**Supplementary Materials**

Tissue-specific transcriptome analysis reveals candidate genes for terpenoid and phenylpropanoid metabolism in the medicinal plant *Ferula assafoetida*

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MATERIAL AND METHODS

**Metabolite analysis**

GC-MS analyses were performed on an Agilent GC model 5977A equipped with a MD800 selective mass detector. Analyses were performed with electron ionization (EI) at 70 eV, an ion-source temperature of 200°C and an interface temperature of 280°C using split-splitless injection (split ratio 1:10) with a 280°C injector temperature and a1 μl injection volume. Separation was achieved using a fused silica column 5% phenyl-poly-dimethyl-siloxane **(**Chrompack CP-Sil 8 CB 50 m x 250 µm x 0.12 µm**)** and the following GC parameters: 50°C for 2 min, 4°C/min to 120°C, 2°C/min to 200 °C, 25°C/min to 280°C with hold for 8 min. Data acquisition was performed with Mass Lab software for the mass ranges 30-600 u with a scan speed of 1 scan/sec**.** Identification of compounds was based on Kovats retention indices as calculated using n-alkanes C11-C28 as reference compounds using three replicated measurements, as well as comparison to reference mass spectral libraries, including Adams (Robert Adams, 2012), US National Institute of Standards and Technology (NIST, USA), WILEY 1996 Ed. mass spectra library, and an in-house library of 600 mass spectra.

For ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QToF-MS) studies, dry plant material was ground to a fine powder. Plant samples (0.5 g) were weighed in a centrifuge tube and 3.5 mL of methanol added before sonication in a water bath for 30 min and centrifugation at 959 *×g* for 15 min. The supernatant was transferred to a 10 mL flask. The extraction procedure was repeated twice and supernatants combined. The final volume was adjusted to 10 mL with methanol and mixed thoroughly. Prior to injection, an adequate sample volume was passed through a 0.45 µm PTFE filter and collected in a LC sample vial. All samples were analyzed on a Waters Acquity UPLC™ system including binary solvent manager, sampler manager, column compartment and a photodiode array (PDA) detector (Waters Acquity model code UPD). Data were acquired and interpreted using MassLynxTM NT 4.1 software. The separation was carried out on a Waters Acquity UPLC™ BEH Shield RP18 column (100 mm × 2.1mm i.d., 1.7 µm) which was equipped with a guard column (Vanguard 2.1 × 5 mm). The sample temperature and column temperature were maintained at 15°C and 35°C, respectively. The mobile phase consisted of water containing 0.05% formic acid (*v*/*v*) (A) and acetonitrile with 0.05% formic acid (B). The analysis was performed using the following gradient elution at a flow rate of 0.30 mL/min: 0-11 min, 30% B to 70% B and 11-15 min, 70% B to 100% B. The column was washed with 100% B for 3 min and re-equilibrated for 3.5 min between runs using the initial conditions. The strong needle wash (90/10; acetonitrile/water, *v*/*v*) and weak needle wash solution (10/90; acetonitrile/water, *v*/*v*) were used. The total run time for analysis was 15 min. Mass spectrometric analyses were performed using electrospray ionization (ESI) on a Waters ACQUITY™ Xevo QToF G2-S mass spectrometer (Waters Corporation, Manchester, UK). The ESI source was operated under the positive ionization mode in the following conditions: capillary of 0.65 kV, cone of 25 V, source temperature of 80°C, desolvation temperature 500°C, desolvation gas flow of 900 L/h, cone gas flow of 50 L/h, and collision energy of 6 eV. Leucine-enkephalin was used for the lock mass at a concentration of 5 ng/mL and flow rate of 5 µL/min. Ions [M+H]+ (*m/z* 556.2771 Da) and fragment ion (*m/z* 278.1141 Da) of leucine-enkephalin were employed to ensure mass accuracy during the MS analysis. The lock spray interval was set at 30 s, and the data were averaged over three scans. The mass spectrometer was programmed to step between low (6 eV) and elevated (10-30 eV) collision energies on the gas cell, using a scan time of 0.5 s per function over a mass range of 50–1200 *m/z*. When data were acquired with MSE, two interleaved scan functions were used. The first scan function acquired a wide mass range using low collision energy. This scan function collected precursor ion information for the sample. The second scan function acquired data over the same mass range; however, the collision energy was ramped from low to high. This scan function allowed for the collection of a full-scan accurate mass of fragments along with precursor ion information. An individual stock solution of standard compounds (umbelliferone, luteolin, and umbelliprenin) was prepared at a concentration of 1.0 mg/mL in methanol. The calibration curves were prepared at seven different concentration levels and listed in Supplementary Table S1.

***De novo* transcriptome assembly and evaluation**

We utilized four different *de novo* transcriptome assembly pipelines to ensure a high quality reference transcriptome was assembled.

First pipeline: Reads from all organs were combined and assembledwith Trinity v2.4.0 with kmer = 25 (Grabherr *et al.* 2011). We refer to this pipeline as “Trinity” pipeline.

Second pipeline: The Khmer v2.0 tools (Crusoe *et al.* 2015) was applied to variable kmer coverage abundance trimming to the reads prior to Trinity assembly. This reduces the computational cost of assembly without negatively affecting the quality of the assembly. We refer to this pipeline as the “Khmer\_Trinity” pipeline.

Third pipeline: The Drap v1.91 was applied as a post processing step after using Trinity to compact and correct the assembled transcriptome (Cabau *et al*. 2017). We refer to this pipeline as the “Trinity\_DRAP” pipeline.

Fourth pipeline: The Drap v1.91 was applied as a post processing step after using Oases v0.2.06 with kmer 25, 31,37, 43, 49 (Zerbino and Birney 2008; Schulz *et al.* 2012). We refer to this pipeline as the “Oases\_DRAP” pipeline.

### Tissue-specific differential expression analysis

The differentially expressed genes of the assembled transcriptome were investigated using the edgeR package in the R statistical environment (FDR <0.05) (Robinson *et al.* 2010b; R Core Team 2016) as follows: The differential expression analysis was calculated from 12 samples from the UC Davis facility (3 samples from 4 different organs) to avoid the possibility of a batch effect altering the expression analysis.We filtered to remove genes with low expression, only retaining genes with more than 10 reads in more than three samples. After this filtering, sample libraries were normalized by calculating the effective library size and normalization factor using the TMM method on counts data (Robinson and Oshlack, 2010a). The model was calculated based on “model.matrix (~organs, data=data)” formula. Organs refer to four organs including roots, flowers, leaves and stems for designing this model. We then identified genes differentially expressed among plant organs using a generalized linear model (glm) in edgeR and multiple-testing correction via the Benjamini and Hochberg (BH) procedure (Benjamini and Hochberg, 1995).

RESULTS AND DISCUSSION

***De novo* transcriptome assembly and evaluation**

By using the Illumina HiSeq platform, the libraries were sequenced, and then after removing low quality reads and adaptor sequences, approximately 218 million reads from roots, 190 million reads from flowers, 123 million reads from stems, and 91 million reads from leaves were generated (Supplemental Table S2). To obtain high quality assemblies, we compared results from four different *de novo* assembly pipelines including Trinity (Grabherr *et al.* 2011), Khmer\_Trinity (Crusoe *et al.* 2015), Trinity\_DRAP, and Oases\_DRAP (Cabau *et al.* 2017) (Supplemental Table S3). The Oases assemblies after DRAP correction produced the longest contigs (N50) in comparison to other pipelines (Supplemental Table S3).

To investigate the quality of the transcriptome assembly we determined the percentage of reads mapping and the completeness of universal single copy orthologs. Using the STAR aligner, we found that over 65% of the reads mapped uniquely to the transcripts produced by Oases\_DRAP (Figure S2). High quality assemblies should have a high representation of universally conserved single copy orthologs; we assessed this metric using BUSCO v3 (Simão *et al.* 2015). The universal single-copy orthologs used were from the OrthoDB database (Zdobnov *et al.* 2016). The transcriptome assembly produced by Oases and further corrected by DRAP contained complete assemblies of over 90% of these genes and more than 80% were present as single copy (Table S4), confirming that this assembled transcriptome was of high quality.

