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Original Research

Identification, Characterization and Expression Profiles of *Dof* Transcription Factors in Common Walnut (*Juglans Regia* L.)

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Abstract

Juglans regia is a hardwood tree of economic importance and produced high-quality wood and edible nuts. *Dof* (DNA binding with one finger) are plant-specific transcription factors (TF) family and has an important role in the development of different tissues. However, no study of the *Dof* gene family in Persian walnut (*J. regia*) has been reported to our knowledge. We identified 39 *JrDof* genes in the genome of *J. regia*. *Dof* family genes clustered into 06 groups based on the phylogenetic tree and contain a highly conserved motif. The motif composition and conserved domain analysis showed that the common walnut *Dof* gene family contains one conserved motif and one conserved domain Zf-*Dof*. Most of the *Dof* genes contain 1 and 2 exons, as revealed by structural analysis. All *JrDof* genes distributed unevenly on 16 chromosomes with the maximum member (4 genes) found on chromosome 3, 4, 6, 7, and 12. High expression of *JrDof12-1*, *JrDof15-3*, *JrDof6-4*, *JrDof5-2*, and *JrDof13-2*, (female flowers), *JrDof10-1*, *JrDof3-3*, and *JrDof14-1*, (male flowers), *JrDof15-3* and *JrDof13-2*, (embryo), *JrDof4-2*, *JrDof19-1*, *JrDof15-2*, and *JrDof12-4*, (leaves, fruit and roots) were identified. RNA-Seq data also confirm that *Dof* genes play a role in the development of *J. regia* male and female flowers.

Keywords: DNA binding with one finger, *Juglans regia*, expression profile, transcription factor, Phylogenetic analysis

Introduction

The *Dof* domain includes a *C2C2* zinc finger motif with 50-52 amino acids, which is characterized by the AAAG- element in the promoter [1, 2]. In plants, *Dof* transcription factor share a highly conserved domain (*zf-Dof*) [3]. The *Dof* domain has bifunctional connections of DNA-protein and protein-protein interactions [4]. *Dof* proteins contain a conserved *Zf-Dof* domain located at the N-terminal region and a transcriptional regulation domain at the C-terminus [5]. The primary *Dof* gene (*ZmDof1*) was first recognized in maize, which is known for its response to light and transcriptional regulation of genes involved in carbon absorption [6, 7]. *Dof* TFs are not found in human beings, yeast, drosophila, and other eukaryotes.

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Transcription factors (TFs) are sequence-specific DNA binding proteins that can hinder or promote gene expression [8]. Thirty-six putative *Dof* genes have been recognized in the genome of *Arabidopsis thaliana* [9]. In *Arabidopsis thaliana,* a total of 9/36 genes were identified that regulate flowering, phytochrome signaling, and seed germination [10, 11]. In *Arabidopsis*, *Dof* genes contain *DAG2* and *DAG1* genes related to seed germination [12]. The genes *CDF1*, *CDF2,* and *CDF3* controlled the photoperiod of flowering plants [13], while phytochrome signaling and cell cycle regulated by *OBP1,* and *OBP3* [10, 14]. In rice, the gibberellin-regulated expression characterized by *OsDof3* [15, 16]. The phosphoenolpyruvate carboxylase gene together with *Dof1* in corn has been revealed as an activator of gene expression related to carbohydrate metabolism [17, 18], and maize *Dof2* seems like a repressor capable of blocking *Dof1* transactivation [19]. The carbon metabolism in wheat is associated with *TaDof1* [20]. The *JrGRAS2* is a beneficial factor for plant high-temperature tolerance associated with *Dof* TF in Persian walnut [21]. *Dof* genes are involved in multiple biological processes such as the regulation of flower development [22], pigmentation and seed proteins [23], nitrogen and carbon assimilation [18], seed germination [4] hormone response [24] and light-mediated regulation [25]. Herbaceous plant species were the main focused of these studies. Contrasting the *Dof* genes of economically important woody and fruit tree species have received less consideration. This study provided valuable clues for the functional characterization of *Dof* gene family members in *J. regia*.

Persian walnut is a diploid (2n = 32), large, wind-pollinated, monoecious, dichogamous, enduring, perennial tree and vital nut tree on earth belonging to the family Juglandaceae [26, 27]. *Juglans* are the most important tree because of its wood and nut since ancient times [28-30]. We used public transcriptome data to examine *Dof* gene expression profiles in different tissues of *J. regia*. In this study, to understand the potential role and characteristics of *Dof* gene family, the phylogenetic analysis, chromosomal locations, conserved domain, gene structure, protein structure, and RNA-Seq data for different tissues were investigated. To know the importance of the *Dof* genes in flowers, we sampled and analyzed the expression level of female and male flowers. This study provides the first genome-wide analysis of the Persian walnut *Dof* gene family, and these findings will be useful for understanding the putative functions of Persian walnut *Dof* genes.

Material and Methods

Dof Transcription Factors Identification in *J. regia*

The Persian walnut whole protein sequence was downloaded from National Center for Biotechnology Information (NCBI). Members of the *Dof* gene family of *Arabidopsis* were downloaded from The Arabidopsis Information Resource (TAIR) website [31]. To search against Persian walnut protein sequences, we used *Arabidopsis Dof* protein sequences as a query using a basic local alignment search tool (BLAST), considering those with an E-value less than 1×10^{-10}. We implemented a profile hidden Markov model (HMM) in HMMER v.3.2.1 for window (http://hmmer.org/download.html/) [32] with default parameters to search for *Dof* proteins and *Dof* domains in the protein family (Pfam) database (http://pfam.xfam.org/) [33].

