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Identification and expression of Aux/IAA, ARF, and LBD family transcription factors in *Boehmeria nivea*

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Abstract

Auxin controls numerous processes in plant development and auxin/indoleacetic acid (*Aux/IAA*), an auxin response factor (*ARF*), and a lateral organ boundaries domain (*LBD*) were considered as early auxin response transcription factors (TFs). Till now, no *Aux/IAA*, *ARF*, and *LBD* TFs were identified in ramie (*Boehmeria nivea* L. Gaud). In this study, we used *Arabidopsis* and mulberry sequences as query to search against the ramie transcriptome database and the searched sequences were analyzed for a full-length coding sequence. In total, we obtained 16 *BnAux/IAA*, 14 *BnARF*, and 16 *BnLBD* TFs on which evolutionary analysis and expression profiling were conducted. Analysis of sequence conservation revealed close evolution relationships between ramie and mulberry. Expression analysis shows these genes were actively expressed in major ramie tissues, and several were auxin responsive. The expressions of these genes were also investigated under drought and a high temperature, main abiotic stresses during ramie life cycle. We found that most genes of the three families were stress-responsive and showed distinct expression patterns under the drought and high temperature stresses.

Additional key words: *Arabidopsis thaliana*, high temperature, indole-acetic acid, mulberry, phylogenetic tree, ramie, water stress.

Introduction

It is well known that auxin controls numerous processes in plant development. The mechanism of auxin signaling and transduction has been continuously revealed in *Arabidopsis thaliana* (Weijers and Friml 2009). In previous studies, three families, *Aux/IAA* (auxin/indoleacetic acid), *ARF* (auxin response factor), and *LBD* (lateral organ boundaries domain), were considered as families of early auxin response genes and consisted of 23, 29, and 42 members, respectively (Abel and Theologis 1996, Ulmasov *et al.* 1999, Reed 2001, Semiarti *et al.* 2001, Iwakawa *et al.* 2002). Auxin/indoleacetic acid proteins interact with *ARF* family transcription factors through conserved domains III/IV that are shared by both types of proteins (Ulmasov *et al.* 1997). The *ARF* transcription factors regulate auxin-response genes by specifically binding to TGTCTC auxin-response elements (Ulmasov *et al.* 1999). The expression of *LBD* genes is enhanced in response to exogenous auxin indicating that the *LBD* gene family may be a target of auxin-mediated *ARF* transcriptional activities (Majer and Hochholdinger 2011, Fan *et al.* 2012). The interaction of these genes regulates an auxin response to maintain natural growth and development.

Molecular researches have provided evidence on *ARF*, *Aux/IAA*, and *LBD* functions in plant growth and development. Genetic manipulations of these genes impede inflorescence development (*AtIAA1*, *AtARF1-4*, *AtLBD3,36*; Chalfun *et al.* 2005, Ellis *et al.* 2005, Ku *et al.* 2009, Finet *et al.* 2013), leaf development (*AtIAA1,29*, *AtARF2*, *AtLOB*, *AtLBD6,12*; Semiarti *et al.* 2001, Shuai *et al.* 2002, Nakazawa *et al.* 2003, Ku *et al.* 2009, Lim *et al.* 2010), root development (*AtIAA12-13*, *AtARF3,7,19*, *AtLBD16,29,33*; Weijers *et al.* 2005, Berckmans *et al.* 2011, Feng *et al.* 2012, Cabrera *et al.* 2014), embryogenesis (*AtIAA10,12*, *AtARF5,7,9*, *AtLBD30*; Hardtke *et al.* 2004, Soyano *et al.* 2008,

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Abbreviations: ARF - auxin response factor; Aux/IAA - auxin/indole-acetic acid; CDS - coding sequence; LBD - lateral organ boundaries domain; PCR - polymerase chain reaction; TF - transcription factor.

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Rademacher *et al.* 2012), metabolism (*AtIAA13,14,28*, *ARF7,19*, *AtLBD37-39*; Falkenberg *et al.* 2008, Albinsky *et al.* 2010, Narise *et al.* 2010), fertility (*AtIAA16*; Rinaldi *et al.* 2012), seed development (*AtARF2,10,16*; Schruff *et al.* 2006, Liu *et al.* 2013), callus formation (*AtLBD16,17,28,29*; Fan *et al.* 2012), and plant defense (*AtLBD20*; Thatcher *et al.* 2012).

