterpene synthases have been identified in plastids and may also be present in the cytosol [11, 12] and several studies have indicated that molecules such as GPP can be exported from the plastids to the cytosol [13]. In addition, sesquiterpenes were first thought to be synthesized exclusively in the cytosol, using precursors generated via the MVA pathway. However, there seem to be some exceptions to this rule, such as snapdragon flowers [14] and tea [15].

Haematococcus pluvialis, a species of green alga, differs from green plants in that it may synthesize isoprenoids exclusively via the MEP pathway [16]. Some diterpenes are also made in the cytosol [17]. All of this indicates that these two pathways (MVA and MEP) are not independent, and cross-talk between them has also been documented. Because the main terpenoids present in German chamomile and Roman chamomile flowers are mainly monoterpenes and sesquiterpenes, the particular pathway from which the precursors are derived is unknown. Our study analyzed transcription of key genes in these two pathways, and deduced the possible synthesis by way of isopentenyl diphosphate (IPP). We performed RNA-seq analyses on disk and ray florets of both German chamomile and Roman chamomile to examine differences in gene expression between the two chamomile species. This comparative transcriptomic analysis provides important insights into the molecular mechanisms that regulate the terpenoid biosynthesis pathways.

Transcriptomic analyses can be used to identify functional elements in the genome, metabolic pathways, and differentially expressed genes in model and non-model organisms [18-21]. The transcriptomes of many important medicinal plants and their individual tissues such as leaves, roots, and stems have been reported [22-26]. Genomic resources and transcriptome sequences for German chamomile and Roman chamomile are very limited at present, and the complex regulatory mechanisms that control carbon flux through the terpenoid biosynthetic pathway and their cooperation in the biosynthesis of volatile terpenoids remain unknown. To facilitate further research on the biosynthesis of secondary metabolites, we focused on the terpenoid metabolic pathways in German and Roman chamomile, including the regulatory relationships between genes for key enzymes and transcription factors. In the current study, we compared the disk floret and ray floret transcriptomes of German chamomile and Roman chamomile using RNA-seq analyses, and identified genes related to terpenoid biosynthesis. This is the first report of the application of RNA-seq to German and Roman chamomile. The data generated in this study will be a useful resource for future genetic and genomic studies in *M. recutita* and *C. nobile*.

Methods

Plant materials

German chamomile and Roman chamomile plants obtained were from YuePing Ltd and grown in the experimental farm at AnHui Agricultural University, HeFei, China. Full-bloom-stage flowers of German chamomile and Roman chamomile were collected from six month-old plants and the disk and ray florets were separated immediately after collection (Supplementary Figure 5). All samples were identified by Prof. Yi Yuan. The essential oil were then extracted from the disk and ray florets of German chamomile and Roman chamomile after drying naturally. A portion of the disk and ray florets of German chamomile and Roman chamomile were flash frozen in liquid nitrogen and stored at -80°C to be used for RNA extraction. The all samples were conducted with three biological replicates which were collected from different plants.

GC-MS (Gas Chromatography-Mass Spectrometry) analysis of the essential oil extracted from German and Roman chamomile

The essential oil of Germany Chamomile and Roman Chamomile flowers were extracted using steam distillation method. 50g dried samples were crushed and then were distilled for 8h in a round bottom flask with 1,000ml of pure water. We determined samples with three biologic replicates. GC conditions: The analytes were separated using an HP-5MS column (30 m × 0.25 mm I.D. × 0.25 µm film thickness; Agilent Technologies, USA); the carrier gas was high-purity helium (99.999%, Airgas Inc.); the flow rate was 1 mL/min; the injector temperature was 280°C; The injection mode was split, and split ratio was 10:1. The oven temperature was maintained at 70°C for 3 min, then programmed to rise from 70°C to 180°C at the rate of 5.5 °C/min, and was then held at 180°C for 4 min. The programmed to rise to 280°C at the rate of 4 °C/min, and was then held at 280°C for 2min. MS conditions: the transfer line temperature was 280°C; ionization mode, electron impact (EI) at 70 eV; the quadrupole temperature was 150°C; scan range, m/z 29–420; scan rate, 3.75 scans/s; with an ion source temperature of 230°C. Compounds were identified by comparing the mass spectra and retention indices with spectra in the NIST database [51]. We used ethyl caprate as the internal standard to calculate relative peak ratios.

RNA extraction, library construction and sequencing

Total RNA was extracted separately from flowers of German chamomile and Roman chamomile using the modified CTAB (Hexadecyl trimethyl ammonium bromide) method with three biological replicates [52]. The yield and quality of RNA was determined using gel electrophoresis and spectrophotometry (Nanodrop 2000). Enrichment of mRNA, fragmentation, cDNA synthesis, adapter addition, fragment size selection, PCR amplification, and RNA-seq were performed at the Beijing Genome Institute (BGI; Shenzhen, China). As a final step, the cDNA libraries were examined using an Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System prior to sequencing on the Illumina HiSeq 4000.

De novo assembly of the chamomile flower transcriptome

Clean reads from the 12 cDNA libraries were obtained by removing low quality reads, adaptor sequences, and reads with a high content of unknown bases (Ns) from the raw reads. *De novo* transcriptome assemblies for the 4 samples were performed separately using the transcriptome assembler Trinity [53], and each sample had three repetitions. Unigenes were generated by removing the redundant sequences and short transcripts. The ORF of Unigenes were predicted and translated by TRANSDCODER (<https://github.com/TransDecoder/TransDecoder/releases>). The German chamomile and Roman chamomile unigenes were then aligned to each other by the RBH method described in Tai et al [18].

Functional annotation and classification of the unigenes

The unigenes were compared against the Nr, Swiss-Prot, COG [54], and Nt sequences databases to retrieve protein functional annotations using Blastx or Blastn with a threshold E-value of 1×10^{-5} . Metabolic pathway assignments for the unigenes were obtained using KEGG annotation [55] to understand the complex biological functions of the gene products. Orthologous gene products can be classified, and the potential functions of the unigenes predicted, using the COG database. Based on Nr searches, the GO classifications of the unigenes were obtained with WEGO software [56, 57] to show the distribution of gene functions in the “Cellular Component”, “Molecular Function”, and “Biological Process” GO domains.

Comparison of nucleotide and protein sequences in German chamomile and Roman chamomile

Protein sequences from German and Roman chamomile were compared using BLAST and MUMmer (<http://mummer.sourceforge.net/>); >80% of the length of each gene in a pair of homologous genes was strictly aligned, and sequences with >70% homology were identified as homologous genes. The remaining sequences were identified as special transcripts in the German and Roman chamomile transcriptomes.

DEGs related to major secondary metabolism pathways

The calculation of relative unigene expression uses the FPKM method (Fragment Per Kilobase of exon model per Million mapped reads) [28]. The identification of DEGs was performed based on “The significance of digital gene expression profiles” [58], which were modified using a rigorous selection criterion ($FDR \leq 0.001$ and $|\log_2 \text{Ratio}| \geq 1$). For each unigene, four FPKM values were generated for each of the four transcriptomes. We used ggplot 2 (http://docs.ggplot2.org/current/geom_point.html) to identify DEGs that play potentially important roles in secondary metabolism [59]. After further investigation, we selected the terpenoid biosynthesis pathways for more detailed analysis. All TF unigenes were annotated in the plantTFDB, and we identified TF genes that showed significant differential expression.

