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ARTICLE

The Effect of Methylation Modification of *MDD* on the Expression of Key Genes in the Process of Saponin Synthesis in *Eleutherococcus senticosus*

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ABSTRACT

Mevalonate pyrophosphate decarboxylase is a kind of key enzyme in the terpenoid synthesis pathway in *Eleutherococcus senticosus*. The results of bisulfite sequencing showed that there were three kinds of samples with a low (0.68%), medium (0.72%) and high (0.79%) DNA methylation ratio in the promoter of *MDD* in *E. senticosus*, respectively. The transcriptome sequencing results showed that the expression of *MDD* in *E. senticosus* was significantly up-regulated in the types with low DNA methylation ratios of *MDD* ($P < 0.05$). There was a significant negative correlation between the saponin content in *E. senticosus* and the DNA methylation ratio of *MDD* promoter ($P < 0.05$). The screening results of differentially expressed transcription factors among the three groups with different DNA methylation ratio showed that a total of 4 transcription factors could bind to 6 CpG sites. Protein-protein interaction analysis showed that *E. senticosus MDD* could interacted with other key enzymes in the process of terpenoid synthesis. In addition, it was found that the DNA methylation of *MDD* promoter was mainly regulated by DNA methyltransferase. These results demonstrated that under the action of DNA methyltransferase, the changes of DNA methylation of *MDD* promoter could regulate its own expression level by affecting the combination ability of transcription factors, and then affected the expression of other genes in terpenoid synthesis pathway. The synthesis and accumulation of saponins in *E. senticosus* was also changed.

KEYWORDS

Eleutherococcus senticosus; mevalonate diphosphate decarboxylase; DNA methylation; transcription factors

1 Introduction

Eleutherococcus senticosus Maxim, one of traditional Chinese medicines, has been used to calm the mind, strengthen the spleen, tonify the kidney and anti-fatigue [1]. Pharmacological studies show that *E. senticosus* contains triterpene saponins, coumarins, lignins, phenols, flavonoids and other secondary metabolites, which have the effects of antioxidation, sedation, hypnosis, hypoglycemia and anti-tumor [2]. So far, 43 saponins have been isolated from *E. senticosus*, of which oleanane type of triterpenoid saponin is one of the main active components [3].

In recent years, it has been a research hotspot to analyze how the diversity of key enzyme affected the content of medicinal components during the synthesis of active components in medicinal plants. Previously, Hwang et al. [4] performed the transcriptome sequencing of *E. senticosus* and analyzed the expression profile of key enzymes

in terpenoid synthesis pathway. The key enzymes in the biosynthesis of saponins, including squalene synthase (SS), squalene epoxidase (SE) and mevalonate pyrophosphate decarboxylase (MDD), have been cloned successively [5]. The analysis of single nucleotide polymorphism (SNPs) showed that there were 6 SNP located in the *SS* gene, but none of them was significantly correlated with the content of total saponins; there were 9 SNPs in *SE* gene, of which 6 SNPs had a significant effect on the content of total saponins [6]. The analysis of the members of the *SS* gene family showed that the expression of *SS*2, the key enzyme in the biosynthesis of triterpene saponins, was significantly correlated to the content of saponins [7]. For *MDD*, there are significant differences between its mRNA expression and the saponin content even in *E. senticosus* planted under the same conditions and from the same clone. This indicated that the analysis only based on DNA sequence variation could not completely reveal the molecular mechanism of the differences in the content of saponins. It must be explored in the field of epigenetics besides DNA sequence variations.

DNA methylation is one of the most important epigenetic modifications [8]. At present, the analysis of DNA methylation mostly derived from model species, and just a few from medicinal plants. The genome-wide DNA methylation analysis demonstrated that the DNA methylation level of *in vitro* cultured embryonic calli of *E. senticosus* was significantly lower than that of non-embryonic calli which have no regeneration capacity [9]. Bisulfite sequencing results showed that several DNA methylation sites were deposited in the promoters of FPS, SS and SE genes of *E. senticosus*, which had a certain effect on the synthesis and accumulation of saponins [4].

In this study, the DNA methylation sites in *MDD* were screened by bisulfite sequencing and the transcriptome sequencing of *E. senticosus* with different DNA methylation types was carried out to analyze the molecular mechanism of different saponin content in *E. senticosus*.

2 Materials and Methods

2.1 The Extraction of Genomic DNA and the Synthesis of cDNA

The leaves of *E. senticosus* with similar growth states, same age and growth potential in Wulingshan National Nature Reserve (Chengde, China) were selected as test materials (n = 100). Total RNA and genomic DNA were extracted by an RNAprep pure plant kit (Beijing Tiangen Biotechnology Co., Ltd., China) and a plant genomic DNA kit (Beijing Tiangen Biotechnology Co., Ltd., China), respectively. Total RNA was then reversely transcribed to cDNA by using RevertAid™ First strand cDNA synthesis Kit (Beijing Tiangen Biotechnology Co., Ltd., China).

2.2 Prediction of CpG Island and Analysis of DNA Methylation Sites in the Promoter of MDD

According to the manufacturer's instruction of the DNA Bisulfite Conversion Kit (TIANGEN Biotech), DNA samples were treated with bisulfite with 3 biological repetitions.

The potential function of the promoter of *MDD* (GenBank: OL467356) was analyzed by PlantCARE online program [10]. Li et al. [11] were used to predict the CpG island in the promoter of *MDD*. To amplify the promoter of *MDD* after bisulfite treatment, the primers were then designed using Prime premier 6 [12]. The primers are listed in Table 1, in which the primers for amplifying the upstream and downstream of CpG island were designated with $CMDDJHS_2$-X_4 and $CMDDJHS_4$-X_6, respectively. The processed DNA was amplified by PCR with a Methylation-Specific Kit (Beijing Tiangen Biotechnology Co., Ltd., China). The total reaction system was 20 μL with 1.0 μL bisulfite-treated DNA (50 ng/μL), 1.0 μL each prime (15 μm), 1.6 μL dNTPs, 0.4 μL MSP DNA poly and 2.0 μL MSP DNA buffer. The amplification conditions of upstream CpG island were pre-denaturation at 94°C for 5 min, then followed by 40 cycles of Denaturation at 94°C for 20 s, annealing at 54°C for 30 s and extension at 72°C for 15 s, and then supplementary extension at 72°C for 5 m. The reaction for amplifying the downstream CpG island was performed with thermal cycling condition of initial denaturation at 95°C for 5 m, then 40 cycles of 94°C for 20 s, 53°C for 30 s, and 72°C for 40 s, and a final extension at 72°C for 5 m.

Table 1: Primers used in the DNA methylation of *E. senticosus MDD* gene promoter for saponin content analysis

Primer name	Primer gene	Sequence (5′-3′)	Expected amplification length
$CMDDJHS_2$	*MDD*	TGTGAAATTGTTAGATTTGTTTAGT	233 bp
$CMDDJHX_4$		TAAATTTACTAATAACACAACAAAACATAA	
$CMDDJHS_4$		TTTTTAGATGTTGTTTTTTATGTATAAGG	709 bp
$CMDDJHX_6$		CCTCAACTCTTAAATATAAACCCACC	
MVKrtS	*MVK*	AAGTGAACCAAGCCAAGTAC	207 bp
MVKrtX		ATAGCATCAAAACCACCTGC	
MDDrtS	*MDD*	CACCACCTCCAGAAATTAAAGA	247 bp
MDDrtX		GTACCCTGAACAGGACCATCTA	
FPSrtS	*FPS*	ATTCACCGAAGAATCCCGAC	182 bp
FPSrtX		CCTGAGCCCACTCAACACAC	
SS1rtS	*SS1*	AGGAGCAGTGAAAATGAGAC	148 bp
SS1rtX		CAAAGTTGCTGTAGCGTTAG	
SS2rtS	*SS2*	ACCAAATAGGCTGTGATTGG	204 bp
SS2rtX		CTCAAACTGGCAATAACAAG	
SErtS	*SE*	TTTGCTGTGGCTATCTACGG	197 bp
SErtX		CTTAGTGAATGAATGGGAGG	
bAS1rtS	*bAS1*	GTCTAATTGCATGTTGGGTG	215 bp
bAS1rtX		CATAAACTGGTGTGCTTTCC	
bAS2rtS	*bAS2*	CCTGATTACTTATGGCTTGC	184 bp
bAS2rtX		CTCCCGATGGATTTTCTTTG	
RGS	*GAPDH*	GCAAGGACTGGAGAGGTGGA	134 bp
RGX		AGTGGGAACTCGGAAGGACA	

After separated by 1.5% agarose gel electrophoresis and purified by an agarose gel DNA Recovery Kit (Beijing Tiangen Biotechnology Co., Ltd., China), PCR products were cloned into PGM-T vector and then transformed into *Escherichia coli* TOP10 competent cells. From each sample, 10 positive strains were selected for sequencing (Beijing Nosai gene Co., Ltd., China).