# Supplementary Figures and Tables

## Supplementary Table

**Supplementary Table S1.** The calibration curves for umbelliferone, luteolin, umbelliprenin.

|  |  |  |  |
| --- | --- | --- | --- |
| **Compound Name** | **Calibration Curve** | **R2** | **Linearity Range  (ng/mL)** |
| umbelliferone | Y = 24.673 \* X – 125.505 | 0.999 | 20-20,000 |
| luteolin | Y = 58.4144 \* X -761.246 | 0.996 | 20-20,000 |
| umbelliprenin | Y = 4.5332 \* X – 26.2643 | 0.994 | 20-20,000 |

**Supplementary Table S2.** RNAseq data obtained from different organs of *F. assafoetida*.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Facility for Sequencing** | **No. of Plants** | **Organ** | **Total No. of Reads after Quality Control** | **Average Read Length (bp)** |
| UC Davis | 3 | Root | 91735578 | 149.31 |
| UC Davis | 3 | Stem | 123539018 | 149.33 |
| UC Davis | 3 | Leaf | 91321958 | 149.41 |
| UC Davis | 3 | Flower | 60283346 | 149.47 |
| Novogene | 2 | Root | 93100182 | 150 |
| Novogene | 2 | Flower | 95622730 | 150 |
| BGI | 1 | Root | 33877268 | 150 |
| BGI | 1 | Flower | 34402438 | 150 |

**Supplemental Table S3.** Summary statistics on *de novo* assembly of *F. assafoetida.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Trinity | Khmer\_Trinity | Trinity\_DRAP | Oases\_DRAP |
| Total transcripts | 329131 | 273777 | 81327 | 60134 |
| Contig N50 (bp) | 1464 | 1250 | 1559 | 1888 |
| Average contig (bp) | 841.37 | 728.51 | 1102.25 | 1241.3 |

**Supplementary Table S4.** Summary evaluation of *F. assafoetida* transcriptome assemblies*.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Trinity | Khmer\_Trinity | Trinity\_ DRAP | Oases\_DRAP |
| Complete BUSCOs (C) | 1272 (88.4%) | 1281 (88.9%) | 1303 (90.5%) | **1315 (91.4%)** |
| Complete and single-copy BUSCOs (S) | 482 (33.5%) | 503 (43.9%) | 1114 (77.4%) | **1176 (81.7%)** |
| Complete and duplicated BUSCOs (D) | 790 (54.9%) | 778 (54.0%) | 189 (13.1%) | 139 (9.7%) |
| Fragmented BUSCOs (F) | 87 (6.0%) | 89 (6.2%) | 47 (3.3%) | 34 (2.4%) |
| Missing BUSCOs (M) | 81 (5.6%) | 70 (4.9%) | 90 (6.2%) | 91 (6.2%) |
| Total BUSCO groups searched | 1440 | 1440 | 1440 | 1440 |

**Supplementary Table S5.** Putative annotation and transcript identities of candidate genes of terpenoid biosynthesis.

|  |  |
| --- | --- |
| **Name in *F. assafoetida*** | **Abbreviation** |
| TRINITY\_DN26504\_c0\_g1\_i1 | *Ferula assafoetida* CPS |
| oases3\_k43\_Locus\_5220\_Transcript\_5\_1 | *Ferula assafoetida* EKS |
| oases3\_k31\_Locus\_43830\_Transcript\_3\_1 | *Ferula assafoetida* TPS1 |
| oases3\_k49\_Locus\_2046\_Transcript\_4\_1 | *Ferula assafoetida* TPS2 |
| oases3\_k49\_Locus\_48413\_Transcript\_4\_1 | *Ferula assafoetida* TPS3 |
| oases6\_k49\_Locus\_56686\_Transcript\_2\_1 | *Ferula assafoetida* TPS4 |
| TRINITY\_DN40361\_c0\_g4\_i2 | *Ferula assafoetida* TPS5 |
| oases3\_k49\_Locus\_1839\_Transcript\_5\_2 | *Ferula assafoetida* TTS1 |
| oases6\_CL5040Contig1\_1 | *Ferula assafoetida* TTS2 |
| oases6\_k25\_Locus\_16826\_Transcript\_4\_1 | *Ferula assafoetida* TTS3 |
| oases6\_k37\_Locus\_1803\_Transcript\_51\_1 | *Ferula assafoetida* TTS4 |
| oases2\_k31\_Locus\_1021\_Transcript\_15\_1 | *Ferula assafoetida* TTS5 |
| oases6\_k49\_Locus\_1643\_Transcript\_11\_1 | *Ferula assafoetida* TTS6 |
| oases6\_k49\_Locus\_26372\_Transcript\_1\_1 | *Ferula assafoetida* TTS7 |
| oases3\_CL17600Contig1\_1 | *Ferula assafoetida* TTS8 |
| oases2\_CL309Contig1\_2 | *Ferula assafoetida* TTS9 |

**Supplementary Table S6**. Putative annotation and transcript identities of candidate genes of coumarin-type phenylpropanoid biosynthesis.

|  |  |
| --- | --- |
| Name in *F. assafoetida* | Abbreviation |
| oases3\_k25\_Locus\_11029\_Transcript\_9\_1 | *Ferula assafoetida* 4CL-like1 |
| oases3\_k25\_Locus\_4163\_Transcript\_5\_1 | *Ferula assafoetida* 4CL-like2 |
| oases3\_k37\_Locus\_4395\_Transcript\_4\_1 | *Ferula assafoetida* 4CL-like3 |
| oases3\_k43\_Locus\_7570\_Transcript\_3\_1 | *Ferula assafoetida* 4CL-like4 |
| oases6\_k25\_Locus\_30933\_Transcript\_3\_1 | *Ferula assafoetida* 4CL-like5 |
| TRINITY\_DN75605\_c0\_g1\_i1 | *Ferula assafoetida* 4CL-like6 |
| oases6\_k37\_Locus\_13492\_Transcript\_4\_1 | *Ferula assafoetida* C2'H |
| oases2\_k37\_Locus\_48872\_Transcript\_1\_1 | *Ferula assafoetida* CHI-like1 |
| oases3\_CL10153Contig1\_2 | *Ferula assafoetida* CHI-like2 |
| oases3\_k49\_Locus\_9379\_Transcript\_12\_1 | *Ferula assafoetida* CHI-like3 |
| oases6\_k25\_Locus\_14487\_Transcript\_14\_1 | *Ferula assafoetida* CHI-like4 |
| oases6\_k37\_Locus\_61759\_Transcript\_1\_1 | *Ferula assafoetida* CHI-like5 |
| oases2\_k25\_Locus\_50568\_Transcript\_1\_1 | *Ferula assafoetida* CHS-like1 |
| oases3\_k25\_Locus\_15765\_Transcript\_6\_1 | *Ferula assafoetida* CHS-like2 |
| oases2\_k25\_Locus\_16219\_Transcript\_3\_1 | *Ferula assafoetida* FH-like1 |
| oases3\_k43\_Locus\_8457\_Transcript\_7\_1 | *Ferula assafoetida* FH-like2 |
| oases6\_k37\_Locus\_57857\_Transcript\_1\_1 | *Ferula assafoetida* FH-like3 |
| oases6\_k49\_Locus\_12143\_Transcript\_7\_2 | *Ferula assafoetida* FNS |
| oases2\_k31\_Locus\_1941\_Transcript\_18\_1 | *Ferula assafoetida* PAL-like1 |
| oases3\_k43\_Locus\_21058\_Transcript\_1\_1 | *Ferula assafoetida* PAL-like2 |
| TRINITY\_DN121792\_c0\_g1\_i1 | *Ferula assafoetida* PAL-like3 |
| TRINITY\_DN38110\_c0\_g8\_i1 | *Ferula assafoetida* PAL-like4 |
| oases2\_k25\_Locus\_8995\_Transcript\_3\_1 | *Ferula assafoetida* PAL-like5 |

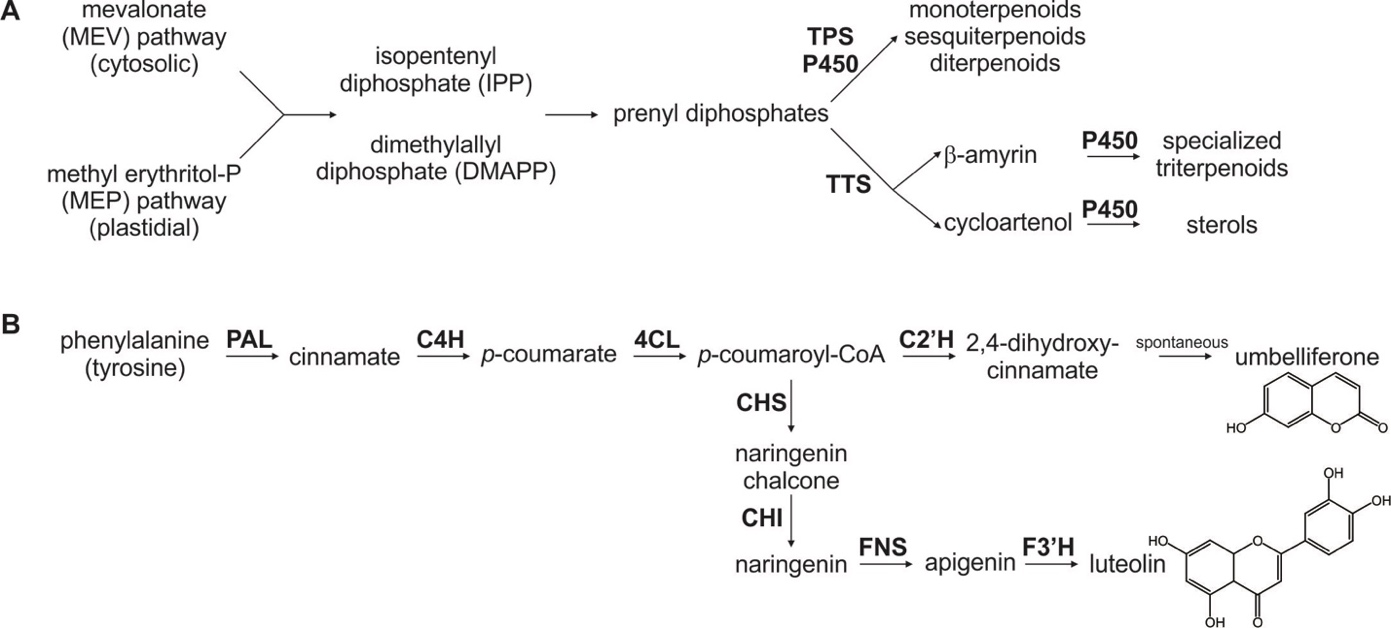
**Supplementary Table S7**. Genes upregulated in sesqui- and tri-terpenoid biosynthesis with ko00909.

