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Genome-wide identification and characterization of *Dof* gene family in *Salvia miltiorrhiza*

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Abstract

Salvia miltiorrhiza is an important medicinal plant, and its main secondary metabolites are tanshinones and phenolic acids. *Dof* transcription factors play an irreplaceable role in regulating plant growth and secondary metabolism. However, the characteristics of *SmDof* genes in *S. miltiorrhiza* have not yet been studied. Based on the whole genome data of *S. miltiorrhiza*, the *SmDofs* family has been systematically explored. A total of 31 *Dof* members have been identified in *S. miltiorrhiza*, and they are clustered into five subgroups according to their evolutionary relationships. Co-expression network results indicated that two *SmDof* genes (*SmDof12* and *SmDof29*) might be involved in modulating the biosynthesis of phenolic acids or tanshinones. To investigate this hypothesis, dual luciferase experiments was introduced to examine the downstream target gene of *SmDof12* and *SmDof29*. Subsequently, it was validated that *SmDof12* inhibited the transcription of the *SmGGPPS* promoter, and *SmDof29* significantly activated the *SmPAL* promoter. The present studies offers important data about the underlying function of *SmDof12* and *SmDof29* involved in the biosynthesis of phenolic acid or tanshinone, and provides valuable insights into further research of the *SmDof* gene families in *S. miltiorrhiza*.

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Introduction

Salvia miltiorrhiza is one of the most commonly used Chinese medical herbs. As a representative species of the *Lamiaceae*, it is widely used in the treatment of cardiovascular and cerebrovascular diseases[1]. Its main active components are phenolic acids and tanshinones, of which phenolic acids consist of salvianolic acid A (Sal A), salvianolic acid B (Sal B), caffeic acid (CA), and rosmarinic acid (RA)[2–4], while tanshinones include dihydrotanshinone (DT), cryptotanshinone (CT), tanshinone I (TI), and tanshinone IIA (TIIA). The above active ingredients have various pharmaceutical values including anti-tumor, antioxidant, and anti-inflammatory effects[5–8]. In recent years, several transcription factors have been reported to participate in and regulate the synthesis of secondary metabolites of *S. miltiorrhiza*[9]. DNA binding with one finger (*Dof*) family is a typical transcription factor (TF) family with zinc finger proteins domain, which is unique to plants and plays an important role in modulating plant growth and development[10]. The *Dof* family has two main regional domains, namely the N-terminal conserved DNA binding domain and the C-terminal transcriptional regulation domain[10]. The N-terminal of the *Dof* protein is usually a highly conserved C2-C2 zinc finger domain consisting of 50–52 amino acids, and it can bind to the AAAG *cis*-acting element in the promoter region of the target gene[11]. The DNA binding domain is a key region, that is considered to be a bidirectional domain and can interact with other proteins[11–13]. The transcriptional regulatory domain of the C-terminal region may perform a variety of functions as it interacts with different regulatory proteins to activate the expression of target genes[11,13].

Dof proteins play a vital role in plant carbon and nitrogen metabolism[14,15], abiotic stress[16], hormone regulation[17], flowering control[18], light responses, and others[19]. *Dof* gene (*ZmDof1*) was first discovered in *Zea mays*[20], and it was thought to participate in the process of carbon metabolism by regulating the expression of the C4 photosynthetic phosphoenolpyruvate carboxylase (*C4PEPC*) gene in *Z. mays*[21]. *JcDof3* interacts with F-box protein to regulate photoperiodic flowering and affect the flowering time[22]. In addition, multiple studies have shown that *Dof* genes are involved in various environmental changes[23]. *OsDof18* is associated with the transport of ammonium salt in rice, thus regulating the utilization efficiency of nitrogen in rice[24] , and it can also restrict the biosynthesis of ethylene and increase prophase primary root elongation[17]. The expression of *ThDof1.4* and *ThZFP1* of *Tamarix ramosissima* can increase the content of proline and enhance the scavenging ability of ROS, thus improving the tolerance of *Tamarix* to salt stress and osmotic stress[25]. In *Arabidopsis thaliana*, *AtCDF3* was highly induced by drought, low temperature, and abscisic acid (ABA), and the overexpression of *AtCDF3* in transgenic plants enhanced their tolerance to drought, cold, and osmotic stress[26]. *SlDof22* is involved in the production of ascorbic acid and the process of tomato salt stress in tomato[27]. These studies uncovered the importance of *Dofs* in the life cycle of plants.

Plant hormones are trace compounds involved in the whole process of plant growth and development, and influence the

growth and development of plants[28]. ABA, as an important plant hormone can accelerate the shedding of plant organs, and can impact the synthesis of secondary metabolites by stimulating the corresponding transcription factors in plants[9]. In *S. miltiorrhiza*, ABA can induce the expression of *SmbZIP1* leading to the upregulation of *SmC4H1* to promote the accumulation of phenolic acids[9]. ABA can also significantly promote the expression of *HMGR*, *FPS*, *CYP71AV1*, and *CPR*, thus increasing the content of artemisinin in *Artemisia annua*[29].

In recent years, the *Dof* gene family has been gradually identified in many plants due to the continuous publication of the high quality of plant genomes. There were 36 *Dof* genes in *Arabidopsis*[30], 103 *Dof* genes in *Camelina sativa*[31], 34 *Dof* genes in melon[32], and 51 *Dof* genes in blueberry[33]. However, the *Dof* family has not been fully explored in the whole genome of *S. miltiorrhiza*. Due to the importance of the *Dof* gene in various physiological processes of plants, it is necessary to study its specific role in *S. miltiorrhiza*. In the present study, genome and transcriptome data of *S. miltiorrhiza* were used to identify the *Dof* genes. Then, multiple sequence matching, evolutionary tree analysis, gene structure, and *cis*-acting element analysis were systematically investigated in the whole genome of *S. miltiorrhiza*. To predict the function of *SmDofs* in regulating the biosynthesis of tanshinones and phenolic acids in *S. miltiorrhiza*, co-expression analysis of the biosynthetic pathway genes related to tanshinones and phenolic acids and the *SmDofs* was performed based on the transcriptome data induced by ABA, and then the target gene of candidate *SmDofs* were validated by the dual luciferase (Dual-LUC) assay. This study enlarges the understanding of the *SmDof* gene family, and reveal the potential molecular mechanism of *SmDofs* in regulating the biosynthesis of tanshinones and phenolic acids in *S. miltiorrhiza*.

Materials and methods

Identification of genes in the genome of *S. miltiorrhiza*

The genome sequences were downloaded from the *S. miltiorrhiza* database[34]. Based on the Pfam database (http://pfam.xfam.org/), the Hidden Markov Model (HMM) file of the *Dof* gene family (PF02701.18) was obtained, and the whole genome of *S. miltiorrhiza* compared using the HMMER search program in HMMER3.0 software package to obtain the gene sequence of the initial screening[35]. SMART (http://smart.embl-heidelberg.de/) and MOTIF Search (www.genome.jp/tools/motif) are employed to predict the structure of the candidate protein domains. ExPASy (http://web.expasy.org/compute_pi/) was used to calculate the sequence length, molecular weight, and isoelectric point[36]. Finally, WoLF PSORT (https://wolfpsort.hgc.jp/) was introduced to predict the subcellular localization of the identified *Dof* proteins[37].

Multiple sequence alignment and phylogenetic tree construction

The conserved domain of *SmDofs* protein was studied by multiple sequence alignment using the DNAMAN 7.0 software. The *AtDof* protein sequences of *A. thaliana* were downloaded from the TAIR database (www.arabidopsis.org)[38]. *AtDofs* and SmDof proteins were analyzed using MEGA 6.0. The phylogenetic tree was constructed using the neighborhood join method (NJ) with the bootstrap value set to 1,000[39].

