Identification of Dof transcription factors in ramie (*Boehmeria nivea* L. Gaud) and their expression in response to different nitrogen treatments

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Abstract

19 DNA binding with one finger (*Dof*) transcription factor genes were identified from genome and transcriptome data of ramie (*Boehmeria nivea* L. Gaud). Chemical character, subnuclear localization, motif analysis, phylogenetic analysis, and tissue-specific analysis were performed. To select *BnDof* genes participating in nitrogen metabolism, we analyzed the expression patterns of *BnDof* genes in different nitrogen conditions (N0, N4, N8, and N12) in different tissues. As a result, eight *BnDof* genes were identified. *BnDof07* (stem) and *BnDof14* (root) had higher expression levels in N0 and N4, and *BnDof15* (stem and leaves) and *BnDof18* (stem) both exhibited the highest expression level in N0, suggesting that these four genes may take part in nitrogen stress. In the leaves, *BnDof01*, *BnDof06*, *BnDof09*, and *BnDof19* expression levels were higher in N8 and N12 but lower in N0 and N4. The present findings provide insights into nitrogen metabolism in ramie highlighting the need to investigate this aspect in further detail in future studies.

Keywords *BnDof* genes · Ramie · Nitrogen treatments · Nitrogen metabolism

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Introduction

DNA binding with one finger (*Dof*) proteins are plant-specific transcription factors (TFs). At the N-terminal, *Dof* proteins contain a conservative *Dof* DNA-binding domain, which has a Cys2/Cys2 Zn²⁺ finger structure that identifies and binds to the cis-element with the sequence 5'-AAAG-3' in the promoter region of the target genes (Plesch et al. 2001; Yanagisawa 2002, 2004). At the C-terminal, *Dof* proteins contain a transcriptional regulation domain, which regulates gene expression (Yanagisawa 2002; Yang and Tuskan 2006). *Dof* proteins participate in various biological processes such as plant flowering (Wu et al. 2017a), plant growth (Yanagisawa et al. 2004), C and N metabolism (Rueda-López et al. 2017), and abiotic stress tolerance (Corrales et al. 2017). For example, *OsDof4* leads to earlier flowering under natural long-day field conditions, with the reverse effect under natural short-day field conditions (Wu et al. 2017a). *ZmDof3* controls starch accumulation and aleurone development in maize endosperm (Qi et al. 2016). *AtDof5.4* negatively regulates cell cycle progression and cell expansion (Xu et al. 2016).

Nitrogen metabolism is crucial for plants and is regulated by multiple genes, including *Dof* genes

(Tsujimoto-Inui et al. 2009), which are involved in nitrogen metabolism. The overexpression of *PpDof5* in Arabidopsis leads to increased lignin content and affects carbon and nitrogen metabolism (Rueda-López et al. 2015). In rice, *ZmDof1* enhances carbon and nitrogen assimilation under low-nitrogen conditions (Kurai et al. 2011). Similarly, *AtDof1* also enhances nitrogen assimilation under low-nitrogen condition (Wang et al. 2009). *Dof* genes affect the absorption capability of nitrogen. The overexpression of *OsDof25* not only affects organic acid metabolism but also affects NH_4^+ uptake in plants (Santos et al. 2012). *OsDof18* increases ammonium uptake in rice (Wu et al. 2017b). *Dof* genes improve the protein content of crops. *OsDof18* improves nitrogen content in rice (Wu et al. 2017b), and *EcDof1* controls grain protein content (Gupta et al. 2012); its expression overlaps the expression of glutamine synthase (*GS*) and glutamate synthase (*GOGAT*) (Gupta et al. 2013). In wheat, the gene expression profile of *TaDof1* also coincides with the enzyme activities of *GS* and *GOGAT* (Kumar et al. 2009).

Many *Dof* genes have been identified in agricultural crops such as barley (Rouhian et al. 2017), maize (Chen and Cao 2015), potato (Venkatesh and Park 2015), soybean (Guo and Qiu 2013), and *Medicago truncatula* (Shu et al. 2015). However, not much is known about *Dof* genes in ramie.