Construction of protein–protein interaction network (PPIN) of the terpenoid biosynthesis pathways

The DEGs between German and Roman chamomile were aligned to STRING database (<http://string-db.org/>) used DIAMOND (<https://github.com/bbuchfink/diamond>) with parameters “-evalue 1e-5 -outfmt 6 -max-target-seqs 1 –more-sensitive). DEGs with a query coverage $\geq 50\%$ and an identity $\geq 40\%$ were identified as the homology interacting protein and were built a protein interaction network. Meanwhile, DEGs related to terpenoid biosynthesis pathways were screened from the interaction networks. Then, these DEGs were enrichment to KEGG pathway (FDR <0.05). PPIN were visualised using Cytoscape [41].

Comparison analysis in German chamomile, Roman chamomile and other the composite family

ORFs of the German chamomile and Roman chamomile were clustered using CD-HIT-EST (v4.6.8), respectively. Proteins of *Lactuca sativa* and *Helianthus annuus* were downloaded from NCBI and the longest isoforms were retained for further analysis. Proteins of *Taraxacum kok-saghyz* were downloaded from BIGD (<http://bigd.big.ac.cn/search?dbId=gwh&q=PRJCA000437>) and proteins of *Chrysanthemum nankingense* were downloaded from Chrysanthemum Genome Database (<http://www.amwayabrc.com/>). Proteins of *Arabidopsis thaliana* were downloaded from TAIR (<http://www.arabidopsis.org/>). The gene families and phylogenetic tree were constructed by orthofinder (v2.2.7)[60]. Briefly, Protein sequences of these seven species were performed by all-vs-all BLAST with e-value threshold of 1e-5. Then the BLAST results were clustered by OrthoMCL [61]. The gene families were visualized by UpSetR (v1.4.0) [62]. The single-copy genes from seven species were aligned by MAFFT (v7.407) [63]. The phylogenetic tree were constructed by FastTree (2.1.10) [64, 65]. The ORFs of German chamomile, Roman chamomile and proteins of other composite family plants were used to predict protein domains based on the Pfam database.

qRT-PCR verification of selected genes

In order to determine the accuracy of the transcriptome sequencing, qRT-PCR analysis was used to assess the quality of the transcriptomic data. Total RNA was extracted from flowers, and first-strand cDNAs used for qRT-PCR analysis were synthesized from total RNA using a Prime-ScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Expression of selected terpenoid pathway genes were monitored by qRT-PCR using the SYBR Green qPCR mastermix (Takara, SYBR Premix Ex TaqIITM), on a Bio-Rad CFX 96TM real-time PCR system (Bio-Rad), according to the manufacturer's instructions. Detailed information about the selected unigenes, including their unigene IDs, and all PCR primers sequences are given in Additional files 1. We used the 18S ribosomal RNA gene as an internal reference for gene expression, and the relative expression levels were calculated using the $2^{-\Delta Ct}$ method [66]. All qRT-PCR analyses were performed using three biological replicates and three technical replicates (means $\pm$ standard deviation).

Results

Determination of essential oil in German and Roman chamomile flowers

GC-MS analysis indicated that the essential monoterpene and sesquiterpene constituents in the flowers of German chamomile and Roman chamomile are significantly different (Figure 1 and Figure 2, Additional file 1). The relative quantities of monoterpenoids and sesquiterpenoids in disk flowers were always higher than in ray flowers in both chamomiles. The main compounds from German chamomile essential oil were sesquiterpenoids, such as α -Bisabolol oxide A, Chamazulene, α -Bisabolol oxide B, α -Bisabolol and Epatulenol. Meanwhile, the major constituents from Roman chamomile essential oil were n-Hexadecanoic acid, linoleic acid and other esters. The contents of α -Bisabolol oxide A, α -Bisabolol oxide B and Chamazulene in German chamomile were 50, 30, 10 times more than that in Roman chamomile. In addition, the content of these compounds in disk flowers were 2-10-fold greater than that in ray flowers in two chamomiles. These results are consistent with the previous findings reported by Yao et al.[27].

De novo transcriptome assembly and comparative RNA-seq analyses

After removing the terminal adaptor sequences, duplicated and ambiguous sequences, and low-quality reads, we generated approximately 106.72 Gb of Illumina RNA-seq data from mRNA extracted from ray flowers and disk flowers of German and Roman chamomile. There were 53.31Gb and 53.41Gb of clean read data used in the assemblies for German and Roman chamomile, respectively. The Q20 and Q30 scores were greater than 98% and 95% for the German and Roman chamomile transcriptome data, respectively. The final German chamomile cDNA assembly consisted of 117,203 unigenes; the average length was 1,056 bp and the N50 length was 1,686 bp. The final assembly for Roman chamomile had 147,616 unigenes with an average length of 914 bp and an N50 length of 1,506 bp (Table 1). There were 50,881 (43.41%) and 48,957 (33.17%) unigenes >1,000 bp in length in German and Roman chamomile, respectively.

Functional annotation and classification

All unigenes from both German chamomile and Roman chamomile were annotated using several public databases: Nr (NCBI non-redundant protein sequences), Nt (non-nucleotide), Swiss-Prot, COG (Clusters of Orthologous Groups of proteins), KEGG (the Kyoto Encyclopedia of Genes and Genomes), and GO (Gene ontology). There were 89,796 (60.83%) and 73,699 (62.88%) sequences annotated for German chamomile and Roman chamomile, respectively. We annotated 68,325 (NR: 58.30%), 47,469 (NT: 40.50%), 48,902 (Swissprot: 41.72%), 29,222 (COG: 24.93%), 52,423 (KEGG: 44.73%), 11,385 (GO: 9.71%), and 51,613 (Interpro: 44.04%) in the Roman chamomile transcriptome. Similarly, 80,594 (NR: 54.60%), 49,537 (NT: 33.56%), 56,793 (Swissprot: 38.47%), 35,889 (COG: 24.31%), 62,060 (KEGG: 42.04%), 13,766 (GO: 9.33%), and 63,945 (Interpro: 43.32%) unigenes were annotated in the German chamomile transcriptome using the seven functional databases (Table 2).

Overall, 29,222 (24.93%) unigenes in the German chamomile transcriptome were assigned to 25 COG categories (Supplementary Figure 1 A). Among these groups, unigenes belonging to “general function prediction” occupied the largest part (8,163), followed by transcription (4,382). In addition, 35,889 of the

total 117,203 Roman chamomile unigenes were classified into 25 COG categories. The assignments (10,007) were mostly enriched in the “general function prediction”, followed by the “transcription” clusters (5,192) (Supplementary Figure 1 B). KEGG pathway analysis was performed to further predict gene function in the biological pathways of the assembled unigenes in the German chamomile and Roman chamomile transcriptomes. In total, 62,060 unigenes in German chamomile were assigned to 135 signal pathways. Among these, 1,520 unigenes were annotated in “Metabolism of terpenoids and polyketides”. Also, 52,423 unigenes in Roman chamomile were categorized into 136 pathways, and 1,633 unigenes were annotated in “Metabolism of terpenoids and polyketides” (Supplementary Figure 2).