BIQ analyzer 2.0 software [13] was used to view and compare the sequences of *MDD* with or without treatment of bisulfite. Type A, Type B and Type C are grouped according to the DNA methylation ratio of *MDD* from low to high.

2.3 RNA-Seq Analysis

According to the DNA methylation type (Types A, B and C) in the promoter of *MDD*, mRNA in mature leaves was extracted and grouped. The cDNA library of each sample with 3 biological replicates was constructed and sequenced on the Nova SEQ 6000 sequencing platform (Illumina, USA). The splice sequences in raw data and low-quality reads were removed to obtain high-quality clean data. Each library sequence was then spiced with the Trinity software (http://trinityrnaseq.github.io/). The transcripts with

low expression were filtered according to FPKM < 1, TPM < 1, or IsoPct < 5.0%, and then clustered into unigenes using Tgicl (v2.0.6) (https://sourceforge.net/projects/tgicl/). Unigene sequence was compared with the data in Non-redundant (Nr), Swiss-Prot, Gene Ontology (GO), Clusters of Orthologous Groups of proteins (COG), euKaryotic Orthology Groups (KOG), the Kyoto encyclopedia of genes and genomes (KEGG), Pfam database to obtain annotation information.

2.4 Analysis of Transcription Factors Binding to MDD Promoter

PlantTFDB [14] and PlantRegMap [15] were used to predict transcription factors that may bind to the promoter of *EsMDD* gene with reference to the transcription factors of *Nicotiana tabacum*. The transcriptome sequencing results of *E. senticosus* were used to screen the differentially expressed transcription factors, which were then mapped by HeatMap function of TBtools [16]. MEGA7 [17] was used for phylogenetic analysis of the transcription factor families and string [18] was used for protein-protein interaction prediction.

2.5 Expression Analysis of Key Genes in the Process of Terpenoid Synthesis

According to the transcriptome sequencing results, the known key enzyme gene sequences of the terpenoid synthesis pathway of *E. senticosus* were compared using BLAST. The key enzyme genes of the terpenoid synthesis pathway in the samples were screened, and qRT-PCR primers were designed (Table 1). According to the qRT-PCR primers of the key enzyme genes of *E. senticosus* terpenoid synthesis pathway in Table 1, the cDNA of the three selected DNA methylation types of *E. senticosus* samples was used as the template for qRT-PCR reaction with Abiprism 7900-HT (Applied Biosystems, Foster City, CA 94404, USA). Each sample was set with 3 biological replicates. The total reaction system was 10 μL. 2 × Talent qPCR premix was 5.0 μL (Beijing Tiangen Biotechnology Co., Ltd., China). Forward primer was 0.3 μL (primer concentration 15 μm), and reverse primer was 0.3 μL. *E. senticosus* cDNA template was 0.5 μL (concentration 50 ng/μL), and 50 × Rox reference dye was 1 μL and RNase free H_2O was 2.9 μL. The reaction conditions were 95°C pre-denaturation for 3 m with denaturation at 95°C for 5 s, annealing at 55°C for 10 s, and supplementary extension at 72°C for 15 s; The reaction was ended after 40 cycles. The gene expression of each sample was calculated by $^{2-\Delta\Delta}$Ct method with SDS 2.4 software.

2.6 Content Determination of Total Saponins of E. senticosus

The leaves of *E. senticosus* with different DNA methylation types were dried and ground, and then shake with 0.5 mL 8% vanillin ethanol solution [19] and 5 mL 72% sulfuric acid solution. The samples were then heated for 10 min at 60°C, and cooled for 15 min in ice bath. The absorbance value of each sample at 534 nm was detected by vanillin-concentrated sulfuric acid coloration method [5]. The total saponin contents were calculated by the regression equation. SPSS 22.0 was used to analyze the correlation between DNA methylation ratio in the promoter of MDD and total saponin content.

2.7 Analysis of DNA Methylation Related Enzymes and Protein Interaction of MDD

According to the transcriptome annotation information, the data of DNA methyltransferase and DNA demethylase genes of *E. senticosus* were also extracted. The results were then identified by the NCBI-CDD function. MEGA7 and DNA methyltransferase and DNA demethylase in other species were used for phylogenetic analysis.

The string was used to analyze the protein-protein interaction between MDD protein in Arabidopsis and other key enzymes in the terpenoid synthesis pathway.

3 Results

3.1 DNA Methylation Analysis of MDD Promoter

The results of Li Lab showed that there were 2 CpG islands in the promoter of *MDD*. The upstream CpG island was in −2265–2148 bp and the downstream CpG island was in −1787–1663 bp. BIQ Analyzer 2.0 software was used to compare the *MDD* promoter the sequence treated with bisulfite with the untreated DNA sequence. The results showed that there were 10 and 18 DNA methylation sites in the upstream and downstream CpG islands of *MDD*, respectively (Fig. 1A), The DNA methylation sites in all samples were located at −2267, −2264, −2214, −2176, −2162, −2157, −2100, −1885, −1849, −1769, −1758, −1742, −1715, −1708, −1644, −1519 bp in the upstream of the transcriptional site. The differential methylation sites were located at −2205, −2196, −2130, −1923, −1572, −1430 bp, the methylation sequencing map of −1923 bp site is shown in Fig. S1 for example. There were 3 types of DNA methylation sites, corresponding to DNA methylation ratios, which were designated as Type A, Type B, Type C. There were 18. 19 and 21 DNA methylation sites in samples of Types A, B and C and the methylation rate of cytosine were 0.68%, 0.72% and 0.79%, respectively (Fig. 1B).

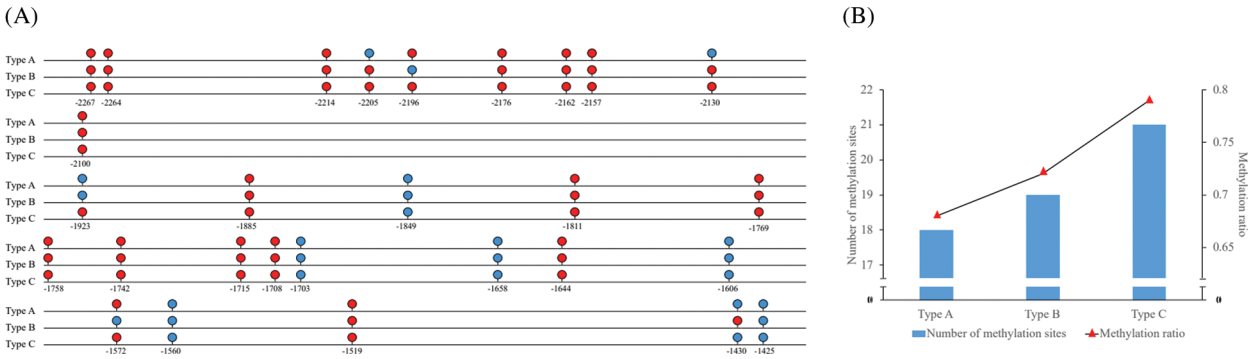

Figure 1: (A): Schematic map of The expression of DNA methylation sites of *MDD* gene promoter of *E. senticosus*. Red site is DNA methylation site, and blue site is DNA unmethylated sites; (B): Schematic diagram of number and ratio of methylation sites

3.2 Determination of Total Saponin Content in E. senticosus

The total saponin contents in the 3 groups of samples of Types A, B and C were determined by vanillin-concentrated sulfuric acid method. According to the average absorbance (0.155 ± 0.000471 for Type A, 0.247 ± 0.000816 for Type B, and 0.338 ± 0.000420 for Type C) the total saponin contents of were calculated as 4.65%, 2.32%, and 1.83% for Types A, B and C, respectively. The correlation analysis showed that there was a significantly negative correlation between the DNA methylation ratio of *MDD* and the saponin content ($P < 0.05$). The correlation equation was $y = -23.532x + 20.112$ ($R^2 = 0.7562$), where "y" standed for the saponin content.