Dof Protein Alignment, Phylogenetic Analysis, Pfam Domain Detection, Chromosome Location, and Protein Domains Analysis of Common Walnut

An unrooted phylogenetic tree was constructed by using MEGA software (Molecular evolutionary genetics analysis, Pennsylvania State University: State College, PA, USA) v.7.0 based on neighbor-joining (NJ) method [29]. The phylogenetic NJ tree was constructed with the pairwise deletion of 1000 bootstraps and a Poisson model [34]. To search for the presence of potential domains, the Pfam web server (http://pfam.xfam.org/) [33], was used, and 39 *Dof* protein sequences were detected. The simple modular architecture research tool (SMART) program (http:/smart.embl-heidelberg.de/) [35], also detected the same domains obtained from Pfam (The large collection of protein family's database) with an E-value cutoff of 1.0 to validate the results. We categorized these sequences into various subfamilies, and their distribution on the 16 chromosomes was visualized using MapChart (http://mapchart.software.informer.com/2.2/) with default parameters [36]. A Conserved Domain Database (CDD) search was conducted in NCBI (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) [37] to confirm the *Dof* domain of each sequence.

Motif, Gene Structure and Protein Structure Analysis of *J. Regia*

To analyze the number of exons and introns, we used the coding sequences (CDS) of *Dof* transcription factors to BLAST against the *J. regia* genome sequences with a top E-value less than $1\times10^{-20.}$ The whole CDS database were downloaded from (https://treegenesdb.org/FTP/Genomes/Jure/v1.1/annotation/). The online gene structure display server was used to illustrate exon and intron structures (http://gsds.cbi.pku.edu.cn/) [38]. The genome browser was used to search for related Persian walnut gene sequences (https://www.ncbi.nlm.nih.gov/genome/). The motif identification used the MEME program with default parameters, the maximum number of motifs (10), and the optimum motif width (30-50) [37].

Plant Materials, Treatments, and Collections

We collected three male and nine female flowers from common walnut individual trees to evaluate the expression of *Dof* transcription factors at various developmental stages growing in the Qinling Mountain. The female flowers were collected on 10 April, 15 April, 22 April, and 1 May respectively as 3, 3, 2, and 1 replicate. The male flowers were collected on 10 April, 11 April, and 2 May, respectively. The first opening of female flowers in this study occurred on April 10, April 15, and April 22, and there was a complete opening of female flowers (specifically, the stigma was not fully developed on April 15 and April 22) and 1 May was the end date. The three respective stages for male flowers appear on April 10, April 11, and May 2. After harvesting, the pericarp was immediately dissected, and the flesh was frozen in liquid nitrogen and stored at −80ºC [39]. To isolate total RNA, we used RNA-prep Pre-Plant Kit (Tiangen, Beijing, China) [40]. Using NEBNext Ultra RNA Library Prep Kit (NEB, Beverly, MA, USA), RNA-seq libraries were produced. The pair end sequence was performed with Novogene (Bioinformatics Technology Co., Ltd., Beijing, China) (www.novogene.cn) on the Illumina HiSeq2500 platform to produce 100 bp reads with default parameters. In Trinity, by using default settings, the de-novo transcriptome was assembled [41] based on the *J. regia* genome reference [42]. We investigated RNA-seq data to identify the spatial and temporal expression patterns of the *Dof* gene family. The transcriptome sequencing datasets were deposited to Bio-Project identifier (ID) PRJNA358784, which was used to conduct RNA-seq of different male and female flowers in *J. regia*. Also, at the initial flowering stage of germination, we analyzed the total RNA seq data from the male and female flowers. We quantified these gene expression patterns using Cufflinks with default parameters based on their fragments per kilobase of exon per million reads mapped (FPKM) values [43] and using Heml 1.0 software with default parameters to represent these results [44]. Additionally, the ggplot2 R package was used for the *Dof* transcription factors expression with error bars [45]. Analysis of differential gene expression (DESeq) was carried out using the package DESeq R v.1.1.1. Genes found by DESeq with adjusted P-value>0.05 were allocated as expressed differentially. We also normalized the number of reads from the RNA-seq information for differential gene expression [44].

Microarray Expression Profiles of *Dof* Transcription Factors

We downloaded public transcriptional raw data to study the expression pattern in different tissues of common walnut (https://treegenesdb.org/FTP/Genomes/Jure.v1.0/transcriptome/rawreads/) [46]. We used the bowtie software (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) with default parameters to align sequences with long reference sequences [47]. The reference sequences were downloaded from (https://treegenesdb.org/FTP/Genomes/*Jure*/v1.4/genome/), using Cufflinks software (http://cufflinks.cbcb.umd.edu/) [48] with default parameters to assemble transcripts, estimate their abundances, and test for differential expression and regulation in RNA sequencing (RNA-Seq), and represented these results using HemI software (Huazhong University of Science and Technology, Wuhan, China, v.1.0) [41]. A hierarchical map based on normalization data was built and viewed with the Mev software (Multiple Experiment Viewer) (George Washington University, Washington, DC, USA, v.4.9.0) [49]. A heat map is a graphical illustration of data that depicts the individual values of a matrix as colors that enable readers to easily comprehend the information [49].

Results and Discussion

Characteristics of *JrDof* Gene Family

Gene family research in recent years is important for understanding gene structure, function, and evolution [50]. The *Dof* genes are plant-specific transcription factors and are widespread in several plant species involved in various biological processes. To date, most work has focused on the functions of *Dof* genes, and previously 36 *AtDof* genes identified in *Arabidopsis* [2, 6], 38 *CcDof* genes in pigeon pea [51], 36 in cucumber [52], 34 *SlDof* genes in tomato [53], 78 *GmDof* genes in soybean [54], 76 *BraDof* genes in Chinese cabbage [55], 35 *StDof* genes in potato [56], 42 in Barrel clover [57], pepper with 33 [58], 30 *OsDof* genes in rice [59], and 20 *Dof* genes in *Chrysanthemum morifolium* [1].