Identification of DEGs and the further analysis on the terpenoids pathway

We identified DEGs by comparing the FPKM (Fragment Per Kilobase of exon model per Million mapped reads) values between the different libraries; thresholds were $\log_2$ fold-change >1 and FDR (False Discovery Rate) ≤ 0.001 [28]. We used the number of DEGs mapping to a pathway/total number of genes mapped to this pathway (rich factors) to estimate the relative degree of enrichment in these pathways. The maximum rich factor (0.5) for pathways in the MC_DF vs. MC_RF comparison was benzoxazinoid biosynthesis, followed by Zeatin biosynthesis (0.38), sesquiterpenoid and triterpenoid biosynthesis (0.35), and flavone and flavonol biosynthesis (0.34). Also, the rich factor of terpenoid backbone biosynthesis was 0.29 and diterpenoid biosynthesis was 0.22. In CN_DF vs. CN_RF, the top three were the ribosome (0.22), vancomycin resistance (0.12), and terpenoid backbone biosynthesis (0.11). In CN_RF vs. MC_RF the top three were benzoxazinoid biosynthesis (0.24), histidine metabolism (0.23), and photosynthesis - antenna proteins (0.22); in addition, carotenoid biosynthesis was 0.19 and limonene and pinene degradation was 0.15. In CN_DF vs. MC_DF, the top three were glucosinolate biosynthesis (0.089), histidine metabolism (0.084), and benzoxazinoid biosynthesis (0.081); terpenoid backbone biosynthesis was 0.057 (Figure 3 and Additional file 2). DEGs identified by comparing RF with DF clustered in the pathways for disease and pest resistance and terpenoid metabolism. The DEGs between German chamomile and Roman chamomile were clustered in disease and pest resistance pathways. A higher rich factor between two different stages in either German or Roman chamomile gives an indication that the secondary metabolism involved in the terpenoid metabolic pathways is different.

Meanwhile, DEGs were identified in the terpenoid biosynthetic pathways of German and Roman chamomile. A schematic representation of the DEGs and annotated genes in the biosynthetic pathways for these compounds is shown in Figure 4. Terpenoid biosynthesis utilizes isoprenoid precursors from terpenoid backbone biosynthesis (MVA and MEP pathways). In the MVA pathway, two AACT, four HMGS, and two HMGR were up regulated in MC-DF vs. MC-RF. In the MEP pathway, one DXS (1-deoxy-D-xylulose-5-phosphate synthase) and two DXR (1-deoxy-D-xylulose-5-phosphate reductoisomerase) genes were down regulated in MC-DF vs MC-RF. Also, DXS and DXR may play rate-limiting roles in controlling metabolic flux through the MEP pathway [29]. A previous study reports that DXS and HDR are both encoded by small gene families in higher plants, and influence the accumulation of downstream isoprenoids [30]. For example, the three DXS genes in maize (*Zea mays*) encode functional enzymes, and two different HDR genes have been identified in loblolly pine (*Pinus taeda*) [31, 32].

In the MC-DF vs. MC-RF comparison, AACT and HMGS are both up-regulated in the terpenoid biosynthesis pathway. There are two and four down-regulated DEGs related to GPPS (geranyl diphosphate synthase) and FPPS (farnesyl pyrophosphate synthase), respectively, and DEGs related to GGPPS (geranylgeranyl pyrophosphate synthase), SS (squalene synthase), and PSY (phytoene synthase) were all up-regulated. Also in MC-DF vs MC-RF, 17 DEGs related to terpene synthase (TPS) were identified, and among them, the relative expression of 15 TPS genes was down-regulated. Terpenoid biosynthesis in plants is catalyzed by a family of enzymes known as terpene synthases that convert prenyl diphosphates to various subclasses of terpenoids [33]. In the CN-DF vs. CN-RF comparison, there were one and three DEGs related to FPPS and TPS that were down-regulated in expression. These results indicate that the gene expression levels of the rate-limiting upstream enzymes and a variety of TPS genes downstream could result in the observed differences in both the variety and contents of terpenoids in flowers of German and Roman chamomile.

TFs play a diverse role in regulating secondary metabolism pathways by turning genes on and off in plants [34]. We searched for candidate TF genes in the transcriptomes of German chamomile and Roman chamomile, and identified 94 differentially expressed transcription factor genes (52 up-regulated and 42 down-regulated) in CN_DF vs. CN_RF. We also identified 59 differentially expressed (31 up- and 28 down-regulated, respectively) transcription factor genes in CN_DF vs. MC_DF, 328 (167 up-regulated and 161 down-regulated) in CN_RF vs. MC_RF, and 479 (267 up-regulated and 212 down-regulated) in the MC_DF vs. MC_RF comparison (Additional file 3).

PPIN construction and analysis between German chamomile and Roman chamomile

A total of 477 and 505 interaction pairs involved in terpenoid biosynthesis pathway were identified from German chamomile and Roman chamomile, respectively. We selected the interaction proteins for further analysis, and the PPIN related to terpenoid biosynthesis pathway were shown in figure 5. And we found these proteins were involved in “Sesquiterpenoid and triterpenoid biosynthesis”, “Brassinosteroid biosynthesis”, “Propanoate metabolism”, “Steroid biosynthesis”, “Carotenoid biosynthesis” and “Valine, leucine and isoleucine degradation” in German chamomile and Roman chamomile. In addition, we also identified three, three and four proteins involved in “Glycosphingolipid biosynthesis - ganglio series”, “Diterpenoid biosynthesis” and “beta-Alanine metabolism” in German chamomile. Meanwhile, we identified three, one, two and one proteins involved in “Butanoate metabolism”, “Stilbenoid, diarylheptanoid and gingerol biosynthesis”, “Flavone and flavonol biosynthesis” and “Tryptophan metabolism” in Roman chamomile.

We found amount of CYPs interaction with protein involved in terpenoid biosynthesis pathway in PPIN of German chamomile and Roman chamomile. And there were more types and amount of CYPs interaction protein in German chamomile than that in Roman chamomile. In addition, we found transcription factors such as MYB, WD-40 repeat and Zinc finger protein in PPIN of German chamomile and Roman chamomile. And the types and amount were also different. Moreover, interactions between proteins

related to terpenoid biosynthesis pathway between German chamomile and Roman chamomile. (Figure 5 and Additional file 4).

Comparison of nucleotide sequence identity and divergence analysis between German and Roman chamomile

A high level of nucleotide sequence similarity was found in genes that are homologous between German and Roman chamomile, with 54% of the genes sharing >75% identity. We obtained 60,338 transcripts that were common to German Chamomile and Roman chamomile. We also detected 56,865 and 87,278 special transcripts in German and Roman chamomile, respectively (Figure 6 A). In addition, the special transcripts identified in the two species were used as queries to search the KEGG databases; we found that 2,960 (43%) and 3,861 (49%) of the unigenes are involved in secondary metabolite biosynthesis in German and Roman chamomile, respectively. Also, 1,000 unigenes (46%) were related to plant-pathogen interactions in German chamomile and 1,295 (50%) in Roman chamomile. A total of 27,886 putative ortholog pairs were identified between German and Roman chamomile. Of these ortholog pairs, 584 had a Ka/Ks value more than 1, suggesting that they were under positive selection. Among these positive selection genes, there were 1 unigenes (CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase) related to terpenoids backbone biosynthesis, 1 unigenes (germacrene D synthase) related to sesquiterpenoids biosynthesis, 2 unigenes ((E)-beta-ocimene synthase and (+)-neomenthol dehydrogenase) related to monoterpenoids biosynthesis and 1 unigenes (*Artemisia annua* cytochrome P450 mono-oxygenase) related to diterpenoids biosynthesis (Table 3). Since the Ka/Ks value is widely used to detect selective pressure (environmental factors) acting on protein-coding sequences, rapid evolution of the genes involved in terpenoids backbone biosynthesis, monoterpenoids biosynthesis, sesquiterpenoids biosynthesis and diterpenoids biosynthesis might be associated with adaptive selection between German and Roman chamomile.