3.3 Assembly and Annotation of Transcriptome Sequencing Results

High-throughput transcriptome sequencing was performed on the 3 groups of *E. senticosus* samples of Types A, B and C on Illumina Nova Seq 6000 platform. After removing junction and low-quality reads, 20.51–29.33 M clean reads and 6.15–8.80 Gb clean bases were obtained. The GC content of all samples was about 43%–45%, and that of all Q30 bases was over 94% (Table 2), which indicated that the quality of sequencing data was acceptable.

Table 2: Statistics of sequencing data after sample filtering

Samples	Clean reads	Clean bases	Error rates (%)	Q20 (%)	Q30 (%)	GC contents (%)
Type A1	20,504,555	6,151,366,500	0.03	98.49%	95.21%	44.60%
Type A2	21,978,379	6,593,513,700	0.03	98.52%	95.33%	44.56%
Type A3	20,968,256	6,290,476,800	0.03	98.53%	95.28%	44.56%
Type B1	27,314,372	8,194,311,600	0.03	98.51%	95.21%	44.14%
Type B2	24,970,311	7,491,093,300	0.03	98.38%	94.89%	43.50%
Type B3	24,162,672	7,248,801,600	0.03	98.51%	95.24%	43.52%
Type C1	21,378,367	6,413,510,100	0.03	98.51%	95.22%	43.29%
Type C2	23,472,929	7,041,878,700	0.03	98.37%	94.78%	43.34%
Type C3	29,333,934	8,800,180,200	0.03	98.45%	95.08%	43.32%

Samples are the sample names; Clean reads are the number of Clean reads; Clean bases are the total number of bases in Clean data; GC is the percentage of GC content in Clean data; ≥Q30 is the percentage of bases whose clean data quality values are greater than or equal to 30. Q30 is the percentage of bases with clean data quality values greater than or equal to 30.

Clean reads generated after filtering the transcriptome sequencing data were assembled using the Trinity program. A total of 374,281 transcripts were generated, of which the longest transcript was 17,973 bp, and the shortest was 197 bp. The average length of the transcripts was 303 bp, and the length of the N50 transcript was 243 bp. Further filtering of the low-expressed transcripts, there were 364,816 unigenes with an average length of 284 bp and an N50 of 240 bp (Table 3). The unigenes with lengths between 200–300 bp accounted for the largest proportion of the total (345,555, 94.72%), and unigenes with lengths between 200–1,000 bp, 1,000–2,000 bp and 2,000–3,000 bp accounted for 97.57% (355,956), 1.51% (5,497) and 0.62% (2,258) of the total, respectively. In addition, the length of 1,105 (0.30%) unigenes were longer than 3,000 bp (Table 3)

Table 3: Statistics of gene results predicted by transcriptome assembly

Types	Unigenes	Transcripts
Total_sequence number	364,816	374,278
Total_sequence length	103,696,934	113,658,066
Maximum length (bp)	17,973	17,973
Minimum length (bp)	197	197
Average length (bp)	284	303
GC%	47.12%	46.56%
N40 (bp)	248	253
N50 (bp)	240	243
N60 (bp)	233	236

Types are the type listed; Unigenes were unigenes obtained after assembly and filtration; Transcripts are transcripts obtained after assembly; Total sequence number is the total number of sequences; Total sequence length is the total length of all sequences; Maximum length is the maximum length of the assembled sequences; Minimum length is the minimum length of the assembled sequences; Average length is the

average length of all sequences obtained from the assembly; GC% is the sum of the number of GC bases as a percentage of the total number of bases; N50: the length of the assembled transcripts sorted from longest to shortest, and the length of the transcripts accumulated to 21%–50% of the total length.

The assembled unigenes sequences were compared with the data in KEGG, NR, Swiss-Prot, GO, COG, KOG and Trembl databases by BLAST program. Amino acid sequences of unigenes were compared with the data from Pfam database using HMMER software to obtain the annotation information. The results are shown in Fig. 2A. A total of 192,820 unigenes were annotated by at least 1 of the above 7 databases, accounting for 52.85% of the total unigenes. In the NR database, 177,470 (48.65%) unigenes were annotated, and the highest matching rate was obtained, and the COG was the least annotated with 64,532 (17.69%). Comparing with the NR database, it was found that the single gene sequence of *E. senticosus* showed the highest match rate (4.69%) with that of *Daucus carota*, and the lowest match rate with *Ramularia collo-cygni* (1.22%) (Fig. 2B).

A total of 364,817 unigenes were divided into 3 groups and 54 classes in the GO database according to their functions. The cellular component group mainly includes cells (cell, 38,186), membranes (membrane, 27,338) and organelles (organelle, 27,408); the molecular function mainly involves the catalytic activity (50,432), binding (42,708) and transporter activity (3,699). The biological process mainly involves metabolic process (50,154), cellular process (42,115) and localization (14,885) (Fig. 2C). Only 13.17% of Unigene annotation information in KOG was annotated to General function prediction, and only 0.09% annotated to cell motility (Fig. 2D).

3.4 Identification and Validation of Differentially Expressed Genes in the Terpenoid Synthesis Pathway

The transcriptome sequencing data were used to screen differentially expressed genes (DEGs) among samples of Types A, B and C with FDR < 0.01 and $|\log2\ FC| \geq 1$ as the screening criteria. A total of 4,618 DEGs were detected in the Type A *vs*. Type C, with 2,203 up-regulated genes and 2,415 down-regulated genes; a total of 345 DEGs were detected in the Type B *vs*. Type C, with 116 up-regulated genes and 229 down-regulated genes; a total of 4,657 DEGs were detected in the Type A *vs*. Type B, including 2,228 up-regulated genes and 2,429 down-regulated genes. Among these, 719 DEGs were differentially expressed in Type A and Type B only, 683 DEGs in Type A *vs*. Type C only, and 25 DEGs in Type B *vs*. Type C only (Fig. 3A). A total of 91 DEGs were differentially expressed in the all 3 types (Fig. 3B). These findings suggested that there was a significant difference in transcript expression among samples of Types A, B and C.

In plants, MVK (Mevalonate kinase), MDD, FPS (Farnesyl pyrophosphate synthase), SS, SE, and bAS (β-amyrin synthase) are key enzymes which involved in the terpenoid synthesis. Eight differentially expressed genes were screened according to the KO numbers of the above 6 genes in 3 groups. The further judgment showed that the sequences of *FPS* and *SS*1 and *SS*2 in the transcriptome sequencing data were the same with those obtained by cloning in our previous study [5]. All seven genes, except *bAS*, were highly expressed in the Type A, but low expressed in Type C. These expression patterns showed a significant negative correlation with the DNA methylation ratio of *E. senticosus MDD* gene promoter ($P < 0.05$). In contrast, among the *bAS* gene family, *bAS*2 was significantly negatively correlated with the DNA methylation ratio of *MDD* ($P < 0.05$) and positively correlated with the saponin content ($P < 0.05$), but *bAS*1 was not (Fig. 3C).

To verify the reliability of the transcriptome data, eight differentially expressed genes involved in terpenoid synthesis were validated by qRT-PCR. The results showed that the trends of the relative expression of differentially expressed genes were all consistent with the transcriptome sequencing data, which indicating the reliability of transcriptome sequencing results (Fig. 3D).