Gene structure analysis and *cis*-elements of *SmDofs* promoters

The organization of exons, introns, and untranslated regions of the *SmDof* genes were analyzed using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/), and visualized by loading the GFF files of *SmDof* genes of *S. miltiorrhiza* to the TBtools (v.2.003) software, which was also used for analyzing and searching for conserved motifs[40]. PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was introduced to study the *cis*-acting elements in a length of 1,500-bp in the upstream of the initiation codon of the 31 *SmDof* genes in *S. miltiorrhiza*. According to the functional annotations of *cis*-acting elements, the candidate elements were gathered for further research[41].

RNA-seq and qRT-PCR detection

Two transcriptome datasets of *S. miltiorrhiza*, of which one is generated from four tissues including flower, stem, leaf, root, and another is collected from hairy roots induced by ABA, were adopted to analyze the expression level of *SmDof* genes[42]. TBtools (v.2.003) software was employed to draw a heat map to exhibit the expression level of the *SmDof* genes derived from transcriptome dataset[40]. To detect the expression profile of candidate *SmDof* genes, hairy roots of *S. miltiorrhiza* were treated with 50 μM ABA and collected after treatment for 0-, 0.5-, 1-, 2-, 4-, and 8-h, respectively[7] . The collected samples were quickly placed in liquid nitrogen and stored in the refrigerator at −80 °C for subsequent RNA extraction. Total RNA was extracted from *S. miltiorrhiza* hairy roots using the Plant Total RNA Extraction Kit (Vazyme Biotech Co., Ltd, China). Meanwhile, the concentration and purity of the extracted RNA was measured by spectrophotometer, and then the RNA integrity was observed by electrophoretic analysis with 1% agarose gel. Reverse transcription was performed with the cDNA Synthesis Kit (Vazyme Biotech Co., Ltd, China), and a total of 100 ng RNA was prepared for cDNA synthesis reaction with a volume of 50 μL[43]. Quantitative primer pairs were designed using the Primer 5.0 software. SuperReal PreMix Plus kit (Vazyme Biotech Co., Ltd, China) was used in ABI Step One Plus real-time PCR System. Quantitative real-time PCR (qPCR) was performed using 10 μL real-time PCR reaction solution, including 1 μL cDNA was used as a template; the upper and downstream primers were 0.2 μL, respectively; 5 μL Taq Pro SYBR qPCR Master Mix and 3.6 μL ddH_2O. The PCR reaction conditions were as follows: 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, a total of 40 cycles, each sample was triply repeated. *SmActin* was used as the internal reference gene to normalize the expression level of *Dof* genes. The method of $2^{-\Delta\Delta CT}$ was used to calculate the relative expression level of *SmDofs*[7].

Co-expression analysis

The co-expression relationship between the *SmDof* genes and the biosynthetic genes involved in tanshinones and phenolic acids biosynthesis was resolved. Pearson correlation coefficient > 0.8 and p-value < 0.05 was set as the cutoff. Then, the co-expression relationship was visualized with the Cytoscape software[44].

Subcellular localization

To dissect the subcellular localization profiles of *SmDof* proteins, the open reading fragment (ORF) cDNA sequences of *SmDof12* and *SmDof29* are amplified and inserted into the

vector of *PHB-YFP* to generate the fusion recombinant of *PHB-SmDof12-YFP* and *PHB-SmDof29-YFP*, and then they are transformed into *Agrobacterium tumefaciens* GV3101 and injected into *N. benthamiana* leaves for transient transformation, respectively[3]. pHB-YFP was used as the negative control. The transgenic *N. benthamiana* leaves were cultivated in the dark for 24 h and then transferred to the light for 24 h. YFP signals from infected *N. benthamiana* leaves were visualized using a high-resolution microscope observation system. The nuclei of epidermal cells of infected *N. benthamiana* leaves were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (10 μg/mL) for 2 h before observation.

Dual-luciferase assay

To investigate the ability of *SmDofs* to transcriptionally activate the tanshinones biosynthetic genes, Dual-luciferase (Dual-LUC) assays were performed as previously reported[45]. Each of the recombinant plasmids of *PHB-SmDof12-YFP* and *PHB-SmDof29-YFP* was introduced into *A. tumefaciens* strain GV3101 to be the effector, and *PHB-YFP* plasmid was used as a negative control. The promoters of *PAL* and *GGPPS* were inserted into pGREEN0800 vector as the reporter constructs to drive the expression of the firefly luciferase gene, respectively. The Renilla luciferase gene driven by CaMV 35S promoter was used as an internal control. And then, each of them was co-transformed into *A. tumefaciens* strain GV3101 with the helper plasmid pSoup19. The reporter strains were mixed with effector strains at a ratio of 1:1 to inject into *N. benthamiana* leaves. Leaves were collected after 48 h for determination of fluorescence values according to the manufacturer's instructions (Promega, Madison, WI, USA)[9]. Three biological replicates were measured for each sample.

Measuring tanshinones and phenolic acids contents by high-performance liquid chromatography

Different tissues including roots, stems, leaves, and flowers of *S. miltiorrhiza* were collected and dried in an oven. The dried tissues were then ground to powder for compound analysis. Extraction of tanshinones and phenolic acids and high-performance liquid chromatography (HPLC) detection were done as the previous report[6,9]. The total content of tanshinones and phenolic acids were quantified by comparing the standard curves and retention times, with solutions without extracts added as the controls.

Statistical analysis

All the detections performed in the present study, including qRT-PCR, HPLC, and Dual-LUC assays, were triply repeated. Gene expression levels, tanshinone contents, and phenolic acid contents were presented as the mean value ± SD. SPSS 16.0 software (SPSS) was employed to analyze statistical significance by single-sample t-test and one-way analysis of variance. p-value < 0.05 was regarded to be statistically significant.

Results

Identification of the *SmDof* genes

The Hidden Markov model (HMM) of the *Dof* domain (PF02701.18) was employed to search for *Dof* genes in *S. miltiorrhiza*. A total of 31 *Dof* genes were detected, and the gene was named *SmDof1-SmDof31*, respectively (Supplementary Table S1). The results of Pfam and SMART analysis showed that all of these proteins contained complete *Dof* domains[23]. The CDS length, protein molecular weight (MW), isoelectric point (pI), and subcellular location of each *SmDof* gene in *S. miltiorrhiza* were further analyzed (Table 1). Of the 31 proteins, *SmDof25* and *SmDof22* had the lowest number of amino acids, decreasing to 168, while *SmDof16* had the highest number of amino acids, reaching to 511. The pI of *SmDofs* ranges from 6.01 (*SmDof5*) to 10.55 (*SmDof17*), and the molecular weight ranges from 18,463.7 (*SmDof22*) to 55,341.6 (*SmDof16*). Subcellular localization prediction revealed that 27 *SmDofs* were located in the nucleus, while four *SmDofs* including *SmDof19*, *21*, *22*, and *25* located in chloroplasts (Table 1).

Sequence alignment and phylogenetic traits of *SmDof* proteins

To dissect the characteristics of the domain within *SmDof* proteins, DNAMAN software was employed to conduct multiple amino acid sequence alignment. The results showed that all the *SmDof* proteins contained a conserved domain in its core sequence, namely CX2CX21CX2C zinc finger structure (Fig. 1). The conserved domain consists of 50 amino acid residues, of them four cysteine residues are relatively conserved within the zinc finger domain in the N-terminal region of *SmDof* proteins[11].

To further explore the evolutionary relationships among the *SmDof* genes, a phylogenetic tree of a total of 67 *Dof* proteins

Table 1. Length, molecular weight, isoelectric point, and subcellular localization of 31 *SmDof* proteins in *S. miltiorrhiza*.