|  |  |  |  |
| --- | --- | --- | --- |
| Name in *F. assafoetida* | KO | EC | Definition |
| oases3\_CL17600Contig1\_1 | K15813 | EC:5.4.99.39 | β-Amyrin |
| oases3\_k49\_Locus\_48413\_Transcript\_4\_1 | K15803 | EC:4.2.3.75 | (-)-germacrene D |
| oases6\_k25\_Locus\_6897\_Transcript\_5\_2 | K15803 | EC:4.2.3.75 | (-)-germacrene D |
| oases6\_k31\_Locus\_55853\_Transcript\_2\_1 | K00801 | EC:2.5.1.21 | farnesyl-diphosphate farnesyltransferase |
| oases6\_k37\_Locus\_56543\_Transcript\_8\_1 | K00801 | EC:2.5.1.21 | farnesyl-diphosphate farnesyltransferase |
| oases6\_k49\_Locus\_18297\_Transcript\_7\_1 | K00801 | EC:2.5.1.21 | farnesyl-diphosphate farnesyltransferase |

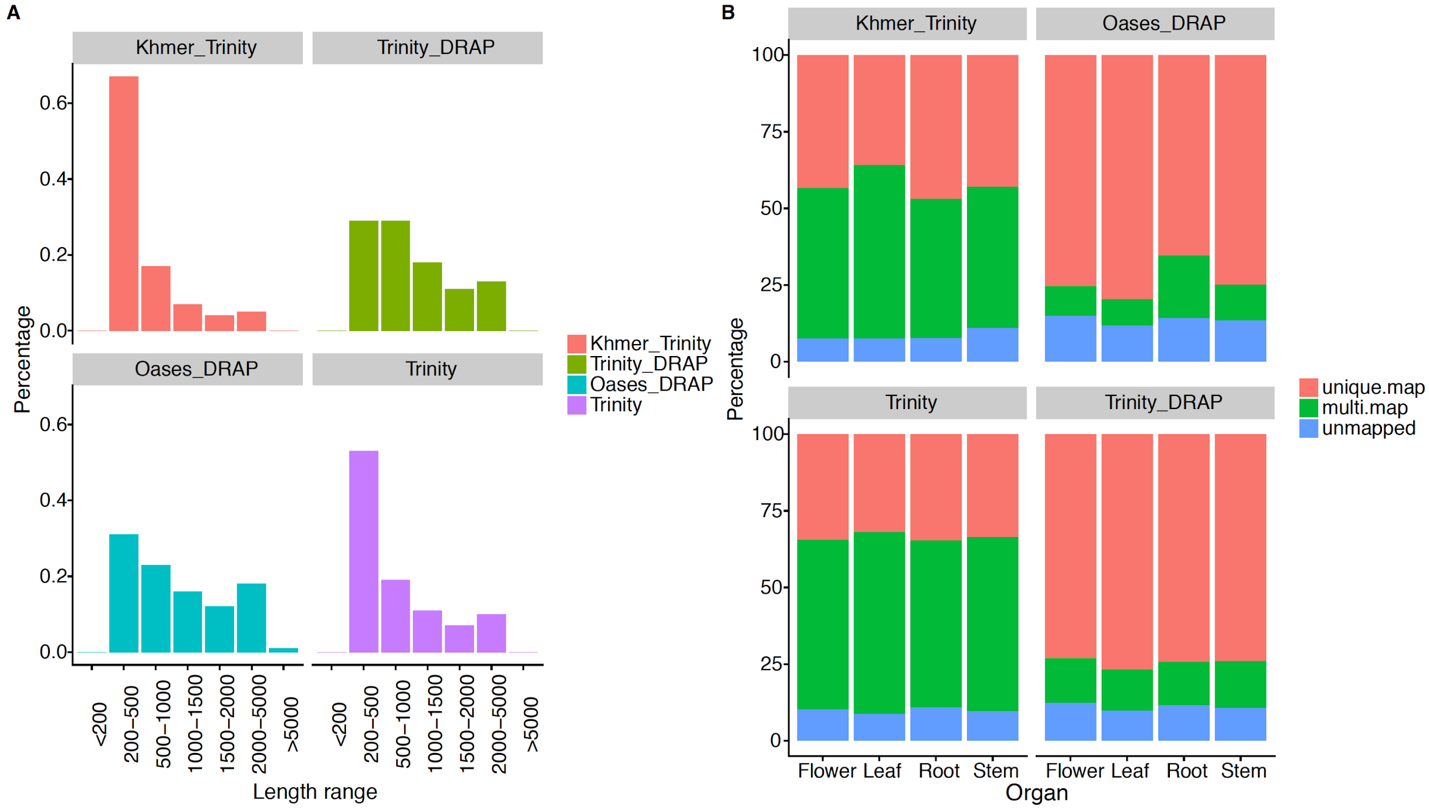
**Supplementary Table S8.** Genes upregulated in flavonoid biosynthesis with ko00941.

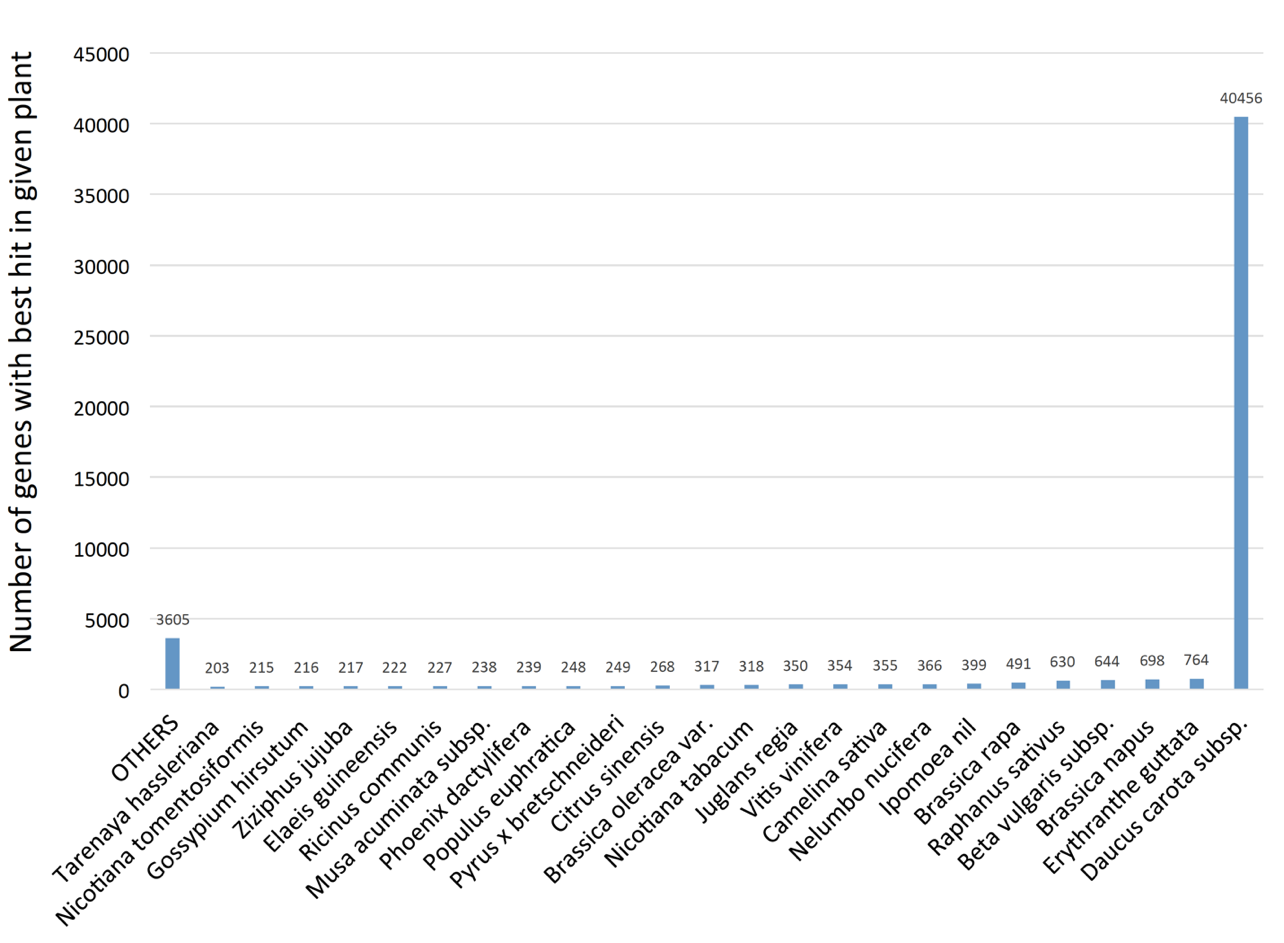
|  |  |  |  |
| --- | --- | --- | --- |
| Name in *F. assafoetida* | KO | EC | Definition |
| oases2\_k25\_Locus\_16219\_Transcript\_3\_1 | K05280 | EC:1.14.13.21 | flavonoid 3'-monooxygenase |
| oases2\_k25\_Locus\_50568\_Transcript\_1\_1 | K00660 | EC:2.3.1.74 | chalcone synthase |
| oases2\_k37\_Locus\_48872\_Transcript\_1\_1 | K01859 | EC:5.5.1.6 | chalcone isomerase |
| oases3\_CL10153Contig1\_2 | K01859 | EC:5.5.1.6 | chalcone isomerase |
| oases3\_k25\_Locus\_52353\_Transcript\_5\_1 | K05278 | EC:1.14.20.6 | flavonol synthase |
| oases3\_k31\_Locus\_19023\_Transcript\_5\_1 | K00475 | EC:1.14.11.9 | naringenin 3-dioxygenase |
| oases3\_k31\_Locus\_19023\_Transcript\_5\_2 | K00475 | EC:1.14.11.9 | naringenin 3-dioxygenase |
| oases3\_k37\_Locus\_50451\_Transcript\_6\_1 | K13082 | EC:1.1.1.219/ EC:1.1.1.234 | bifunctional dihydroflavonol 4 reductase/  flavanone 4-reductase |
| oases6\_k25\_Locus\_14487\_Transcript\_14\_1 | K01859 | EC:5.5.1.6 | chalcone isomerase |
| oases6\_k49\_Locus\_12143\_Transcript\_7\_2 | K00475 | EC:1.14.11.9 | naringenin 3-dioxygenase |