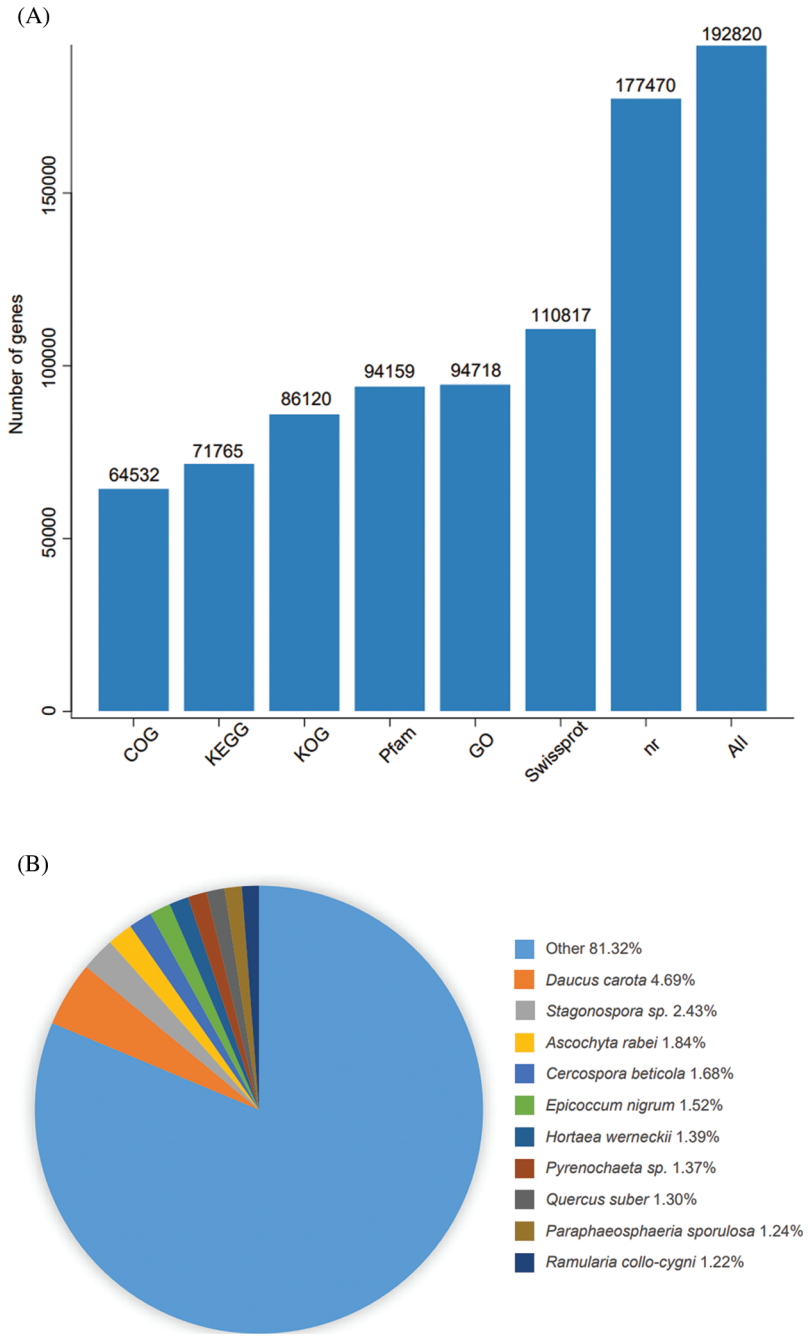

Figure 2: (Continued)

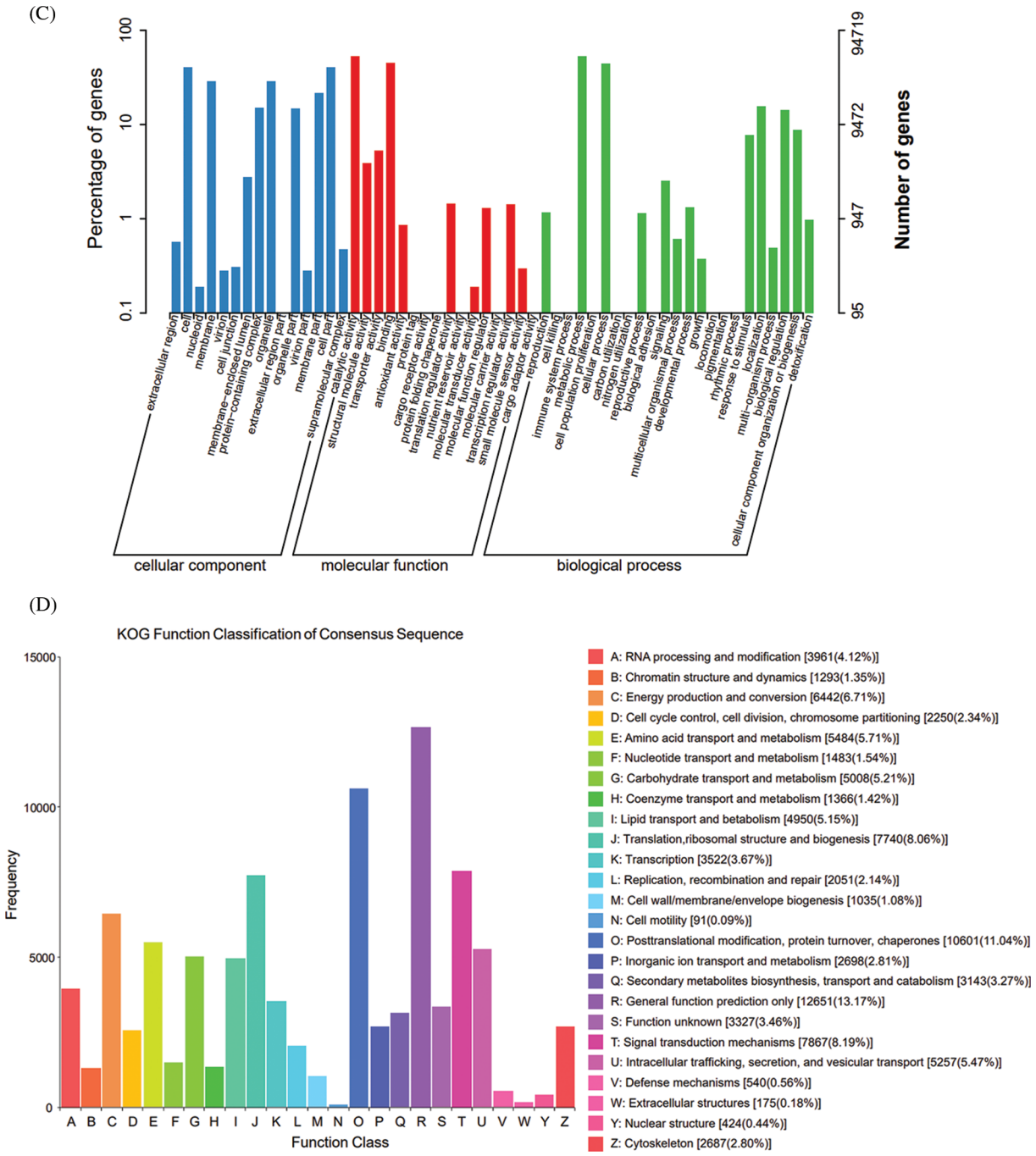

Figure 2: Annotation map of the transcriptome of *E. senticosus*. (A): annotation of Unigenes in 7 databases; (B): annotation results of Nr database; (C): annotation results of GO database; (D): annotation results of KOG database

