Transcriptome profiling on Euphorbia kansui elicited with methyl jasmonate

Peng Li
Xi'an International University
Xiaoqing Liang
Xi'an International University
Min Zhang
Xi'an International University
Xiaoai Fang
Xi ' an Botanical Garden of Shaanxi Province (Institute of Botany of Shaanxi Province)
Xia Cai
caix@nwu.edu.cn

Northwest University) Ministry of Education

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Abstract

Euphorbia kansui Liou of *Euphorbia* (Euphorbiaceae) is an endemic traditional

medicine in China, and exerted effective antitumor activity due to its specific metabolic

composition, especially terpenoid.This study was designed to illustrate the detailed effects of MeJA on *E. kansui* through transcriptome before and after MeJA treatment. 96,040 unigenes with an average length of 812 bp were identified, among which 53,822 unigenes were annotated according to function database. Of note, we noted that MeJA treatment significantly altered the transcription pattern of *E. kansui*, especially at 24 hat of MeJA treatment. MeJA treatment mainly altered the expression of genes that functioned in biosynthesis of cutin, wax, flavonoid and terpenoid represented by MVA pathway in *E. kansui*. Typically, MeJA treatment mainly activated the expression of genes for terpenoid biosynthesis in EK at 36 hat. Further qRT-PCR verified that exogenous application of 20 μM MeJA greatly upregulate the expression levels of genes encoding enzymes in the terpenoid biosynthesis in *E. kansui* at 36 hat. Importantly, the contents of total terpenoid in *E. kansui* at 36 hat of MeJA treatment were promoted to 2.80 times of those without MeJA treatment. our results highlighted that MeJA could promoted the accumulation of terpenoid in E. kansui, which provided basis for cultivating high quality of E. kansui with higher levels of

bioactive compounds.

Introduction

Euphorbia kansui Liou is an endemic perennial herb of Euphorbia (Euphorbiaceae) in China (*Li et al., 2018;* Liu et al., 2018). Its dried root, as a common traditional Chinese medicine, has been listed in the Chinese Pharmacopoeia (*Guo et al., 2015*). E. kansui extracts in folk are widely used for the treatment of tumor (*Wu et al., 1991; Wang et al., 2018*). The main chemical constituent of E. kansui is terpenoid with numerous medicinal bioactivities, including anti-tumor, anti-cancer, anti-fertility, anti-virus, anti-proliferative, anti-inflammatory and immunomodulatory activities (Hsieh et al., 2011; *Z*hao et al., 2014; Kim et al., 2015; Fei et al., 2016; Shen et al., 2016; Young et al., 2018; Zhou et al., 2021; Guo et al., 2021). Terpenoids derived from E. kansui exerted significant inhibition effects on the excessive proliferation of embryonic cells and intestinal epithelioid cells (Miyata et al., 2006; Cheng et al., 2015). The ethanol extract of E. kansui has the ability to activate lymphocytes, which can remove virus-infected cells (Zheng et al., 1998). However, how to cultivate E. kansui with high level of bioactive terpenoids to promote the medicinal bioactivity of E. kansui remain unclear.

Terpenoids based on isoprene unit are the largest and structurally diverse class of natural compounds in plants, and have been reported to function for plant defense against biotic and abiotic stresses (Tholl 2015). It has been well documented that plant-derived isoprenoid are synthesized via the cytosolic mevalonate (MVA) and plastidic methyl-D-erythritol-4- phosphate (MEP) pathways (Rohmer et al., 1993; Chappell et al., 1995; Laule et al., 2003) (Fig. 1). MVA pathway is recognized as the main pathway for synthesizing terpenoid-like compounds (Huang et al., 2018). IPP (isopentenyl pyrophosphate) is the main intermediate product of both MVA and MEP, after the IPP polymerized, C_5 chain continuing to extend and forming a variety of compounds (Haralampidis et al., 2002; Kim et al., 2010). Thus, activation of MVA pathway might be a potential method for cultivating high quality of *E. kansui*.

The methyl jasmonate (MeJA), a volatile organic compound, has been identified as a vital cellular regulator that mediates diverse physiological processes in plant growth and development (*Cheong et al., 2003; Adil et al., 2022*). MeJA can activate plant defense against biotic and abiotic stimuli (Fonseca et al., 2009; Zhao et al., 2012, Zhang et al., 2015a). Simons et al. (2008) reported that MeJA upregulated the defensive compounds when plants attacked by herbivores. MeJA has also been used to elicit defense responses in several species through effectively promoting the synthesis of the secondary metabolites, especially terpenoids (Vasconsuelo and Boland 2007; Kuźma et al., 2009; Cheng et al., 2013; Zhang et al., 2014), including tropane alkaloids in *Hyoscyamus niger* (Zhang et al., 2007b) and *Scopolia parviflora* (Kang et al., 2004), tanshinone in *Salvia castanea* diels f. *tomentosa* stib (Li et al., 2016), triterpene in *Euphorbia pekinensis* (Zhang et al., 2015b), volatile terpenoids from *Amomum villosum* (Wang et al., 2014), phenolic acids in *Salvia miltiorrhiza* (Xiao et al., 2010). Adil et al. (2022) demonstrated the deterrent effects of MeJA in inducing defense responses in cotton plants to sucking pests. Thus, Adil et al. (2022) suggested that MeJA could be exploited as a non-toxic pest management tool. Thus, these results implied that exogenous application of MeJA might be an effective strategy to increase the accumulation of terpenoid in *E. kansui*, then promote the medicinal quality of *E. kansui*.

Improvement in de novo assembly of high-throughput sequencing data can provide accurate estimation of gene expression levels, so this approach becomes powerful in quantifying gene expression (Sun et al., 2013). To date, next-generation sequencing technologies have been used to analyze the genome and transcriptome in some species. At the transcriptional level, several genes have been reported to be regulated by extensively applied JA or MeJA (Link et al., 1999; Duan et al., 2010; Li et al., 2013; Liao and Peng 2013; Hao et al., 2014; Hao et al., 2015; Long et al., 2015; Liu et al., 2016). Comparison of the gene expression profiles after MeJA treatment also can be used to unveil the relation between gene and metabolism, find key genes involved in the biosynthesis of active compounds and conduct metabolic engineering (Xiao et al., 2010; Chen et al., 2013; Chen et al., 2015). Whereas, no MeJA elicited transcriptomic information was previously reported on *E. kansui*. In present study, to illustrate the effects of MeJA on affecting terpenoid biosynthesis in *E. kansui*, the detailed transcriptome profiles of *E. kansui* after MeJA treatment were sequenced and analyzed. Pairwise comparisons on the gene expression, especially the differentially expressed genes (DEGs) profiles, provide insights into the regulation mechanism of MeJA to *E. kansui*. The finding will be very useful for improving the production of terpenoid from *E. kansui* and for further functional research on this plant.

Results

Constructing of transcriptome profiles of Euphorbia kansui after MeJA treatment.

Exogenous MeJA treatments on *Euphorbia kansui* (EK) seedlings were conducted in three different concentrations, including 200 μM, 20 μM and 2 μM of MeJA. Casbene and AACT were chosen to evaluate the effects of different concentration of MeJA on causing changes of gene expression level in EK. In all spraying experiments, the expression levels of casbene and AACT increased at 24 h, 36 h after MeJA treatment (hat), but did not exhibite significant change at 12 hat, then slightly reduced at 48 hat (Figure S1). However, only 20 μM of exogenous MeJA treatment resulted in significant increases in expression levels of both genes at 24 hat, 36 hat, and significantly decreased at 48 hat (Figure S1; $P < 0.05$). Thus, 20 μM MeJA spraying experiments at 0 (CK), 24 (T1), 36 (T2), 48 (T3) hat were chosen for transcriptome sequencing.

For transcriptome profiles of EK following MeJA treatment, three biological replicates from each experimental group were collected for sequencing. Through raw reads filtering and trinity assembly, we obtained 96,040 unigenes with an average length of 812 bp (Table 1, Figure S2). Then, we analyzed the function of these unigenes using numerous function annotation databases, including Nr, Swiss-Prot, KEGG and KOG databases (Figure S3). Typically, among all unigenes, 52,142 unigenes showed significant similarities to the known proteins in the Nr database, 40,079 had BLAST hits in the Swiss-Prot database, 20,761 in the KEGG database and 36,331 in KOG database (Figure S3). In the Nr and Swiss-Prot databases, most of the matched sequences (69.87% in Nr and 67.69% in Swiss-Prot) had *E*-values between 0 and 1×10^{-20}, while the rest 30.13% in Nr and 32.31% in Swiss-Prot had *E*-values from 1×10^{-20} to 1×10^{-5} (Fig. 2A, B). Homologies among different species are illustrated in Fig. 2C, where out of the matched 52,142 unigenes in Nr, 17,511 (33.58%) were matched to *Jatropha curcas* followed by *Populus euphratica* (3107; 5.96%) and *Theobroma cacao* (2661; 5.10%), suggesting that *E. kansui* was genetically more related to *Jatropha curcas*.