Ramie (*Boehmeria nivea*) is a traditional fiber crop that is cultivated mainly China. Over the past few years, ramie has also been used as animal feed because of its high protein and amino acid content and rapid growth rate (Zhu Taotao et al. 2016). The biological yield of ramie is high, and its nitrogen demand is large. However, the nitrogenous fertilizer absorbed by ramie is only a small part of the amount applied, leading to fertilizer wastage and environmental contamination (Robertson and Vitousek 2009). Therefore, improving the nitrogen use efficiency of ramie is one of the breeding goals for this crop. A high protein content is an essential characteristic for forage crops. A previous study showed ramie's protein content to be related to nitrogen metabolism. Zheng et al. (2015) founded tobacco protein content to be higher when *BnGS1-2* was overexpressed in tobacco. The protein content varies in ramie grown under different nitrogen treatments (Diluo 2012; Chen et al. 2017). However, little is known about the function of *BnDof* genes in nitrogen metabolism. Using genomics and transcriptomics approaches, we attempted to identify the *BnDof* genes regulating nitrogen metabolism in ramie with the aim of improving nitrogen use efficiency and protein content. Chemical characterization, protein location, motifs, and tissue-specific expression were investigated. We also analyzed the expression patterns of *BnDof* genes under different nitrogen treatments. In a word, this study provided evidences for further study the candidate genes which control nitrogen use efficiency and protein content in ramie.

Table 1 Primers were used for qRT-PCR

Name	Primer
Dof.01F	CGAGTCGCTCAACACCAAG
Dof.01R	GCCAGCAGGGAGCTGAAA
Dof.02F	TTCAAGAACTATTGGGAGGGACT
Dof.02R	GCCAGAGTTATTCGCGGCCGCGGCT
Dof.03F	CGCTGAAGAAGCCAGACAA
Dof.03R	GAGATGGTGATGTGACGGTAA
Dof.04F	CCCGAAGAAGCTCCAAGC
Dof.04R	CCATCTCGTCCAACCCGTAT
Dof.05F	AAGAACAAGCGCTCCAAGAAA
Dof.05R	CATGTGTTGTCCAGTTCGGG
Dof.06F	AATAGGCTTCGCTGTTGATGG
Dof.06R	TTTCCTTGCCCTTTGTTCTG
Dof.07F	TCGCTCACCTAAGTGGTCTCA
Dof.07R	ATGCCCAAATCTCTCTCC
Dof.08F	TGCCTTTCGAGTACGGTCAT
Dof.08R	CGCACTGTTGCCAATCCC
Dof.09F	GAATAAGCCTAGCGTTGTTGAG
Dof.09R	TGGGAGGCAGTGGCAGTA
Dof.10F	CATGAGCTACTGGGGGTTT
Dof.10R	CCAGTGATGATCTTGCTGATG
Dof.11F	CAGGTAACACTGCCAGCATCT
Dof.11R	AATTCCCGCGAAGTTCATAG
Dof.12F	CACAACCCTAATATCTCACCATCA
Dof.12R	TCCATCGCCGAGACTTGC
Dof.13F	ATGTCCTCCGCCAACAAAC
Dof.13R	GGGTGAGGCTGTAGTTGTTG
Dof.14F	TTTCCGATGCCATTCTACCC
Dof.14R	AGAGTCCTCCTCCGCTGATT
Dof.15F	GACGATAGCGTCTTTGCTCG
Dof.15R	TGCCCATCTTCCAACCTTCACT
Dof.16F	GTCAACTCCGTCGTCACCG
Dof.16R	CCGCCGAAGAGCAAGAA
Dof.17F	CCACCATCACCTTCATCAGC
Dof.17R	AACTCGTCCCTTACCACAA
Dof.18F	TCATACCGTGCCCTAGATGC
Dof.18R	CTCGTGGTGTGCTGAGAATT
Dof.19F	TCATACCAGTCGGATCAATG
Dof.19R	GTTCTCAGAAATGCCCAAGC
18sF	TGACGGAGAATTAGGGTTCCGA
18sR	CCGTGTCAGGATTGGGTAATTT

