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Research article

Transcriptome-wide analysis reveals core transcriptional regulators associated with culm development and variation in *Dendrocalamus sinicus*, the strongest woody bamboo in the world

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HIGHLIGHTS

- Transcription factors have different functions in culm development of bamboo.
- The signalling mechanism is most obviously regulated by transcription factors.
- MYB, C3H, and ARF are core transcriptional regulators in culm development.
- Fifty target genes regulated by 9 TFs are candidate genes for culm development.
- Eighteen target genes regulated by 7 TFs play key roles in culm differentiation.

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ABSTRACT

Transcription factors (TFs) play indispensable roles in plant development and stress responses. As the largest woody bamboo species in the world, *Dendrocalamus sinicus* is endemic to Yunnan Province, China, and possesses two natural variants characterized by culm shape, namely straight or bent culms. Understanding the transcriptional regulation network of *D. sinicus* provides a unique opportunity to clarify the growth and development characteristics of woody bamboos. In this study, 10,236 TF transcripts belonging to 57 families were identified from transcriptome data of two variants at different developmental stages, from which we constructed a transcriptional regulatory network and unigene-coding protein-TFs interactive network of culm development for this attractive species. Gene function enrichment analysis revealed that hormone signaling and MAPK signaling pathways were two most enriched pathways in TF-regulated network. Based on PPI analysis, 50 genes interacting with nine TFs were screened as the core regulation components related to culm development. Of them, 18 synergistic genes of seven TFs, including nuclear cap-binding protein subunit 1, transcription factor GTE9-like, and ATP-dependent DNA helicase DDX11 isoform X1, involved in culm-shape variation. Most of these genes would interact with MYB, C3H, and ARF transcription factors. Six members with two each from ARF, C3H, and MYB transcription factor families and six key interacting genes (IAA3, IAA19, leucine-tRNA ligase, nuclear cap-binding protein subunit 1, elongation factor 2, and coiled-coil domain-containing protein 94) cooperate with these transcription factors were differentially expressed at development stage of young culms, and were validated by quantitative PCR. Our results represent a crucial step towards understanding the regulatory mechanisms of TFs involved in culm development and variation of *D. sinicus*.

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1. Introduction

Transcription factors (TFs), which depend on internal and external signal molecules to regulate the synthesis of mRNA, are key determinants of gene expression, and play an essential role in plant development and stress responses [1, 2]. By combining with some upstream elements, TFs temporarily turn on or off transcription of their target genes [3, 4]. During plant growth and development, a large number of TFs are involved in different signal pathways. Meanwhile, functional regulation of target genes by TFs is complex and is associated with the TF dose, binding-site affinity, protein dimerization, etc. [2, 5]. Due to the lack of large-scale, high-quality transcriptional regulatory networks, a comprehensive understanding of the contribution of TFs to regulatory systems in plants has long been hindered.

Given importance of TFs in gene regulation, the underlying mechanisms of TFs on plant stress responses, growth and development have attracted much attention, and significant progress and achievements were made increasingly in recent years. TF families, including MYB, ARF, C3H, Aux/IAA, NAC, bHLH, and WRKY, etc., regulate the expression levels of functional genes and promote plant development, secondary metabolism, and plant resistance, such as tolerance to drought, salt, and low temperatures [6, 7, 8]. MYB are widely distributed and conserved TFs with diverse functions in almost all aspects of plant development and metabolism, and play important regulatory roles in cell differentiation, cell cycle, and plant morphogenesis in higher plants [6]. Auxin is the first plant hormone to be shown to promote cell division and cell elongation [7]. ARF, C3H and Aux/IAA families involved in auxin synthesis, polarity transport, and signal transduction regulate plant growth in *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), and rice (*Oryza sativa*) [9]. As the plant-specific TF evolving during the transition from aquatic to terrestrial plant life, NAC not only regulate genes involved in water conductance and cell support but also control flower and fruit formation [10]. Regulation of the bHLH and WRKY families in plant development is supposed to be related to GA signals [2, 11]. Not only are that, the co-expression of MYB and bHLH, and formation of the MYB-bHLH-WD40 (MBW) complex are responsible for a wide range of biological events [12, 13]. With the development of identification methods and gene research, the classes and number of identified plant TFs were constantly updated. By 2019, 1678 TFs of *Arabidopsis* TFs have been reported to be used to construct a universal gene expression regulation network using EXPLICIT approach [14]. In the same year, 2296 TFs from 58 families were recorded in the Plant Translation Factor Database (http://planttfdb.gao-lab.org/) [4]. The identification of TFs for many plants were based on these TFs in *Arabidopsis*.