Ramie (*Boehmeria nivea* L. Gaud) is important natural fiber crop of the *Urticaceae* family and is widely cultivated in China, India, and other Southeast Asian and Pacific Rim countries. Till now, genome-wide and evolutionary analyses of *Aux/IAA*, *ARF*, and *LBD* have been conducted in model plants such as *Arabidopsis*, rice and maize (Wang *et al.* 2010b, Majer and Hochholdinger 2011, Finet *et al.* 2013). However, very few ramie *Aux/IAA*, *ARF*, and *LBD* genes have been identified even if their importance in plant growth and development (Huang *et al.* 2014). Previous studies revealed that auxin acts as important regulator in stem development and environment adaptation of ramie (Wolters and Jurgens 2009, Schuetz *et al.* 2013). Our study on auxin-related TFs brings a new perspective to ramie researches. In this study, we used published sequences in the three families to search against the transcriptome database (Chen *et al.* 2014, Huang *et al.* 2014, An *et al.* 2015). The identification of full-length coding sequence (CDS) *BnAux/IAA*, *BnARF*, and *BnLBD* TFs and the characterization of their expression pattern provide a basis for future clarification of their functions in ramie growth and development.

Materials and methods

To identify *ARF*, *Aux/IAA,* and *LBD* TFs in mulberry, *BLAST* searches were performed in the *Morus* genome database (http://morus.swu.edu.cn/morusdb/) using the protein sequences of 29 *Aux/IAA*, 23 *ARF*, and 42 *LBD* TFs from the database of *Arabidopsis* transcription factors (http://datf.cbi.pku.edu.cn/) as query sequences. All *Arabidopsis* and mulberry protein sequences of the three families were used to search against three ramie transcriptome databases, respectively (Chen *et al.* 2014, Huang *et al.* 2014, An *et al.* 2015). Obtained sequences from the three databases were aligned based on the nucleotide sequence using *Clustal X* (Thompson *et al.* 1997). The genes from the three different databases that had an overlap of more than 50 bp were further assembled. If two or three genes from the three databases overlapped completely, the gene with a longer nucleotide sequence was reserved. Additionally, the genes which appeared only in a single database were set aside. Finally, all genes that were assembled and set aside were analyzed to obtain CDS by using an open reading frame finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Obtained CDS sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) under accession numbers given in Table 1 Suppl.

A neighbor-joining phylogenetic tree was constructed for Aux/IAA, ARF, and LBD proteins using the *MEGA 5.0* software (Tamura *et al.* 2011). The most parsimonious tree with bootstrap values from 1 000 trials was used. *Arabidopsis* was selected as model system, and mulberry was selected as closely-related species for phylogenetic comparisons. Multiple expectation maximization for motif elicitation utility was used to investigate motifs of these proteins (http://meme.nbcr.net/meme/; Bailey *et al.* 2009).

Boehmeria nivea L. Gaud cv. 1504 was grown in the Ramie Germplasm Repository of the Huazhong Agricultural University (Wuhan, Hubei Province, China). Leaves, shoots, roots, and stem bark were separately sampled from three 2-month-old plants. For indole-acetic acid (IAA) and abiotic stress treatments, young plants (about 0.15 m from the top) were cut off and notches were soaked into 0.02 g dm^{-3} $KMnO_4$ for 2 d and then in tap water for rooting. After rooting, all plants were grown in Hoagland's nutrient solution. Leaves of plants cultured without soil for 7 d were: sprayed with 0.05 M IAA (*Sigma-Aldrich*, Saint Louis, MO, USA) and then sampled at 15 and 60 min intervals (Wu *et al.* 2012); treated with 20 g dm^{-3} polyethylene glycol 6000 as drought treatment and sampled after 8 h (An *et al.* 2015); grown in an incubator at 40 °C as high temperature treatment and sampled after 24 h. Untreated leaves were sampled as control. All the samples were immediately frozen in liquid nitrogen and stored at -80 °C. Three samples for each tissue and treatment were separately stored as three biological replicates.