Table 1
Assembly statistics

Type	Amount	GC%	N50	Min Length	Mean Length	Mac Length	Assembled Bases
Genes	96040	39.1749	1066	300	812	12511	78037302
Transcripts	170087	38.8688	1116	300	865	12511	147240952

Subsequently, we annotated the 52,142 unigenes using GO, KEGG and KOG databases (Figure S4, S5). The results showed that GO assignments to the biological processes were the majority, followed by molecular function and cellular component (Figure S4; Additional file 2: Table S2). Meanwhile, the results of KOG showed that 36,331 unigenes were assigned to the KOG classifications (Figure S5; Table S3). Of note, both KEGG and KOG annotation showed that the clusters of secondary metabolites biosynthesis, lipid transport and metabolism, Terpenoid backbone biosynthesis, Flavonoid biosynthesis and Sesquiterpenoid and triterpenoid biosynthesis were the major groups, showing the ability of EK in producing secondary metabolites represented by terpenoids (Figure S5; Additional file 3: Table S3; Additional file 4: Table S4).

MeJA treatment caused dramatic changes in transcription pattern of Euphorbia kansui.

To investigate the variations in transcription pattern of EK following MeJA treatment at different time points, we performed principal component analysis (PCA) on all transcriptome profiles. As shown in Fig. 3A, the top 2 principal component were used to construct the PCA plots (Fig. 3A). The PCA plots showed that all biological replicates from the same experimental group were clustered together, and clear separated with other groups, suggesting that MeJA treatment caused significant changes in the transcription pattern of EK (Fig. 3A). The principal component 1 (PC1) explained 59.5% variations in transcription pattern of all samples, while these variations were mainly caused by MeJA treatment (Fig. 3A), supporting the effects of MeJA in altering transcription pattern of EK. Typically, the distance between TI and CK was larger than that between T2/T3 and CK, suggesting that exogenous application of MeJA mainly caused transcription variations in EK at 24 hat. Then, we analyzed the differentially expressed genes (DEGs) in each comparison using fold change ≥ 2 and a *p* (P-Value) < 0.05 (Fig. 3B-C; Figure S6). Totally, we identified 12,106 upregulated and 19,952 downregulated DEGs T1 vs. CK comparison, and 5,330 upregulated and 9,953 downregulated DEGs in T2 vs. CK, 4,281 upregulated and 8,185 downregulated DEGs in T3 vs. CK, respectively (Fig. 3B-C; Figure S6). Of note, the number of DEGs in T1 vs. CK was more than that in other two pairwise comparisons (Fig. 3B-C; Figure S6), supporting the phenomenon that exogenous application of MeJA mainly caused transcription variations in EK at 24 hat. Additionally, there are also plenty of DEGs in T1 vs. T2 and T1 vs. T3 comparisons, as shown in (Figure S6).

Subsequently, the Short Timeseries Expression Miner (STEM) cluster was used to directly compare the expression patterns of these DEGs in response to MeJA treatment of 0 h (CK), 24 hat (T1), 36 h (T2) and 48 h (T3). Seven significant gene profiles (P-Value ≤ 0.01): STEM profile 5 (13,286 genes), profile 14 (5,124 genes), profile 11 (4,424 genes), profile 18 (4,128 genes), profile 12 (2,402 genes), profile 4 (2,339 genes) and profile 16 (2,208 genes) were identified as significant changes (Figure S7). It could be found that in profile 11, 18 and 12, after MeJA treatment 36 hat, the gene expression levels reached the peaks, after MeJA treatment 48 hat, gene expression decreased in profile 11 and 18 and kept unchanged in profile 12 (Figure S7). While in profile 14 and 16, gene expression levels got to the highest at MeJA treatment 24 hat, and then decreased at treatment 36 hat and 48 hat, respectively (Figure S7). In profile 4 and 5, gene expression levels get to the lowest at MeJA treatment 24 hat, and then got back to that before MeJA treatment at MeJA 36 hat (Figure S7). Taken together, these results suggested that exogenous MeJA application dramatically altered the transcription pattern in EK, especially at 24 hat.

MeJA treatment altered the expression of genes with function relevant to terpenoid biosynthesis in Euphorbia kansui.

To investigate the function of DEGs associated with MeJA treatment at different time points in EK, we performed GO enrichment analysis on the DEGs in T1 vs. CK, T2 vs. CK and T3 vs. CK comparisons. In “Biological Process” between T1 and CK, we found the DEGs were mainly enriched in plastid organization, cell wall organization or biogenesis, shoot system morphogenesis, leaf morphogenesis, external encapsulating structure organization, reactive oxygen species metabolic process, carbohydrate metabolic process, succinate metabolic process, photosynthesis, cell wall organization, leaf development, triterpenoid biosynthetic process, alpha-amino acid metabolic process, lysine biosynthetic process, monosaccharide metabolic process, indole-containing compound metabolic process (Table S8). Then, we found the terms relevant to cell wall organization, anion transport, external encapsulating structure organization, inorganic anion transport, cell wall organization or biogenesis, ion transport, triterpenoid metabolic process, regulation of localization, regulation of transport, male meiosis, succinate metabolic process, uridine metabolic process, photoperiodism, triterpenoid catabolic process, pentacyclic triterpenoid catabolic process, triterpenoid biosynthetic process, terpenoid catabolic process, terpenoid biosynthetic process and tetracyclic triterpenoid metabolic process were significantly enriched based on DEGs from T2 vs. CK (Table S8). In “Biological Process” between T3 and CK, we found the DEGs in gene expression, cell wall organization, polysaccharide catabolic process, carbohydrate catabolic process, triterpenoid metabolic process, cell wall organization or biogenesis, succinate metabolic process, triterpenoid biosynthetic process, triterpenoid catabolic process, pentacyclic triterpenoid catabolic process, pentacyclic triterpenoid metabolic process, pentacyclic triterpenoid biosynthetic process, inorganic anion transport, external encapsulating structure organization, anion transport, male meiosis and phloem or xylem histogenesis all showed significiant differences. (Table S8; Additional file 5: Table S5). Of note, the number of terms relevant to terpenoid synthesis in T2 vs. CK and T3 vs. CK were higher than that in T1 vs. CK, suggesting that MeJA might mainly caused terpenoid synthesis at 36 or 48 hat in EK.

MeJA altering the transcription pattern of genes involved in numerous pathways represented by terpenoid biosynthesis in Euphorbia kansui.

To investigate the effects of MeJA in affecting the expression of genes involved in biosynthesis in EK, all DEGs were then mapped in the KEGG database (Fig. 4A). For T1 vs. CK comparison, the DEGs mainly involved in Cutin, suberine and wax biosynthesis, Plant hormone signal transduction, Photosynthesis - antenna proteins, alpha-Linolenic acid metabolism, Ribosome, Porphyrin and chlorophyll metabolism, Photosynthesis, Glycosaminoglycan degradation, Phenylalanine, tyrosine and tryptophan biosynthesis, Zeatin biosynthesis, Ubiquinone and other terpenoid-quinone biosynthesis, Cyanoamino acid metabolism, RNA transport, Starch and sucrose metabolism and Phenylalanine metabolism (Fig. 4A; Table S9). Typically, no terms relevant to terpenoid biosynthesis were significantly enriched in T1 vs. CK comparison, suggesting that MeJA treatment did not affect terpenoid biosynthesis at 24 hat in EK (Fig. 4A; Table S9). Then, we found that the DEGs in T2 vs. CK comparison mainly involved in Ribosome, Cutin, suberine and wax biosynthesis, alpha-Linolenic acid metabolism, Fatty acid elongation, Vitamin B6 metabolism, Sesquiterpenoid and triterpenoid biosynthesis, Terpenoid backbone biosynthesis, Diterpenoid biosynthesis and Monoterpenoid biosynthesis (Fig. 4A; Table S9; Additional file 6: Table S6). Numerous terpenoid-related biosynthesis including Sesquiterpenoid and triterpenoid biosynthesis, Terpenoid backbone biosynthesis, Diterpenoid biosynthesis and Monoterpenoid biosynthesis were identified in T2 vs. CK comparison, suggesting that 36 hat of MeJA treatment could triggered the terpenoid biosynthesis in EK. For T3 vs. CK comparison, 12 pathways were significantly enriched, including Ribosome, Fatty acid elongation, Cutin, suberine and wax biosynthesis, Pentose and glucuronate interconversions, Ether lipid metabolism, Vitamin B6 metabolism, Sesquiterpenoid and triterpenoid biosynthesis, Photosynthesis-antenna proteins, Histidine metabolism, Flavonoid biosynthesis, Photosynthesis and Glycerolipid metabolism (Fig. 4A; Table S9). This results about T3 vs. CK also showed that 48 hat of MeJA treatment did not exerted effective activation on terpenoid biosynthesis in EK (Fig. 4A; Table S9). Additionally, KEGG analysis on DEGs from T2 vs. T1 showed that 32 (0.7%) DEGs were identified in “Sesquiterpenoid and triterpenoid biosynthesis”, 37 (0.81%) DEGs were in “Cutin, suberine and wax biosynthesis”, 36 (0.79%) DEGs were in “Terpenoid backbone biosynthesis”, 126 (2.76%) DEGs were in “Plant-pathogen interaction”, 15 (0.4%) DEGs were in “Flavonoid biosynthesis” (0.45%) (Additional file 7: Table S7). Importantly, as shown in Fig. 4B, we found that after 36 hat MeJA treatment, besides Unigene079832, most DEGs encoding key enzymes involved in MVA-like terpenoid biosynthesis were upregulated in EK in comparison with samples from other three experimental groups.