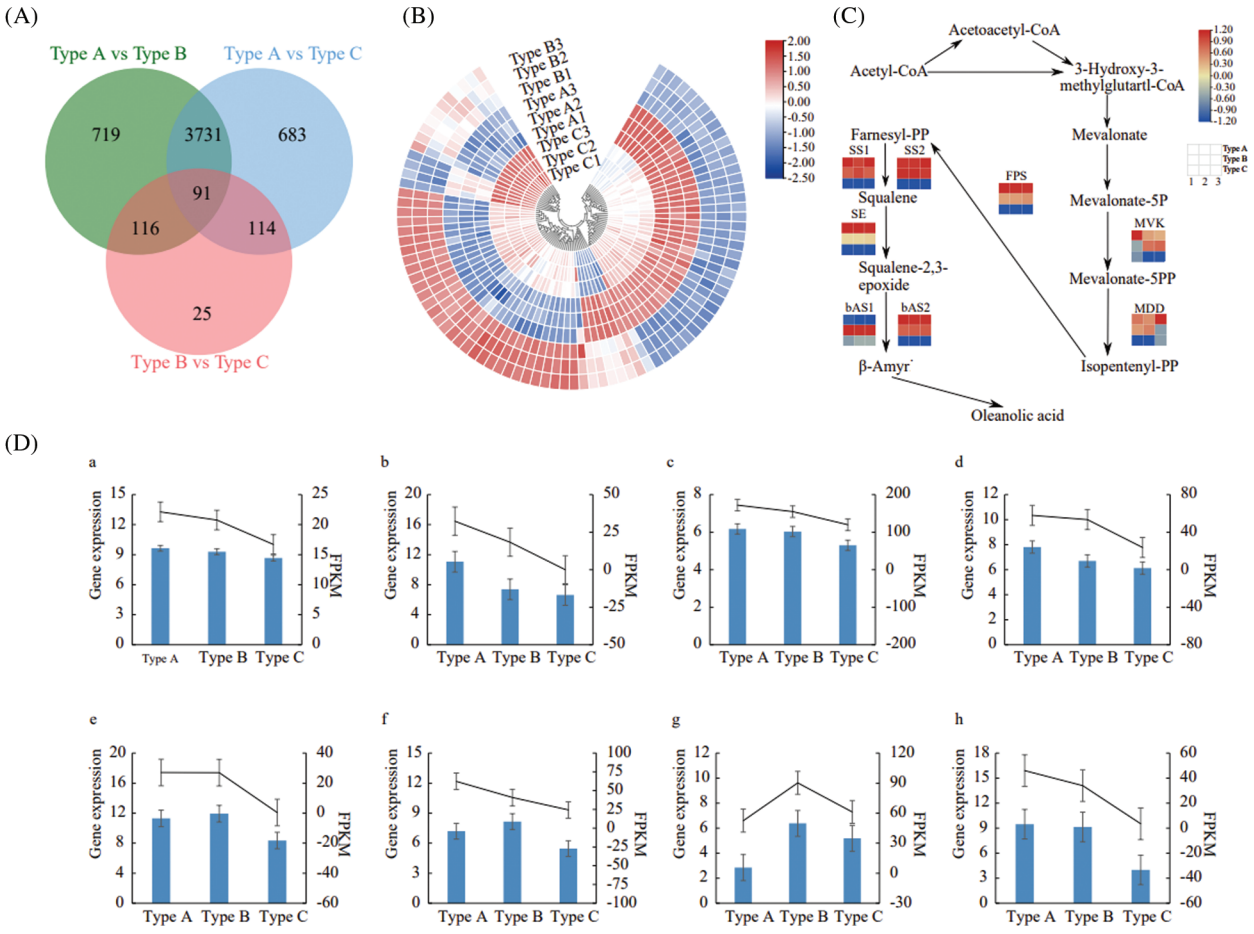

Figure 3: Effect of *E. senticosus MDD* promoter DNA methylation on gene expression. (A): statistical map of differential genes of the transcriptome; (B): clustering map of 91 DEGs differentially expressed in all 3 groups of samples; (C): analysis of gene expression of key enzymes of the transcriptional terpenoid synthesis pathway; (D): qRT-PCR validation results of key enzyme genes of terpenoid synthesis pathway: blue bars are qRT-PCR gene expressions, and black lines are the FPKM value of the relative gene expression of the transcriptome. (a): *MVK*; (b): *MDD*; (c): *FPS*; (d): *SS1*; (e): *SS2*; (f): *SE*; (g): *bAS1*; (h): *bAS2*

3.5 Analysis of the E. senticosus MDD Gene Promoter Cis-Acting Element and Binding Prediction of Differentially Expressed Transcription Factors

The results of predicted *cis*-acting elements in the promoter of *MDD* are shown in Table 4. In addition to TATA-box and CAAT-box, the largest number of elements were related to light response, such as TCT-motif and Box-4. Other *cis*-acting elements were responded to drought, gibberellin, methyl jasmonate, and abscisic acid (Fig. 4).

PlantTFDB was used to predict the transcription factor binding sites in the promoter region of *E. senticosus MDD* with *Nicotiana tabacum* as the reference. The results showed that the promoter region of *MDD* could bind to 34 of 324 transcription factors. Among them, 15 can bind to DNA methylation sites in CpG island (Fig. 4). Nineteen of 120 differentially expressed transcription factors binding to *MDD* promoter were found among samples with different DNA methylation types of *MDD*. Sixty-two were positively correlated with the DNA methylation ratio, while 58 were negatively related with it (Fig. 5A).

Table 4: Predicted *cis*-acting elements of the promoter of *E. senticosus MDD*

Cis-acting element names	Binding sequences	Functions
TCT-motif	TCTTAC	Light responsiveness
Box 4	ATTAAT	Light responsiveness
GT1-motif	GGTTAA	Light responsiveness
G-box	CACGTT	Light responsiveness
MRE	AACCTAA	Light responsiveness
GATA-motif	GATAGGA	Light responsiveness
MBS	CAACTG	Drought-inducibility
MYB	TAACCA	Drought-inducibility
TATC-motif	TATCCCA	Gibberellin responsiveness
GARE-motif	TCTGTTG	Gibberellin responsiveness
TGACG-motif	TGACG	MeJA responsiveness
CGTCA-motif	CGTCA	MeJA responsiveness
ABRE	ACGTG	Abscisic acid responsiveness

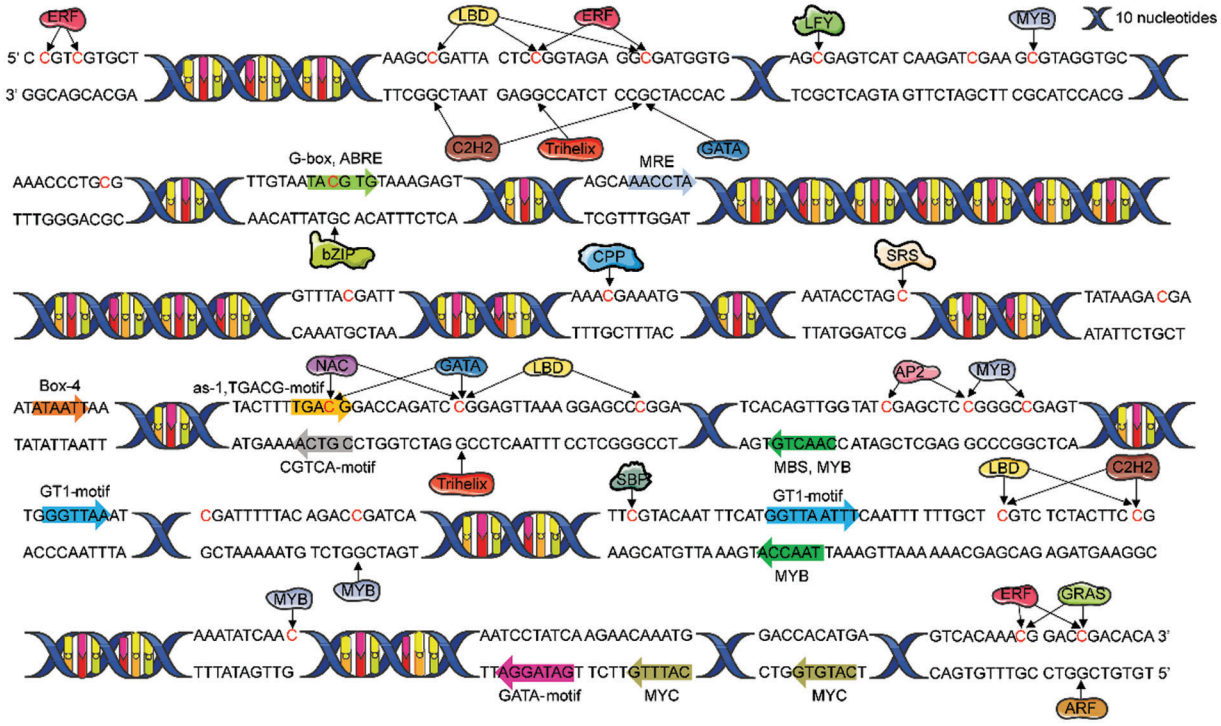

Figure 4: Binding prediction of transcription factors with *E. senticosus MDD* promoter region and *cis*-acting elements prediction (−2268 to −1419 bp)