Dof Gene Family Identification and Phylogenetic Relationship

We identified a total of 39 *Dof* genes based on the *J. regia* whole reference genome (Fig. 1). In this study, the number of *JrDof* genes identified was slightly higher than that of *Arabidopsis*, rice, sorghum, and *poplar* [2, 5]. Furthermore, conserved domain analysis also confirmed that all *JrDofs* at their N-terminus contain *Zf-Dof* domain that is the basic characteristics of *Dof* gene family (Fig. 2) [60]. The neighbor-joining (NJ) phylogenetic tree showed that the *JrDof* genes are divided into six subfamilies (A to F) in Persian walnut (Fig. 1), and according to previous studies, this classification was performed [1, 34, 55]. The *Dof* members from *Arabidopsis* and rice were classified into four groups (a, b, c and d), which is slightly distinct from the present phylogenetic analysis with *Dofs* of *J. regia* [61]. In the phylogenetic tree analysis, subfamilies “A” with 12 *Dof* family members were

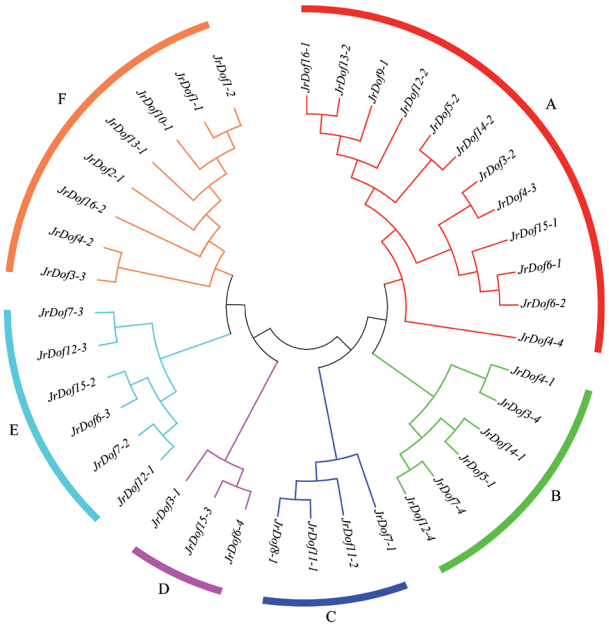

Fig. 1. Phylogenetic analysis of *Dof* gene family in common walnut (39). A neighbor-joining (NJ) tree was constructed using 39 *Dof* sequences. The tree further clustered into 06 groups.

the largest clades, and "D" with three *Dof* family members, were the smallest clades (Fig. 1).

Dof Genes Motifs Identification and Conserved Motifs Analysis

Putative motifs were predicted by the MEME program to reveal the diversification of *Dof* genes in Persian walnut, and 10 conserved motifs were identified. Graphical view of the motifs identified was presented in Fig. 3(a, b). Motifs have different structural variations that provide more information about its involvement in many biological processes (Fig. 3a, b). Among 10 motifs identified, the widely distributed Motif 1 belongs to the *Dof* domain, which is involved in protein interaction as well as DNA binding [62, 63]. TFs sometimes contain multiple DNA-binding domains. For example, plant-specific *WRKY* TFs possess different numbers of *WRKY* DNA-binding domains, which allows the proteins to be classified into subgroups [64]. However, in the case of *Dof* proteins, only a single copy of the *Dof* domain can consistently be found in their N-terminal regions (Fig. 3a, b). In the *J. regia Dof* gene family, Motif 1 has been identified in all genes, while Motif 10 belongs only to group B members, which indicates that Motif 10 in members of group B is specific to the evolution (Fig. 3a). Motifs 6, 8, 4, 9, 3, 5, and 2 are specific to group F. To understand the *Dof* genes in Persian walnut, and the structural information provides further insight into these motifs (Fig. 3b). However, high divergence