The total RNA was isolated and reverse transcribed separately using an *RNA Prep Pure* plant kit (*Tiangen Biomart*, Beijing, China) and a *GoScript* reverse transcription system (*Promega*, Madison, WI, USA) according to the manufacturer's instructions. Real time quantitative polymerase chain reaction (qPCR) analysis was conducted by an optical 96-well plate *iQ5 multicolor* real time PCR system (*Bio-Rad*, Hercules, CA, USA). Each 0.02 cm^3 reaction volume was mixed with 0.001 cm^3 of a cDNA template, 0.01 mM gene-specific primers, 0.01 cm^3 of *iTaq* universal *SYBR Green Supermix* (*Bio-Rad*), and 0.007 cm^3 of double distilled H_2O. The ramie glyceraldehyde-3-phosphate-dehydrogenase gene was selected as endogenous control (Kong *et al.* 2014). Gene-specific primers (Table 2 Suppl.) were online designed (http://primer3.ut.ee/) and commercially synthesized (*Sunny Biotech*, Shanghai, China). A thermal cycle used was as follows: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. After amplification, a dissociation stage was carried out to detect any complex products. The mean and the standard error of nine Ct values for each sample (*i.e.*, three biological replicates and each replicate performed three times in qRT-PCR analysis) were calculated. Relative expression was calculated according to Livak and Schmittgen (2001).

Results

By searching against the *Morus* genome database (He *et al*. 2013), 19 *Aux/IAA*, 17 *ARF*, and 32 *LBD* sequences were found in the mulberry genome (Table 3 Suppl.). By searching against the three ramie transcriptome databases, we found 18, 17, and 20 *Aux/IAA* homologs, 14, 20, and 14 *ARF* homologs, 23, 11, and 20 *LBD* homologs (Table 4 Suppl.). After further assembly and CDS prediction, 16 *Aux/IAA*, 14 *ARF*, and 16 *LBD* TFs contained full CDSs,

Table 1. Numbers of *Aux/IAA*, *ARF*, and *LBD* transcription factors in *Arabidopsis*, mulberry, and ramie.

Groups	IAA-I	IAA-II	ARF-Ia	ARF-Ib	ARF-II	ARF-III	ARF-IV	LBD-Ia	LBD-Ib	LBD-II
Arabidopsis	15	14	5	8	2	5	3	26	10	6
mulberry	11	8	5	1	2	5	4	21	6	5
ramie	12	4	4	0	2	5	3	14	0	2