Materials and methods

Identification and characterization of *Dof* TFs

Putative *BnDof* genes were identified from ramie the genome and transcriptome of ramie (variety, Zhongzhu 1). We performed sequences alignment in SwissProt, KEGG, GO, and

Table 2 Information of *BnDof* genes

<i>Dof</i> gene	GenBank accession NO.	<i>pI</i> isoelectric point	Molecular weight (Da)	Protein length (aa)	Subnuclear localisation
<i>BnDof01</i>	MH374296	9.4	20253.4	194	Nucleus
<i>BnDof02</i>	MH374297	8.86	42490.8	416	Nucleus
<i>BnDof03</i>	MH374298	6.11	46041.4	435	Nucleus
<i>BnDof04</i>	MH374299	6.7	24275.54	242	Nucleus
<i>BnDof05</i>	MH374300	8.66	31873.49	296	Nucleus
<i>BnDof06</i>	MH374301	9.22	28967.53	267	Nucleus
<i>BnDof07</i>	MH374302	7.1	36210.01	339	Nucleus
<i>BnDof08</i>	MH374303	7.25	33408.65	321	Nucleus
<i>BnDof09</i>	MH374304	6.77	32654.22	297	Nucleus
<i>BnDof10</i>	MH374305	9.07	32165.11	302	Nucleus
<i>BnDof11</i>	MH374306	9	39859.92	374	Nucleus
<i>BnDof12</i>	MH374307	8.22	44105.04	403	Nucleus
<i>BnDof13</i>	MH374308	9.36	39391.49	370	Nucleus
<i>BnDof14</i>	MH374309	5.22	54583.02	506	Nucleus
<i>BnDof15</i>	MH374310	7.63	44948.59	421	Nucleus
<i>BnDof16</i>	MH374311	5.57	22511.39	208	Nucleus
<i>BnDof17</i>	MH374312	10.09	22201.81	202	Nucleus
<i>BnDof18</i>	MH374313	9.55	20852.58	183	Nucleus
<i>BnDof19</i>	MH374314	9.48	38060.02	359	Nucleus

Trembl databases to select putative sequences. Multiple alignments were performed using Blastn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to avoid repetition. If the alignment result showed the same sequence among putative *BnDof*, we selected the longest one as the representative. Finally, the putative sequences were submitted to NCBI Conserved Domain Research (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to ensure that the putative sequences contain the Dof domain. ExPASy (https://web.expasy.org/compute_pi) was used to analyze the isoelectric point (*pI*) and molecular weight (MW) of BnDof proteins (Gasteiger et al. 2005). Using PredictProtein (<https://www.predictprotein.org>) predicted protein location (Rost et al. 2004).

Phylogenetic analysis and motif analysis of *BnDof* genes

Dof protein sequences were used for phylogenetic analysis. Protein sequences of Dof family from Arabidopsis, maize, rice, and *M. truncatula* were retrieved from NCBI (<https://www.ncbi.nlm.nih.gov/>); the sequences are shown in Supplementary file 1. Multiple sequence alignments were performed using ClustalW and phylogenetic trees were constructed by MEGA 6.0 using the neighbor-joining method and *p*-distance model; partial gap deletion was selected and the bootstrap value was 1000.

Using MEME (<http://meme-suite.org>), we identified the conserved motifs of BnDof proteins. The motif discovery

mode was “Classic mode.” The site distribution selected “Any number of repetitions,” and the maximum number of motifs was set to 15.