In addition, from KEGG analysis, we found some unigenes in the “Cutin, suberine and wax biosynthesis”, “Flavonoid biosynthesis” pathway expressed differently under MeJA elicitation. Typically, 25 (0.71%), 28 (0.74%), 11 (0.79%), 37 (0.81%), 20 (0.77%) and 28 (0.89%) DEGs respectively in “Cutin, suberine and wax biosynthesis” pathway through pairwise comparison (CK-vs-T1, CK-vs-T2, CK-vs-T3, T1-vs-T2, T1-vs-T3, T2-vs-T3) (Table 2). From Table 3, we found the expression levels of unigenes coding cytochrome P450-like gene including unigene016395, unigene016396, unigene053539 and unigene010561 are all upregulated after MeJA treatment 36 h. Interestingly, we found “Flavonoid biosynthesis” pathway, genes coding chalcone–flavonone isomerase (CHI), shikimate O-hydroxycinnamoyltransferase (HCT), caffeoyl CoA 3-O-methyltransferase (CCOMT) and flavanone 3-hydroxylase (F3H) are all downregulated after MeJA treatment 24 h, however, upregulated at 36 h (Table 3). The expression levels of CHI and F3H reached the highest at 48 h. Taken together, these results suggested that exogenous application of MeJA treatment mainly triggered the terpenoid biosynthesis, especially MVA pathway in EK at 36 hat, while MeJA treatment also altered the transcription pattern of genes involved in biosynthesis of cutin, wax and flavonoid, implying the effects of MeJA in promoting the accumulation of bioactive metabolites in EK.

Table 2
The number of MeJA-responsive DEGs in terpenoid biosynthesis, “Cutin, suberine and wax biosynthesis”, “Flavonoid biosynthesis” pathway of KEGG annotation

Pathway	DEGs with pathway annotation					
	CK-vs-T1	CK-vs-T2	CK-vs-T3	T1-vs-T2	T1-vs-T3	T2-vs-T3
	3539(100%)	3781(100%)	1394(100%)	4568(100%)	2605(100%)	3161(100%)
Terpenoid backbone biosynthesis	40(1.13%)	45(1.19)	18(1.29%)	36(0.79%)	28 (1.07%)	33 (1.04%)
Ubiquinone and other terpenoid-quinone biosynthesis	21(0.59%)	39 (1.03%)	8 (0.57%)	39 (0.85%)	28 (1.07%)	35 (1.11%)
Steroid biosynthesis	25 (0.71%)	31 (0.82%)	5 (0.36%)	36 (0.79%)	22 (0.84%)	32 (1.01%)
Sesquiterpenoid and triterpenoid biosynthesis	14 (0.4%)	26 (0.69%)	8 (0.57%)	32(0.7%)	16 (0.61%)	25 (0.79%)
Diterpenoid biosynthesis	8 (0.23%)	7 (0.19%)	4 (0.29%)	11 (0.24%)	5 (0.19%)	5 (0.16%)
Monoterpenoid biosynthesis	4 (0.11%)	3 (0.08%)	1 (0.07%)	5 (0.11%)	3 (0.12%)	1 (0.03%)
Cutin, suberine and wax biosynthesis	25 (0.71%)	28 (0.74%)	11 (0.79%)	37 (0.81%)	20 (0.77%)	28 (0.89%)
Flavonoid biosynthesis	11 (0.31%)	15 (0.4%)	14 (1%)	21 (0.46%)	13 (0.5%)	12 (0.36%)

Table 3

KEGG pathway analysis of the MeJA-responsive differential expressed genes in "Terpenoid biosynthesis", "Cutin, suberine and wax biosynthesis" and "Flavonoid biosynthesis" pathway

pathway Enzyme	Unigen ID	Enzyme ID	Pathway ID	fpmk changes of DEG			
Terpenoid biosynthesis							
Terpenoid backbone biosynthesis							
MVA				CK 24h 36h 48h			
acetyl-CoA acetyltransferase	Unigene064228	EC:2.3.1.9	map00900	0.2	0.68	2.47	0.35
acetyl-CoA acetyltransferase	Unigene022256	EC:2.3.1.9	map00900	0.2	0.68	2.47	0.35
cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase	Unigene028845	EC:2.3.3.10	map00900	0.19	0.86	3.025	0.55
3-hydroxy-3-methylglutaryl-coenzyme A reductase 1-like	Unigene016616	EC:1.1.1.34	map00900	22.65	49.635	168.25	41.005
probable phosphomevalonate kinase	Unigene078778	EC:1.1.1.34	map00900	0.001	0.085	1.08	0.09
hydroxymethylglutaryl-CoA reductase	Unigene090341	EC:1.1.1.34	map00900	0.195	0.41	1.345	0.36
phosphomevalonate kinase isoform X2	Unigene078776	EC:2.7.4.2	map00900	0.11	0.705	1.5	0.25
GHMP kinase family protein	Unigene083442	EC:2.7.4.2	map00900	1.42	1.315	5.21	1.955
phosphomevalonate kinase-like	Unigene083438	EC:2.7.4.2	map00900	1.895	2.575	6.52	4.105
mevalonate pyrophosphate decarboxylase	Unigene040754	EC:4.1.1.33	map00900	0.105	0.525	2.105	0.365
isopentenyl diphosphate isomerase	Unigene011459	EC:5.3.3.2	map00900	0.175	0.615	2.585	0.385
isopentenyl pyrophosphate	Unigene019565	EC:5.3.3.2	map00900	0.001	0.915	3.16	0.585
geranyl diphosphate synthase	Unigene037391	EC:2.5.1.1	map00900	0.001	0.001	0.785	0.001
decaprenyl-diphosphate synthase subunit 1	Unigene003040	EC:2.5.1.1	map00900	0.001	0.14	0.465	0.14
farnesyl pyrophosphate synthase	Unigene028555	EC:2.5.1.1	map00900	0.045	0.515	1.55	0.285
farnesyl pyrophosphate synthase 1-like isoform X1	Unigene079082	EC:2.5.1.1	map00900	7.955	5.275	28.985	12.605
MEP							
probable 1-deoxy-D-xylulose-5-phosphate synthase, chloroplastic isoform X3	Unigene044979	EC:2.2.1.7	map00900	0.27	0.001	1.46	0.185
probable 1-deoxy-D-xylulose-5-phosphate synthase 2	Unigene042075	EC:2.2.1.7	map00900	28.01	11.845	6.415	4.815
probable 1-deoxy-D-xylulose-5-phosphate synthase 2	Unigene093405	EC:2.2.1.7	map00900	70.425	26.575	20.93	10.91
probable 1-deoxy-D-xylulose-5-phosphate synthase 2	Unigene089680	EC:2.2.1.7	map00900	76.88	25.2	25.19	11.31

pathway Enzyme	Unigen ID	Enzyme ID	Pathway ID	fpmk changes of DEG			
Terpenoid biosynthesis							
Terpenoid backbone biosynthesis							
1-deoxy-D-xylulose-5-phosphate synthase 2	Unigene089682	EC:2.2.1.7	map00900	163.78	64.69	39.105	30.01
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	Unigene056879	EC:1.17.7.1	map00900	262.015	137.31	125.12	148.96
4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Unigene003422	EC:1.17.7.2	map00900	1.915	0.3	0.001	0.69
4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Unigene003418	EC:1.17.7.2	map00900	361.155	237.77	171.695	195.195
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	Unigene024815	EC:2.7.7.60	map00900	1.76	0.001	0.85	0.2
geranylgeranyl pyrophosphate synthase	Unigene016497	EC:2.5.1.1	map00900	138.865	20.76	24.02	15.325
farnesyl diphosphate synthase	Unigene004541	EC:2.5.1.1	map00900	25.005	12.3	11.39	9.685
Sesquiterpenoid and triterpenoid biosynthesis							
squalene synthase	Unigene032992	EC:2.5.1.21	map00909	0.08	0.39	1.47	0.19
sucrose synthase	Unigene043983	EC:2.5.1.21	map00909	31.355	19.67	40.29	16.84
squalene monooxygenase 1,	Unigene014678	EC:1.14.13.132	map00909	0.17	0.895	2.525	0.38
Squalene monooxygenase	Unigene076528	EC:1.14.13.132	map00909	2.42	10.805	2.62	3.985
squalene epoxidase	Unigene048572	EC:1.14.13.132	map00909	3.095	7.005	3.085	2.945
Squalene monooxygenase	Unigene025852	EC:1.14.13.132	map00909	0.23	0.05	2.78	0.51
squalene monooxygenase-like	Unigene041706	EC:1.14.13.132	map00909	0.965	0.72	2.175	1.13
beta-amyrin synthase-like	Unigene022072	EC:5.499.39	map00909	2.575	25.455	2.73	4.57
Steroid biosynthesis							
squalene synthase	Unigene032992	EC:2.5.1.21	map00100	0.08	0.39	1.47	0.19
sucrose synthase	Unigene043983	EC:2.5.1.21	map00100	31.355	19.67	40.29	16.84
mitogen-activated protein kinase kinase kinase YODA	Unigene043985	EC:2.5.1.21	map00100	19.57	18.49	27.07	24.29
squalene monooxygenase 1, partial	Unigene014678	EC:1.14.13.132	map00100	0.17	0.895	2.525	0.38
Squalene monooxygenase	Unigene076528	EC:1.14.13.132	map00100	2.42	10.805	2.62	3.985
squalene epoxidase	Unigene048572	EC:1.14.13.132	map00100	3.095	7.005	3.085	2.945
N(6)-adenine-specific DNA methyltransferase 2	Unigene072562	EC:1.14.13.132	map00100	0.12	0.395	1.07	0.265
N(6)-adenine-specific DNA methyltransferase 2	Unigene002085	EC:1.1.1.170	map00100	55.41	251.2	42.07	82.475
delta(24)-sterol reductase-like	Unigene014530	EC:1.3.1.72	map00100	0.155	0.845	2.135	0.375