**1.2 Supplementary Figures**

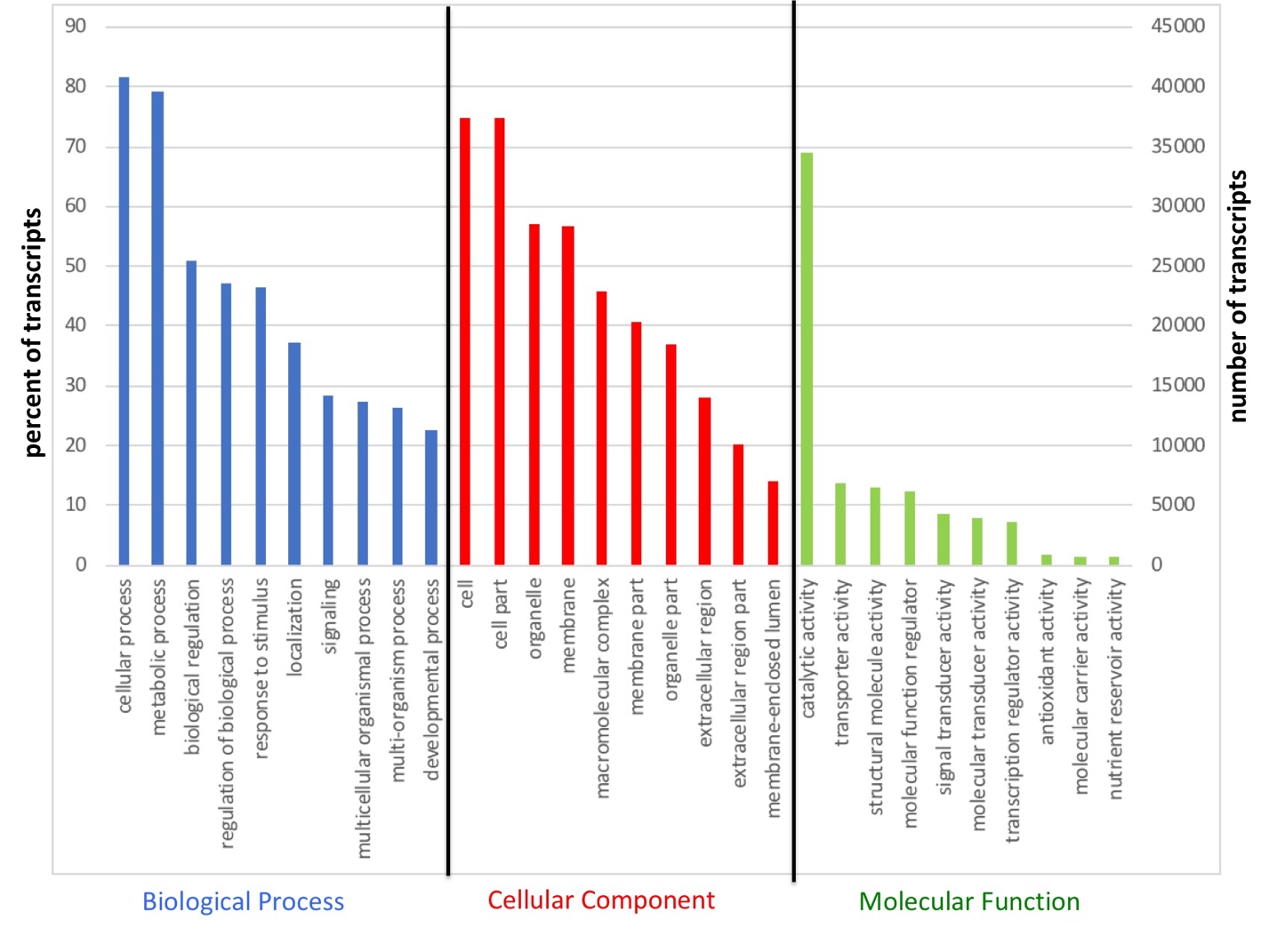
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**Supplementary Figure S1.** Schematic overview of core pathway reactions in the biosynthesis of terpenoids (**A**) and phenylpropanoids (**B**) targeted in this study as relevant bioactive constituents in *Ferula assafoetida*. Key enzymes are highlighted in bold: TPS, terpene synthase; TTS, triterpene synthase; P450, cytochrome P450-dependent monooxygenase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA-ligase; C2′H, p‐coumaroyl-CoA 2′‐hydroxylase; CHS, chalcone synthases; CHI, chalcone isomerases; FNS, flavone synthases; F3’H, flavanone 3-hydroxylase.

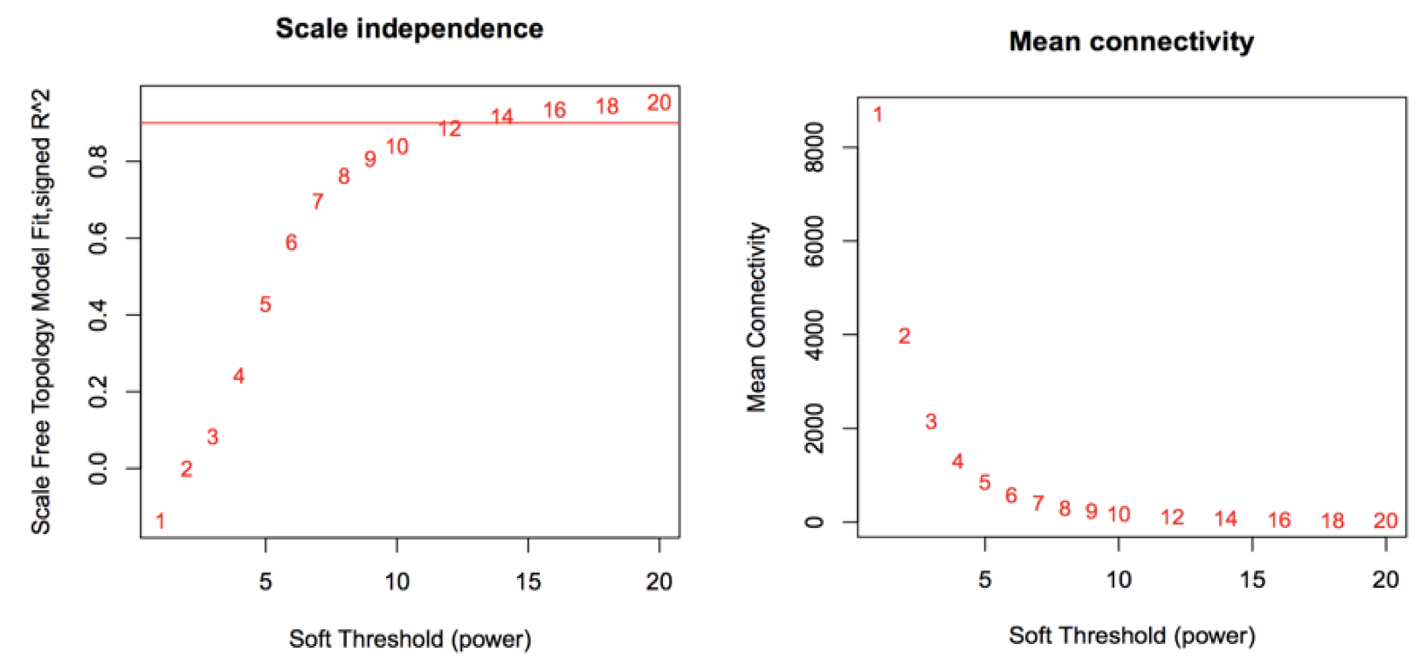
**Supplementary Figure S2.** Length distribution of transcripts of assembled transcriptomes (**A**) and fraction of the RNAseq reads uniquely mapped to the assembled transcriptome by STAR 2.5.2b (**B**).