As the largest subfamily of Gramineae, Bambusoideae includes ca. 1500 species and are native to all continents except Antarctica and Europe. They are not only an important wood source, raw material of architecture and pulp industry, but also are used in landscaping and environmental protection [15, 16]. Tropical woody bamboos (Bambuseae) are evolutionary groups in Gramineae, originating from differentiation within herbaceous and woody bamboo lineages. Compared to other gramineous plants, they are an excellent model system for studying plant growth due to their long vegetative growth period, unpredictable flowering period, and rapid shoot growth [16, 17, 18]. To date, research on TFs' regulation in bamboo growth and stress responses is relatively scarce. Although the importance of TFs in bamboo development has been emphasized, main concerns have been focused on expression analysis and identification of single TF families, such as bZIP, Aux/IAA, and CBF, based on high-throughput sequencing [19, 20, 21].

Dendrocalamus sinicus is a large-scale tropical sympodial bamboo that is endemic to Yunnan Province in China. As the largest bamboo species documented in the world, its culms can grow up to 30 cm in diameter and 30 m in height, with an amazing daily growth as much as 100 cm at young shoot stage [22, 23]. Within natural distribution area of *D. sinicus*, there are two stable variants based on culm shapes (namely, straight or bent culms), making this species an ideal model for comparative studies of culm development and morphological variation in woody bamboos [24]. However, the lack of genomic information limits study on molecular mechanisms of culm development and variation in this important and unique woody bamboo. In the present research, we performed transcriptome sequencing of two variants at different development stages to investigate the functions and characteristics of TFs in culm development of *D. sinicus*. We obtained 10,236 unigenes from 57 TF families and construct the transcriptional regulation network which provide a solid foundation for further research on the regulatory mechanisms of culm development in woody bamboos.

2. Materials and methods

2.1. Plant materials

The samples used in the present study were collected on 30 June, 2020 from Ximeng (99°32′35 E, 22°43′51 N) and Menglian (99°39′53 E, 22°26′61 N) Counties, Yunnan Province, respectively. For straight and bent culm type, four shoots from each of three clumps, representing the different developmental stages of shoots at 0, 5, 15, and 30 days after shooting, were sampled for transcriptome sequencing. Each sample was collected in three biological replicates marked as S0, S5, S15, S30, and B0, B5, B15, B30. Totally, 24 RNA samples were collected from the basal 1/3 of shoots after removing the sheath. The samples were frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA isolation, library construction, and sequencing

Total RNA of bamboo shoots or young bamboo samples were isolated using a plant RNA isolation kit (Aidlab Biotechnologies, Beijing, China). Qualified RNA samples were used to construct RNA-sequencing (RNA-seq) libraries, and RNA-seq was performed using the BGISEQ-500 sequencing platform by BGI Life Tech Co., Ltd. (Shenzhen, China). Clean reads were obtained after removing low-quality sequences from the raw data. The RNA-seq clean data from the 24 samples were deposited in the China National GeneBank DataBase (CNGBdb) of the Chinese National Standard Agency (CNSA) (https://db.cngb.org/cnsa/) with accession number CNP0000900, and accession number PRJNA610455 in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA).

2.3. De novo transcriptome assembly and functional annotation

De novo assembly was performed using Trinity v2.0.6 [25]. Unigene open reading frames (ORFs) were detected using getorf (http://emboss.bioinformatics.nl/cgi-bin/emboss/getorf) and the ORFs were then aligned to TF protein domains (data from TFs) using HMMER software (http://hmmer.org). Unigenes were then identified based on the characteristics of the TF families described in the plant TF database (PlantTFDB) [26]. The gene functions related to the TFs were examined by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis with Phyper function in the R package [27]. The *P* value can be calculated by hypergeometric distribution (https://en.wikipedia.org/wiki/Hypergeometric_distribution). All *P* values were false discovery rate (FDR) corrected to obtain FDR-corrected *P* values (Q values), and a Q value of $\leq$0.05 was treated as significant.