pathway Enzyme	Unigen ID	Enzyme ID	Pathway ID	fpmk changes of DEG			
Terpenoid biosynthesis							
Terpenoid backbone biosynthesis							
3beta-hydroxysteroid-dehydrogenase/decarboxylase isoform	Unigene022670	EC:1.1.1.170	map00100	0.185	0.9	2.615	0.29
cycloartenol-C-24-methyltransferase 1-like	Unigene036530	EC:ERG6	map00100	0.185	1.04	2.53	0.33
7-dehydrocholesterol reductase	Unigene020078	EC:1.3.1.21	map00100	0.565	2.4	8.355	1.245
acyl-coenzyme A:cholesterol acyltransferase	Unigene013755	EC:2.3.1.26	map00100	0.07	0.355	0.98	0.305
cycloartenol synthase	Unigene050717	EC:5.4.99.8	map00100	2.63	7.885	2.565	1.845
cycloartenol-C-24-methyltransferase 1-like	Unigene036530	EC:SMT1	map00100	0.185	1.04	2.53	0.33
Obtusifoliol 14alpha-Demethylase	Unigene061050	EC:CYP51G1	map00100	0.075	0.615	2.245	0.265
C-4 sterol methyl oxidase (ISS)	Unigene082000	EC:SMO2	map00100	0.11	0.875	3.055	0.485
C-5 sterol desaturase	Unigene002420	EC:1.14.21.6	map00100	0.195	0.445	1.795	0.265
triacylglycerol lipase	Unigene081843	EC:3.1.1.13	map00100	0.001	0.915	2.215	0.001
triacylglycerol lipase	Unigene031929	EC:3.1.1.13	map00100	0.13	0.42	1.045	0.16
esterase / lipase	Unigene009853	EC:3.1.1.13	map00100	0.12	0.12	0.91	0.1
Monoterpenoid biosynthesis							
F-box/kelch-repeat protein At3g23880-like	Unigene086409	EC:1.1.1.206	map00902	0.001	1.265	0.001	0.001
probable terpene synthase 11	Unigene055596	EC:4.2.3.25	map00902	0.965	0.001	0.13	0.001
(+)-neomenthol dehydrogenase-like isoform X1	Unigene019800	EC:1.1.1.208	map00902	1.89	0.19	2.155	2.265
(+)-neomenthol dehydrogenase-like	Unigene092575	EC:1.1.1.208	map00902	2.32	0.26	6.075	1.835
Diterpenoid biosynthesis							
ent-kaurene oxidase	Unigene014957	EC:1.14.13.78	map00904	0.11	0.33	1.13	0.205
cytochrome P450 82G1-like	Unigene019714	EC:CYP82G1	map00904	0.72	0.145	2.66	0.68
ent-kaur-16-ene synthase	Unigene023377	EC:4.2.3.19	map00904	47.29	2.56	1.875	3.81
gibberellin 3-beta-dioxygenase 1	Unigene040056	EC:1.14.11.15	map00904	0.14	0.14	1.725	0.425
gibberellin 2-beta-dioxygenase-like	Unigene068133	EC:1.14.11.13	map00904	32.09	48.075	15.57	31.55
ent-copalyl diphosphate synthase	Unigene083812	EC:5.5.1.13	map00904	3.34	0.16	0.245	0.195
ent-kaurene synthase	Unigene091248	EC:4.2.3.19	map00904	4.795	0	2.17	1.82

pathway Enzyme	Unigen ID	Enzyme ID	Pathway ID	fpmk changes of DEG			
Terpenoid biosynthesis							
Terpenoid backbone biosynthesis							
Ubiquinone and other terpenoid-quinone biosynthesis							
homogentisate geranylgeranyl transferase	Unigene042099	EC:25.1.115	map00130	1.11	3.485	0.38	1.08
homogentisate solanesyltransferase, chloroplastic	Unigene080040	EC:25.1.117	map00130	5.025	4.71	12.035	10.125
37 kDa chloroplast inner membrane protein	Unigene059865	EC:2.1.1.295	map00130	1	0.915	11.99	2.625
37 kDa inner envelope membrane protein	Unigene039484	EC:2.1.1.295	map00130	2.32	0.72	10.325	4.495
2-methyl-6-phytyl-1,4-hydroquinone methyltransferase, chloroplastic	Unigene077544	EC:2.1.1.295	map00130	14.835	3.505	44.745	22.845
gamma-tocopherol methyltransferase	Unigene025639	EC:2.1.1.95	map00130	8.485	5.305	17.89	10.695
probable tocopherol O-methyltransferase, chloroplastic isoform X2	Unigene069994	EC:2.1.1.95	map00130	18.325	18.54	36.685	26.95
4-hydroxybenzoate polyprenyl transferase	Unigene067103	EC:Coq2	map00130	0.07	0.375	0.71	0.12
2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial	Unigene080019	EC:Coq5	map00130	0.001	2.675	0.275	0.001
2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial	Unigene056630	EC:Coq5	map00130	0.13	0.465	1.525	0.14
ubiquinone biosynthesis protein COQ7	Unigene084930	EC:Coq7	map00130	0.08	0.575	1.91	0.375
retrovirus-related Pol polyprotein from transposon TNT 1–94	Unigene063000	EC:6.2.1.26	map00130	0.77	2.17	0.15	0.62
1,4-dihydroxy-2-naphthoyl-CoA synthase, peroxisomal-like	Unigene070519	EC:4.1.3.36	map00130	10.16	26.055	11.115	10.055
Hexaprenyldihydroxybenzoate methyltransferase	Unigene013837	EC:2.1.1.114	map00130	0.18	0.63	1.2	0.001
protein PHYLLO, chloroplastic isoform X3	Unigene024447	EC:5.4.4.2	map00130	0.245	0.62	2.8	0.775
protein PHYLLO, chloroplastic	Unigene095857	EC:5.4.4.2	map00130	0.82	0.52	3.74	1.305
protein PHYLLO, chloroplastic	Unigene064679	EC:5.4.4.2	map00130	0.72	0.41	1.98	0.74
isochorismate synthase 2, chloroplastic-like isoform X1	Unigene012041	EC:5.4.4.2	map00130	29.525	22.26	105.14	51.745

pathway Enzyme	Unigen ID	Enzyme ID	Pathway ID	fpmk changes of DEG			
Terpenoid biosynthesis							
Terpenoid backbone biosynthesis							
isochorismate synthase 2, chloroplastic	Unigene012036	EC:5.4.4.2	map00130	20.345	9.515	45.455	29.97
isochorismate synthase 2, chloroplastic	Unigene012037	EC:5.4.4.2	map00130	24.01	13.685	51.935	40.1
geranylgeranyl diphosphate reductase	Unigene043490	EC:1.3.1.83	map00900	46.05	27.115	101.745	92.595
Cutin, suberine and wax biosynthesis							
cytochrome P450 86A8-like	Unigene061395	EC:CYP86A4	map00073	0.63	2.755	10.23	1.745
cytochrome P450 86A8-like	Unigene061396	EC:CYP86A4	map00073	1.935	5.065	24.21	2.73
Os04g0560100	Unigene010561	EC:CYP86A4	map00073	0.14	0.001	1.355	0.06
cytochrome P450 86A7	Unigene053539	EC:CYP86A4	map00073	1.465	3.84	10.82	1.67
Flavonoid biosynthesis							
chalcone–flavonone isomerase	Unigene040233	EC:5.5.1.6	map00941	19.97	7.475	26.43	27.19
shikimate O-hydroxycinnamoyltransferase	Unigene048505	EC:2.3.1.133	map00941	2.825	0.415	22.77	6.655
shikimate O-hydroxycinnamoyltransferase	Unigene022901	EC:2.3.1.133	map00941	4.48	0.665	13.565	8.785
shikimate O-hydroxycinnamoyltransferase	Unigene070081	EC:2.3.1.133	map00941	1.43	0.115	1.73	3.33
caffeoyl CoA 3-O-methyltransferase	Unigene071049	EC:2.3.1.74	map00941	1.525	0.001	1.78	0.975
flavanone 3-hydroxylase	Unigene054810	EC:1.1.4.11.9	map00941	26.505	20.38	49.09	61.335

MeJA treatment activated terpenoid biosynthesis and promoted the accumulation of terpenoid in Euphorbia kansui.

In order to experimentally verify the DEG profile obtained by sequence data in terpenoid biosynthesis associated with MeJA treatment, qRT-PCR analysis was conducted on genes relevant to terpenoid biosynthesis in EK following MeJA treatment. A total of 18 genes coding key enzymes in terpenoid biosynthesis pathway, including AACT, HMGS, HMGR, PMK, GHMP, MDC, DXS, MCS, HDS, HDR, IDI, DPPI, FPPS, GPPS, GGPPS, GGPPR, SUS and N6AMT2 (Table 3) were selected to analyze the gene expression pattern under different MeJA treatment (Fig. 5). The gene and *β-ACTIN*-specific primers for qRT-PCR were listed in Additional file 1: Table S1. The results suggested that the expression levels of the majority of the genes verified were consistent with those from RNA transcriptome data (Fig. 5). Most of these genes relevant to terpenoid biosynthesis were upregulated in EK by MeJA treatment at 36 hat (Fig. 5). Overall, qRT-PCR verified the effects of MeJA treatment in activating the transcription of terpenoid biosynthesis in EK at 36 hat.