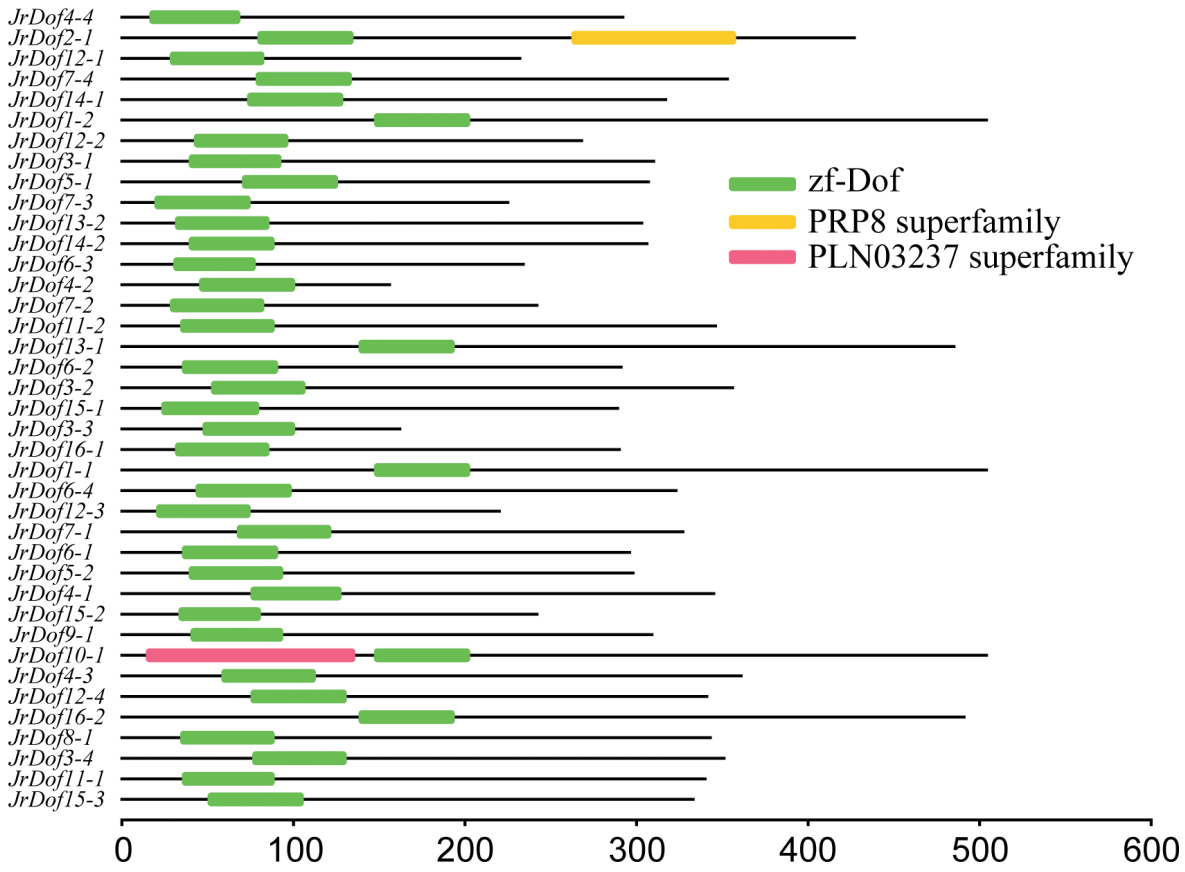

Fig. 2. Whole conserved domains of the Dof gene family of common walnut.

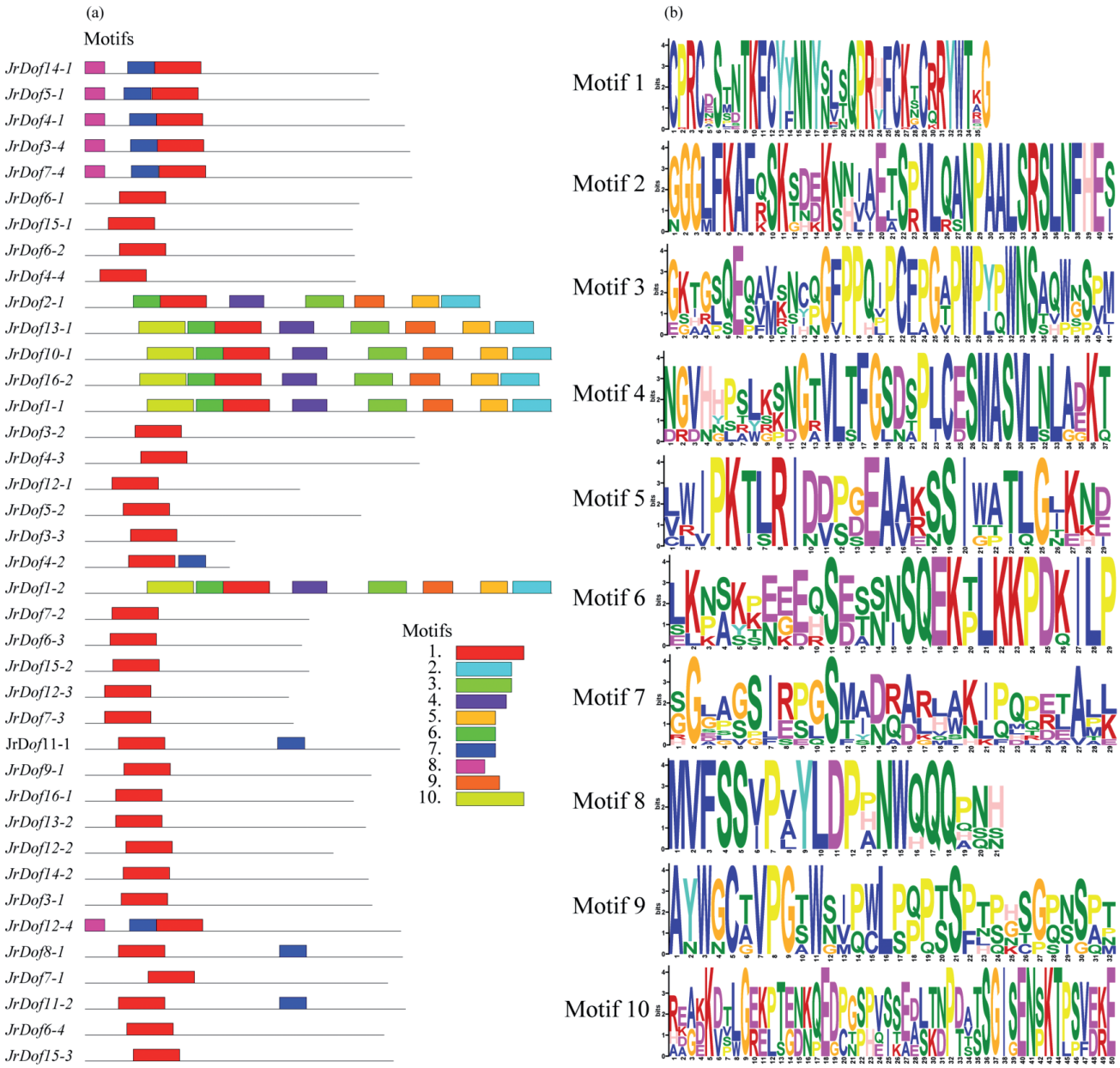

Fig. 3. a) Multiple sequence alignment of Dof proteins in J. regia. Motifs with specific colors can be found on respective genes, b) Motif structure of Dof proteins. The MEME search tool was used to make motif structures.