Comparison analysis in German chamomile, Roman chamomile and other composite family plants

A total of 92,393 gene families were identified by orthofinder, among them 1,232 single copy genes were identified. There were 6 gene families specifically in German chamomile, and 8 gene families specifically in Roman chamomile. In gene families which specifically from German chamomile, we found one gene family related to disease resistance. A total of 5,718 gene families were specifically both in German chamomile and Roman chamomile (Figure 6B). The KEGG enrichment results showed that gene families specifically both in German chamomile and Roman chamomile were mainly enriched in 'Metabolism', and 472 gene families were enriched in 'Biosynthesis of secondary metabolites' (Additional file 5). The phylogenetic tree of single copy genes showed that German chamomile and Roman chamomile had a high identity to *C. nankingense* (Figure 6C). In addition, we analyzed unigenes of German chamomile, Roman chamomile and other composite family plants based on the Pfam database, and the results showed that unigenes related to TFs such as WD-repeat and Zinc-finger were more than other composite

family plants. Furthermore, there were more MATE and ABC transporter in German chamomile and Roman chamomile (Figure 6D).

qRT-PCR analysis and correlation analysis

We used qRT-PCR (quantitative real-time PCR) analyses to examine the expression levels of 21 DEGs from the transcriptomic libraries of German chamomile (14 DEGs) and Roman chamomile (7 DEGs). Of these genes, 19 showed differential expression levels that matched the RNA-seq data. The agreement rate was 90.48% (Supplementary Figure 3 and Additional file 6). These results verified the accuracy of the transcriptome data. The three samples of German chamomile and Roman chamomile showed good correlation; the correlation index was >0.84 for CN-DF-1, CN-DF-2 and CN-DF-3, and it was >0.99 for CN-RF-1, CN-RF-2, and CN-RF-3; MC-DF-1, MC-DF-2 and MC-DF-3; and MC-RF-1, MC-RF-2 and MC-RF-3, which indicated that the transcriptome data was relatively accurate. However, the correlation index between MC-DF and CN-DF was ~ 0.8 , and the correlation index between MC-RF and CN-RF was ~ 0.85 . The correlation index between MC-DF and MC-RF was only 0.48-0.49, and between CN-DF and CN-RF it was 0.5-0.63 (Supplementary Figure 4).

Discussion

Of the many species of chamomile, two of the most popular are German and Roman chamomile, and both species are in the Asteraceae family. German chamomile is more widely grown than Roman chamomile [35, 36]. The medicinal value of German chamomile is related to the content of the essential oil that is mainly produced in the flowers. The main components of the essential oil are monoterpenoids and sesquiterpenoids, such as (E)- β -farnesene, terpene alcohol, chamazulene, α -bisabolol (4.8-11.3%), and α -bisabolol oxides A and B. The levels of α -bisabolol and α -bisabolol oxides A and B in the flowers peak at full bloom and then decline [37]. The composition of essential oils is quite different between Roman and German chamomile. As discussed above, the essential oil of German chamomile is composed mainly of sesquiterpenoids such as chamazulene, bisabolol, and its oxide, while the major constituents of Roman chamomile oil are angelates, such as angelic acid and its esters, and monoterpenoids such as α -pinene [38] [39]. In this study, we analyzed and compared the differences in the compounds between the two species of chamomile. Our results showed that there were more kinds of monoterpenoids and sesquiterpenoids in German Chamomile than that in Roman Chamomile, and that the content was higher. In addition, there were more kinds monoterpenoids and sesquiterpenoids, and the content was higher, in disk florets than in ray florets.

There are at least two ways in which the biosynthesis of terpenoids can be regulated: the level of terpenoid backbone biosynthesis and the level of substrates for TPSs. Previous studies have suggested that in the terpenoid backbone biosynthesis pathway, the rate of metabolic flux through the MEP pathway is controlled by DXS and DXR [29]. Furthermore, DXS and HDR are encoded by small gene families; there are three functional DXS genes in maize [31], and two different HDR genes in *Pinus taeda* [32]. In this study, we found 11 DXS and 4 DXR genes in the MC-DF vs MC-RF comparison. Meanwhile, two AACTs,

two HMGRs and four HMGSs were found to be up regulated. We therefore speculated that increased gene expression in the MVA pathway could provide more substrate for the downstream pathway. The gene expression level of key genes (such as DXS, DXR, MDC, SS, and PSY) is higher in MC-DF vs. MC-RF. We also found that expression level of genes for FPS and TPS was higher in CN-DF vs. CN-RF. While TPS gene regulation has previously been shown to be important [40], our results indicate that up-regulated TPSs and higher expression level of key genes in the disk florets might lead to the higher contents of monoterpenoids and sesquiterpenoids found in German chamomile. Meanwhile, we identified seven transcripts under accelerated evolution with ratios of Ka/Ks >1 in terpenoids biosynthesis pathway. These fast evolving transcripts may be the one reason result to the difference between German and Roman Chamomile.

Transcription factors (TFs), which are generally DNA binding proteins, use a variety of mechanisms to regulate gene expression in response to changes in environmental conditions, during development, in response to stress, and in defense responses through interactions directed towards plant pathogens [41]. WRKY, NAC, DOF, MYB, the Zinc finger family, F-Box Homeobox, the WD40 repeat family, bHLH, pathogenesis-related/ERF, and AP2 are commonly-identified classes of TFs [42-44]. Spyropoulou et al. identified a pool of TFs involved in terpene synthesis using RNA sequencing in *Solanum lycopersicum* [45], and Suttipanta et al. found that in *Catharanthus roseus*, ORCA2 and an AP2 family member, MYC2, a bHLH family member, and WRKY1 together regulate indole alkaloid and terpenoid biosynthesis [46, 47]. In this study, we compared genes of German chamomile and Roman chamomile, and other composite family plants based on Pfam database, the results showed that unigenes related to TFs such as WD-repeat and Zinc-finger in German chamomile and Roman chamomile were more than other composite family plants. Moreover, there were more MATE and ABC transporter in German chamomile and Roman chamomile.

CYPs are not only plays an indispensable role in primary metabolism of plants but also in secondary metabolism. Recently, researches show that CYPs play as modification enzymes in terpenoid biosynthesis [48, 49]. In addition, we identified many CYPs interaction with protein involved in terpenoid biosynthesis pathway in PPIN of German chamomile and Roman chamomile, and found there were more CYPs interaction proteins in German chamomile compared to Roman chamomile. This result reinforced our finding that the terpenoid biosynthesis pathway in German chamomile is more active that in Roman chamomile, and more expression level of DEGs, the regulation of, TFs and CYPs might result in the observed differences in terpenoid content between German and Roman chamomile.

Conclusions

Previous studies have shown that essential oil compositions vary in German chamomile as compared to Roman chamomile [27, 50]. In this study, we found there are more monoterpenoids and sesquiterpenoids in flowers of German chamomile compared to Roman chamomile, and these compounds were more prevalent in disk florets than in ray florets. Furthermore, we obtained a large number of unigenes by transcriptome sequencing in German and Roman chamomile. We identified many candidate unigenes

related to terpenoid biosynthesis, and the majority were more highly expressed in MC-DF than in MC-RF, especially the key enzyme genes in this pathway. The DEGs, TFs and CYPs involved in the terpenoid biosynthesis pathway were more numerous in German chamomile than in Roman chamomile, meanwhile, even transcripts related to terpenoid biosynthesis pathway under accelerated evolution were identified, which could explain the differences of chemical compounds in metabolite biosynthesis between the two species. The phylogenetic tree of single copy genes showed that German chamomile and Roman chamomile had a high identity to *C. nankingense*. Our study is the first to report that a comparative transcriptome analysis between German chamomile and Roman chamomile. The transcriptomic data characterized in this research will be an invaluable resource for further studies of the functional genomics, molecular biology, and breeding in German chamomile and Roman chamomile.