Subsequently, we determined the content of the leaf total terpenoid at different MeJA treatment by ultraviolet-visible spectrophotometry. The euphadienol served as standard substance. The results showed that the content of total terpenoid was higher at 24 h 20 μM MeJA treatment to CK, and reached the highest at 36 h μM MeJA treatment, and with the time of MeJA treatment prolonged to 48 h, the content of the total terpenoids decreased (Fig. 6). Compared with the control, the content of total terpenoid was on average about 2.38 times at 24 h MeJA treatment and 2.80 times at 36 h MeJA treatment (Fig. 6). The statistical analysis at MeJA treatments 36 h showed significant differences ($P < 0.05$) to the control (Fig. 6). Thus, these results confirmed that

exogenous application of MeJA could activate terpenoid biosynthesis and promote the accumulation of terpenoid in EK at 36 hat, then leading to high quality of EK for usage.

Discussion

The methyl jasmonate (MeJA) has been identified as a vital cellular regulator that mediates the synthesis of numerous secondary metabolites, especially terpenoids (Zhang et al. 2007b, Kang et al. 2004, Li et al. 2016, Zhang et al. 2015b, Wang et al. 2014, *Xiao et al.2010, Adil et al. 2022*). Liu et al. (2018) reported that MeJA can trigger reprogramming of gene expression relevant to many secondary metabolic pathways. Moreover, exogenous MeJA activated the accumulation of a range of secondary metabolites that have pharmacological effects for human health, and play important roles in the adaptation of plants to a particular environment (Choi et al. 2005, Cao et al. 2015). In the present study, we constructed the RNA-seq library of *E. kansui* leaves after treating with 20 μM of MeJA at 0, 24, 36 and 48 hat to investigate the effects of MeJA in affecting terpenoid synthesis in this traditional Chinese medicinal plant. Due to the lack of available genome sequence for *E. kansu*i, by using a series of bioinformatics tools, we identified 96 040 unigenes including differentially expressed genes in response to MeJA. Typically, MeJA treatment activated the expression of genes involved terpenoid biosynthesis represented by MVA pathway in *E. kansu*i to promote the accumulation of terpenoid.

It has been reported in other species that exogenous application of MeJA could upregulate the expression of genes involved in secondary metabolites, such as taxol synthesis in *Taxus chinensis* (Li et al. 2012) and tanshinone biosynthesis in *Salvia Miltiorrhiza* (Hao et al. 2014). Most of the known genes (*AACT*, *HMGS*, *HMGR*, *PMK*, *GHMP* and *MDC*) involved in the terpenoid biosynthesis have also been identified in our present RNA-Seq data and upregulated by MeJA treatment. It is well known that all terpenoid are derived from the common precursor IPP which can be synthesized via two different pathways (Zhang et al. 2007a). From then on, the intermediate diphosphate precursors, including geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), are synthesized under the catalysis of related diphosphate synthases (PPSs) in the monoterpene, triterpenoids and sesquiterpene, and diterpene biosynthetic pathways, respectively (Ma et al. 2012). Typically, triterpenoids and sesquiterpenoids are synthesized by MVA pathway, while monoterpenoids, diterpenoids, tetraterpenoids are exclusively synthesized by MEP pathway (Lichtenthaler et al. 1997, *Mccaskill et al. 1998*). In *E. kansui*, terpenoids are biologically active components; especially triterpenoid with anti-tumor and anti-viral bioactivities, and Euphadienol is a major triterpenoid compound (Yasukawa et al. 2000, Guo et al. 2010, *Z*hao et al. 2014). Our results suggested that the exogenous application of MeJA could upregulate the expression of genes encoding enzymes for the MVA terpenoid backbone biosynthesis, and increase the expression levels of genes encoding FPPS, SQS (squalene synthase) and SUS (sucrose synthase) which are the key enzymes in “Sesquiterpenoid and triterpenoid biosynthesis” pathway. MVA and MEP pathways were considered to perform mevalonate biosynthesis in the cytosol and plastid, respectively (Hemmerlin et al. 2012). Our result supports the view that production and accumulation of metabolites in a compartment-dependent manner enable their cost-effective utilization and channeling of resources to either growth or defense, thus increasing fitness and competitive ability (Kliebenstein 2004).

Here, the genes that encoded all of the enzymes except MK in MVA pathway and FPPS were upregulated in *E. kansu*i by 20μM of MeJA elicitation at 36 hat,. Therefore, we conjecture that these two pathways may be negatively regulated after exogenous MeJA application in *E. kansui*. Similarly, levels of transcripts for MEP pathway enzymes are upregulated by light during seedling deetiolation (Ghassemian et al. 2006, Rodríguez-Concepción 2006, Cordoba et al. 2009, Meier et al. 2011), while, MVA pathway genes of dark-grown seedlings are downregulated by illumination (Ghassemian et al. 2006, Rodríguez-Concepción 2006). Shi et al. (2015) also reported that the expression levels of DXS, GGR, GGPPS, HMGR and HDR in MEP pathway increased by approximately 2-4-fold by MeJA treatment in tea leaves. It has been commented that the few connections between MVA and MEP pathways are negative, e.g., transcriptional activation of genes in one pathway is correlated with the repression of genes in the other pathway (Vranová et al. 2013, Rodríguez-Concepción and Boronat 2015). Moreover, not all genes within the MVA or MEP pathway and genes for relative branch-point enzymes were interconnected, demonstrating that there is no en bloc transcriptional regulation of all genes coding enzymes (Vranová et al. 2013). Our results might supply an evidence for the comments that MVA- and MEP-pathway may be negatively regulated after exogenous MeJA application, and not all genes coding enzymes within these two pathways were interconnected. While this comments still requires molecular biology verification.

Cutin and suberine play important roles in protecting plants from biotic and abiotic stresses (Pollard et al. 2008). The surface wax can protect tissue from environmental stresses, limit nonstomatal water loss, and help to prevent germination of pathogenic

microbes (Raven and Edwards 2004, Riederer 2007, Samuels et al. 2008). In our study, we found cytochrome P450-like genes in “Cutin, suberine and wax biosynthesis” pathway, e.g., unigene016395, unigene016396, unigene053539 and unigene010561, and their expressions level are all upregulated after MeJA treatment 36h. In addition, exogenous MeJA had a positive effect on the gene expression in “Flavonoid biosynthesis” pathway, then promoted flavonoid accumulation in *Scutellaria lateriflora* hairy roots, such as baicalin, baicalein and wogonin (Tuan et al. 2016). Exogenous application of MeJA could activate the flavonoid pathway genes, then promote the resistance against blue mold and reduce the symptoms resulting from inoculating apples with *Penicillium expansum* (Ahmadi-Afzadi et al. 2018). Flavanone 3-hydroxylase (F3H) and HCT is a crucial enzyme involved in the flavonoid biosynthesis (Zhang et al 2018, Shoeva and Khlestkina 2018). MeJA induced CCAMT expression upregulated (Wang et al. 2017). Our results showed F3H and CCAMT were upregulated at MeJA treatment 36 h.

Conclusions

Our in-depth transcriptome profiles of *Euphorbia kansui* showed that genes responsible for terpenoid biosynthesis, especially MVA pathway were activated in *E. kansui* plant after exogenous application of 20 μM MeJA. Moreover, 20 μM MeJA effectively increased the content of terpenoids in *E. kansui* at 36 hat. Additionally, MeJA also reprogrammed the transcription pattern of biosynthesis of flavonoid, cutin and wax in *E. kansui*. These results might supply resources for genetic engineering research on *E. kansui*.

Materials and methods

Plant materials and MeJA treatment

Healthy *Euphorbia kansui* plants were grown at the Botanical Garden of Northwest University in Shaanxi Province, which were growing in a natural environment (Shaanxi, People’s Republic of China).

The plants at the same vegetative growth stage in September were assigned into four groups, sprayed with 200 μM, 20 μM, 2 μM MeJA (in Milli-Q water) (Sigma, St. Louis, MO, USA) only once, and Milli-Q water control, respectively. The MeJA solution was sprayed as a fine mist to completely wet the adaxial side of each leaf. Apical leaves from control group and three groups treated with MeJA of different concentration were collected separately at 12, 24, 36 and 48 h after treatments and directly into liquid nitrogen, three repetitions were taken for each treatment and time point. All samples with treatment of different concentration and time were prepared in triplicate. All materials were stored at -80°C until analysis.

Extraction and reverse transcription of total RNA

Total RNA was separately isolated from leaves using RNAprep Pure Plant Kit (DP441, TIANGEN, Beijing, China), according to the manufacturer's instructions. Gel electrophoresis was used to detect the integrity of the RNA. First-strand cDNA was synthesized from 1 to 2 μg of total RNA using M-MLV Reverse Transcriptase and oligo (dT) 18 primers following manufacturer's instructions (TaKaRa, Dalian, China).