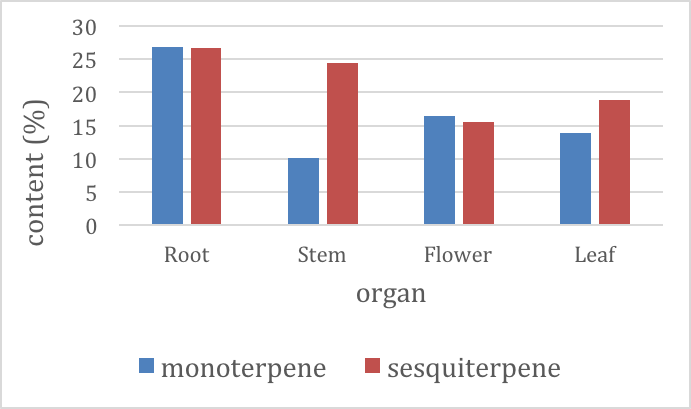
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**Supplementary Figure S3.** Distribution of assembled transcriptome best hits to different plants as a result of blastx against all RefSeq plant databases.

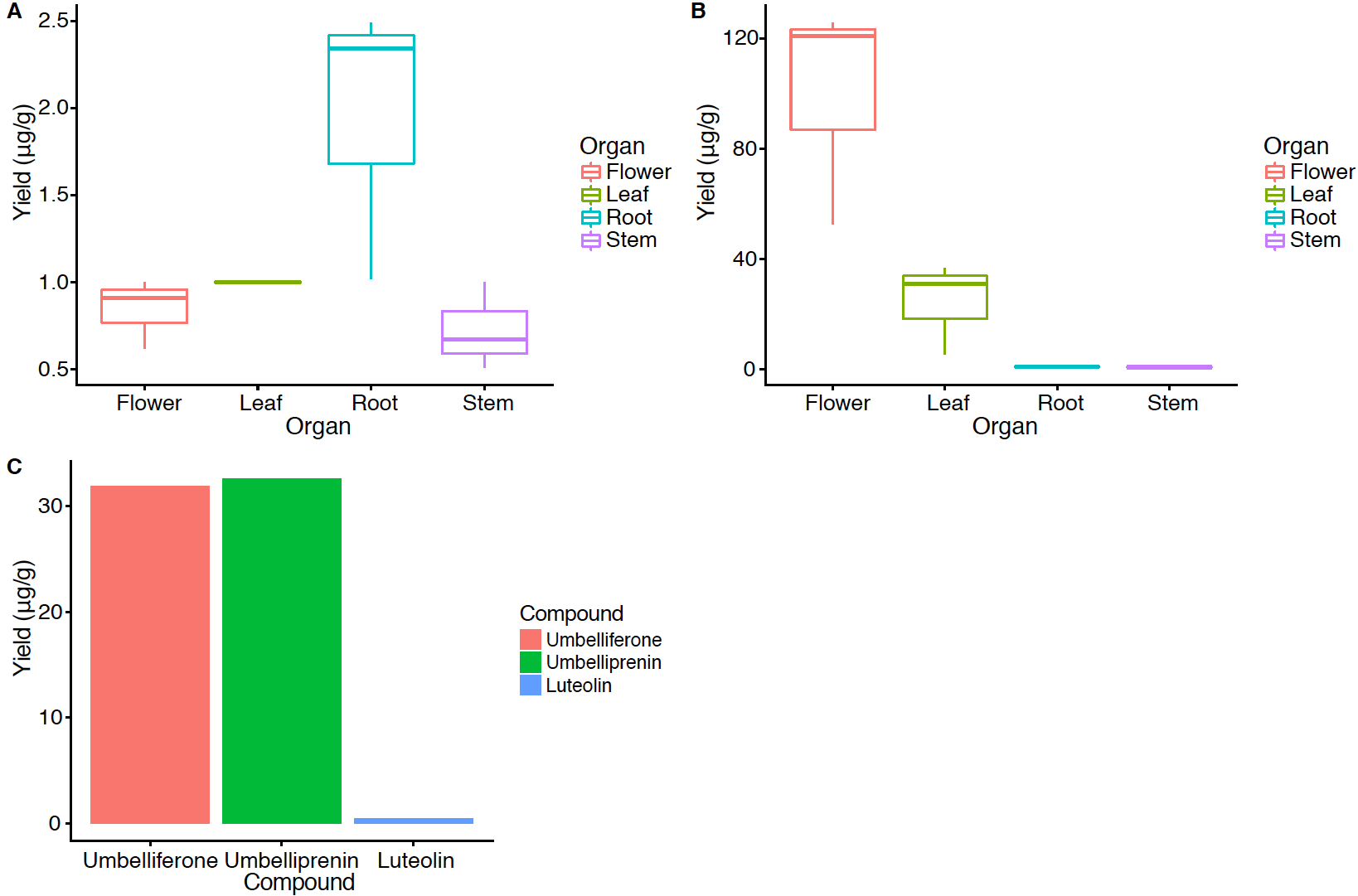
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**Supplementary Figure S4.** Gene ontology (GO) classification of the transcripts derived from *F. assafoetida.*

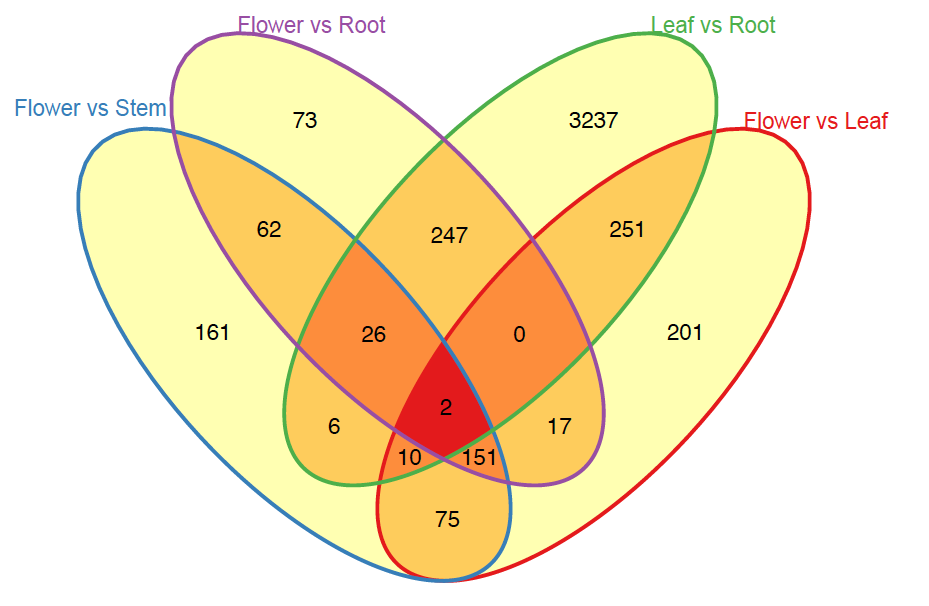
**Supplementary Figure S5.** Determination of soft threshold (power) for network construction.