Among the 28 DNA methylation sites, 5 sites did not have bindable transcription factors (−2162, −1923, −1811, −1742, −1658 bp). Among the remaining 23 sites, only 4 sites (−2205, −2196, −1572 and −1430 bp sites) have differences in methylation status across three types, which was likely to bind to four gene families, including *ERF* (Ethylene-responsive factor), *LBD* (Lateral Organ Boundaries Domain), *C2H2* (Cys2/His2-type zinc finger protein) and *GRAS*. Among them, 18 members of *ERF* gene family mainly bind to −2205 bp and −2196 bp sites, and a small number of them bind to −1430 bp sites. There were 9 members of *LBD* family, mainly binding to −2205 bp and −2196 bp sites, and a small number of them binding to −1572 bp site. In addition, 1 member of the *C2H2* gene family is combined with −2196 bp and −1572 bp sites, and 1 member of the *GRAS* gene family is combined with −1430 bp site.

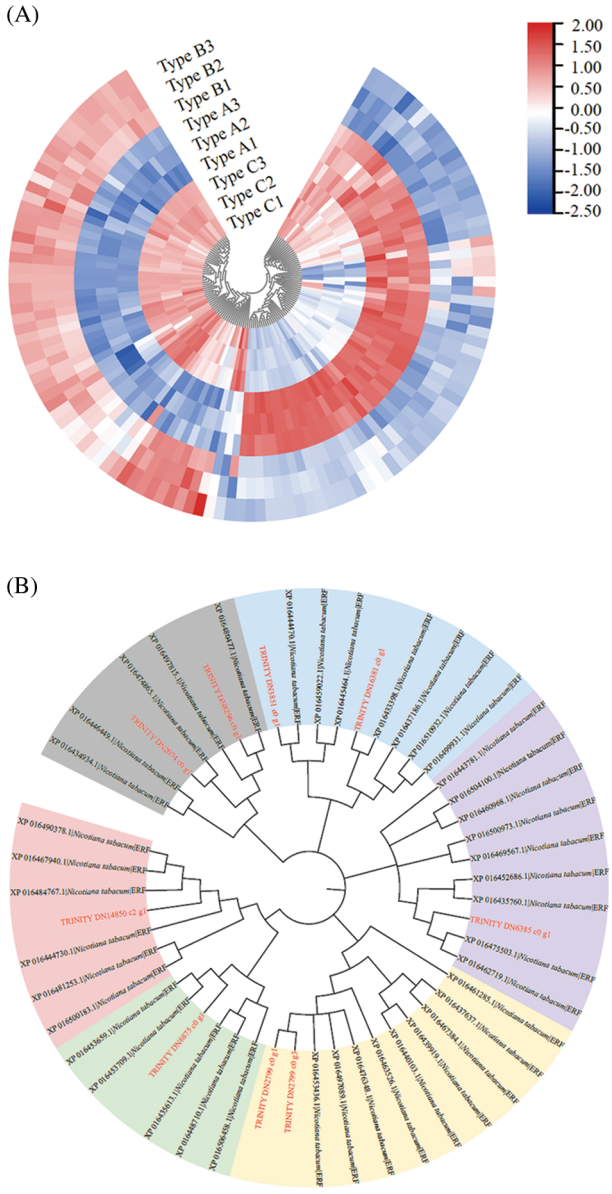

Figure 5: (Continued)

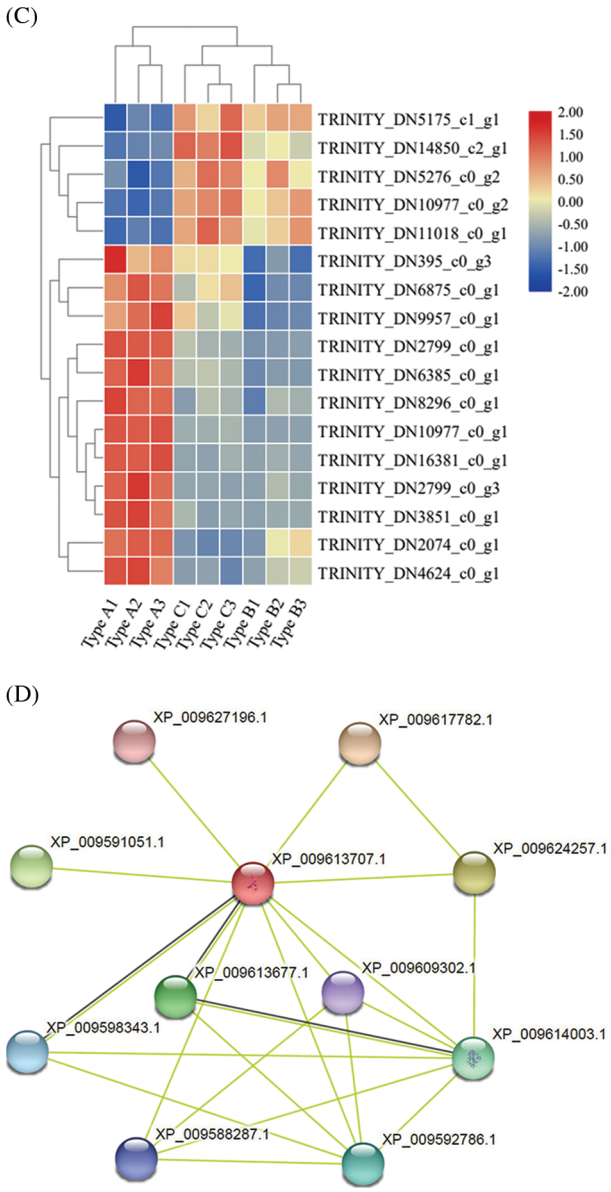

Figure 5: Effect of *E. senticosus MDD* promoter DNA methylation on transcription factor expression and protein interactions analysis (A): Differential transcription factor analysis of 3 *E. senticosus MDD* promoter DNA methyl types; (B): Cluster analysis of differentially expressed transcription factors in promoter methylation sites of *E. senticosus MDD* gene; (C): Clustering diagram of *ERF* genes differentially expressed in *E. senticosus MDD* promoter region and *N. tabacum ERF* gene family. ERF genes differentially expressed in the *E. senticosus MDD* promoter region are labeled in red; members of the *N. tabacum ERF* family are labeled in black, and different colored regions represent *ERF* members of different subfamilies; (D): Protein interaction analysis of *LBD* transcription factor at −1572 bp site of *E. senticosus MDD* promoter. Green lines represent interaction and black lines represent co-expression

The cluster analysis showed that the above 4 differentially expressed transcription factors were significantly different across different DNA methylation types of *MDD* ($P < 0.05$), which were mainly divided into 2 expression patterns. The first one was high expression in Type A and low expression in Type C. The second one was low expression in Type A and high expression in Type C. *ERF* and *GRAS* mainly showed in the second expression pattern, while *LBD* and *C2H2* mainly showed in the first expression pattern. But there are also some exceptions. For example, *EsERF*1 (TRINITY_DN14850_c2_g1), which belongs to *ERF* family, appeared in the first expression pattern; *EsLBD*1-1 (TRINITY_DN10977_c0_g1) and *EsLBD*1-2 (TRINITY _ DN10977_c0_g2), which were *LBD* family members, showed in the second pattern and the first pattern, respectively (Fig. 5B).