Plant treatments

Ramie plants (Zhongzhu 1) were grown on normal nutrient solution, after which uniformly growing plants were transferred to different nutrient solutions with varying nitrogen contents till the reproductive stage. The nitrogen contents were as follows: N0 (0 mM N), N4 (4 mM N), N8 (8 mM N), and N12 (12 mM N) (Diluo 2012), and the nitrogen form was nitrate. The experimental group contained six plants, and the nutrient solution was replaced every 4 days. We sampled root, stem, and leaves on the 18th day.

Quantitative real-time PCR (qRT-PCR) assays

Tissue-specific analysis was carried on out the root, stem, leaves, male flowers and female flowers, which were sampled from field. The expression patterns of *BnDof* genes in response to different nitrogen treatments were studied in root, stem, and leaves. Total RNA was extracted from samples using the EASYspin Plus Plant RNA Rapid Extraction Kit (Aidlab, China), and used to synthesize first-strand cDNA by the ReveatAid First-Strand cDNA Synthesis Kit (Thermo, America). The primers were designed by Primer 6; all the primer sequences are shown in the Table 1. qRT-PCR

Fig. 1 Phylogenetic relationships among Arabidopsis, maize, rice, *Medicago truncatula*, and ramie Dof families. Yellow circle represented Arabidopsis, violet circle represented rice, light green circle

represented maize, dark green circle represented *Medicago truncatula*, and red circle represented ramie

was carried out with Mx3000P (Genetimes Technology, China), and each final 20 μ L volume contained 10 μ L $2 \times$ Sybr Green qPCR Mix (Aidlab, China), 1 μ L primer (4 μ M), 0.8 μ L DNA template, and 8.2 μ L H_2O . The qRT-PCR procedure was as follows: the initial denaturation at 94 $^{\circ}$ C for 3 min, and 40 cycles of 94 $^{\circ}$ C for 20 s, 57 $^{\circ}$ C for 20 s, and 72 $^{\circ}$ C for 30 s. All qRT-PCR experiments were carried out thrice in the same conditions. The 18 s rRNA gene was used as the reference gene (Zheng et al. 2015); the primers are shown in Table 1. Relative gene expression was calculated

by $2^{-\Delta\Delta C_t}$ method. The heat maps were constructed by Morphueus (<https://software.broadinstitute.org/morpheus/>).

Results

Identification of *BnDof* genes in ramie

In all, 19 *BnDof* genes were identified on the basis of ramie genomics and transcriptomics; 13 *BnDof* genes (*BnDof01–BnDof13*) by genomics analysis, 6 (*BnDof14–BnDof19*) by transcriptomics analysis. Information about *BnDof* genes, such as gene ID, length of coding

Fig. 2 Phylogenetic analysis of 19 ramie Dof gene family protein sequences and the conserved motifs of the proteins

sequences, *pI*, MW, and subnuclear localization, is presented in Table 2. The length of coding sequences ranged from 183 to 506 aa; *pI* value, from 5.22 to 10.09; MW, from 20.25 to 54.59 kDa. Prediction of protein location showed them all to be localized in the nucleus.

Phylogenetic analysis and conserved motif analysis

To understand the phylogenetic relationships among Arabidopsis, maize, rice, *M. truncatula*, and ramie Dof genes, we performed multiple sequence alignment using protein sequences with 31, 30, 18, 42, and 19 sequences of the respective species. An unrooted phylogenetic tree was constructed (Fig. 1). Dof proteins were divided into six groups, A, B, C, D, E, and F. All groups contained AtDof, OsDof, ZmDof, MtDof, and BnDof protein. Relative to the Dof genes of Arabidopsis, rice, and maize, BnDof genes were more closely related to the Dof genes of *M. truncatula*, which suggested that BnDof might share similar evolutionary patterns with MtDof genes. The other phylogenetic tree was constructed with BnDof protein sequences (Fig. 2), and four groups were obtained.