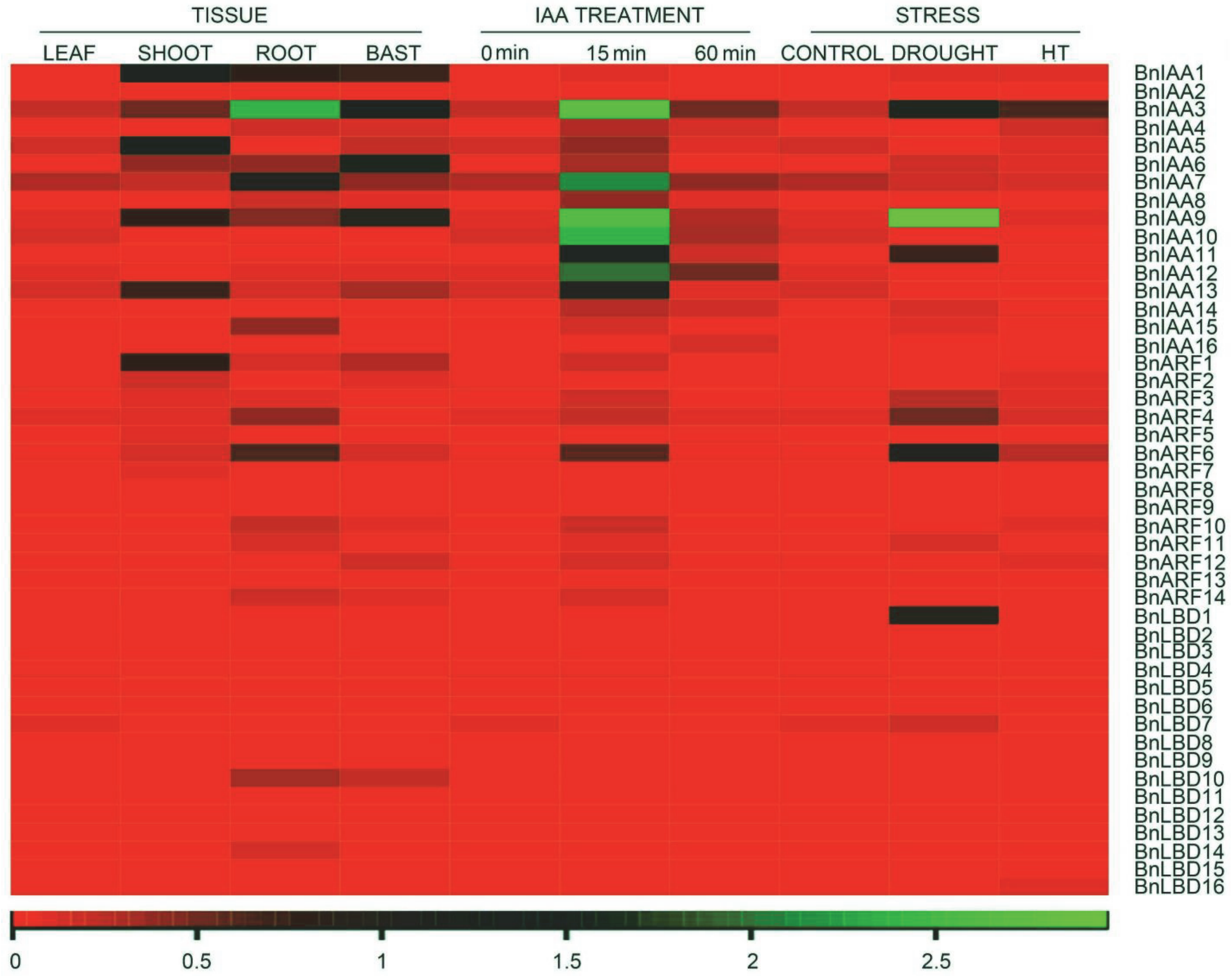

Fig. 1. The expression pattern of *BnAux/IAA*, *BnARF*, and *BnLBD* transcription factors in different ramie tissues [leaf, shoot, root, and stem bark (BAST)], under indole-acetic acid (IAA) treatment (after 0, 15, and 60 min) and abiotic stresses [DROUGHT (20 % polyethylene glycol for 8 h) and high temperature (HT; 40 °C for 24 h)]. The colour bar at the bottom indicates degree of expression: red - low expression; green - high expression.

Table 2. *BnAux/IAA*, *BnARF*, and *BnLBD* transcription factor expressions in major tissues and responses to indole-acetic acid (IAA), drought (D), and high temperature (HT). The *up* and *down arrows* represent an increase and decrease in expression, respectively. The *arrow* without an *asterisk* represents more than 3-fold and the *arrow* with an *asterisk* more than 10-fold changes.

Gene	Tissue	IAA response		Stress response	
		15 min	60 min	D	HT
BnIAA1	shoot	↑	↓	↑	↑
BnIAA2	shoot		↑	↓*	
BnIAA3	root	↑*	↓	↑	↑
BnIAA4	root	↑	↓		↑
BnIAA5	shoot	↑	↓	↓	
BnIAA6	bark	↑	↓	↑	
BnIAA7	root	↑	↓		
BnIAA8	root	↑	↓		
BnIAA9	bark	↑*	↓*	↑*	
BnIAA10	leaf	↑*	↓*		
BnIAA11	bark	↑*	↓*	↑*	
BnIAA12	constitutive	↑*	↓		
BnIAA13	shoot	↑	↓	↓	↓
BnIAA14	leaf	↑*		↑	↑
BnIAA15	root	↑	↓	↑	
BnIAA16	bark	↑	↑*	↓*	
BnARF1	shoot	↑	↓		
BnARF2	shoot			↓*	
BnARF3	shoot	↑	↓	↑	
BnARF4	root			↑	
BnARF5	shoot				
BnARF6	root	↑	↓	↑*	↑
BnARF7	shoot				
BnARF8	leaf			↓	
BnARF9	shoot			↓	
BnARF10	root	↑	↓		↑
BnARF11	root	↑		↑*	↑
BnARF12	bark			↓	
BnARF13	root				↑
BnARF14	root	↑	↓		
BnLBD1	leaf	↑	↓	↑*	↓
BnLBD2	root	↑	↓	↓	↓
BnLBD3	leaf	↑	↓	↓*	↓*
BnLBD4	leaf		↓	↓*	↓*
BnLBD5	constitutive		↓	↑	↑*
BnLBD6	leaf	↑	↓	↓	↓
BnLBD7	leaf	↓*			↓*
BnLBD8	root		↓*	↓*	↓*
BnLBD9	leaf	↓	↓		↓*
BnLBD10	root		↓	↑	
BnLBD11	leaf	↓	↓	↓*	↓*
BnLBD12	root	↓	↓*	↓	↓*
BnLBD13	leaf	↓	↓		↓*
BnLBD14	root		↓*		↓*
BnLBD15	leaf/root	↓*		↑	↑
BnLBD16	leaf	↓*		↓*	