Abbreviations

MC: German chamomile; CN: Roman chamomile; RF: ray florets; DF: disk florets; MEP: methylerythritol phosphate; MVA: mevalonate; FPKM: Fragment Per Kilobase of exon model per Million mapped reads; Nr: Non-redundant protein database; Nt: Non-redundant nucleotide database; Swiss-Prot: Annotated protein sequence database; KEGG: Kyoto encyclopedia of genes and genomes; COG: Clusters of orthologous groups of proteins; GO: Gene ontology; DEGs: Differentially expressed genes; TFs: Transcription factors; qRT-PCR: quantitative real-time polymerase chain reaction; IPP: isopentenylpyrophosphate; GPP: geranyl pyrophosphate; DMAPP: Dimethylallyl diphosphate; FPP: farnesyl-PP; AACT: acetyl-CoA C-acetyltransferase; HMGS: hydroxymethylglutaryl-CoA synthase; DXS: 1-deoxy-D-xylulose-5-phosphate synthase; DXR: 1-deoxy-D-xylulose-5-phosphate reductoisomerase; HDR: 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase; TPS: terpene synthase; GPPS: geranyl diphosphate synthase; GGPPS: geranylgeranyl diphosphate synthase; SS: squalene synthase; PSY: phytoene synthase; FPPS: farnesyl diphosphate synthase; IPT: isopentenyl transferases; HDS: 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; MCS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; PMK: phosphomevalonate kinase; MPD: diphosphomevalonate decarboxylase; HMGR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase; GC-MS: Gas Chromatography-Mass Spectrometer; FDR: False Discovery Rate; PPIN: protein-protein interaction network

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of supporting data

The Illumina RNA-seq data generated from flowers of *Matricaria recutita* L. and *Chamaemelum nobile* L. are available in the NCBI SRA (<http://trace.ncbi.nlm.nih.gov/Traces/sra>) under accession number PRJNA382469. The sequences will be submitted to the NCBI database under project number accession (PRJNA382469), if the manuscript is accepted for publication in BMC Genomics, prior to publication.

Competing interest

The authors declare that they have no competing interests.

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Authors' Contributions

YLT designed the experimental plan and wrote the manuscript. YY conceived and revised the manuscript. XJH performed the experiments. CL interpreted the sequence data. JMS, CCL, CXW and CXG participated in sample collection and RNA preparation. HHW, LS, XYS, and WJ were actively involved in manuscript revision and data analysis. GFP provided valuable comments and suggestions for improving the quality of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Summary of RNA-seq and unigene data from German and Roman chamomile

	German chamomile	Roman chamomile
Total Raw Reads (Gb)	55.9	56.3
Total Clean Bases (Gb)	53.31	53.41
Clean Reads Q20 (%)	>98%	>98%
Clean Reads Q30 (%)	>95%	>95%
Average unigene length	1056	914
Total number of unigenes	117,203	147,616
N50 value	1,686	1,506
GC (%)	40.12	40.86

Table 2. Functional annotation of floret unigenes in German and Roman chamomile

Values	German chamomile		Roman chamomile	
	Number	Percentage	Number	Percentage
Total	147,616	100%	117,203	100%
Nr-Annotated	80,594	54.60%	68,325	58.30%
Nt-Annotated	49,537	33.56%	47,469	40.50%
Swissprot-Annotated	56,793	38.47%	48,902	41.72%
KEGG-Annotated	62,060	42.04%	52,423	44.73%
COG-Annotated	35,889	24.31%	29,222	24.93%
Interpro-Annotated	63,945	43.32%	51,613	44.04%
GO-Annotated	13,766	9.33%	11,385	9.71%
Overall	89,796	60.83%	73,699	62.88%

Table 3. Genes related to terpenoids pathway with Ka/Ks value more than 1

Pathway	GenePair (MC)	GenePair (CN)	Ka	Ks	ka/ks	Description
Terpenoid backbone biosynthesis	CL6716.Contig2	Unigene23705	0.0094	0.0088	1.0681	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
Monoterpenoid biosynthesis	Unigene31629	Unigene603	0.0093	0.0085	1.0941	(E)-beta-ocimene synthase
Sesquiterpenoid biosynthesis	Unigene29935	CL9420.Contig2	0.0122	0.0034	3.5882	Germacrene D synthase
Monoterpenoid biosynthesis	CL6029.Contig1	CL15218.Contig2	0.0059	0.0049	1.204	(+)-neomenthol dehydrogenase
Limonene and pinene degradation	CL15540.Contig9	CL3089.Contig1	0.0082	0.0047	1.7446	Parthenolide synthase
Limonene and pinene degradation	Unigene10231	CL15190.Contig1	0.0054	0.0049	1.102	Aldehyde dehydrogenase
Diterpenoid biosynthesis	CL14129.Contig2	CL8947.Contig2	0.0037	0.0014	2.6428	Artemisia annua cytochrome P450 mono-oxygenase