in their structures was found between the different groups, reflecting the complex nature of the function of *Dof* proteins in *J. regia*. In the same group, the majority of *JrDof* genes shared similar motifs indicating that these conserved motifs are closely related and implying functional similarities between the proteins [65]. In total, 41 conserved motifs were identified in poplar, *Arabidopsis*, and rice *Dof* protein sequences [1]. The motif distribution indicated that the genes containing the same motifs were likely produced via gene expansion within the same groups. Conserved motifs were further evaluated to analyze the structural characteristics of *Dof* genes. The results of our analysis showed that the *Dof* gene family contained at least one conserved domain (*Zf-Dof*) and two specific domains (*PRP8* and *PLN03237*) (Fig. 2) [5, 66]. All the *JrDof*s contained the *Zf-Dof* conserved domain, the *JrDof 2-1* contains the *PRP8,* and *JrDof 10-1* contains *PLN03237* specific domain, respectively (Fig. 2). Gene structure analysis and position of conserved motif provide further details about this family evolutionary relationships in *J. regia* [54].

Dof Genes Chromosomal Locations and Exon/Intron Structure

Locations of *Dof* genes and genetic linkage on the chromosome were identified using MapChart [36]. The *JrDof* genes were then renamed according to their location on the chromosome (Fig. 4) [53]. Our results

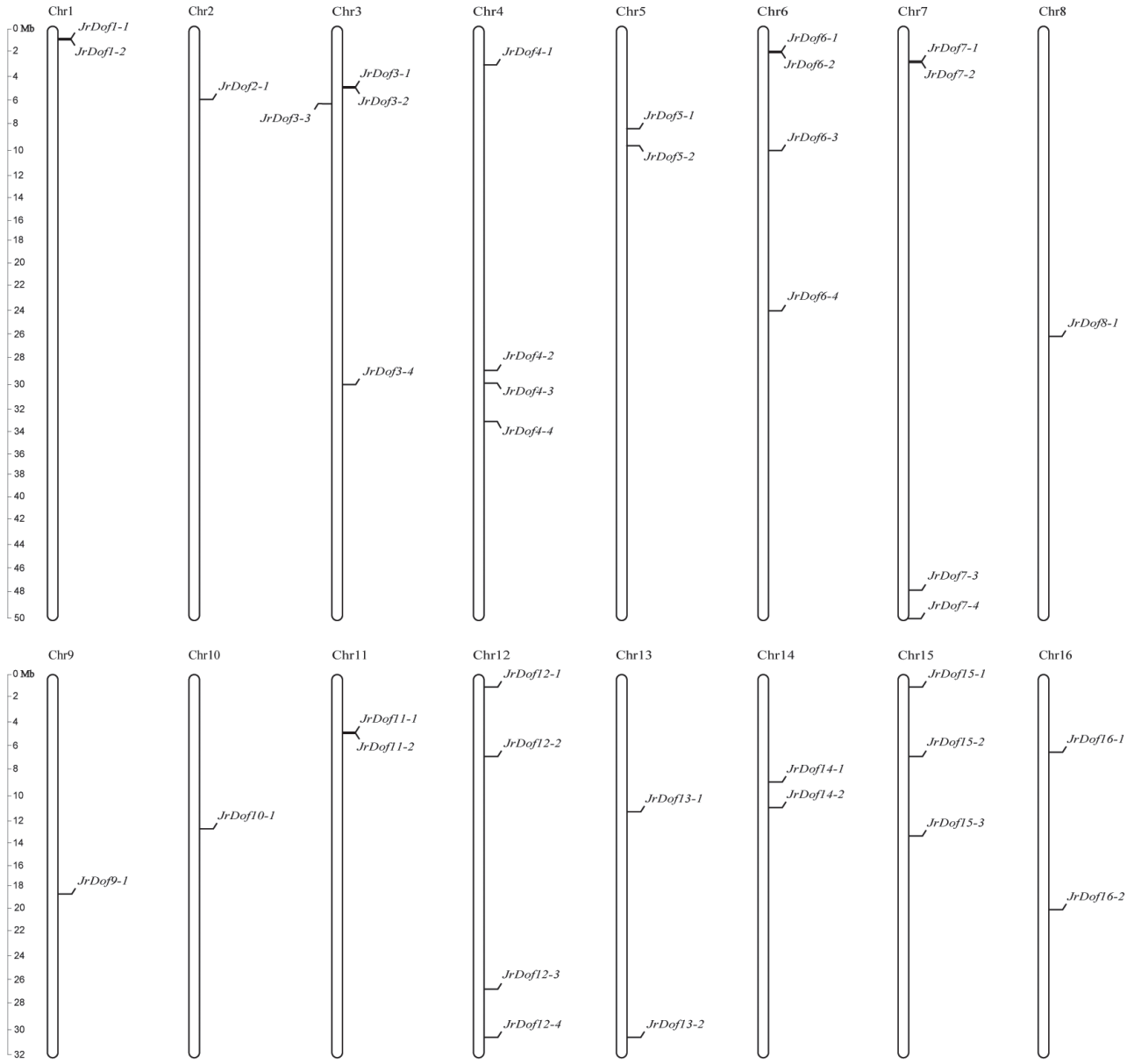

Fig. 4. Distribution of Dof genes on 16 chromosomes. MapChart was used to locate genes on chromosomes. Gene start point is shown on chromosome while genes size is shown in Mbs against each gene.

showed that the *Dof* genes distributed unevenly on 16 chromosomes of Persian walnut, and clustered only on few chromosomes in many plant species (Fig. 4) [51, 55]. The chromosomal location and length of *Dof* genes are shown in Fig. 4. The highest number of *Dof* genes (four genes) was found on chromosome 3, 4, 6, 7, and 12, followed by three genes on chromosome 15. In contrast, chromosomes 2, 8, 9, and 10 contain only 1 *Dof* genes, respectively (Fig. 4). As four *Juglans Dof* was located on individual chromosomes (Fig. 4), the same phenomenon was observed in barrel clover [57].