2.4. Co-expression analysis of the differentially expressed genes

Gene expression levels were determined and analyzed by RSEM v1.2.8 [28]. Differentially expressed genes (DEGs) between different growth stages among the straight or bent culm type, and between two culm types at the same growth stage were then identified using DEGseq with fold change $\geq$2 and adjusted using a P value $\leq$0.001 based on a Poisson distribution [29]. DEGs with a maximum fragments per kilobase of transcript per million mapped reads (FPKM) value $<$10 in comparisons

were removed. A co-expression network of TFs and related genes was constructed using the WGCNA R-package with the parameters power = 8, merge cut height = 0.15, and weight threshold = 0.25 [30]. The gene co-expression network was then visualized in Cytoscape software [31] to discover key TFs in the development of *D. sinicus*.

2.5. Prediction of TF interacting proteins

DEGs were compared by BLASTX (E-value was set to 1e-10) in the STRING protein interaction database (http://string-db.org/) to identify protein-protein interaction network (PPI network) [32]. The lower string score limit of protein interaction in the database was set to 900. Subsequently, fuzzy clustering via mufzz was used for soft clustering of gene-expression data during the development of *D. sinicus* [33]. Clusters with increased or decreased expression during the development of the two types of *D. sinicus* were selected to screen related TFs and their interacting proteins.

2.6. Validation of the predicted core transcriptional regulators using qRT-PCR

The CFX96 Real-Time PCR Detection System (Bio-Rad) was used to perform validation for the predicted 12 core regulators, including 2 in each of ARF, C3H, and MYB and their target genes that were differentially expressed during the development of *D. sinicus* in transcriptome data. Gene-specific primers for qRT-PCR were designed using Primer 3 (Table S1). cDNA was synthesised from 10 μg of total RNA using TransScript II One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China). The amplifications were performed using the SYBR Green I method with TransStart Top Green qPCR SuperMix (TransGen Biotech, China). All reactions were performed with three biological replications. *Actin* gene was used as an internal control for normalization and data were quantified by using the $2^{-\triangle\triangle Ct}$ method [34].

3. Results

3.1. Putative TF profiles in the development of D. sinicus

The hmmsearch program in the HMMER3.0 was used to identify unigenes encoding TFs based on the TF prediction server of the PlantTFDB. In total, 10,236 unigenes from 57 TF families were identified (Table 1). Among them were 1173 unigenes of the MYB family and more than 700 members of the bHLH, C3H, and AP2-EREBP families. These four TF families comprised 33.6% of the total unigenes, indicating their importance in *D. sinicus* culm development. The first 15 types of TF families with more than 200 members contained 7251 unigenes, accounting for 70.8% of all unigenes.

3.2. GO enrichment of putative TFs in the development of D. sinicus

GO term enrichment analysis revealed 57 TF families involved in 569 GO terms (Table 1), of which 346 were involved in biological processes, 79 and 144 were enriched in cell components and molecular function, respectively. Among them, 1173 unigenes from the MYB family participated in the most GO terms (134). Respectively, 1113 and 969 unigenes were enriched in DNA binding (GO: 0003677) and nucleus (GO: 0005634) terms, which also enriched many unigenes from other TF

Table 1. Putative TF family profiles in the development of *D. sinicus*.