Figures

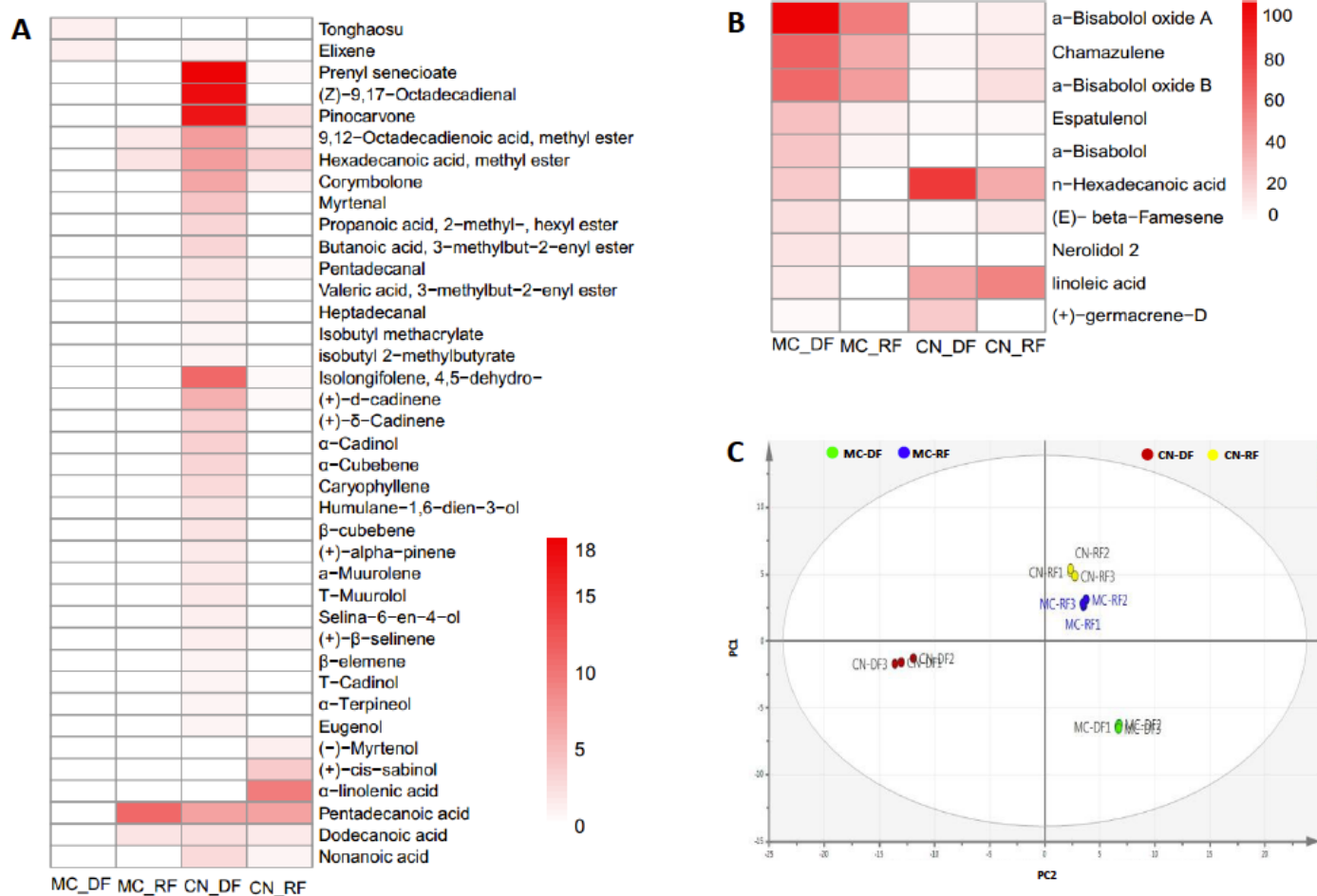


Figure 1

Heat map of essential oil content (A and B) and principal component analysis (OPLS-DA) plot (C) of data from essential oil of two different kind flowers from German Chamomile (MC) and Roman chamomile (CN) detection by GC-MS.

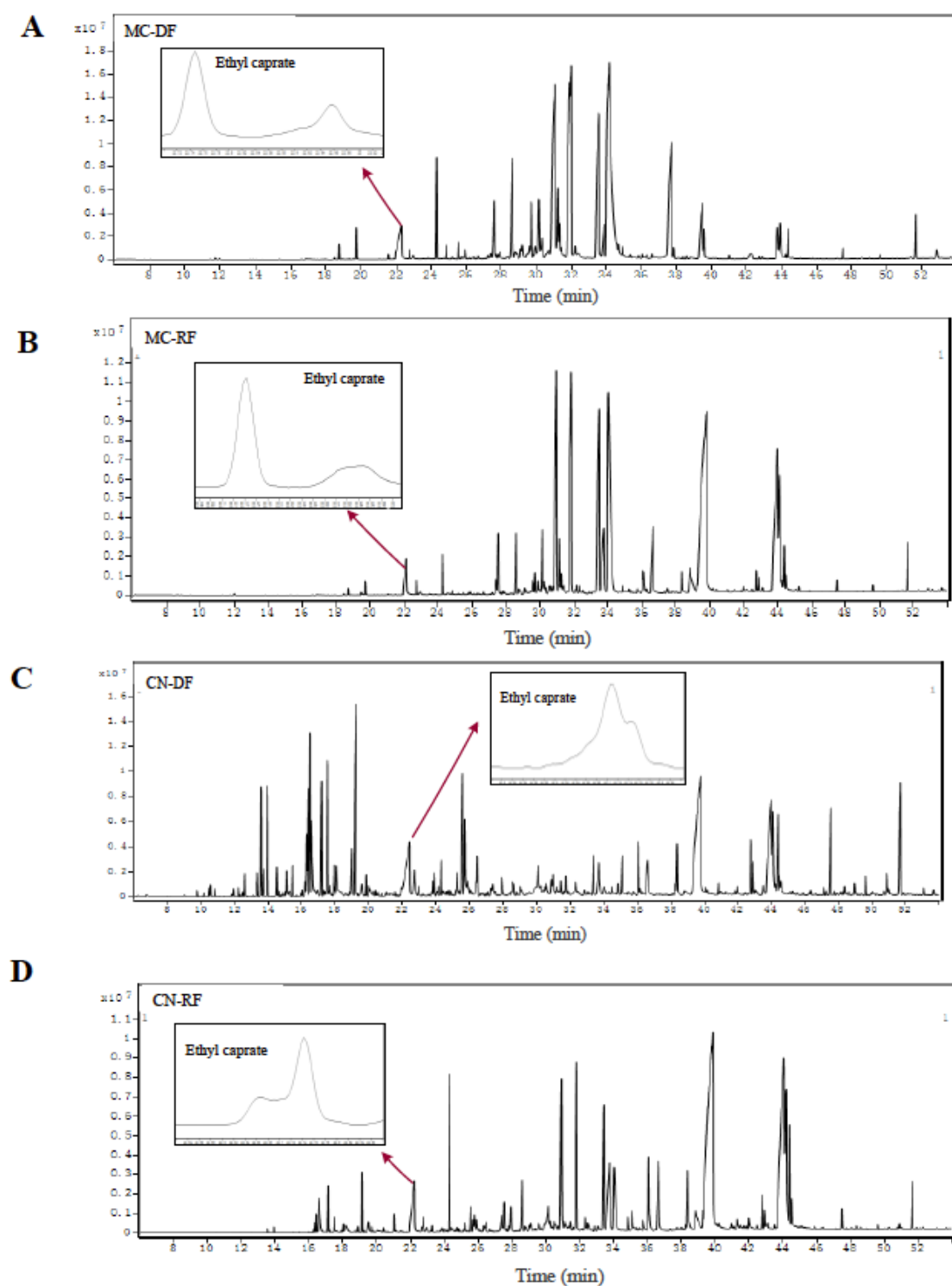


Figure 2

Total Ion Chromatography of essential oil from disk florets of German chamomile (A), ray florets of German chamomile (B), disk florets of Roman chamomile (C) and ray florets of Roman chamomile (D).