which were named *BnIAA1* to *BnIAA16*, *BnARF1* to *BnARF14*, and *BnLBD1* to *BnLBD16*, respectively (Table 1 Suppl.). According to the *BLAST* result against GenBank, all 46 ramie TFs marked homology with known functions in other species.

The phylogenetic analysis divided all the proteins into established families (Majer and Hochholdinger 2011, Ha *et al.* 2013). There were nearly equal amounts of genes from the three species in groups IAA-I, ARF-Ia, ARF-II, ARF-III, and ARF-IV (Table 1, Fig. 1 Suppl.). Less or none ramie genes were clustered in groups IAA-II, ARF-Ib, LBD-Ia, LBD-Ib, and LBD-II. We further investigated the functional domains of ramie proteins (Fig. 2 Suppl.). Domains I - IV in Aux/IAA proteins, B3 and auxin response factor domains in ARF proteins, and lateral organ boundary domain in LBD proteins were all found in these proteins of the three families, respectively (Ha *et al.* 2013, Shen *et al.* 2014, Zhang *et al.* 2014).

In order to characterize the expression patterns in different organs, the relative expressions of all *BnIAA*, *BnARF*, and *BnLBD* TFs were analyzed in leaf, shoot, root, and stem bark tissues. The results indicate that there were five *BnIAA*, six *BnARF*, and five *BnLBD* genes with a higher expression in root than the other tissues (Table 2, Fig. 1). Two *BnIAA*, one *BnARF*, and nine *BnLBD* were expressed at higher levels in leaf, whereas four *BnIAA* and six *BnARF* genes had higher expressions in shoot. However, only four *BnIAA* and one *BnARF* gene were highly expressed in stem bark. Additionally, *BnLBD*15 showed a similar expression pattern in leaf and root. The remaining two genes, *BnIAA12* and *BnLBD5*, showed a constitutive expression in every tissue.

The response to the IAA treatment was also investigated in this study. The results show that six *BnARF*, all *BnIAA*, and *BnLBD* TFs had up- or down-regulated expressions by more than 3-fold when IAA treated indicating they were highly sensitive to IAA (Table 2, Fig. 1). Among these TFs, 13 *BnIAA*, 5 *BnARF*, and 4 *BnLBD* were up-regulated at 15 min and down-regulated at 60 min after the IAA treatment. Moreover, 12 *BnLBD* TFs were down-regulated, whereas 3 *BnIAA* and 1 *BnARF* TF were up-regulated during the process. The rest eight *BnARFs* were less sensitive than the other *BnARF* TFs.

Drought and high temperature treatments were conducted as stress in this study. The results show that 12 *BnIAA*, 10 *BnARF* and all *BnLBD* were up- or down-regulated by more than 3-fold when the plants were stressed, and so were considered to be stress-responsive TFs (Table 2, Fig. 1). Among these 39 TFs, the expressions of 11 *BnIAA*, 8 *BnARF*, and 12 *BnLBD* TFs were regulated by the drought, whereas 5 *BnIAA*, 4 *BnARF*, and 10 *BnLBD* TFs were regulated by the high temperature. There were 4 *BnIAA*, 2 *BnARF*, and 10 *BnLBD* TFs regulated by both the drought and the high temperature – up-regulated under the drought and down-regulated under the high temperature except *BnLBD1*. Overall, 15 (*BnIAA1,3,6,9,11,14,15, BnARF3,4,6,11, BnLBD1,5,10,15*) and 10 (*BnIAA1,3,4,14, BnARF6,10,11,13, BnLBD5,15*) stress-responsive TFs were up-regulated under the drought or the high temperature; 16 (*BnIAA2,5,13,16, BnARF2,8,9,12, BnLBD2,3,4,6,8,11,12,16*) and 13 (*BnIAA13, BnLBD1-4,6-9,11-14*) stress-responsive TFs were down-regulated.