	Family name	Unigene Number	KEGG number	GO number		Family name	Unigene Number	KEGG number	GO number
1	MYB	1173	25	134	30	Tify	78	2	14
2	bHLH	819	22	33	31	CPP	74	0	3
3	C3H	739	32	92	32	HB	74	3	12
4	AP2-EREBP	713	10	63	33	TIG	73	0	4
5	NAC	477	27	38	34	SRS	72	8	9
6	ARF	467	2	24	35	LOB	64	9	22
7	SBP	429	12	4	36	BBR/BPC	57	1	4
8	G2-like	423	17	20	37	ARR-B	52	1	9
9	WRKY	362	12	40	38	RWP-RK	48	5	7
10	FAR1	355	22	49	39	OFP	45	16	8
11	GRAS	302	19	29	40	C2C2-YABBY	44	4	20
12	Trihelix	262	22	40	41	TAZ	43	4	13
13	C2H2	256	18	47	42	EIL	41	2	11
14	TUB	255	8	16	43	BES1	38	2	11
15	C2C2-Dof	219	12	10	44	C2C2–CO-like	31	5	12
16	ABI3VP1	199	26	23	45	zf-HD	31	6	7
17	MADS	195	0	20	46	VOZ	29	3	7
18	bZIP	181	8	26	47	CAMTA	22	1	7
19	mTERF	171	1	23	48	VARL	20	2	3
20	Alfin-like	158	0	21	49	CSD	19	0	13
21	C2C2-GATA	157	11	8	50	PLATZ	14	3	3
22	FHA	146	8	37	51	PBF-2-like	13	1	6
23	LIM	131	1	11	52	S1Fa-like	10	0	4
24	GRF	117	10	10	53	ULT	9	0	3
25	TCP	106	8	19	54	DBP	8	0	6
26	E2F-DP	105	0	21	55	Sigma70-like	8	3	14
27	HSF	105	4	12	56	HRT	4	0	0
28	GeBP	97	8	20	57	LFY	1	0	0
29	BSD	95	12	9					

Notes: KEGG and GO numbers represent the numbers of KEGG pathways and enriched GO terms in the annotated unigenes, respectively.

families. The nucleus term was the most abundant, accounting for 6246 unigenes in 49 TF families, followed by DNA binding enriched in 4828 unigenes from 34 TF families.

Because only a few GO terms were significantly enriched, the 10 most significantly enriched of the 15 TF families with the most annotated unigenes were selected for analysis (except for the SBP TF, in which only four GO terms were enriched), using a total of 93 selected GO terms. As shown in Figure 1, there were 60 enriched GO terms in biological processes, most of which were related to plant development processes, with 9 related to stress-response processes. However, the other terms enriched fewer unigenes, except for transcription regulation DNA-dependent (GO: 0006355) and auxin-activated signalling pathways (GO: 0009374) related to ARF.

The GO enrichment analysis revealed the involvement of TFs in plant-development regulation. In addition to participating in a wide range of biological processes, MYB regulated cell proliferation and differentiation; bHLH was involved in regulating protein dimerization activity; C3H, SBP, C2C2-Dof, and FAR1 were involved in regulating metal ion binding; and ARF, WRKY, and GRAS were involved in plant hormone signalling, such as IAA, GA, ABA, brassinosteroid (BR), and SA. In addition, as a