Gene ID	Name	Length (aa)	MW (Da)	pI	Subcellar localization
SMILT016590.1	*SmDof1*	304	33,168.7	8.66	nucleus
SMILT016591.1	*SmDof2*	246	26,202.9	9.8	nucleus
SMILT016651.1	*SmDof3*	242	26,184.9	8.96	nucleus
SMILT021318.1	*SmDof4*	225	23,560.2	8.6	nucleus
SMILT032678.1	*SmDof5*	224	24,689.4	6.01	nucleus
SMILT003591.1	*SmDof6*	241	25,256.1	4.66	nucleus
SMILT009582.1	*SmDof7*	306	32,436.3	4.69	nucleus
SMILT017417.1	*SmDof8*	301	33,248.9	6.7	nucleus
SMILT020107.1	*SmDof9*	332	36,827.9	7.94	nucleus
SMILT023380.1	*SmDof10*	318	34,176.8	9.72	nucleus
SMILT025505.1	*SmDof11*	283	30,690.9	8.48	nucleus
SMILT025760.1	*SmDof12*	274	30,006.1	8.47	nucleus
SMILT028288.1	*SmDof13*	249	27,303.1	8.99	nucleus
SMILT030586.1	*SmDof14*	334	36,582.9	6.92	nucleus
SMILT031093.1	*SmDof15*	230	23,444.9	8.49	nucleus
SMILT000323.1	*SmDof16*	511	55,341.6	5.23	nucleus
SMILT000784.1	*SmDof17*	265	27,611.4	10.55	nucleus
SMILT000789.1	*SmDof18*	198	22,350	9.04	nucleus
SMILT001058.1	*SmDof19*	190	20,795.1	9.27	chloroplast
SMILT001687.1	*SmDof20*	216	24,023.7	9.28	nucleus
SMILT002891.1	*SmDof21*	268	29,359.3	4.54	chloroplast
SMILT004451.1	*SmDof22*	168	18,463.7	8.83	chloroplast
SMILT005491.1	*SmDof23*	266	29,274.4	9.31	nucleus
SMILT007077.1	*SmDof24*	251	27,427	9.06	nucleus
SMILT007580.1	*SmDof25*	168	18,625.9	9.22	chloroplast
SMILT009335.1	*SmDof26*	191	21,529.9	9.5	nucleus
SMILT010473.1	*SmDof27*	240	24,863.9	7.82	nucleus
SMILT012697.1	*SmDof28*	248	26,508.7	9.28	nucleus
SMILT019592.1	*SmDof29*	283	30,740.7	8.39	nucleus
SMILT023561.1	*SmDof30*	337	35,816.5	9.51	nucleus
SMILT024154.1	*SmDof31*	258	27,841.9	8.06	nucleus

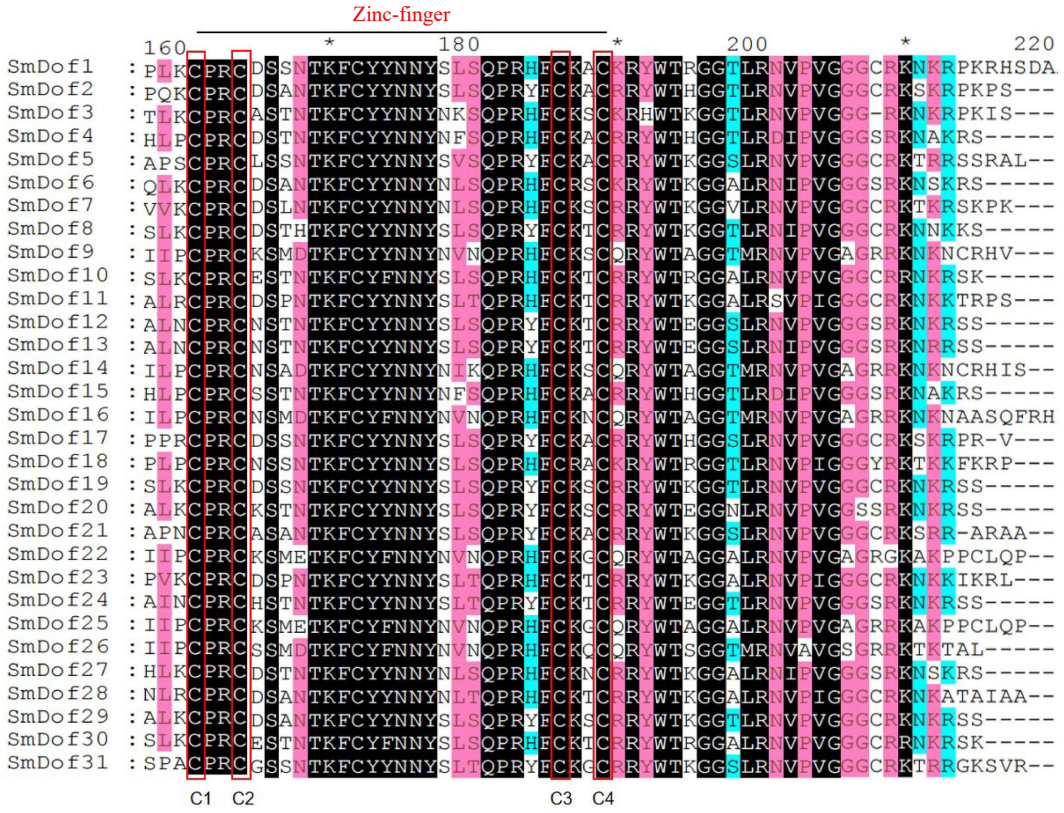

Fig. 1 Multiple sequence alignment of the 31 *SmDof* proteins. Different colors represent identical and conserved amino acid residues, and the red box shows the conserved zinc-finger domain.

(Supplementary Table S2) in *Arabidopsis* (36 members) and *S. miltiorrhiza* (31 members) were constructed. The total number of *Dof* genes in *S. miltiorrhiza* and *A. thaliana* is comparatively secure, and it indicates the conservative features of this gene family. Sixty seven *Dof* proteins are divided into five groups based on the branch of the tree, Groups I–V (Fig. 2). There are 31 *SmDof* gene families in *S. miltiorrhiza*, among them, six *SmDofs* are distributed in Group I and Group IV, 11 in Group II, and eight in Group III. In *Arabidopsis*, Groups I to IV contain 7, 0, 0, 10, and 19 *Dof* genes, respectively. The variable number of the five subgroups is beneficial for us to evaluate the degree of gene expansion or loss during the evolution of the two species.

Structural feature of *SmDof* genes

To further investigate the functional regions of *SmDof* proteins, the conserved motif was predicted by the MEME program utilizing a two-component finite mixture model. In total, 15 motifs were identified in all the *SmDof* proteins, and we found that many groups of *SmDofs* shared a similar conserved motif. As shown in Fig. 3a and b, motif 1 is included in all *SmDof* proteins. Among all groups, Group I contained the most *SmDof* members being consisted of motifs 1, 2, 3, 8, and 15. The common motifs among the *SmDof* proteins are indicative of conserved evolutionary relatedness and similar biological functions.

To study the structure of *SmDof* genes, the full-length cDNA sequences of all *SmDof* genes with the corresponding genomic DNA were aligned (Fig. 3c). The number of exons in *SmDof* ranged from 1 to 2. There were no more than two introns in each *SmDofs*. The variation in the number of exons may indicate that the *SmDof* genes may have diverse functions related to the medicinal substance biosynthesis, growth, or development in *S. miltiorrhiza*.