Discussion

The new genome sequence of mulberry provided us a closely-related reference for evolutionary comparison (He *et al*. 2013). We found 19 *Aux/IAA*, 17 *ARF*, and 32 *LBD* TFs in the mulberry genome. In ramie, we successfully obtained 16 *BnIAA*, 14 *BnARF*, and 16 *BnLBD* TFs with full CDSs for the first time. According to the phylogenetic analysis, *Urticales* plants might possess smaller gene scales for *Aux/IAA*, *ARF*, and *LBD* families, especially the specific gene expansion of *Arabidopsis ARF* genes in group *ARF-Ib* (Table 1, Fig. 1 Suppl.). The phenomenon might be caused by a whole genome duplication (De Bodt *et al*. 2005). There were much less or none ramie genes in group IAA-II, ARF-Ib, LBD-Ia, LBD-Ib, and LBD-II, which suggests that our work might not cover the whole genome and the availability of ramie genome would be required in future studies.

The RT-PCR revealed a high expressions of *BnIAA* and *BnARF* TFs in leaf, shoot, root, and stem bark tissues (Table 2), which might indicate diverse functions of IAA and ARF TFs in plant growth and development (Liscum and Reed 2002). Interestingly, *BnLBD* TFs were highly expressed in leaf and root tissues. These genes might share the same functions with *AtLOB* and *AtLBD6,12* in leaf (Semiarti *et al*. 2001, Shuai *et al*. 2002, Nakazawa *et al*. 2003) and *AtLBD16,29,33* in root (Berckmans *et al*. 2011, Feng *et al*. 2012, Cabrera *et al*. 2014).

Under the IAA treatment, *BnARF*, *BnIAA*, and *BnLBD* TFs showed significantly or slightly differential expressions (Table 2, Fig. 1). The result indicates that these genes were actively expressed in auxin signaling transduction. In addition, increased or decreased expressions of eight *BnARF* TFs were between 1- to 3-fold, which was not as remarkable as *BnIAA* and *BnLBD*. This might be caused by a less sensitivity of *BnARF* than *BnIAA* or *BnLBD* to exogenous IAA. Similarly, sorghum *ARF* TFs are less sensitive to exogenous IAA compared with *IAA* TFs (Wang *et al*. 2010a).

Ramie is mainly cultivated in subtropics where drought and high temperature are main abiotic stresses during its life cycle. To better understand a relationship between auxin and abiotic stresses, we here investigated the expressions of Bn*IAA*, *BnARF*, and *BnLBD* TFs in regulating drought and high temperature stress tolerance. The differentially expressed *BnIAA*, *BnARF*, and *BnLBD* TFs indicate interrelations between auxin signaling and abiotic stresses. The expression patterns of *BnIAA*, *BnARF*, and *BnLBD* TFs regulated by drought and high temperature together or separately suggests the similarity or distinction of response regulations between the two stresses even if they are commonly caused by a hot weather. The *Aux/IAA* and *ARF* TFs have been proven to function in regulating drought and high temperature tolerance (Chen *et al*. 2013, Du *et al*. 2013). Our identification of *BnIAA* and *BnARF* TFs provides a basis for further study in ramie. The *LBD* TFs mainly regulate auxin dependent lateral organ formation (Majer and Hochholdinger 2011), only a few researches investigated *LBD* functions in response to abiotic stresses (Wang *et al*. 2010a). In this study, most *BnLBD*s were greatly down-regulated (over 10-fold) when the plants were stressed, which is similar to sorghum *LBD* TFs (Wang *et al*. 2010a). Interestingly, *BnLBD1* showed opposite expressions under the drought and high temperature stresses, which indicates that *BnLBD1* might perform distinct functions with other *BnLBDs*. We here report for the first time that 16 *BnLBDs* regulated high temperature stress tolerance, which provides a new perspective in high temperature tolerance of ramie.

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