To know about the gene structure of the *Dof* family in the genome of *J. regia*, we compared diverse exon-intron organizations of the *Dof* family. To determine the structural heterogeneity of *J. regia Dof* genes, we analyzed the characterization of the exon-intron structure in the genomic DNA sequences. The number of exons predicted among the *Dof* genes were comparatively lower, varying from one to three, 19 genes having one exon and no introns, other 19 genes have 2 exons [67] while one member (*JrDof 9-1*) having three exons (Fig. 5) [56, 68]. The diverse status of exon and intron splicing might be meaningful for the *JrDof* gene evolution [54]. The *Dof* genes of the same group also have similar gene structures, such as intron number and exon length. The similar structural features in the Persian walnut genome may be related to their similar functions [68]. The intron number and intron-exon organization of *Dof* genes in the *J. regia* genome were quite like *Arabidopsis* and rice [17], soybean [69],

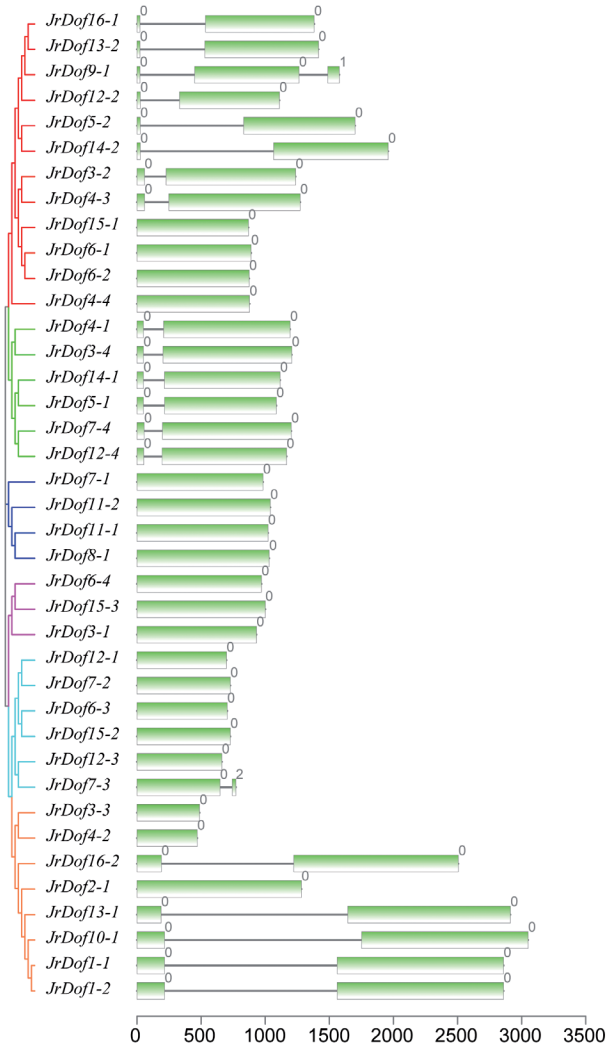

Fig. 5. Intron-Exon structure of 39 *Dof* genes in *J. regia* genome. Colored boxes indicate Exons, and gray lines represent Introns; 0, 1, and 2 represent different types of phases. Phase 0: located between two consecutive codons; Phase 1: splitting codon between the first and second nucleotides; Phase 2: between the second and third nucleotides of a codon.

and tomato [53]. The number of introns in *J. regia Dof* genes ranging from zero to two (Fig. 5). The number of introns in soybean and Barrel clover, with only 0 to 1, was reported to be very small [57]. The *Dof* genes in Cucumber also have 0 to 2 introns that predict structural similarity with other species [52]. *Dof* proteins found to be highly conserved and comparable to previous *Dof* gene studies in other species.

Expression Profiles of *Dof* Genes in Different *J. regia* Tissues

It was important to answer the question, whether the *JrDof* protein structure had any correlation with their functions in different tissues/organs. Based on the sequence similarity of conserved motifs, a total of 10 conserved motifs including the *zf-Dof* domain were identified (Fig. 2 and 3a) [70, 71]. Gene expression analysis in many plant species has been performed at various developmental stages for different tissues through sequencing that is publicly available. RNA-Seq data for the analysis of gene expression profiles is a convenient tool. We carried out RNA-Seq analysis for various *J. regia* tissues, including root, somatic embryo, embryo, young leaf, leaves, hull dehiscing, hull cortex, hull peel, immature hull, vegetative buds, and fruit (Fig. 6).