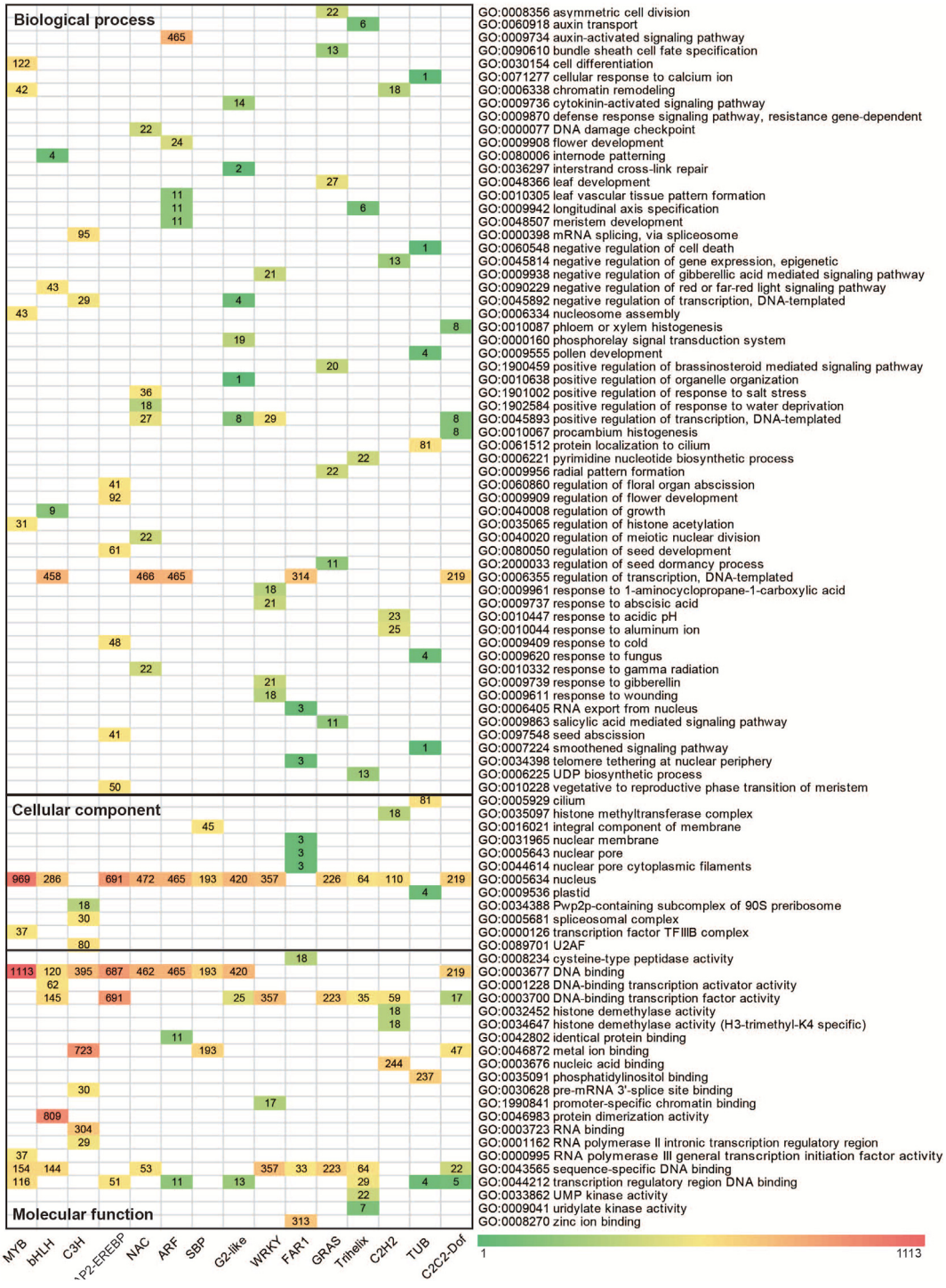

Figure 1. The 10 most significantly enriched GO terms of the 15 TF families with the most annotated unigenes. The number in the grid represents the number of unigenes in a TF family participating in the corresponding GO term.

plant-specific TF, GRAS also regulated asymmetric cell division, bundle sheath cell-fate specification, and radial pattern formation, all of which are important in bamboo development.

3.3. KEGG enrichment of putative TFs in the development of D. sinicus

The KEGG enrichment analysis showed that 57 TF families were enriched in 87 KEGG pathways in the development of *D. sinicus* (Table 1). Among them, the top 15 TF families with the largest numbers of unigenes were enriched in 75 KEGG pathways, including 31 pathways with Q values <0.01 (Figure 2). The C3H family was enriched in the most KEGG pathways, and 461 of 739 unigenes were enriched in 32 KEGG metabolic pathways. NAC, ABI3VP1, and MYB were enriched in 27, 26 and 25 KEGG pathways, respectively.

The plant hormone signal transduction (ko04075) pathway enriched the largest number of TFs, including 2330 unigenes from 16 TF families. Of these, 585 were from bHLH family members, followed by 463 from ARF and 381 from SBP. The other four KEGG pathways contained more than 500 unigenes, including the MAPK signalling (ko04016, 722), RNA transport (ko03013, 750), circular rhythm (ko04712, 715), and mRNA surveillance (ko03015, 574) pathways. Some KEGG pathways involved many kinds of TF families. For example, the mRNA surveillance pathway (ko03015) involved up to 29 TF families, followed by the plant MAPK signalling pathway (ko04016), which involved 27 TF families. In addition, the phenylpropanoid biosynthesis (ko00940, 26), RNA transport (ko03013, 25), amino sugar and nucleotide sugar metabolism (ko00520, 23), and spliceosome pathways (ko03040, 22) all involved more than 20 TF families. However, 24 TF families enriched in amino sugar and nucleotide sugar metabolism (ko00520) contained only 134 unigenes.