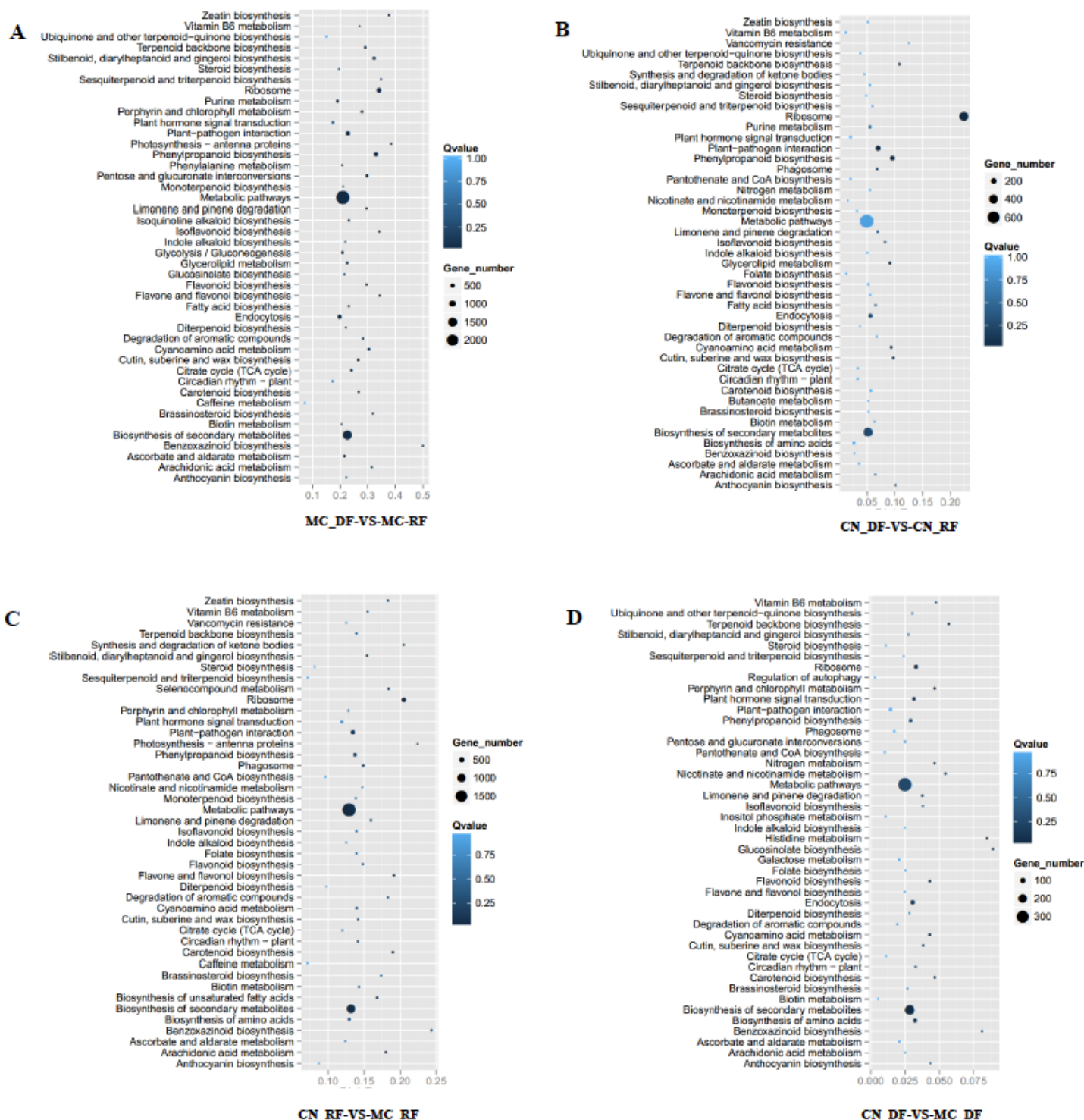


Figure 3

Pathway enrichment analysis by tissue pair comparisons The ratio between the number of DEGs mapped to a pathway and the total number of genes mapped to that pathway are indicated by rich factors. A larger rich factor indicates greater intensiveness. The Q values were calculated using a hypergeometric test with the Bonferroni Correction. The Q value is a corrected p value that ranges from 0-1, and lower Q

values mean greater intensiveness. "Gene number" refers to the number of DEGs mapped to a given pathway.

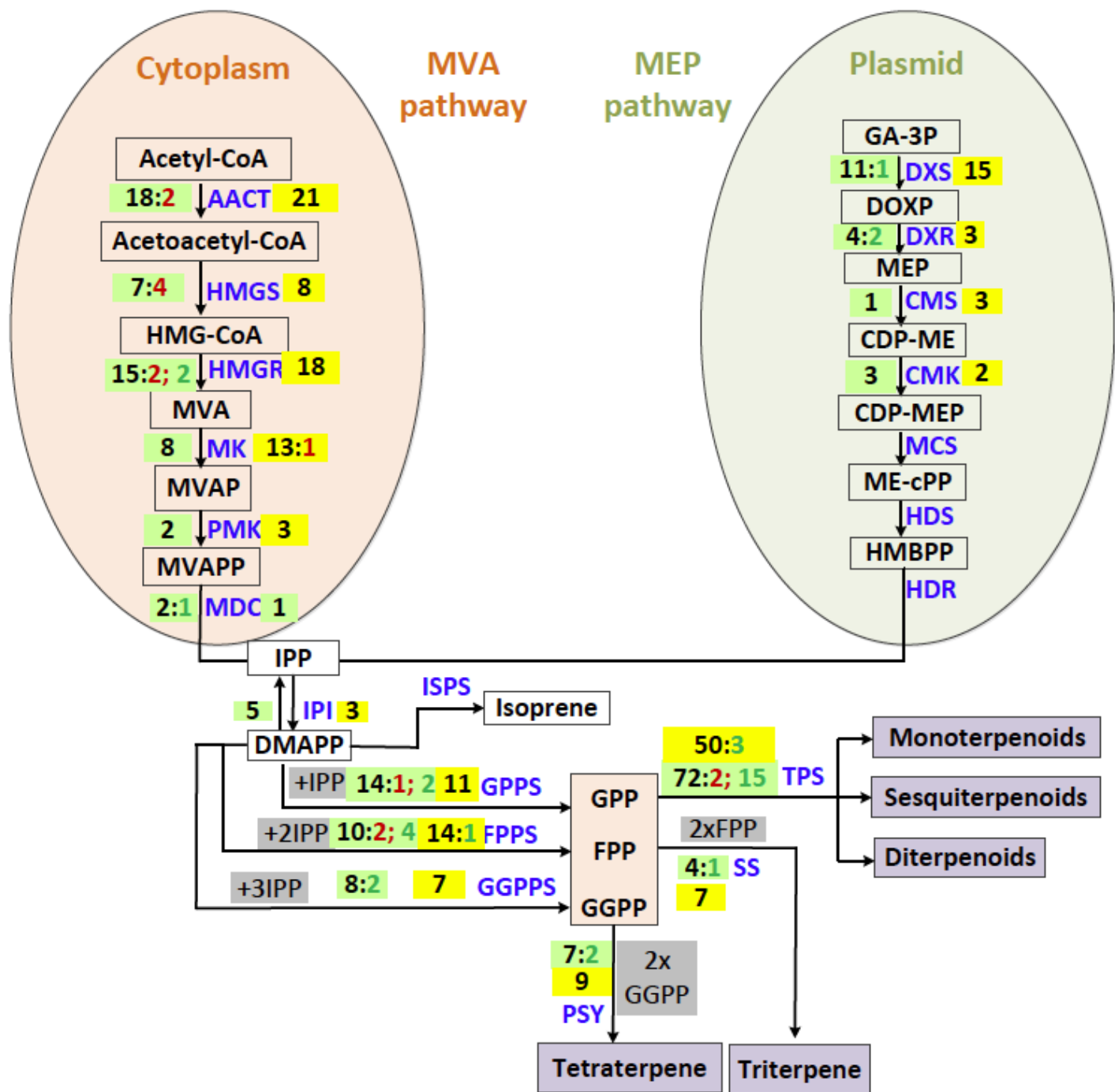


Figure 4

Number of annotated genes and DEGs related to terpenoid biosynthetic pathway in German chamomile (green) and Roman chamomile (yellow) Compound names are shown below each arrow. Abbreviations beside the arrows indicate the enzymes catalyzing the reaction. The numbers written in black indicate the

total number of genes in this pathway; numbers in red show the number of up-regulated genes, and those in green show the number of down-regulated genes.