Real-time Quantitative PCR (qRT-PCR) analysis and statistical analysis

Expression levels of genes selected to verification were further investigated by quantitative real-time PCR (qRT-PCR) using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) with a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc, Singapore). The PCR analysis was performed in 25 μL reactions using the method described in the manufacturer’s protocol. The gene-specific primers for qRT-PCR were listed in Additional file 1: Table S1. The *β-ACTIN* gene was used as an internal reference. The reaction system was as follows: 25.0 μL of reaction mixture contained 12.5 μL SYBR Premix ExTaq (Takara, Dalian, China), 1.0 μL of each primer at 10 μM, 8.5 μL ddH_2O, and 2.0 μL (80 ng) cDNA. *ACTIN*-F and *ACTIN*-R primers were used to be a loading control to normalize samples in separate tubes. PCRs were performed by using the SYBR Premix ExTaqII Kit (Takara, Dalian, China). The PCR conditions consisted of denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, then dissociation at 95°C for 5 s, 58°C for 30s and 72°C for 1 min. Expression levels of genes were verified in triplicate and calculated using the $2^{-\Delta\Delta CT}$ method. The statistical results are presented as means^SE. Statistical analysis using ANOVA and Tukey’s test was carried out with a 5% level of confidence using SPSS18.0 software.

RNA Library construction and sequencing

Control group (treatment with water) and three treatment groups (24 h, 36 h and 48 h of 20 μM MeJA spraying) were used to construct a broad RNA-seq library. The experiments were performed with two biological replicates.

After total RNA was extracted, mRNA was enriched by Oligo (dT) beads. Then the enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse transcripted into cDNA with random primers. Second-strand cDNA were synthesized by DNA polymerase I, RNase H, dNTP and buffer. Then the cDNA fragments were purified with QiaQuick PCR extraction kit, end repaired, poly (A) added, and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified, and sequenced using Illumina HiSeq™ 4000 by Sagene Biotech Co. Ltd (Guangzhou, China).

Raw reads filtering and sequence assembly

Reads obtained from the sequencing machines were further filtered according to the rules of removing reads containing adapters; removing reads containing more than 10% of unknown nucleotides (N) and removing low quality reads containing more than 50% of low quality (SQ ≤ 10) bases. Transcriptome de novo assembly was carried out with short reads assembling program –Trinity (Grabherr et al. 2011). The sequences obtained from "Trinity" are called unigenes. The unigene expression was calculated and normalized to RPKM (Reads Per kb per Million reads) (Mortazavi et al. 2008). The formula is as follow:

$$RPKM = \left(1000000 * C\right)/\left(N * L/1000\right))$$

Let RPKM to be the expression of Unigene A. C is the number of reads that are uniquely mapped to Unigene A. N is the total number of reads that are uniquely mapped to all unigenes. L is the length (base number) of Unigene A.

Protein coding sequence (CDS) prediction

BLASTx (v 2.4.0) (http://www.ncbi.nlm.nih.gov/BLAST/) (*E*-value < 0.00001) was used to compare unigenes to NCBI non-redundant protein (Nr) database (http://www.ncbi.nlm.nih.gov), SwissProt protein database (http://www.expasy.ch/sport), KOG database (http://www.ncbi.nlm.nih.gov.COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) databases. The sequence with the best comparison result of each unigene in the Nr library was taken as the corresponding homology sequence and then the species to which the homologous sequence belonged was determined. According to the Nr annotation information, we can get the Gene Ontology (GO) functional annotation by Blast2GO software (v2.3.5) (Li et al., 2009). Functional classification of unigenes was performed by using WEGO software (*Conesa et al,. 2005*).

Differentially expressed genes analysis and advanced annotation of unigenes

To identify DEGs, the edgeR package (http://www.r-project.org/) was used. We identified genes with a fold change ≥ 2 and a *p* (P-Value) < 0.05 in a comparison as significant DEGs. DEGs were then subjected to enrichment analysis of GO functions and KEGG pathways. GO enrichment analysis provides all GO terms that significantly enriched in DEGs comparing to the genome background, and filter the DEGs that correspond to biological functions. Firstly all DEGs were mapped to GO terms in the Gene Ontology database (http://www.geneontology.org/), gene numbers were calculated for every term, significantly enriched GO terms in DEGs comparing to the genome background were defined by hyper geometric test. The calculating formula of P-value is as follows (N represents the number of all genes with GO annotation, n represents the number of DEGs in N, M is the number of all genes that are annotated to the specific GO terms; m represents the number of DEGs in M). The calculated *p*-value was gone through FDR Correction, taking FDR ≤ 0.05 as a threshold. This analysis can recognize the main predicted biological functions for DEGs.

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}$$

Genes usually interact with the other to play roles in certain biological functions. KEGG is the public pathway-related database (Li et al., 2009). Pathway enrichment analysis identified significantly enriched metabolic pathway or signal transduction pathways in

DEGs comparing with the whole genome background. The calculating formula is the same as that in GO analysis (N represents the number of all genes that with KEGG annotation, n represents the number of DEGs in N, M is the number of all genes annotated to specific pathway, m is the number of DEGs in M). The calculated *p*- value was gone through FDR Correction, taking FDR ≤ 0.05 as a threshold. Pathways meeting this condition were defined as significantly enriched pathways in DEGs.

Determination of total terpenoids by ultraviolet-visible spectrophotometry

The Euphadienol standard (obtained from Chinese National Institute for the Control of Pharmaceutical and Biological Products) was put into a 10 mL volumetric flask and dissolved in methanol. Then, the master solution was diluted into different concentration gradient solution (0.75, 0.5, 0.25, 0.1 and 0.075 mg/mL) with methanol separately. Finally, the different concentration gradient solution and mother liquor were determined by 754C Ultraviolet-visible Spectrophotometer (Jinghua Instruments, Shanghai, China). The detective wavelength was 230 nm. The regression equation between absorbance (Y) and the quantity of Euphadienol (X) was: $Y = 0.8171X + 0.0388$, $r = 0.9949$.

Dried leaf powder (2.0 g) was put into a conical flask. After 25 mL ethyl acetate was added, it was weighed and ultrasonically extracted for 40 min (Power 250 W; Frequency 50 KHz) at room temperature. The ethyl acetate was added to make up the lost weight after cooling, and then was shaken well. Then 10 mL filtrate was measured and streamed dry. Then the brown residue on the evaporating dish was dissolved with methanol and transferred to a 10 mL volumetric flask and shaken vigorously after adding methanol to its scale. Finally, according to the standard curve method, 1000 μL of the sample solution was used for reaction and then the content of total terpenoids was qualitatively detected.

Abbreviations

AACT
-acetyl CoA hydrolase
HMGS
-hydroxymethylglutaryl-CoA synthase
HMGR
-hydroxymethylglutaryl-CoA reductase
MK
-mevalonate kinase
PMK
-phosphomevalonate kinase
GHMP
-GHMP kinase family protein
MDC
-mevalonate pyrophosphate decarboxylase
DXS
-1-deoxy-D-xylulose-5-phosphate synthase
DXR
-1- deoxy-D-lxylulose 5-phosphate reductoisomerase
CMS
-4-diphosphocytidyl-2-C-methyl-D-erythritol synthase
CMK
-4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
MCS
-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
HDS
-4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
HDR
-4-hydroxy-3-methylbut-2-enyl diphosphate reductase
IDI

-isopentenyl diphosphate isomerase
DPPI
-dimethyllallyl pyprophosphate isomerase
GPPS
-geranyl diphosphate synthase
FPPS
-farnesyl pyrophosphate synthase
IPK
-isopentenyl monophosphate kinase
GGPPS
-geranylgeranyl pyrophosphate synthase
PPSs
-diphosphate synthases
SUS
-sucrose synthase
N6AMT2
-N(6)-adenine-specific DNA methyltransferase 2
CHI
-chalcone–flavonone isomerase
HCT
-shikimate O-hydroxycinnamoyltransferase
CCOMT
-caffeoyl CoA 3-O-methyltransferase
F3H
-flavanone 3-hydroxylase
MVA
-Mevalonate 5-phosphate
MEP
-2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
IPP
-isopentenyl diphosphate
DMAPP
-dimethylallyl diphosphate
DXP
-1-deoxy-D-xyulose 5-phosphate
GPP
-geranyl diphosphate
FPP
-farnesyl diphosphate
GGPP
-geranylgeranyl diphosphate
GGPPR
-geranylgeranyl diphosphate reductase
GGR
-geranylgeranyl reductase
STB
-Sesquiterpenoid and triterpenoid biosynthesis
SB
-Steroid biosynthesis
MB

-Monoterpenoid biosynthesis
DB
-diterpenoid biosynthesis
UTB
-Ubiquinone and other terpenoidquinone biosynthesis

Declarations

Author contributions

PL conducted most of the experimental work. PL, QZ and YZ composed the manuscript. MW, XF and WS read and edited the manuscript. XC conceived the idea, planned the work and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

The raw RNA-Seq reads have been deposited into the National Center for Biotechnology Information (NCBI) and can be accessed in the sequence read archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) with accession number PRJNA421567, SRA number SRP126436.

Competing Interests

The authors declare that they have no competing interests.