After that, the second most differentially expressed transcription factors, *ERF* and *LBD*, were also analyzed. Phylogenetic analysis was performed using the *N. tabacum ERF* gene family in PlantTFDB with differentially expressed *ERF*s in the promoter region of the *E. senticosus MDD*. After the comparison with ClustaW, the NJ method is used 1500 times to construct the phylogenetic tree, and the results are shown in Fig. 5C. The *ERF*s of *E. senticosus* were divided into several different subfamilies, in which *EsERF*1 was a member of a different subfamily from the other *E. senticosus ERF* members. *EsLBD*1-1 and *EsLBD*1-2, which belong to the *LBD* family and are different transcripts of the same gene, were identified to have a binding site at −1572 bp after comparing with the *N. tabacum LBD*. Protein-protein interaction analysis was performed on the *LBD* members bound to this site, and the results were shown in Fig. 5D. The members at this site interacted with *bHLH* (Basic helix-loop-helix, XP_009609302.1), *ERF* (XP_009588287.1) and *KNOX* (Knotted1-like homebox, XP_009613677.1, XP_009598343.1, XP_009617782.1).

3.6 Analysis of DNA Methyltransferase and Demethylase in E. senticosus

DNA methylation is affected by many factors, among which DNA methyltransferase and DNA demethylase have a direct effect on it. Due to the lack of key information of DNA methyltransferase in *N. tabacum*, the DNA methyltransferase gene of *A. thaliana* and the DNA demethylase of *Lonicera japonica* are used as the identification standard. The comparison method is ClustaW, and the test is carried out 1500 times by using the NJ method to construct the phylogenetic tree. The results are shown in Fig. 6A. A total of 1 DNA methyltransferase gene, named *EsMT*0 (TRINITY_DN4200_c0_g1), and 1 DNA demethylase gene, named *EsDM*0 (TRINITY_DN722_c1_g1), were identified in *E. senticosus*. Clustering analysis of the 2 genes showed that *EsMT*0 clustered with the DNA methyltransferase of *A. thaliana*, while *EsDM*0 clustered with the DNA demethylase of *L. japonica*.

Analysis of the differential expression of these 2 genes in the 3 groups of samples revealed that the DNA methyltransferase gene *EsMT*0 was significantly correlated with the methylation ratio ($P < 0.05$), and it was significantly low expressed in Type A and significantly high expressed in Type C. Its expression was significantly and positively correlated with the methylation ratio; while *EsDM*0 did not show a significant correlation with DNA methylation ratio (Fig. 6B). Meanwhile, another 22 methyltransferases and 20 demethylases were found in the proteome of *E. senticosus* (Table S1, Table S2), but the genes corresponding to these enzymes were not differentially expressed in the transcriptome.

The protein-protein interaction analysis of MDD in *A. thaliana* was performed using String program, and the results were shown in Fig. 6C. MDD interacts with multiple genes in the terpenoid synthesis pathway, including *HMGS*, *HDR*, *MVK* in the upstream, *IPP*1 and its homologous genes *IPP*2, *FPS*1, *FPS2* and *SS*1 in the downstream. AT1G31910 and AT1G26640 are unclassified proteins.

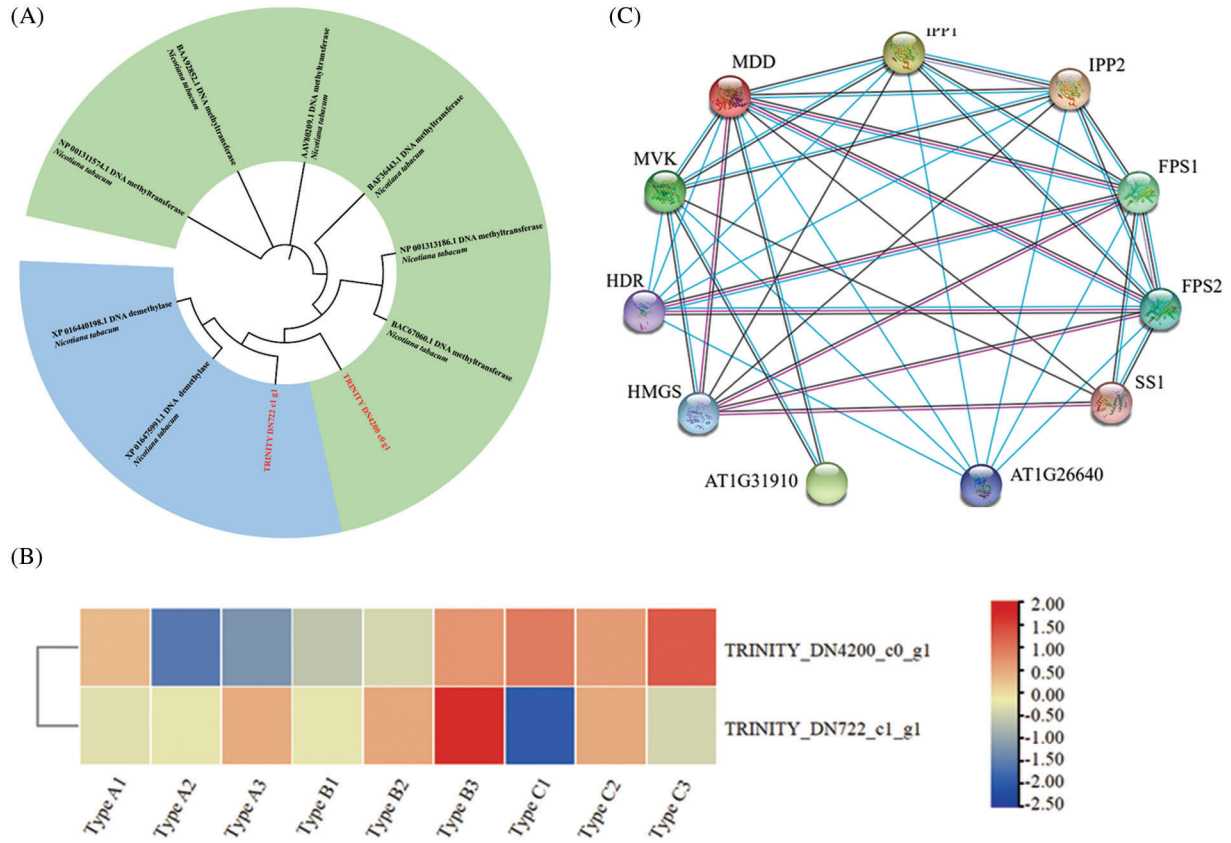

Figure 6: DNA methyltransferase/demethylase analysis and protein interaction analysis of *MDD* in *A. thaliana* (A): Identification and analysis of DNA methyltransferase in *E. senticosus*; (B): Heat map of DNA methyltransferase and demethylase expression in *E. senticosus*; (C): MDD protein interaction in *A. thaliana*. Red lines represent experimentally validated interactions; blue lines represent interactions validated by databases; black lines represent co-expression, and purple lines represent homology

4 Discussion

This study discovered the effect of DNA methylation in the promoter region of *E. senticosus MDD* on the expression of key genes related to saponin synthesis and the content of saponin. The results showed that with the increase of DNA methylation ratio, the expression of *E. senticosus MDD* was significantly down-regulated, and the expression of key genes related to terpenoid synthesis pathway, such as *EsFPS*, *EsSS* and *EsSE*, were also significantly reduced, and the content of saponin was also decreased, which was basically consistent with the results of Wang et al. [4]. The expression of *bAS*1 was not significantly correlated with the methylation ratio of *MDD*, while that of *bAS*2 was significantly correlated with the ratio, which was consistent with previous studies on the correlation between *bAS* expression and saponin content in *E. senticosus* [20].