Similar to the results of GO enrichment by TFs, KEGG enrichment also showed that various TFs are involved in the regulation of bamboo development. Taking the 15 most abundant TFs as an example (Figure 2), the plant hormone signal transduction pathway was mainly enriched with six TF categories: bHLH, ARF, SBP, G2-like, FAR1, and GRAS. The plant MAPK signalling pathway was mainly enriched with bHLH, WRKY, and FAR1. The plant circadian rhythm signalling pathway was mainly enriched with MYB, bHLH, and C2C2-Dof. C3H, NAC and Trihelix were mainly enriched in amino acid metabolism and genetic information processing, such as RNA transport and mRNA surveillance. C2H2 was enriched mainly in the synthesis and metabolism of nutrients.

3.4. Analysis of differentially expressed genes co-expressed with putative TFs during the development of D. sinicus

Using RNA-seq data, the whole-transcriptome gene co-expression network at different development stages of *D. sinicus* was established. The gene co-expression modules containing TFs were obtained by network division. To focus the scope of the TF families, the members in the same gene family were clustered together in Figure 3. There were 1487 co-expression relationships with 20 TF families in the network. The non-TFs genes co-expressed with these TFs were automatically divided into two clusters (A and B) from Cytoscape software. It can be seen from Figure 3 that 16 TFs—including ARF, C3H, GRAS, bHLH, SRS, C2C2-GATA, NAC, and RWP-RK—were mainly co-expressed with Cluster A (138 unigenes), while four TFs—NAC, MYB, WRKY and AP2-EREBP—were mainly co-expressed with Cluster B (1531 unigenes). In addition, the TFs also interacted with each other, as shown in Figure 3. These results indicated that genes involved in the development of the culm of *D. sinicus* may intensively be regulated by members from the four TFs of NAC, MYB, WRKY, and AP2-EREBP.

The GO and KEGG enrichment analysis for genes in the co-expression network showed that some genes in Clusters A and B were enriched in the same GO terms or KEGG pathways (Figure 4). For example, PSII associated light-harvesting complex II catabolic process, cholorophyll (ide) b reductase activity, the chlorophyll catabolic process, the autophagosome membrane, and the cytoplasmic vesicle had significant enrichment in both clusters (Figure 4a and b). And, 13 significantly enriched KEGG pathways were related to metabolism and the degradation of various amino acids, as well as the synthesis and metabolism of biological macromolecule in both clusters (Figure 4c and d). However, based on their enriched GO terms, the genes in Cluster A were concentrated mostly in the biological process related to plant growth and development, such as beta-alanine metabolic and biosynthetic processes, binding and response to metal ions, as well as the basic activities of cell development such as dioxygenase, fucosyltransferase, peptidase, and oxidoreductase activity, which were mainly regulated by ARF, C3H, GRAS, bHLH, C2C2,