We analyzed the conserved motifs of the 19 BnDof proteins using the MEME program (Fig. 2). Sequences of the motifs are shown in Fig. 3. Ten motifs were founded, and the E value of each motif was lower than 0.05. Every protein had motif1 with Zn finger. Motif2 and motif4 were only in group C, and motif7 was only in group B1.

Tissue-specific expression of BnDof genes

To investigate the expression patterns of BnDof genes in root, stem, leaves, and flowers, we generated a heatmap based on qRT-PCR data (Fig. 4). Most BnDof genes had low expression in female flowers, except for BnDof08 and BnDof12; furthermore, BnDof12 had low expression in other tissues. BnDof03, BnDof08, and BnDof16 were highly expressed both in the root and male flowers. Three genes (BnDof02, BnDof17, and BnDof18) were highly expressed in the leaves, and BnDof07, BnDof10, and BnDof19 were highly expressed in the stem. BnDof01, BnDof13, and BnDof15 were expressed at high level in the roots, stems, and leaves.

Expression patterns of BnDof genes in response to different nitrogen treatments

We analyzed the expression patterns of BnDof genes in the root, stem, and leaves under different nitrogen treatments, and a heatmap was constructed (Fig. 5).

In the roots (Fig. 5a), BnDof01 expression increased from N0 to N4, to N8; however, it reduced in N12. BnDof14 expression was higher in low-nitrogen conditions (N0 and N4), but was low in high-nitrogen conditions (N8 and N12). BnDof19 expression was higher in high-nitrogen conditions, low in low-nitrogen conditions. The expression was the highest in N8.

In the stems (Fig. 5b), BnDof07 expression was higher in low-nitrogen conditions. BnDof15 and BnDof18 both had the highest expression in N0.

Fig. 4 Tissue-specific analysis of *BnDof* genes. The *BnDof* genes were listed on the right, and the colour scale was shown at the top. The higher expression of each gene was presented in red and the opposite was presented in blue. The capital letters indicated the following: R=root, S=stem, L=leaves, M=male flowers and F=female flowers

one based on Dof protein sequences in ramie, rice, maize, Arabidopsis, and *M. truncatula* (Fig. 1), and the other based on the alignment of *BnDof* protein sequences (Fig. 2). In Fig. 2, six pairs of paralogous *BnDof* proteins were identified, such as *BnDof06/BnDof12* and *BnDof02/BnDof13*, which are crucial to demonstrate the evolutionary mechanisms for more recent duplications. However, the duplications of those genes warrant further study, because the chromosomal distribution of *BnDof* genes is not known. In Fig. 1, *BnDof* genes were closest to *MtDof* genes, followed by Arabidopsis. The potential reasons are that ramie, *M. truncatula*, and Arabidopsis are all dicotyledons, and ramie and *M. truncatula* are both perennial plants.

Conserved motifs of *BnDof* proteins

We identified 10 motifs of *BnDof* proteins, which showed that *BnDof* genes participate in multifarious biological processes. *BnDof* proteins had similar motifs in the same group, which suggested that the proteins had similar functions in the same group. Nevertheless, high divergence was found in different groups. For instance, motif2 and motif7 were only present in group C. This finding demonstrated that different *BnDof* genes performed different functions. All sequences contained only one Zn finger, which is a DNA-binding domain. Chrysanthemum and Arabidopsis (Song et al. 2016), chickpea (Nasim et al. 2016), maize (Chen and Cao 2015), poplar (Wang et al. 2016), potato (Venkatesh and Park 2015), and Dof proteins have been found to contain one DNA-binding domain, as well, which suggests that motif1 is a conserved motif of *Dof* genes.