Plant growth and development processes are closely related to DNA methylation, and in DNA methylation studies on *Glycine max* [21], *A. thaliana* [22] and *Citrus reticulata* [23], DNA methylation ratios were found to be positively correlated with their growth and development. Studies on *Brassica rapa* showed that DNA methylation modifications are necessary for plant growth [24]. One of the factors affecting DNA methylation/demethylation is temperature. The trend of DNA methylation/demethylation varies among different species, even among different parts within the same species and tissues. For

example, the ratio of DNA methylation was increased in *Fragaria* x *ananassa* under the low-temperature stress [25], while decreased in *A. thaliana* under the same condition [26]. After low-temperature treatment on 4 varieties of *Cannabis sativa* [27], it was found that there were differences in DNA methylation among different varieties. The study of *Oryza sativa* [28] showed that there were differences in DNA methylation among different tissues. As one of the environmental signals, the temperature may lead to DNA methylation changes in some sensitive genes [29]. Therefore, it is speculated that *MDD* may be a low-temperature sensitive gene. The regulatory mechanism derived to enhance the tolerance to low temperature can be seen in the 2 calmodulin-binding transcription activator CAMTA transcription factors (TRINITY_DN15573_c0_g3, TRINITY_DN45924_c0_g4) that bind to the promoter region of *E. senticosus MDD*. This transcription factor has been confirmed to be involved in cold adaptation regulation [30]. The low temperature-responsive element as-1 was found in CpG island region and covered –1796 bp DNA methylation site. That may explain why there were still significant differences in DNA methylation status and saponin content among samples with similar growth status, age and growth potential. Hwang and his colleges demonstrated that the expression of *SE*, *bAS*, *CYP* (Cytochrome P450s), *UGT* (UDP-glycosyltransferases), which involved in the process of terpenoid synthesis, were significantly up-regulated after treatment with MeJA [7]. In present study, MeJA responsive elements were also found in the promoter regions of *MDD* in *E. senticosus*, which suggesting that MeJA might be a key element in regulating terpenoid synthesis.

One of the determinants of gene expression is the transcription factor, which selects genes for transcription or inhibition by recognizing the base sequence of the gene promoter region. DNA methylation in the promoter CpG island region is thought to inhibit the binding of transcription factors to the promoter, silencing gene expression [31]. However, recent studies have shown that DNA methylation might inhibit, promote or have no effect on the binding capability of transcription factor [32]. In this study, transcription factors *ERF*, *LBD*, *GRAS*, and *C2H2* was significant correlated with the the DNA methylation. The expression of ERF and GRAS was significantly inhibited by DNA methylation, while LBD and C2H2 tended to be promoted by it. *LBD* is involved in the growth and development of plant organs, and its expression was positively correlated with the growth in *Phyllostachys heterocycle* [33], which indicating *LBD* might be positively correlated with the DNA methylation ratio. *EsLBD*1-1 and *EsLBD*1-2 are different transcripts in *LBD* family; however, their expression patterns are opposite, which may be due to protein interaction with bHLH transcription factor. In plants, bHLH plays an important role in signal transduction, growth, development and stress responses [34,35]. In recent years, bHLH has been proved to be involved in regulating the biosynthesis of secondary metabolites in medicinal plants. The full length of bHLH in various medicinal plants have been cloned, including *Panax ginseng* [36] and *Panax notoginseng* [37], which belong to Araliaceae, the same family with *E. senticosus*. The study of LBD in *A. thaliana* showed that bHLH could reduce the binding ability of LBD to DNA and inhibit its expression through protein interaction [38]. In the prediction of transcription factors, it was also found that there was a binding site of bHLH in the promoter region of *E. senticosus MDD*, which was located in the middle and lower reaches near 3' end. Through the prediction of Strings, it was also found that there was a protein-protein interaction between LBD and bHLH, which indicated that bHLH might indirectly affect the expression of *E. senticosus MDD* by affecting LBD.

ERF belongs to AP2/ERF superfamily. ERF is mainly involved in various stress responses of plants, such as low-temperature stress [39], drought stress [40], and salt stress [41]. After *Gossypium hirsutum* ERF was introduced into *A. thaliana* for overexpression, transgenic *A. thaliana* plants grew slowly and showed dwarf phenotype [42], which indicated that ERF might be negatively correlated with plant growth. With the relationship between DNA methylation and plant growth, it can be speculated that ERF was mainly negatively correlated with DNA methylation ratios, while *EsERF*1 showed the opposite expression pattern. In the study of *Carica papaya*, it was found that there were multiple subfamilies of

ERF, and the expression patterns were different among different subfamilies [43]. Therefore, *EsERF*1 may present different expression patterns with other *ERF* subfamilies.

Studies based on methyl-SELEX and bisulfite-SELEX analysis of the sensitivity of human transcription factors to DNA methylation showed that most *C2H2* and *GATA* family members preferred to recognize binding sites with DNA methylation modification, which indicated that C2H2 and GATA transcription factors might be positively correlated with DNA methylation ratios. Besides, DNA methylation in the binding region decreases the binding capability of *MYB*, *bZIP*, and *HSF* families to the recognized sequences [44]. TRINITY_DN5175_c1_g1 and TRINITY_DN5276_c0_g2 belong to *C2H2* and *GATA* family, respectively. They both had a positive correlation with DNA methylation ratios. The binding sequence of MYB was near the 3' of non-CpG island region, and the bZIP binding sequence was located in the 5' of non-CpG island region. The HSF binding region was between –2264 bp and –2214 bp, without covering the DNA methylation site. The binding tendency of the above transcription factors is consistent with previous studies.

In the analysis of DNA methyltransferase and DNA demethylase in *E. senticosus*, it was found that there was no significant correlation between DNA demethylase and methylation ratio, while DNA methyltransferase *EsMT*0 showed a significant positive correlation. In the study of *Citrus reticulata*, it was found that the increase in DNA methylation level was due to the decrease of demethylase rather than the increase of methyltransferase [23]. Therefore, it was speculated that these two enzymes had different methylation regulating modes in different species. In other words, some species were mainly affected by DNA methyltransferase, some were mainly affected by demethylase, and some might work together. Based on this study, it could be inferred that the process of DNA methylation in *E. senticosus* was mainly regulated by DNA methyltransferase.

Previous studies in plants showed that the expression levels of the key genes on terpenoid synthesis pathway, such as *Hevea brasiliensis* [45] and *Picrorhiza kurroa* [46], changed in the same trend. In recent studies, it was found that the expression levels of key enzymes in the terpenoid synthesis pathway of plants and animals such as *Dendroctonus* and *armandi* also changed in the same trend [47]. The analysis of protein-protein interaction with MDD in *A. thaliana* showed that there was interaction and co-expression relationship between the key enzymes in the terpenoid synthesis pathway, which may explain the above expression trend. It can be speculated that the *MDD* with different methylation status affected its own expression and regulated the expression of other key genes in the process of terpenoid synthesis through protein-protein interaction and co-expression.

5 Conclusions

The CpG island of *MDD* promoter in *E. senticosus* was subjected to DNA methylation at specific sites, which affected the binding activity of specific transcription factors and regulated the expression level of *MDD*. In this study, 28 DNA methylation sites were found in the promoter of *MDD*, of which −2205, −2196, −1572, −1430 bp sites were differentially methylated. The DNA methylation rates of cytosine were 0.68% for Type A, 0.72% for Type B and 0.79% for Type C. The content of saponins was significantly negatively correlated with the methylation ratio. Except for *bAS1*, the key genes in terpenoid synthesis pathway were significantly negatively correlated with the methylation ratio. The 4 transcription factors can bind to 4 methylation modification sites, and the expression of them was significantly correlated with DNA methylation ratios and might be affected by the protein interaction of other transcription factors. Analysis of DNA methyltransferases/demethylases in *E. senticosus* suggested that methyltransferases might play a dominant role in the process of methylation of *MDD* promoter, and the change of DNA methylation status might affect the expression of other genes in the terpenoid biosynthesis pathway through protein-protein interaction.

Authorship: The authors confirm contribution to the paper as follows: study conception and design: Zhaobin Xing, Yuehong Long; data collection: Minghui Cui, Jie Zhang, Xin Song, Shuo Wang, Jing Dong, Xuelei Zhao; analysis and interpretation of results: Minghui Cui, Limei Lin; draft manuscript preparation: Minghui Cui, Limei Lin, Zhaobin Xing, Yuehong Long. All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Appendix

Supplementary Table 1: Sequence information of *Eleutherococcus senticosus* methyltransferase protein

Supplementary Table 2: Sequence information of *Eleutherococcus senticosus* demethylases protein