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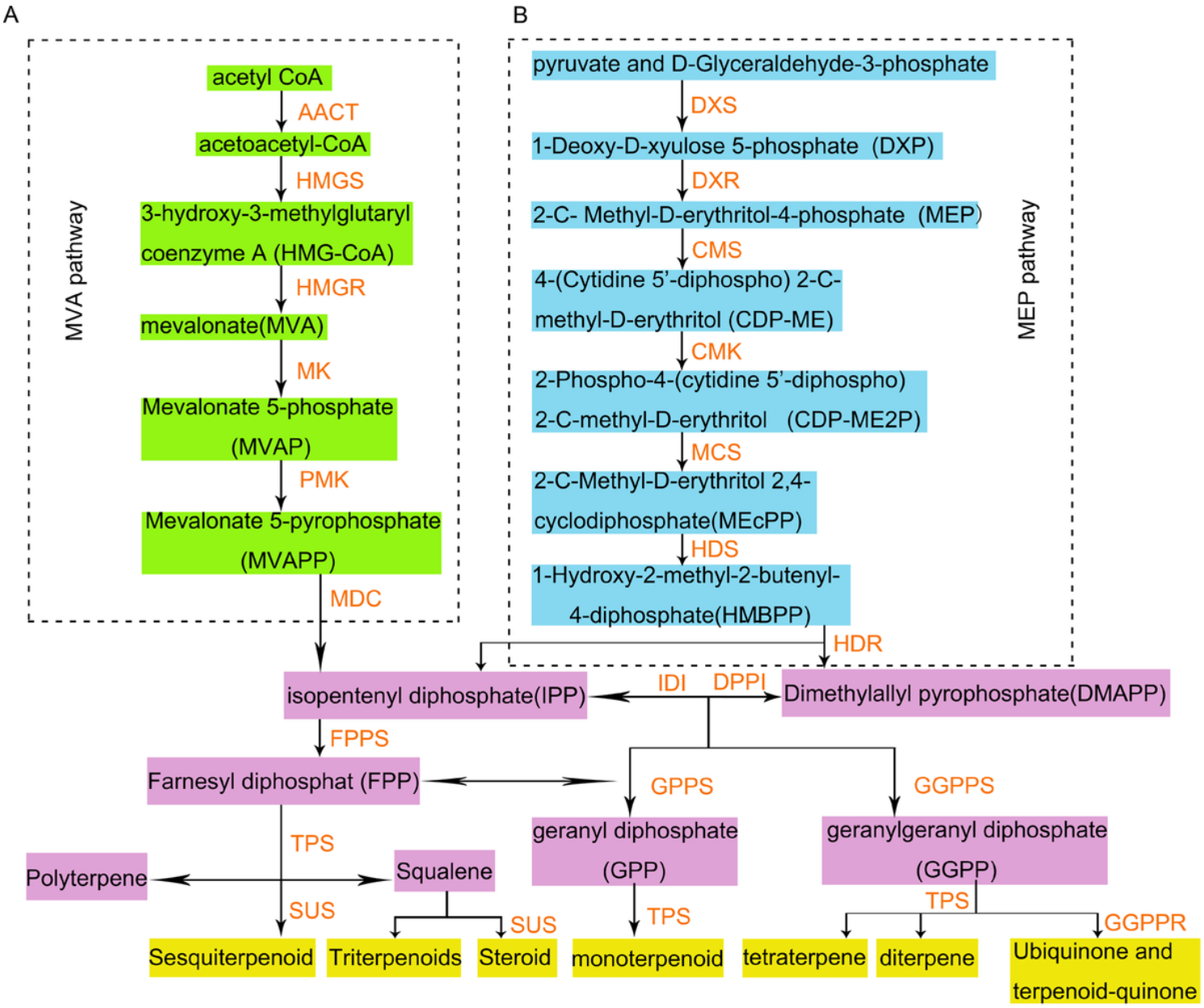
Additional Tables

Additional Tables 1 and 9 are available in the Supplementary Files section

Figures



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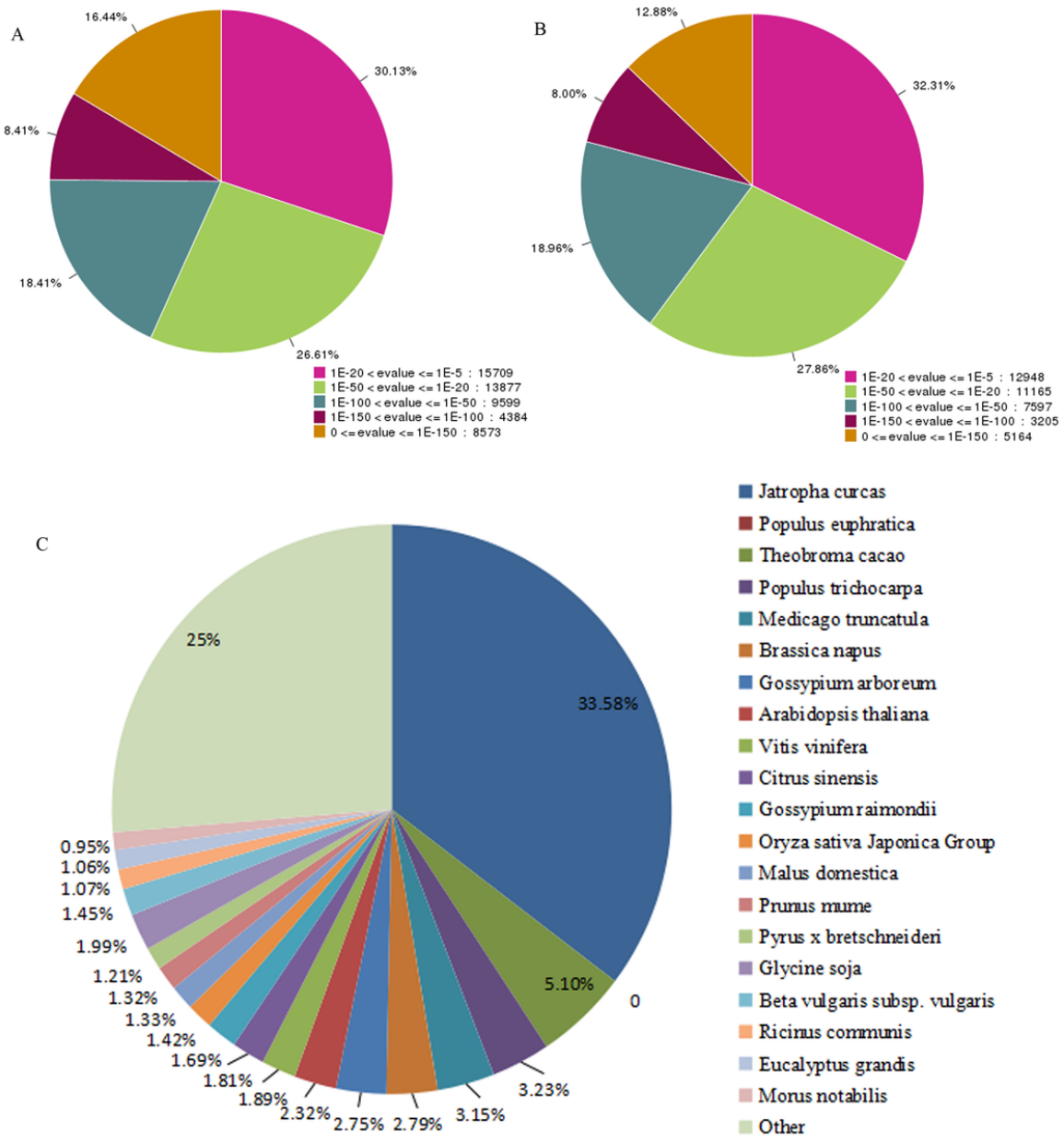

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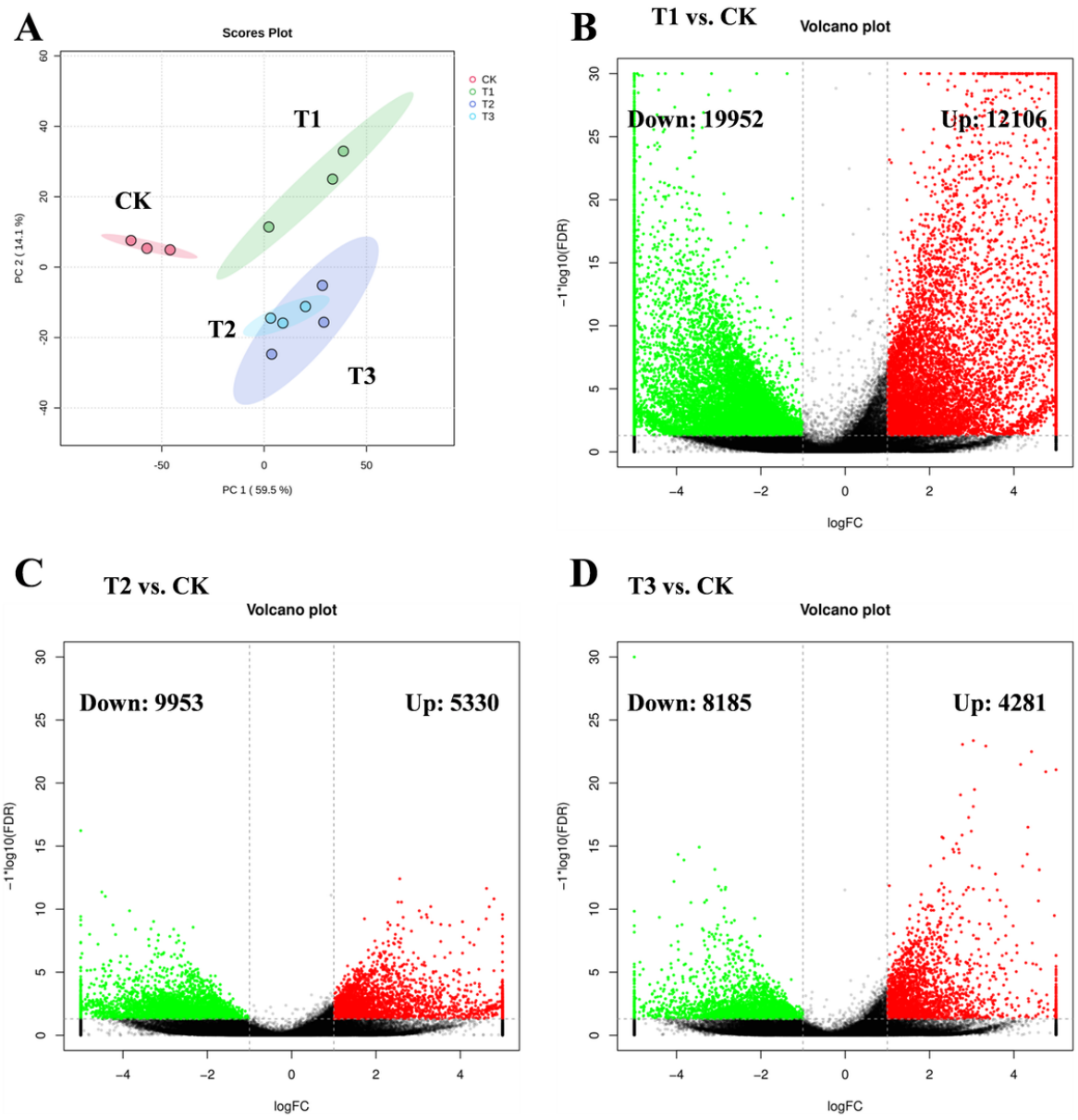