Cis-acting elements in the promoter region of *SmDof* genes

PlantCARE was introduced to analyze the promoter sequence of 31 *SmDof* genes from the translation initiation site (ATG), and 55 *cis*-acting elements were identified. Among of them, they were related to plant cell development, plant hormones, environmental stress, and light response, respectively (Fig. 4). The results show that 22 light-responsive elements get the most abundant compared to other elements, and 31 *SmDof* genes have light-responsive elements like Box 4, MRE, GT1-motif. In addition, 12 *cis*-acting elements related to plant hormones were identified. In addition, there are five *cis*-acting elements associated with cell development, like CAAT-box, HD-Zip 1, MBSI, CCAAT-box, and MSA-like. There are four *cis*-acting elements associated with environmental stress, like TC-rich repeats, AT-rich element, LTR, and MBS (Fig. 4). It is implied that most of the *SmDofs* may play an important role in response to plant hormones and are light responsive. This is in agreement with the previous studies on *Dof* gene families in sugarcane, which is thought to be involved in light response, metabolism, and other functions[19].

Expression patterns of *SmDofs* and synthetase genes involved in tanshinones and phenolic acids biosynthesis pathway in various tissues and under ABA treatment

To gain a deeper understanding of *SmDof* expression patterns, four tissues including root, stem, leaf, and flower were

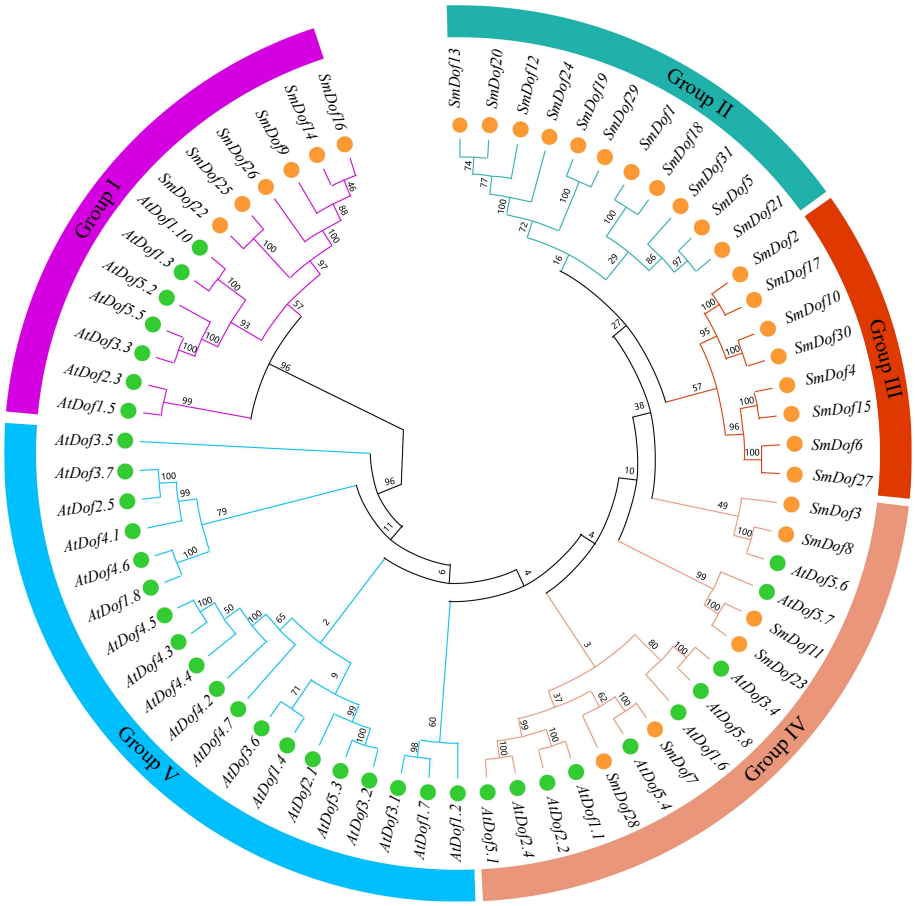

Fig. 2 Evolutionary relationship of *SmDof* proteins in *S. miltiorrhiza* and *Arabidopsis*. Varied colors represent different groups. There were five groups, Groups I–V, with the green circles representing the *SmDof* proteins of *Arabidopsis* and the orange circles representing the *Dof* proteins of *S. miltiorrhiza*.

collected to measure the total content of tanshinones and phenolic acid by HPLC, and subjected to transcriptome sequencing to investigate the expression of *SmDofs*. The results showed that the total phenolic acids and tanshinones content were all highest in root compared to other tissues (Fig. 5a), and a total of five genes (*SmDof6*, *12*, *13*, *27*, *29*) were highly expressed in the root, which is harvested in practice as the medicinal tissue[3] (Fig. 5b & Supplementary Table S3).

To mine the candidate *SmDofs* in response to ABA treatment, the expression variation of candidate *SmDof* gene exhibiting at least a 2-fold increase more than the control was set as the cutoff. In total, 11 *SmDof* genes including *SmDof9*, *16*, *18*, *21*, *22*, *23*, *24*, *25*, *26*, *28*, and *29* showed an obvious increase compared to the control, among which three *SmDofs* (*SmDof22*, *25*, and *26*) exhibited the highest increase reaching to a 17-fold increase over the control. Whereas, seven *SmDof* gens including *SmDof4*, *6*, *12*, *14*, *15*, *18*, and *20* downregulated the expression levels more than 2-fold than the control (Fig. 5c &Supplementary Table S4). Moreover, qRT-PCR was employed to examine the expression level of synthetase genes involved in the tanshinones and phenolic acids biosynthesis pathway. As shown in Fig. 5d, several genes were revealed including *PAL*, *C4H*, *TAT*, *RAS1*, and *CYP98A14* in phenolic acid biosynthesis pathway and *GGPPS* in tanshinone biosynthesis pathway upregulated significantly under the induction of exogenous ABA, in particular, *PAL*, and *GGPPS* were the most up-regulated. Therefore, the above results provide a valuable dataset for mining functional *SmDof* genes in regulating medicinal substance metabolite synthesis in *S. miltiorrhiza*.

Co-expression relationship of *SmDofs* with the biosynthetic genes involved in tanshinones and phenolic acids biosynthesis in *S. miltiorrhiza*

As reported by Shi et al., ABA can affect the expression of the biosynthetic genes involved in tanshinones and phenolic acids biosynthesis, thereby promoting the medicinal metabolites accumulation in *S. miltiorrhiza* hairy roots[42]. Therefore, the co-expression relationship between the 31 *SmDofs* with the biosynthetic genes related to the biosynthesis of tanshinones and phenolic acids in *S. miltiorrhiza* was dissected. The results showed that 15 *SmDofs* (including *SmDof4*, *5*, *8*, *10*, *12*, *13*, *14*, *15*, *17*, *19*, *20*, *28*, *29*, *30*, *31*) co-expressed with *SmRAS*, *SmHPPR*, *SmC4H*, *Sm4CL*, *SmCYP98A14*, *SmPAL*, or *SmTAT* genes, respectively, with the Pearson correlation coefficient > 0.8 and *p*-value < 0.05. Moreover, 15 *SmDofs* (including *SmDof4*, *6*, *9*, *10*, *11*, *12*, *13*, *14*, *15*, *17*, *20*, *27*, *28*, *29*, *30*) exhibited a co-expression pattern with *SmCYP76AH1*, *SmKSL*, *SmCPS*, *SmGGPPS*, *SmDXR*, or *SmDXS2* genes, respectively, and the correlation coefficient was greater than 0.8. It is noteworthy that *SmDof4*, *10*, *12*, *13*, *14*, *15*, *17*, *20*, *28*, *29*, *30* not only co-express with tanshinones biosynthetic genes, but also co-express with phenolic acids biosynthetic genes, implying that the above 11 *SmDof* genes may play