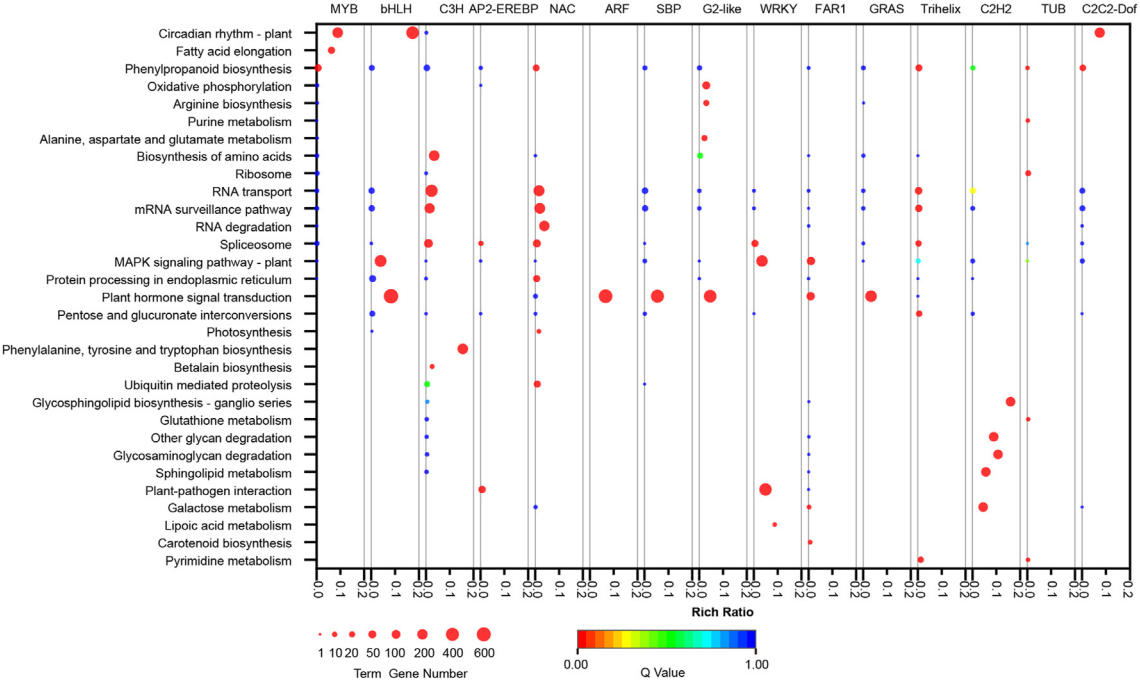

Figure 2. KEGG pathways with Q values <0.01 from the 15 TF families with the most annotated unigenes.

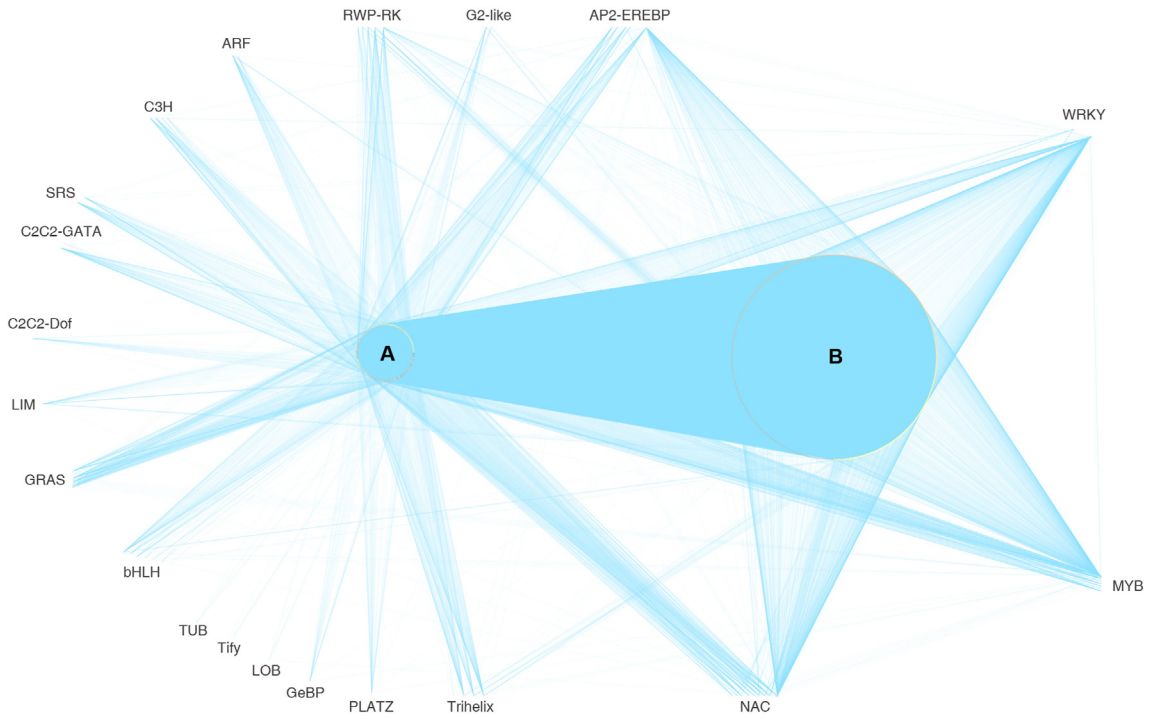

Figure 3. Co-expression network of *D. sinicus* based on DEGs with a highlight of putative regulating TF families. The same transcription factors were displayed together, each transcription factor had several points representing a single gene, respectively.