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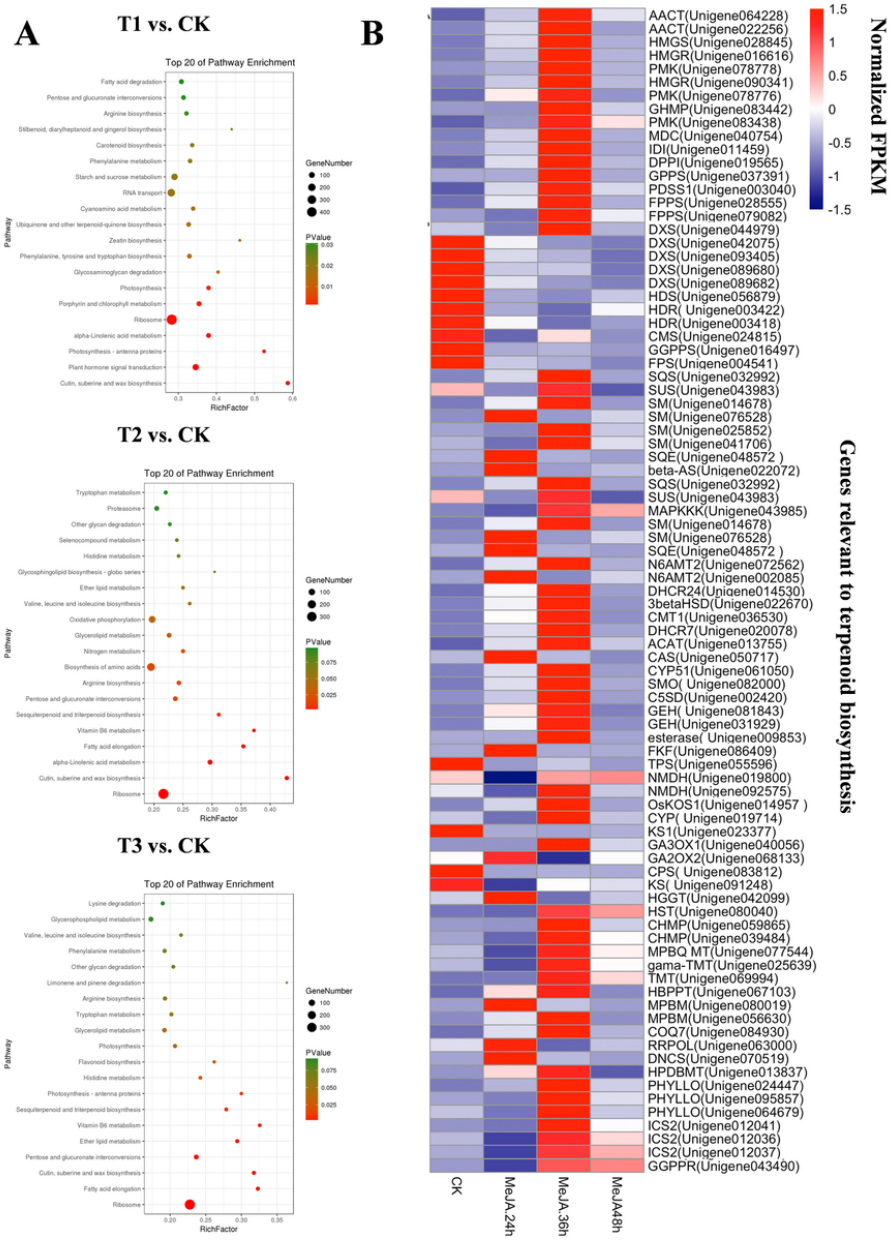

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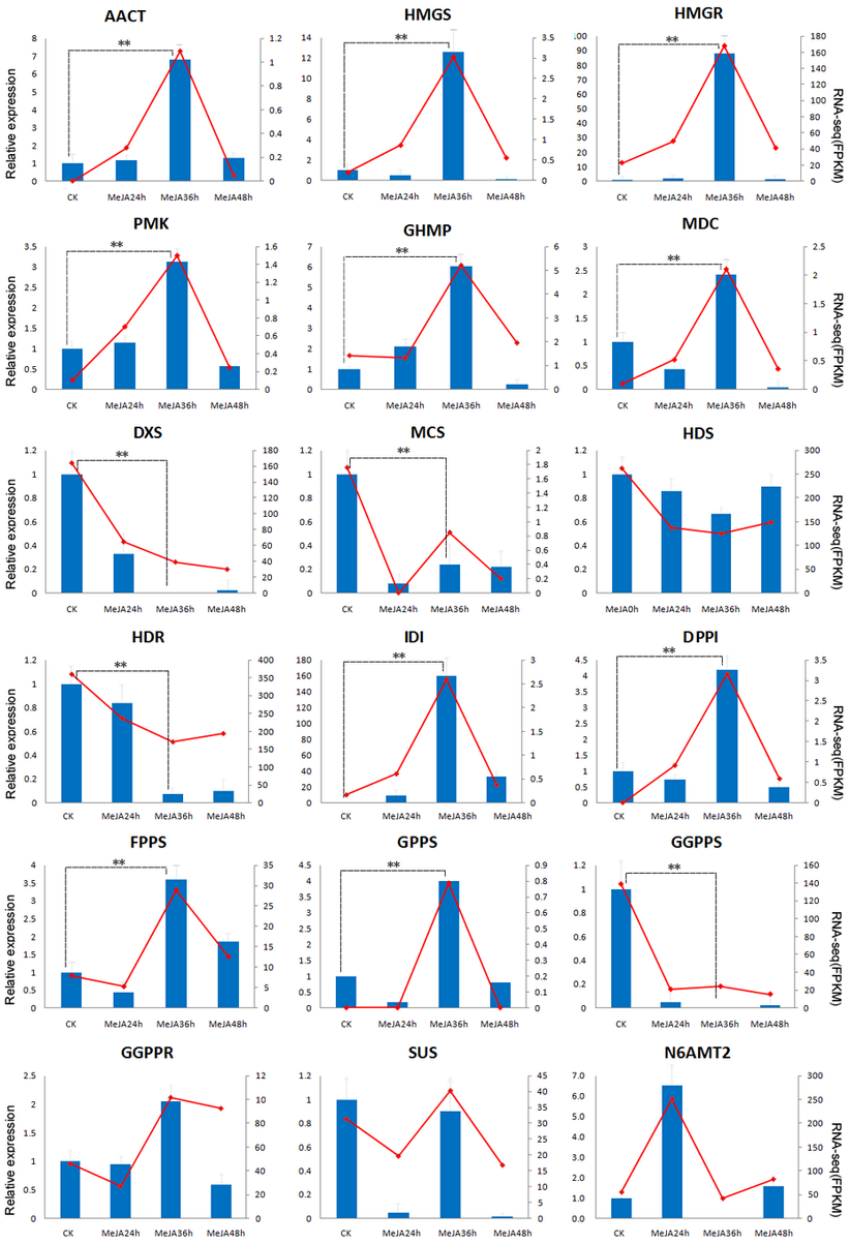

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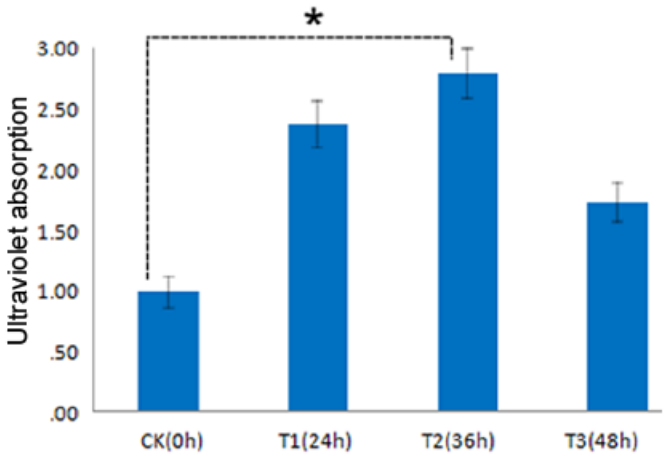

Figure 6

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