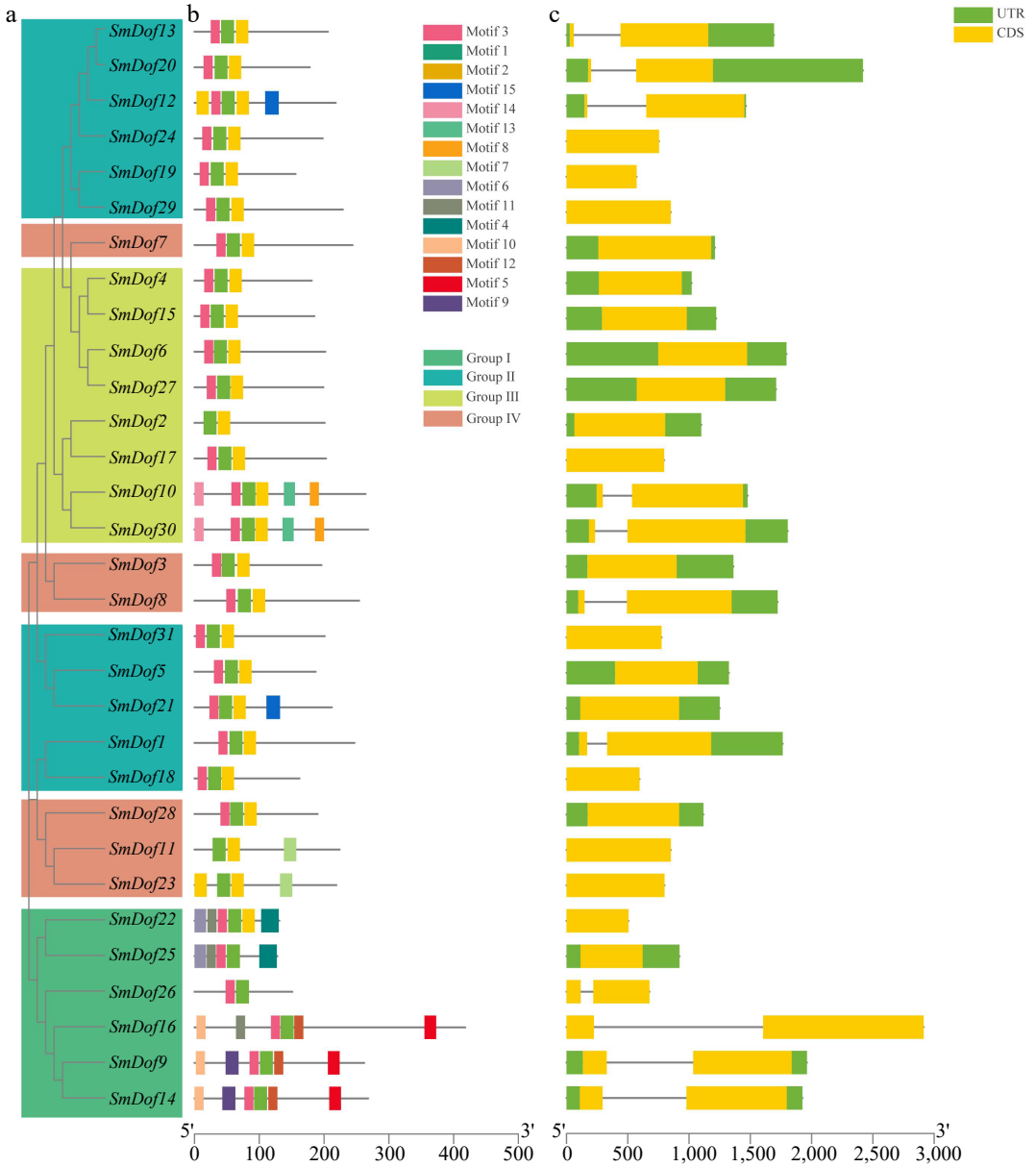

Fig. 3 Phylogeny, conserved motifs and gene structure of *SmDof* proteins in *S. miltiorrhiza*. (a) *SmDof* proteins evolutionary tree. (b) Conserved motifs of the 31 *SmDof* proteins. Different colors represent 15 different motifs, and the bottom line represents the length of the sequence. (c) Exon/intron structures of *SmDofs*. Green represents UTRs and yellow represents CDS.

a vital role in promoting the accumulation of the above two types of medicinal substances.

Expression pattern and subcellular localization analysis of *SmDof12* and *SmDof29* genes

Transcriptome dataset and co-expression analysis were integrated to mine the candidate *SmDof* genes in association with the biosynthesis of tanshinones and phenolic acids in *S. miltiorrhiza*. By the transcriptome dataset from various tissues, five *SmDof* genes were found including *SmDof6*, *12*, *13*, *27*, and 29 all expressed vigorously in the root (Fig. 5b), which is thought to be the main tissue to accumulate the medicinal substances in practice[3]. According to the results of co-expression analysis, *SmDof12* had the highest negative correlation coefficient (reaching −0.917) with the *SmGGPPS* gene related to the biosynthesis of tanshinones. Whereas, *SmDof29* got the highest correlation with the *SmPAL* gene involved in the phenolic acids biosynthetic pathway, with the correlation coefficient of 0.912 (Fig. 6). Those results push us to validate the expression profile of the two *SmDof* genes. As expected, the expression profiles of *SmDof12* and *SmDof29* detected by qRT-PCR analysis were consistent with the transcriptomic dataset (Fig. 7a, b & Supplementary Table S5), of which indicated the reliability of the transcriptome dataset derived from four types of tissues and hairy root lines treated with ABA in *S. miltiorrhiza*.

And then, the subcellular localization of the *SmDof12* and *SmDof29* in epidermal cells from 45-day-old *N. benthamiana* leaves were studied by transient expression analysis of the two genes fused with YFP, respectively. Robust fluorescence was observed only in the nuclei in *35S-SmDof12-YFP* and *35S-SmDof29-YFP*, while the *35S-YFP* control displayed fluorescence throughout the whole cell (Fig. 7c), suggesting that the