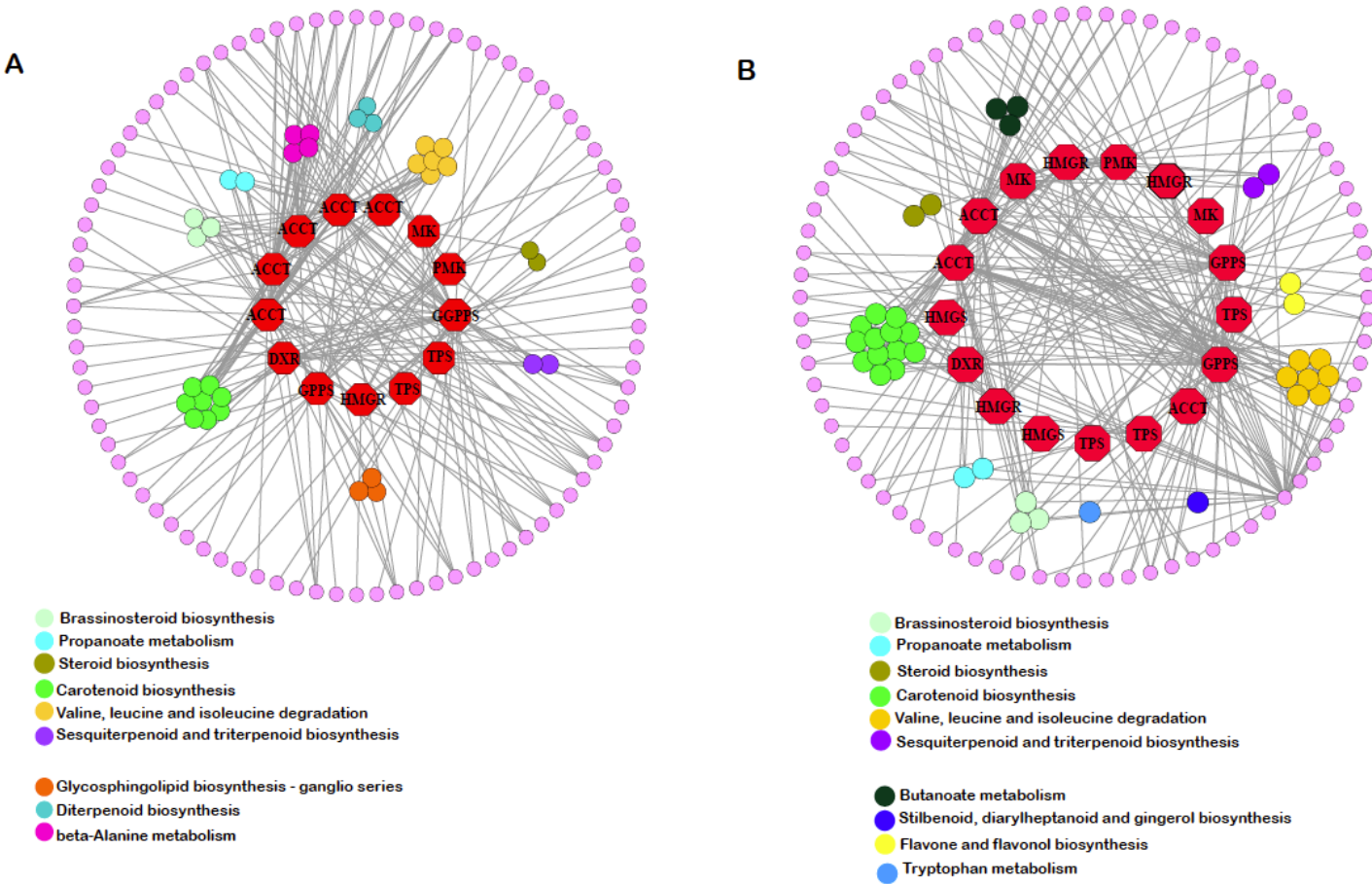


Figure 5

PPIN involved in terpenoid biosynthetic pathway in German chamomile (A) and Roman chamomile (B).

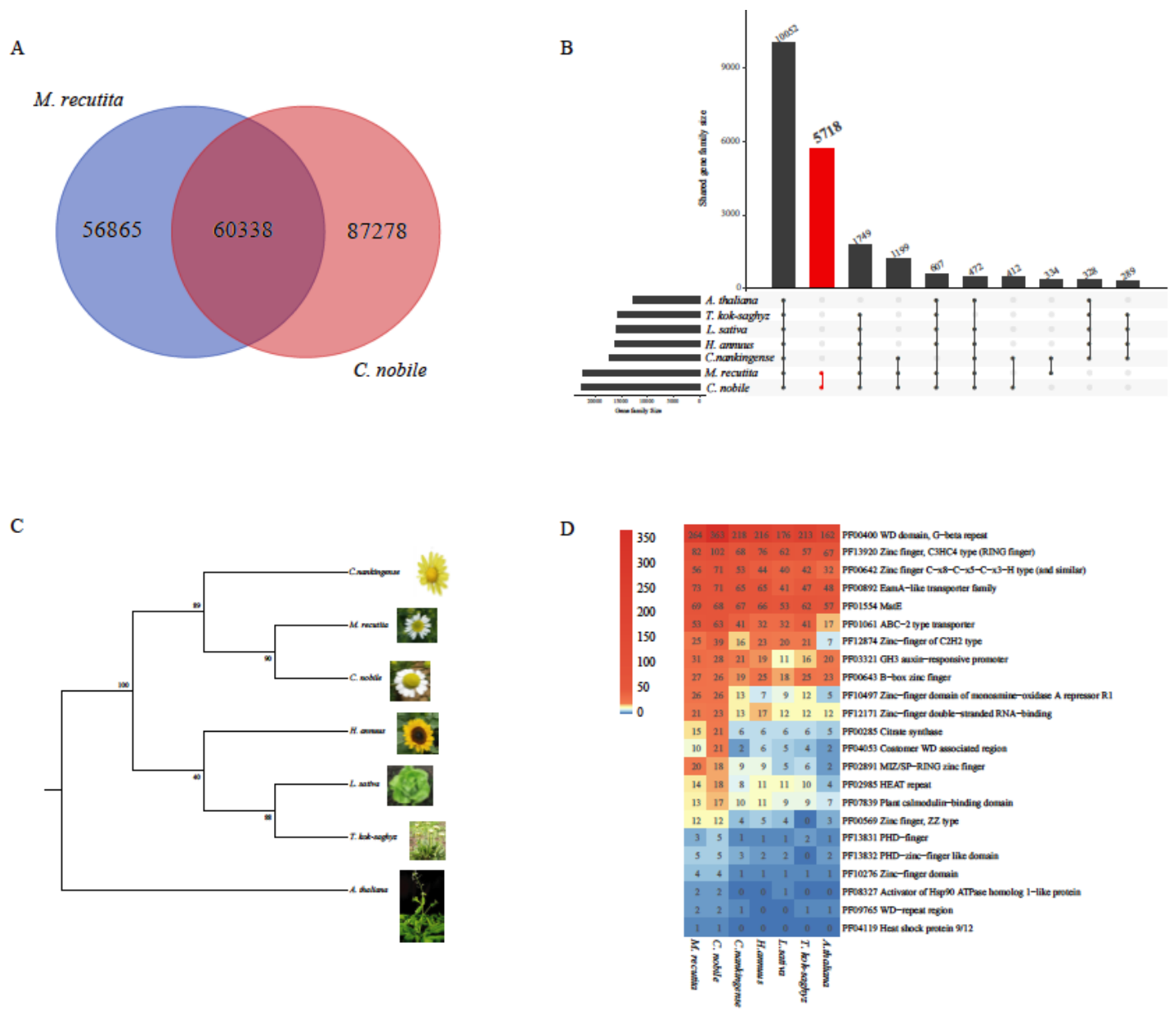


Figure 6

Comparison analysis in German chamomile, Roman chamomile and other the composite family (A) Unigenes comparison between German chamomile and Roman chamomile. (B) The analysis of gene families in two chamomiles and other the composite family. (C) Phylogenetic tree of two chamomiles and other the composite family. (D) Pfam statistics in two chamomiles and other the composite family.

Supplementary Files

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