Analysis of the expression profile showed a higher expression of some *Dof* genes in multiple tissues. For instance, *JrDof10-1*, *JrDof7-1*, *JrDof12-1*, *JrDof15-3*, *JrDof13-2*, *JrDof4-3*, *JrDof6-3*, *JrDof6-4*, *JrDof14-2*, *JrDof5-2*, and *JrDof16-1* were highly expressed in female flowers, while *JrDof10-1*, *JrDof6-4*, *JrDof12-1*, *JrDof15-3*, *JrDof3-3*, *JrDof5-1*, *JrDof9-1*, *JrDof14-1*, *JrDof3-2*, *JrDof14-2*, *JrDof13-2*, and *JrDof16-1* expressed highly in male flowers (Fig. 6) [1, 62, 72]. Previous studies showed that *HbDof12a* is the most expressed gene in both female and male flowers, while *HbDof08b* the most expressed in roots [68]. In male flowers, on May 2, all *Dof* genes have a high level of expression than in the other two periods (Fig. 6). In total, 23 *Dof* genes expressed highly in the female and male flowers (reproductive tissues) (Fig. 6). Importantly, we also noticed 14 *Dof* genes (*JrDof13-1*, *JrDof4-2*, *JrDof15-2*, *JrDof6-3*, *JrDof15-3*, *JrDof7-1*, *JrDof13-2*, *JrDof11-2*, *JrDof9-1*, *JrDof16-1*, *JrDof11-1*, *JrDof12-4*, *JrDof5-2*, and *JrDof14-1*) highly expressed in somatic embryo, embryo, vegetative buds, young leaf, leaves, hull cortex, immature hull, hull dehiscing, hull peel, root, and fruit (vegetative tissues) (Fig. 6) [73]. *Dof* genes that differ in the pattern of expression have also been reported in other species, e.g. *Arabidopsis*, rice, poplar, and Chinese cabbage [55, 74]. These results are consistent with previous results that *Dof* genes were ubiquitously expressed in all tissues with possible redundant functions in higher plant tissues [75, 76]. High expression in root tissues predicting its key role in the development of root, these findings are consistent with previous studies in which *Dof* genes high expression were observed in roots of soybean and other plants [2, 7, 22, 50, 54, 77]. Expression profiles showed that there is no high and specific expression of *Dof* genes in leaves, suggesting that *Dof* gene family had little or no role in the development of leaves, which is in contrast with the previous study in which *HbDof14b* was highly expressed in leaf (Fig. 6) [78, 79]. *Dof* gene expression in flower tissues was also observed in barrel clover [80]. Previous studies also showed that *Dof* genes play an important role in the reproductive and vegetative tissues in different plants [19, 27, 52, 58, 77, 78]. Analysis of transcript expression of various Persian walnut tissues (female and male flowers, embryo, leaves, hull, fruit, and leaves) showed a high level of expression in females and male flowers (reproductive tissues), while other vegetative tissues showed a comparatively low degree of expression as compared to reproductive tissues (Fig. 7). The expression of *CmDOF20* and *CmDOF21*

was significantly higher in reproductive organs than that in vegetative organs based on the previous report which supports our results [1]. The highly expressed or differentially expressed *JrDof* genes reported in this study play a regulatory role in *J. regia* development (Fig. 6 and 7). However, additional research is needed to determine the functions of the *JrDof* genes.

Conclusions

In Persian walnut (*J. regia*), we identified a total of 39 *Dof* genes and clustered into six groups (A-F). *Dof* transcription factors were further characterized according to the phylogenetic analysis, gene organization, conserved motifs, and *Dof* domain.