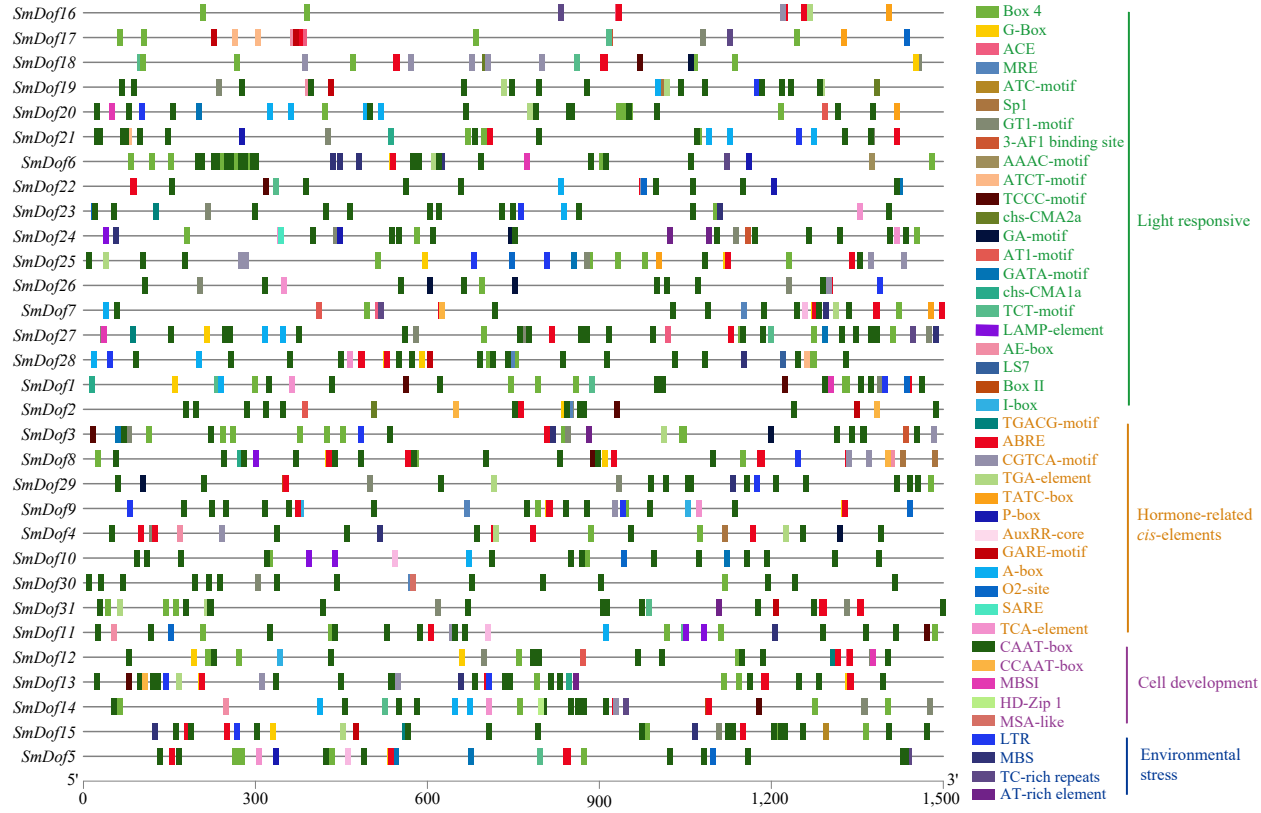

Fig. 4 *Cis*-acting elements of *SmDof* promoters in *S. miltiorrhiza*. *Dof* family *cis*-acting element of *S. miltiorrhiza*. Different colors represent different classes of *cis*-acting elements and motifs. Green represents *cis*-acting elements associated with light, yellow represents *cis*-acting elements associated with plant hormones, purple represents *cis*-acting elements associated with cell development, and blue represents environmental stress.

SmDof12 and *SmDof29* proteins are all localized in the nuclei in *S. miltiorrhiza*.

Validation of the target genes of *SmDof12* and *29* proteins by Dual-LUC assay

According to the results of co-expression analysis, it pushes the exploration of the underlying mechanism of *SmDof12* and *SmDof29* in regulating tanshinones and phenolic acids. By Dual-LUC assay (Fig. 7d), it was revealed that *SmDof12* could uniquely inhibit the transcription of the *SmGGPPS* promoter, leading to a 3-fold decrease compared to the *35S-YFP* control, whereas, *SmDof29* significantly activated the *SmPAL* promoter up to 1.69-fold compared to the control. Those results indicated that *SmDof12* might inhibit the biosynthesis of tanshinones by decreasing the activity of the *SmGGPPS* promoter, while *SmDof29* activated the transcription of *SmPAL* to increase the production of phenolic acids in *S. miltiorrhiza*.

Discussion

Dof genes widely exist in plants and have been validated to participate in diverse biological functions[14,15]. *S. miltiorrhiza* is a valuable traditional Chinese herbal plant and has been used widely in clinic treatments[1]. Genome-wide identification of *SmDof* gene lays a foundation for the subsequent study of its function. In the present study, a total of 31 *Dof* genes were identified in *S. miltiorrhiza*, and the number of *SmDof* genes was comparative to that of *A. thaliana* (36 members)[30], rice (30 members)[32], and tomato (34 members)[46]. The genome size of the above plants varied greatly, but the number of *Dof* proteins was not related to the size of the genome thus implying its conserved function in the above plant species.

Multiple sequence alignment uncovered the conserved domain within the *SmDof* proteins in *S. miltiorrhiza*. Phylogenetic tree construction showed that the *SmDofs* got low homology with *Arabidopsis*, and only Group I together with Group IV had more than six *Dof* genes getting high sequence similarity between *S. miltiorrhiza* and *Arabidopsis*. Previous studies have confirmed that phylogenetic analysis can provide a valuable theoretical basis for functional prediction of similar genes in different species[46]. Genes clustering in the same subgroup are relatively conserved in gene structure, gene expression patterns, and functional evolution[47]. By phylogenetic tree construction, it was found that *AtDof5*.4 and *SmDof7* had high homology, and they were grouped into the same branch. Previous studies have verified that *AtDof5*.4 is a negative regulator modulating cell proliferation and expansion in *Arabidopsis*[48], so it is speculated that *SmDof7* may also have the same function as *AtDof5*.4. Indeed, *SmDof7* got the highest expression level in the stem and root, indicating that *SmDof7* might regulate the cell proliferation and expansion in stem and root of *S. miltiorrhiza*.

The diverse structure and organization of the *Dof* genes, is associated with the evolution and functional differentiation of this gene family in certain species[49]. Gene structures analysis of all the *Dof* genes in *S. miltiorrhiza* exhibited visible variation between different subgroups, while similar structures were