Fig. 5 Expression patterns of *BnDof* genes under nitrogen treatments. The colour scale was shown at the top. **a** Represented root, **b** represented steam, and **c** represented leaves. Nitrogen treatments included

N0 (0 mM nitrogen), N4 (4 mM nitrogen), N8 (8 mM nitrogen), and N12 (12 mM nitrogen). A1 and A2 represented two biological repeats

Fig. 6 Phylogenetic relationship among BnDof proteins which may take part in nitrogen metabolism and other Dof proteins of rice (*OsDof25*), Arabidopsis (*AtDof1.7*), maritime pine (*PpDof5*), wheat (*TaDof1*), maize (*ZmDof1*), and finger millet (*EcDof1*, *EcDof2*) that involved in nitrogen metabolism

Different tissue-specific expression levels of *BnDof* genes

Because gene expression patterns can provide clues to study gene functions, we analyzed *BnDof* expression patterns in the root, stem, leaves, male flowers, and female flowers at the reproductive stage. The results exhibited spatial variations of *BnDof* genes. There were 4 pairs of paralogous *BnDof* genes, and 3 pairs (excluding *BnDof03* and *BnDof16*) showed different expression patterns, suggesting functional divergence after duplication events. *BnDof08* and *BnDof12* were highly expressed high in flowers. In rice, *OsDof4* regulates flowering time and was expressed widely in young panicles (Wu et al. 2017a). Furthermore, *BnDof08*, *BnDof12*, and *OsDof4* were in the same group. As a result, we could predict that *BnDof08* and *BnDof12* regulate flowering in ramie. For the differential expression of *BnDof* genes in organs, we could predict that they play role in controlling ramie plant development. However, further research is required for confirming this.

BnDof genes involved in the nitrogen metabolism of ramie

To identify putative *BnDof* genes, controlling nitrogen metabolism, we analyzed the expression patterns of *BnDof*

genes under different nitrogen levels in ramie root, stem, and leaves (Fig. 5). We identified that *BnDof01*, 14, and 19 in the roots; *BnDof07*, 15, and 18 in the stems; *BnDof01*, 06, 09, 14, 15, and 19 in the leaves might be participating in ramie nitrogen metabolism. We also analyzed the evolutionary relationship among these eight putative *BnDof* genes and other *Dof* genes involved in nitrogen metabolism based on protein sequences (Kumar et al. 2009; Kurai et al. 2011; Santos et al. 2012; Wang et al. 2009; Gupta et al. 2014; Rueda-López et al. 2015) (Fig. 6). *BnDof01*, *BnDof07*, and *AtDof1.7*, *BnDof14*, *BnDof18*, *PpDof5*, and *EcDof1* were in the same branch. Therefore, *BnDof01*, and *BnDof07*, and *BnDof14* and *BnDof18* are worth more investigation. Of these four genes, *BnDof07* and *BnDof14* (in the roots) were highly expressed in N0 and N4, and *BnDof01* was highly expressed in N8 and N12 conditions; *BnDof18* had the highest expression in N0. Thus, *BnDof07*, *BnDof14*, and *BnDof18* may regulate nitrogen metabolism under low-nitrogen conditions and might, therefore, be taking part in nitrogen stress. *BnDof01* may regulate nitrogen metabolism under high-nitrogen conditions, which may improve the ability to absorb nitrogen. For the other four genes, we are not sure that they could not regulate nitrogen metabolism due to species specificity. We could find many *BnDof* genes expressed at the highest level in N8 (Fig. 5). Diluo (2012) found that ramie grew better in the N8 condition compared to the other conditions (N0, N4, and N12). Therefore, in this study, although certain *BnDof* genes showed the highest expression levels in this condition, they may not be involved in nitrogen metabolism.

Conclusions

In this study on ramie, 19 *BnDof* genes were identified. Conserved motif analysis, phylogenetic analysis, and tissue-specific expression of *BnDof* genes in different tissues were performed. All *BnDof* proteins contained motif1, which harbored the Dof domain. Different *BnDof* genes showed diverse expression patterns in different tissues. We also analyzed the expression patterns of *BnDof* genes under different nitrogen treatments and identified eight *BnDof* genes that might control nitrogen metabolism in ramie. Our study provided clues for us to select *BnDof* genes involved in nitrogen metabolism and controlling nitrogen use efficiency and protein content in ramie.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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