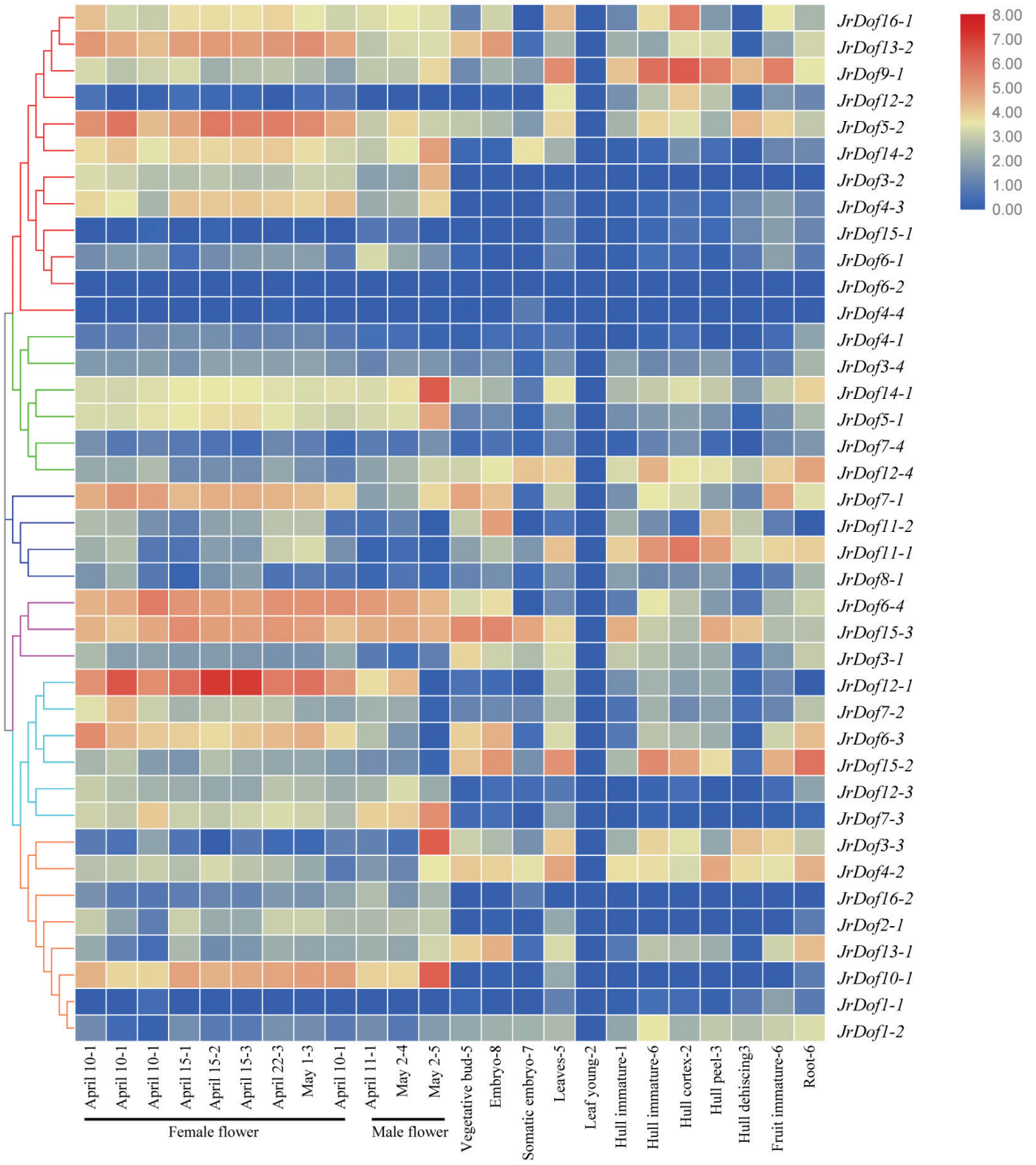

Fig. 6. Expression pattern of multiple tissues of common walnut based on *Dof* transcription factor. Analysis of expression patterns of all tissues in common walnut using RNA sequencing (RNA-Seq). The heatmap was drawn in log-10 transformed expression values. Red represents relatively high, and the green represents relatively low expression.

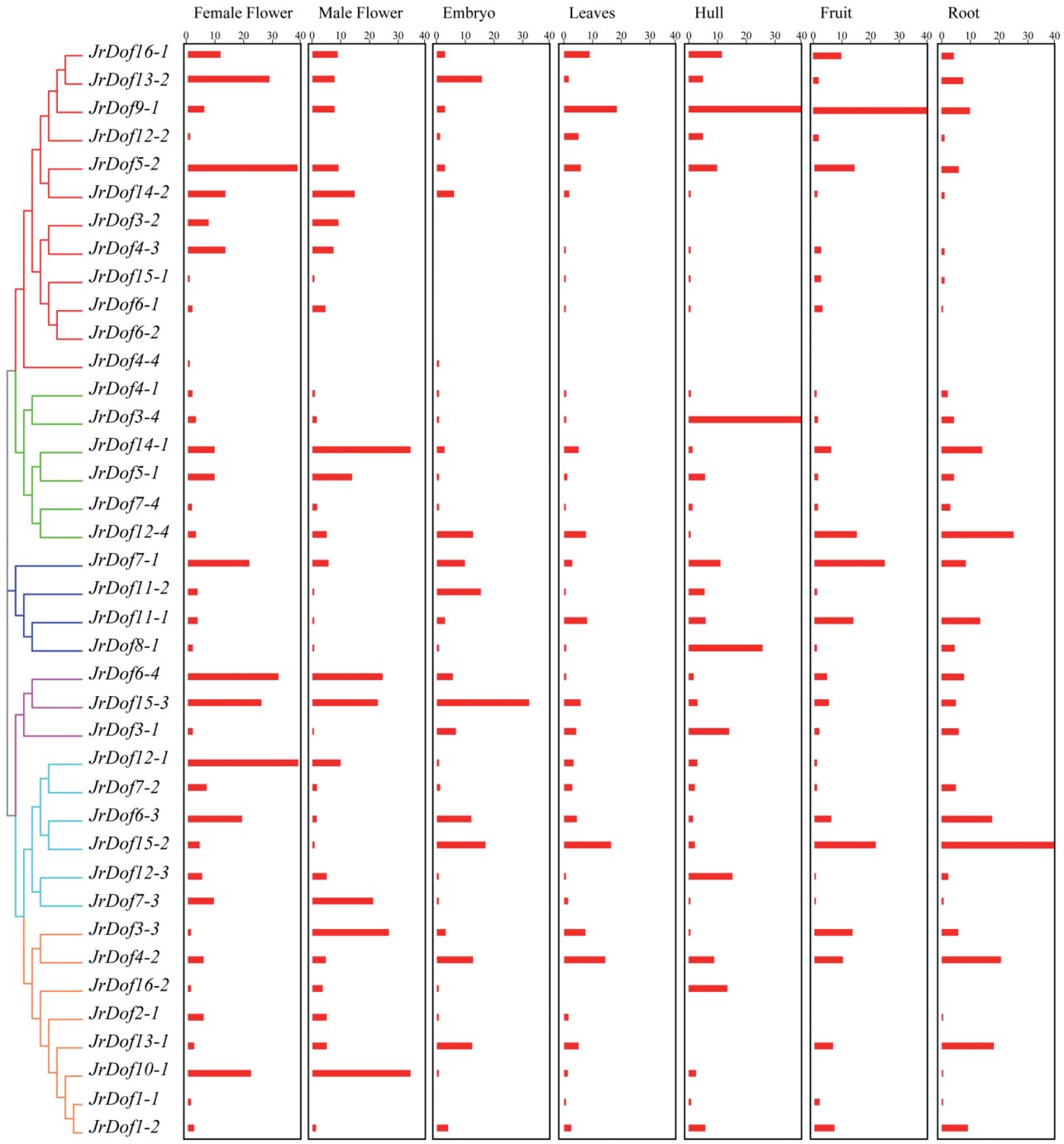

Fig. 7 The values of Dof gene family member expression in different tissues of common walnut.

Based on the conserved domains, *Dof* genes contain a conserved domain (*Zf-Dof*) in all genes. Moreover, the analysis of the expression profile based on RNA-Sequencing showed that the *Dof* transcription factors reveal diverse patterns of expression in different Persian walnut tissues. Most of the common walnut *Dof* transcription factors expressed highly in male and female flowers. A total of 16 *Dof* genes were highly expressed in female and male flowers, while others in reproductive tissues were low in expression or no expression. Also, a total of 14 *Dof* genes highly expressed in vegetative tissues. In the development of female and male flowers, the important role of *Dof* genes was observed, that can be further studied and used for the improvement of *J. regia* development in term of nuts. In conclusion, these results provide a base for studying the potential function of Persian walnut *Dof* transcription factors.

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Conflicts of Interest

The authors declare no conflict of interest.

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