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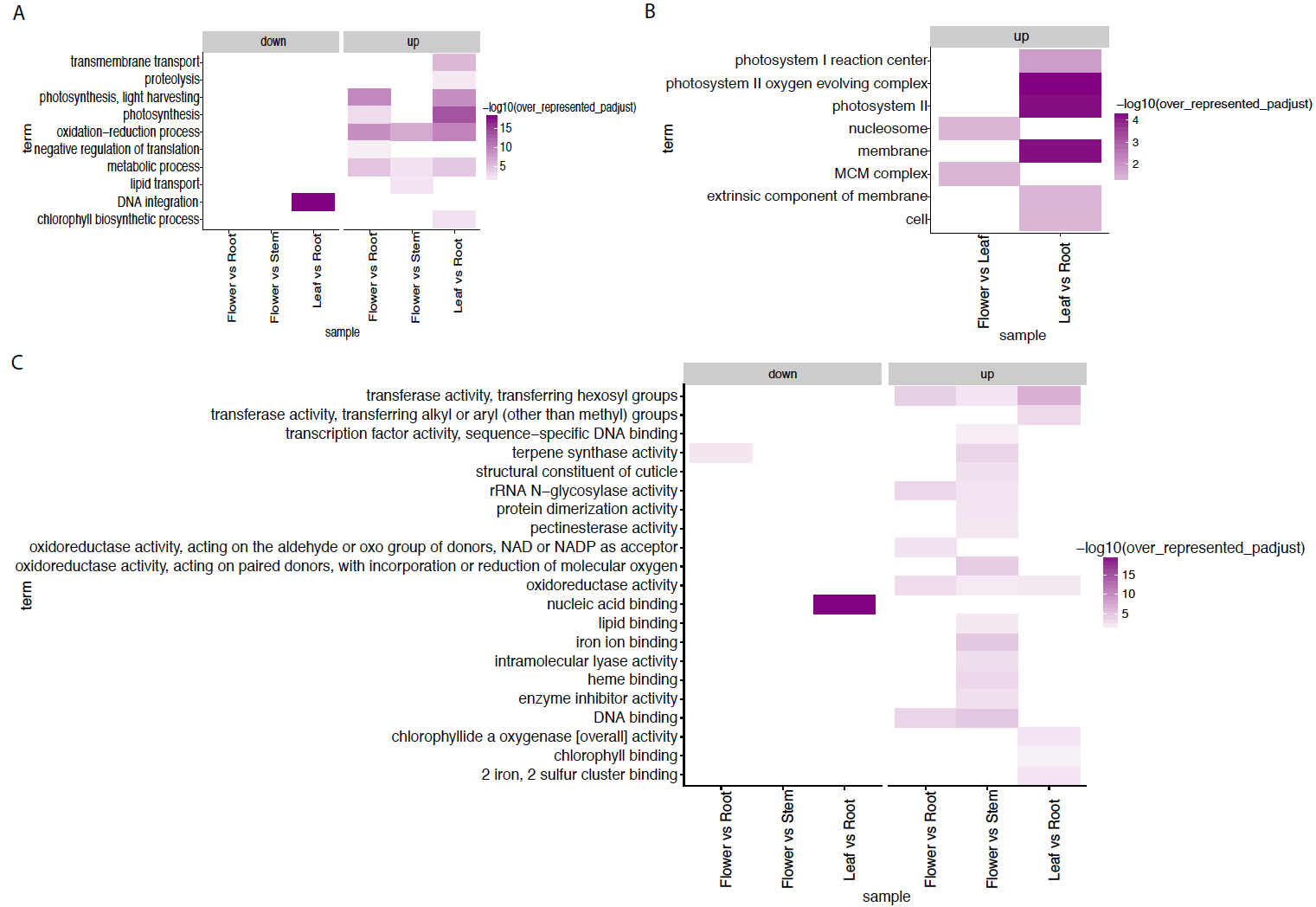
**Supplementary Figure S6.** Relative terpenoid composition in the essential oils of different organs.

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**Supplementary Figure S7.** Quantification of the amount of umbelliferone (**A**) and luteolin (**B**) among different organs. Quantification of the amount of umbelliferone, umbelliprenin and luteolin (**C**) in oleo-gum-resin. The yield was microgram per gram dry weight (µg/g) of these compounds for each sample. Each value represents the average of two injections for each sample by LOD (limits of detection) at 10 ng/ml and LOD at 20 ng/ml.

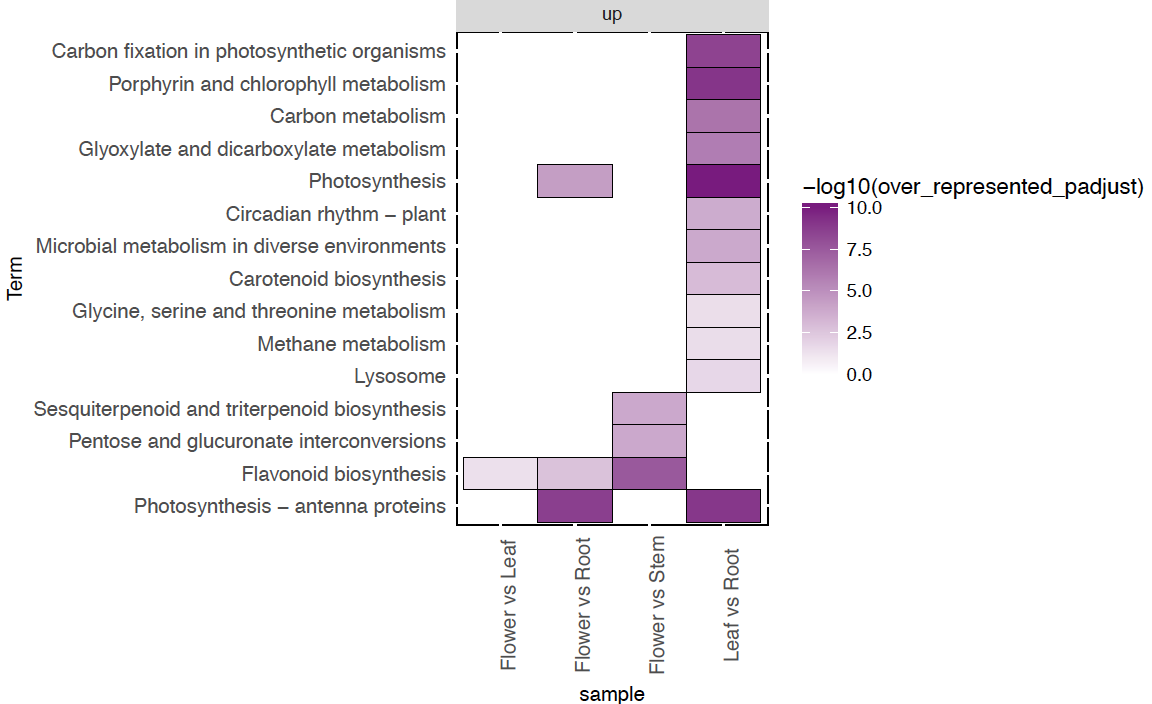
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**Supplementary Figure S8.** Venn diagram of differentially expressed genes used in pairwise statistical tests and contrast matrix of different organs (Significance level: FDR <0.05).

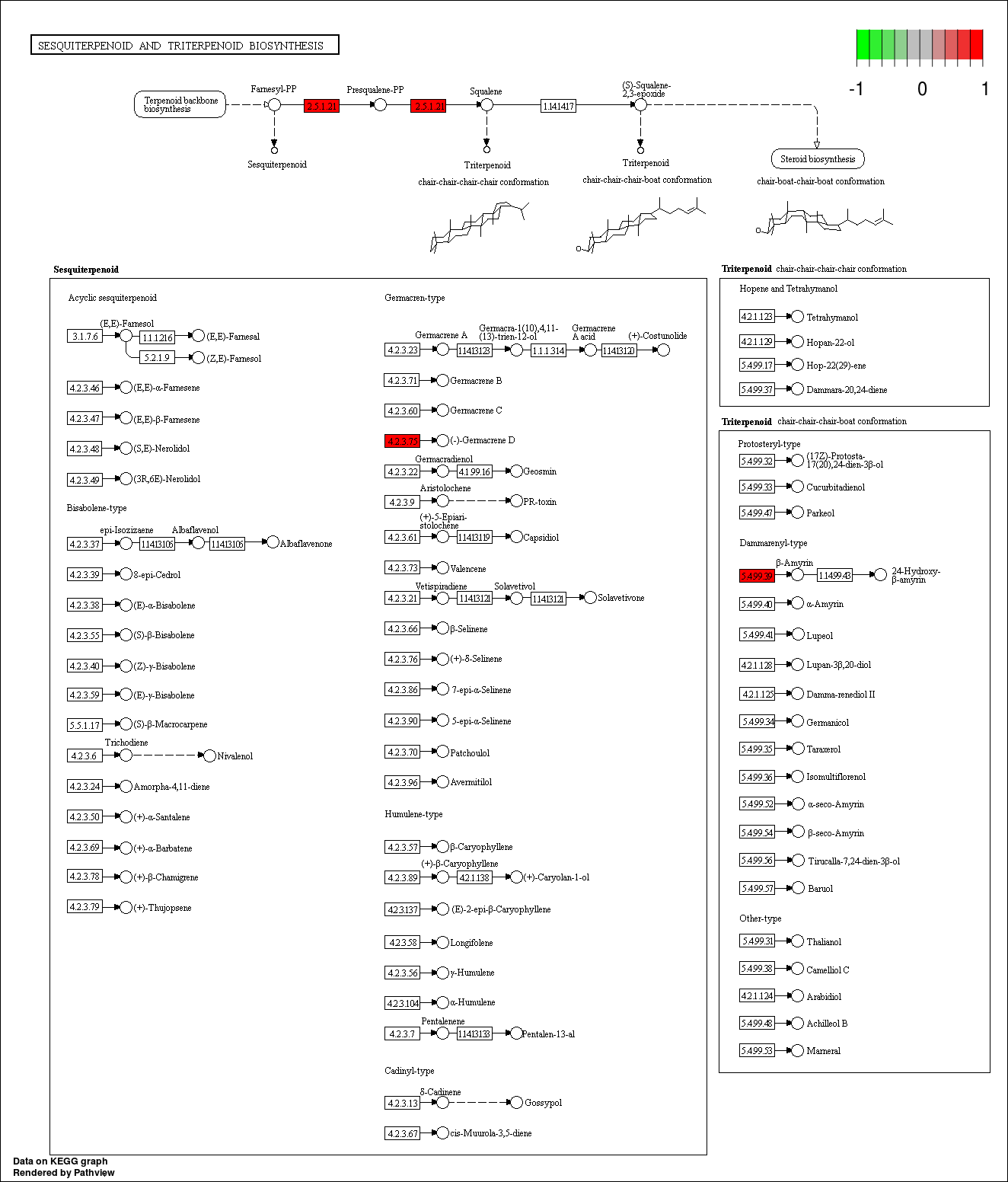
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**Supplementary Figure S9.** Over-represented GO terms in differential expressed genes between pairwise comparison organs for three ontologies biological process (**A**), cellular component (**B**) and molecular function (**C**). “down” in Flower vs Root means higher expression in Root; “down” in Flower vs Stem means higher expression in Stem; “down” in Leaf vs Root means higher expression in Root.