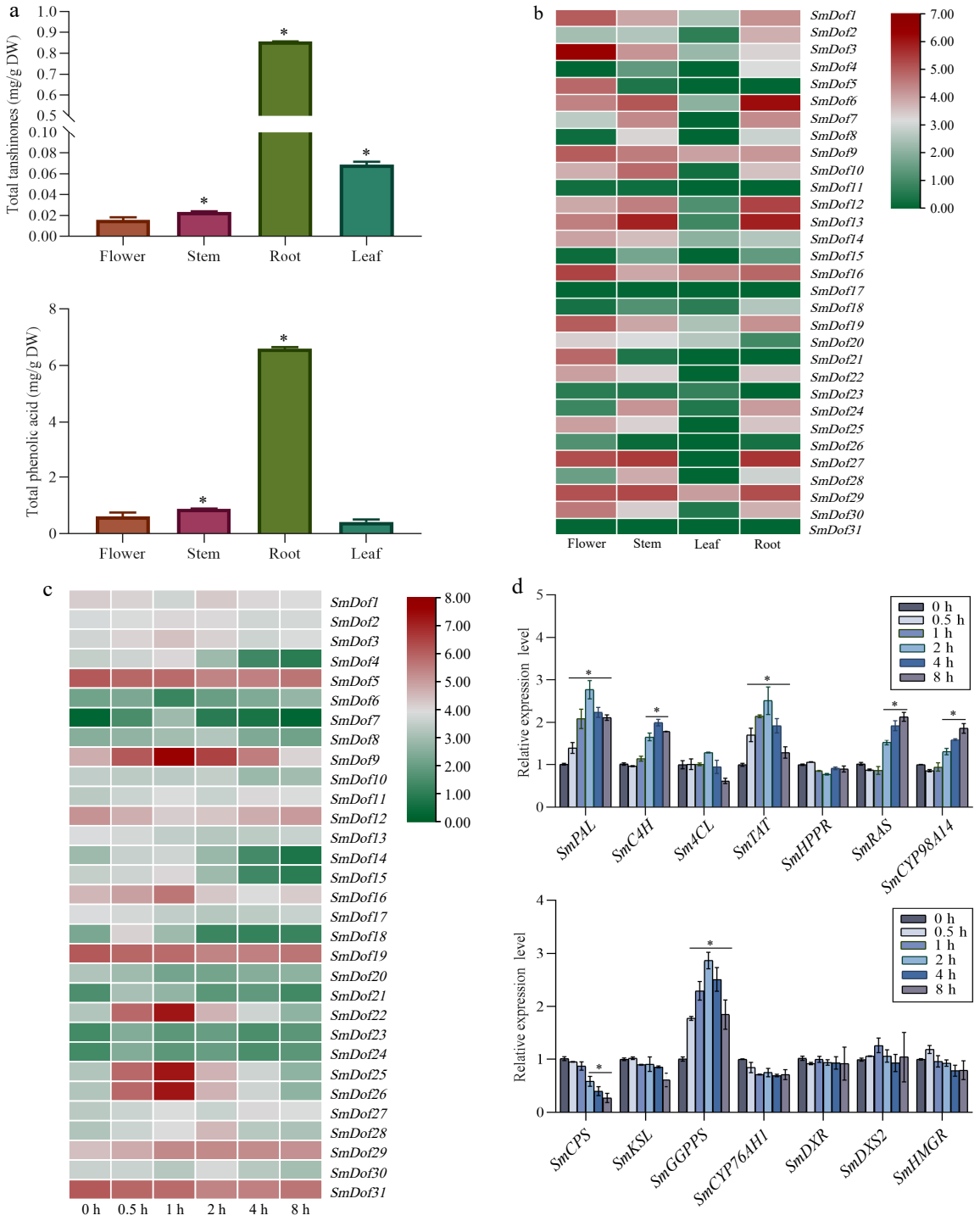

Fig. 5 Expression profiles of *SmDof* genes and synthetase genes involved in tanshinones and phenolic acids biosynthesis pathway. (a) Contents of tanshinones and phenolic acids in different tissues of *S. miltiorrhiza*. (b) Expression profiles of *SmDof* gene in various tissues of *S. miltiorrhiza*. (c) Expression profile of *SmDof* genes under the treatment of ABA induction based on the transcriptome dataset. Red and blue boxes indicate high and low expression levels of *SmDofs*, respectively. (d) Expression profiles of synthetase genes involved in tanshinones and phenolic acids biosynthesis pathway under the induction of exogenous ABA detected by qRT-PCR. Three biological replicates were performed and the mean ± SD was taken, SD was represented by the error line. * indicates significant difference in *t*-test (*$p < 0.05$).

observed within the same subgroup (Fig. 3c). In general, the structure of the 31 *SmDof* genes was relatively simple and contained one or two exons, of which it was similar to the previous studies on melon[15]. However, 11 *SmDof* genes (including *SmDof 11*, *16*, *17*, *18*, *19*, *22*, *23*, *24*, *26*, *29*, *31*) had only one intron or even no intron (Fig. 3c). As previously reported, the intron-less genes may be associated with the quick stress response[50].

In previous reports, many *Dof* genes have been validated to be a key regulatory center involved in secondary metabolic

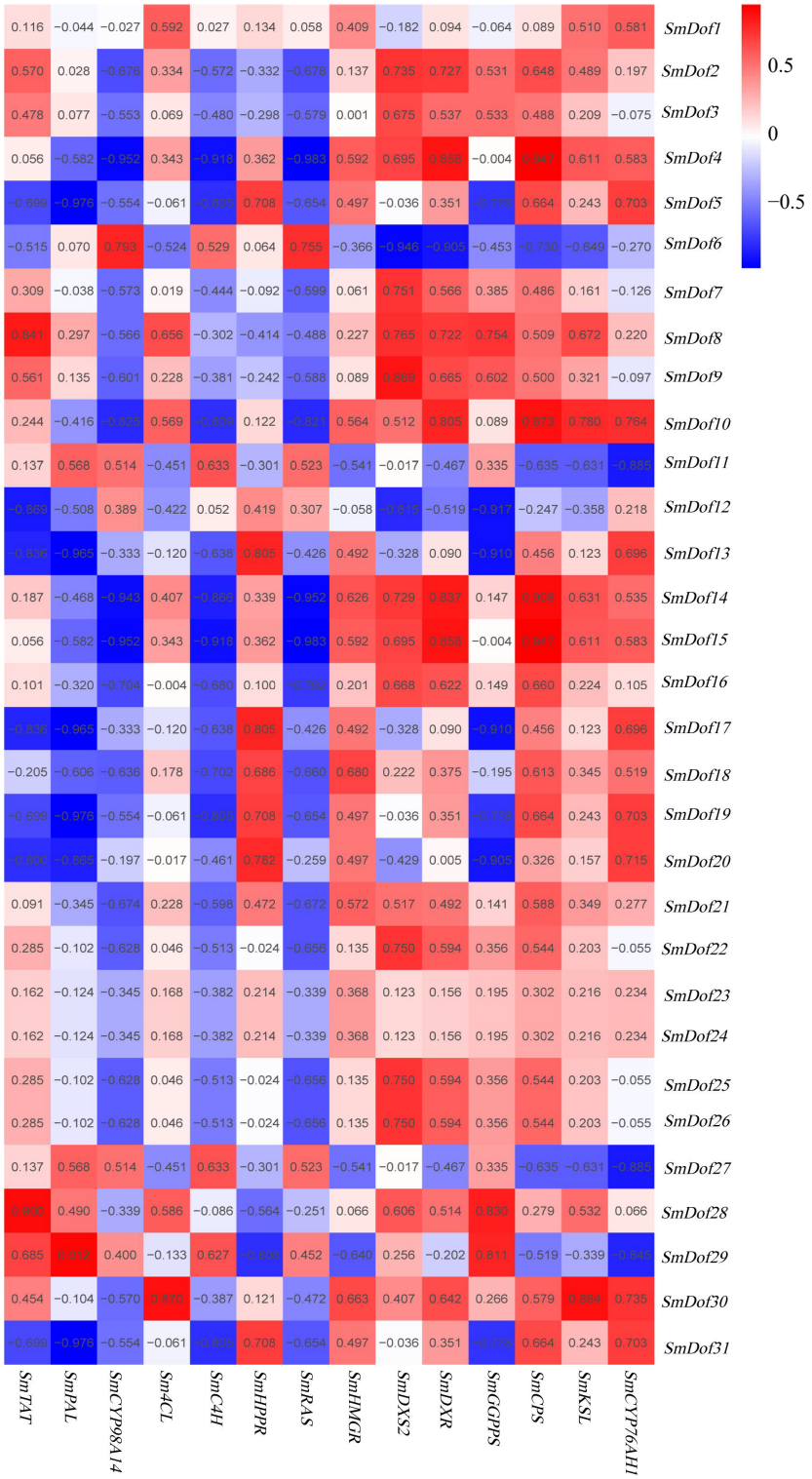

Fig. 6 Co-expression analysis of *SmDof* genes and the genes involved in the biosynthetic pathway of phenolic acids and tanshinones. R > 0.5 indicates a positive correlation; R < –0.5 indicates a negative correlation. The data were obtained from the ABA transcriptome dataset.

synthesis, abiotic stress response, and hormone regulation pathway[23,51,52]. In grape (*Vitis vinifera* L.), *VyDof8* was validated to be induced by a variety of abiotic stress. Overexpression of *VyDof8* in tobacco (*Nicotiana tabacum*) significantly elevated ABA accumulation and drought tolerance during prolonged droughts compared to the control plants[53]. The expression pattern of candidate genes in a certain tissue or under stress signal is often closely related to the function of these genes[54]. Therefore, in this study, the transcriptome dataset of *S. miltiorrhiza* was introduced to dissect the expression profiles of *SmDof* gene families in root, stem, leaf, and flower. Most of the *SmDof* genes (*23* out of *31*) are expressed in the root of *S. miltiorrhiza*. It