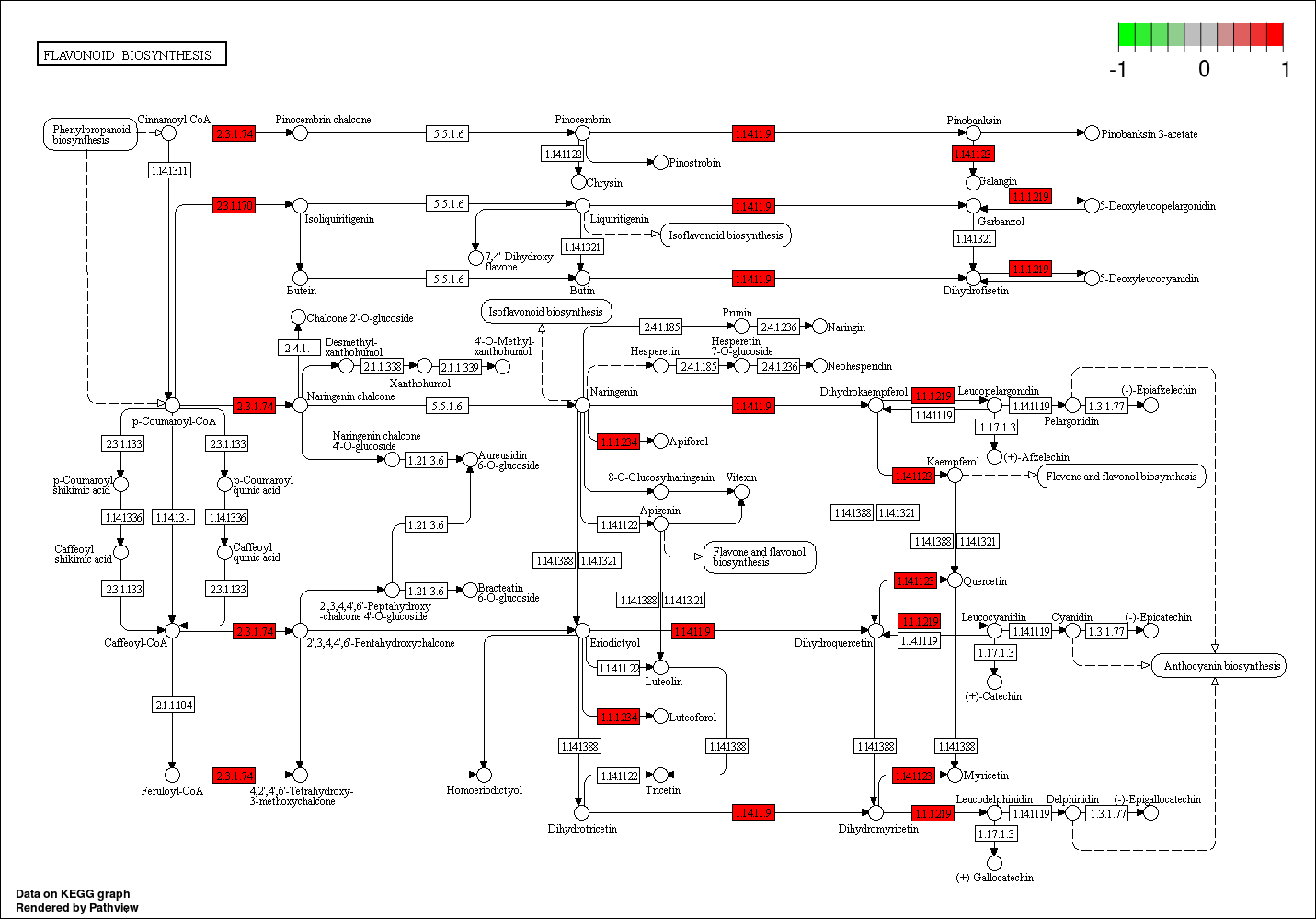
“up” in Flower vs Root indicates higher expression in Flower; “up” in Flower vs Stem indicates higher expression in Flower; “up” in Leaf vs Root indicates higher expression in Leaf; “up” in Flower vs Leaf indicates higher expression in Flower; “up” in Leaf vs Root means higher expression in Leaf.

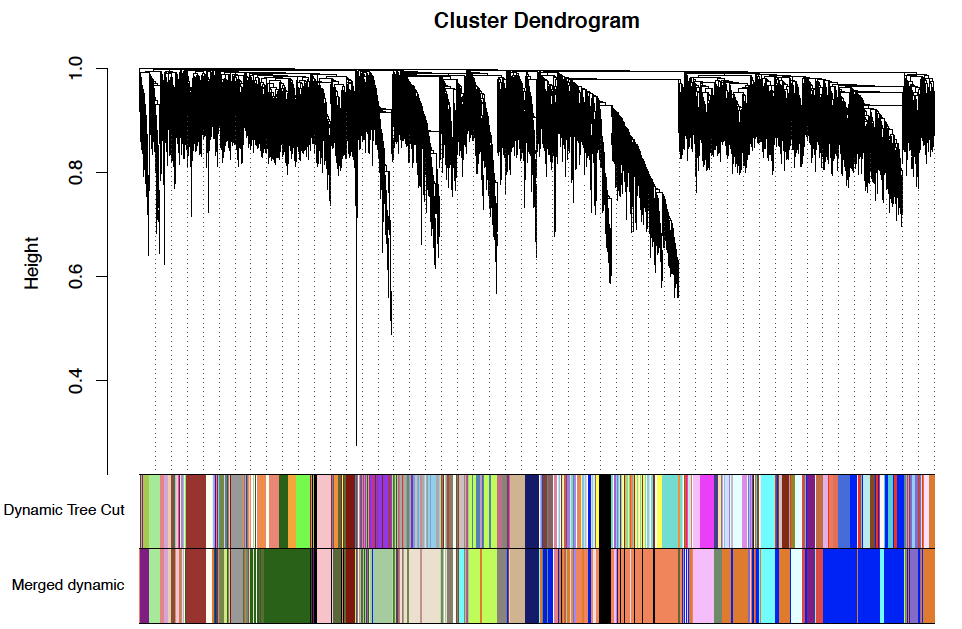
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**Supplementary Figure S10.** Pathway analysis result for finding over-represented KEGG orthology by using the KEGG database. “up” in Flower vs Leaf indicates higher expression in Flower; “up” in Flower vs Root indicates higher expression in Flower; “up” in Flower vs Stem indicates higher expression in Flower; “up” in Leaf vs Root indicates higher expression in Leaf.

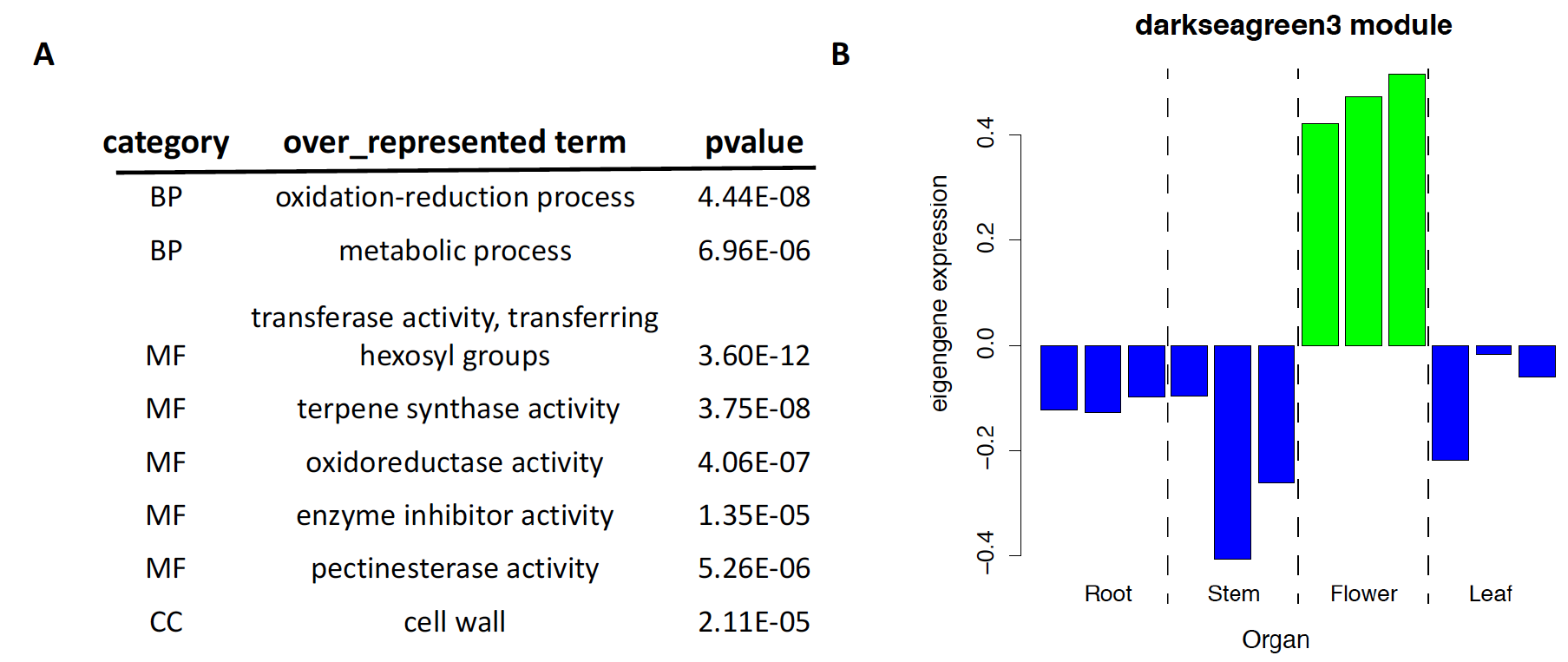


**Supplementary Figure S11.** Visualization of terpenoid pathway branches that were identified as significantly enriched in flowers versus stems of *F. assafoetida*. *F. assafoetida* transcripts significantly mapped to sesquiterpenoid biosynthesis are highlighted in red.

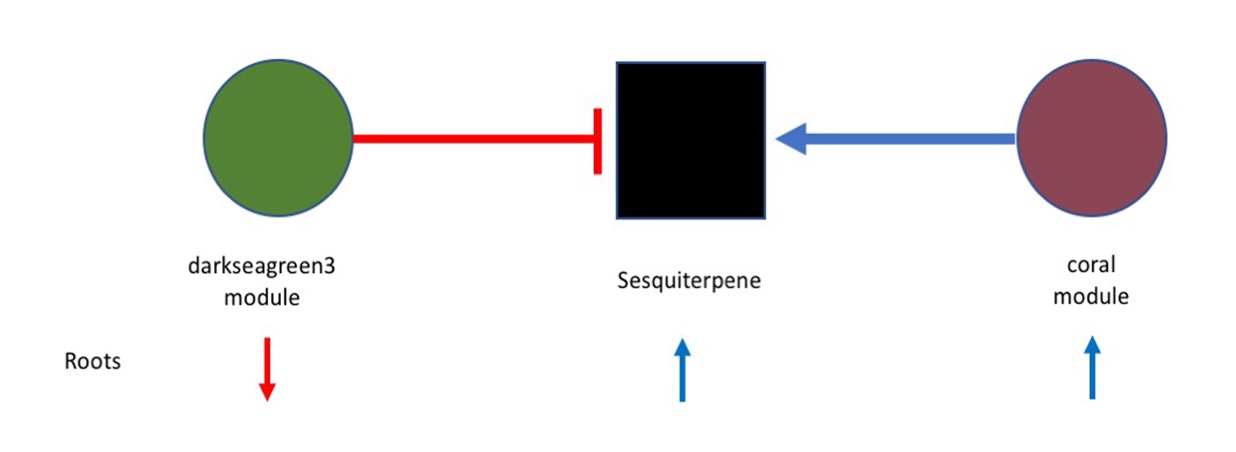
**Supplementary Figure S12.** Visualization of phenylpropanoid pathway branches that were identified as significantly enriched in flowers versus stems of *F. assafoetida*. *F. assafoetida* transcripts significantly mapped to phenylpropanoid and flavonoid biosynthesis are highlighted in red.

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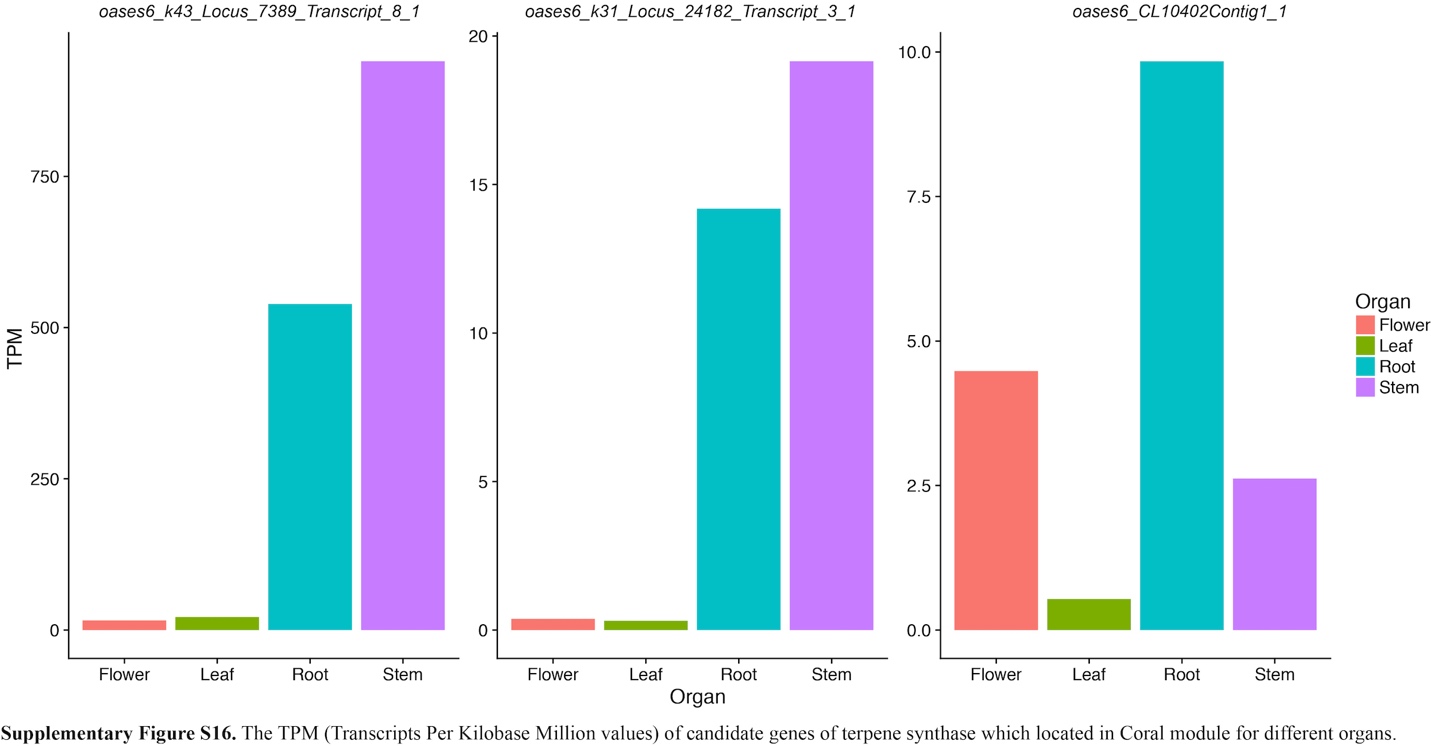
**Supplementary Figure S13.** Gene network of *F. assafoetida* was constructed using WGCNA by setting the soft power to 12, type to signed hybrid, minModuleSize to 30, dissimilarity threshold to 0.2, and deepslit to 2.

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**Supplementary Figure S14.** Over-representation analysis (ORA) of darkseagreen3 module (**A**). The eigengenes expression value of darkseagreen3 module across different organs (**B**).

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**Supplementary Figure S15.** The contribution of the darkseagreen3 and coral modules in sesquiterpene biosynthesis. The co-expression analysis indicates that the darksagreen3 module is negatively correlated with production of sesquiterpene in roots while the coral module is positively correlated with production of sesquiterpene.



**Supplementary Figure S16.** The TPM (Transcripts Per Kilobase Million values) of terpene synthase candidate genes located in the coral module for different organs.

REFERENCES

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