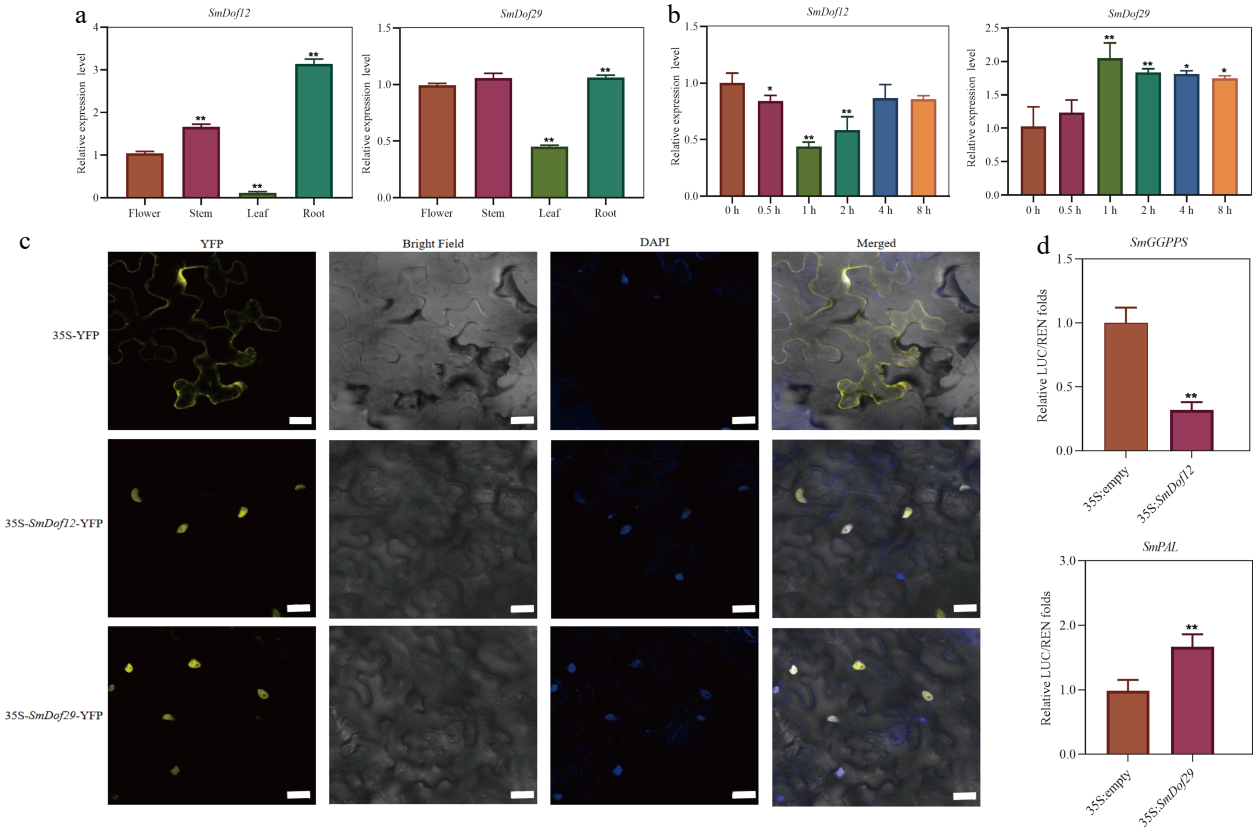

Fig. 7 Functional characterization of *SmDof12* and *SmDof29*. (a) Expression patterns of *SmDof12* and *SmDof29* in four tissues of *S. miltiorrhiza*. The fold changes of the relative gene expression level in the other three tissues are all normalized to the expression level in flower. (b) Expression patterns of *SmDof12* and *SmDof29* in hairy roots of *S. miltiorrhiza* treated with ABA. The fold changes in the relative gene expression level were all normalized to the control expression without induction at the 0 h time point. (c) Subcellular localization of *SmDof12* and *SmDof29* in tobacco. 35S-YFP is the control group, yellow is the fluorescence of YFP, and blue is the nucleus. Scale bar = 50 μm. (d) Dual-Luc assay showed that *SmDof12* could inhibit the activity of *SmGGPPS* promoter and *SmDof29* could promote the activity of *SmPAL* promoter. Three biological replicates were performed and the mean ± SD was taken, SD was represented by the error line. * indicates significant difference in *t*-test (* $p < 0.05$, ** $p < 0.01$).

is speculated that some members of the 23 *SmDof* genes may be related to the growth and development of *S. miltiorrhiza* roots. It was also revealed that *SmDof3* and *16* are highly expressed in flower (Fig. 5b). As a *Dof* gene, *CDF3* in tomato (*Lycopersicon esculentum*) getting high gene sequence homology with *SmDof3* and *16* were validated to regulate the flowering time through modulating the expression of FT-like genes[55]. Therefore, the research project whether overexpression of *SmDof3* or *16* in *S. mitiorrhiza* has a significant impact on regulating its flowering time or growth is worthy of inquiry. The varied expression profiles of *SmDof* genes in various tissues provide basic data to explore their functions.

By investigating *cis*-acting elements within promoters, it indicates that the *SmDof* genes are related to light response, hormone-related response, cell development, and environmental stress (Fig. 4). In the present study, several *SmDof* genes (eg. *SmDof12* and *SmDof29*) were found to contain ABA-responsive elements (ACGTG) in putative promoter regions. Through the transcriptome dataset together with gene expression validation detected by qRT-PCR, it was confirmed that *SmDof12* and *29* expressed vigorously in *S. miltiorrhiza* root (Fig. 5b; Fig. 7a, b). Furthermore, *SmDof12* and *29* were verified to co-express with the metabolic pathway genes involved in tanshinones or phenolic acids biosynthesis (Fig. 6). This pushes the exploration of the underlying molecular mechanism of how *SmDof12* and *29* to regulate the expression of the downstream target gene to modulate the tanshinones and phenolic acids biosynthesis. Subsequently, *SmGGPPS* and *SmPAL* were validated to be the target of *SmDof12* and *SmDof29* by Dual-LUC assay, respectively (Fig. 7d). Through the same strategy, in *Scutellariae baicalensis*, it is verified that *SbNAC25* reduces the synthesis of flavonoid by downregulating the expression of *FNSII-2, OMT2, CHI*, and *F6H2* genes[56]. The fact that the gene expression profile in special tissues and under certain stress treatment detected by transcriptome sequencing and qRT-PCR validation in combination with gene co-expression analysis is a quite valid strategy to mine the candidate regulatory genes and their downstream target genes involved in the biosynthetic pathway of secondary metabolite in many plants[57,58].

Conclusions

In the present study, the *SmDof* gene families in *S. miltiorrhiza* were characterized based on the whole genome, transcriptome dataset, and qRT-PCR expression analysis. Two *SmDof* genes (*SmDof12* and *SmDof29*) were mined by gene co-expression strategy from the identified 31 *SmDofs*, and their target genes of *SmDof12* and *SmDof29* were validated by

Dual-LUC experiments. This study is the first comprehensive analysis of the *SmDof* gene families in *S. miltiorrhiza*, and provides valid data for further exploring the underlying molecular mechanism of *SmDofs* in response to ABA induction. It may also be beneficial to elucidate the diverse biological functions of *Dof* genes in other plants.

Author contributions

The authors confirm contribution to the paper as follows: study conception and supervision: Zhou W, Kai G, Zhu J; study design: Wang X, Wang Q, Hao S; experiment performance and data analysis: Wang X, Wang Q, Hao S; manuscript suggestions: Wang X, Zhou W; draft manuscript preparation: Wang X, Zhou W. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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