RWP-RK, etc. In Cluster B, most of the GO terms were closely related to molecular function in the regulation of plant growth and development, mainly in cell responses to various signals including nitrogen starvation, ABA, bacterium, sucrose, and cold, as well as the regulation of various biological processes including iron ion transport, meiotic nuclear division, meristem development, ruffle assembly, germination, and catalytic activity. According to the GO and KEGG enrichment of co-expression network, there were more co-expressed genes be regulated by NAC, MYB, WRKY, and AP2-EREBP.

3.5. Screening of interacting proteins in the culm development and variation of D. sinicus

Through PPI network analysis of DEGs, 477 proteins were predicted to be associate with 29 TF families in the development of *D. sinicus* (Table S2 and Figure S1). There were at least 50 key TF nodes with more than 5 interacting proteins. MYB and C3H comprised the majority of nodes in this network, suggesting that they are more likely to be crucial transcriptional regulators. In conjunction with the fuzzy clustering results, 58 proteins interacted with 16 TF families were selected because of continuously increasing expression with the development of at least one type of *D. sinicus* (Figure 5a and Table S3). Some of these proteins were coordinately regulated with different TF families, such as transcriptional corepressor LEUNIG (XP_015634285.1), which possesses the WD40-repeat domain interacting with different proteins to regulate multiple developmental processes in angiosperms [35], interacted with AP2-EREBP, C2H2, and MYB in *D. sinicus*. Some proteins were interacted with different members of the same TF family, such as auxin-responsive protein IAA10 (A2XB18.2) and DNA topoisomerase 2 (XP_015627338.1), which interacted with two unigene-coding proteins of the ARF and MYB families, respectively. Some TFs interacted with multiple target proteins. For example, MYB (CL6131.Contig11) interacted with pre-mRNA-processing factor 19-like (XP_020169930.1), coiled-coil domain-containing protein 94 (XP_006644321.1), pre-mRNA-splicing factor SLU7 (XP_020147738.1), zinc finger CCCH domain-containing protein 49-like (XP_020090512.1), CDT1-like protein (XP_020183286.1), the expression of which increased with the development of *D. sinicus*. Similarly, the expression of three interacting protein of BSD (CL11103.Contig1), and two of C3H (Unigene81229), E2F-DP (Unigene164424), and VARL (Unigene95837) also increased with the development of *D. sinicus* (Table S3).

On the contrary, expression of 42 genes which would interact with 13 TF families decreased with the development of at least one type of *D. sinicus* (Figure 5b and Table S4). The genes had the reverse regulation effect in the development of *D. sinicus*. Similar to the control of up-regulated gene expression during development, they also interacted with different TF families or different members of the same family (Table S4). ATP-dependent helicase BRM (XP_006648224.1) was linked with five unigenes of LFY and MYB. As the key enzymes in ubiquitination during plant development [36], E3 ubiquitin-protein ligase RBBP6 (EMS56750.1) interacted with C3H and Trihelix. The seven unigenes of C2H2 family were all involved in the interaction of PP2C and CNBD-containing protein (RLN07474.1).

Based on co-expression network and expression clustering analysis (Figures 3 and 5), we determined 50 key regulatory genes of culm development and predicted nine TFs in the co-expression network that regulate them (Table S5). Amazingly, 40 of them participate in the function of ARF, C3H and MYB, indicating their pivotal roles in transcriptional regulation of TFs. Among them, three genes, nuclear cap-binding protein subunit 1 (XP_003557925.1), transcription factor GTE9-like (XP_006647466.1), and ATP-dependent DNA helicase DDX11 isoform X1 (XP_025881112.1), would function together with C3H family, showed the opposite trend with the development between straight and bend culm types of *D. sinicus* (Figure 5). Similarly, there were 15 other genes (Table S6), such as luc7-like protein 3 (XP_015695268.1), transcription and mRNA export factor SUS1 (XP_006645241.1), auxin-responsive protein IAA10 (A2XB18.2), etc., 11 of which would work together with MYB, C3H, and ARF. They could be screened as candidate genes for key synergistic factors in studies for culm variation of *D. sinicus*.

3.6. qRT-PCR verification of the co-expressed and interacting genes

Combined with the level and pattern of gene expression, 6 co-expression genes (auxin-responsive protein IAA3, IAA19, leucine-tRNA ligase, nuclear cap-binding protein subunit 1, elongation factor 2, and

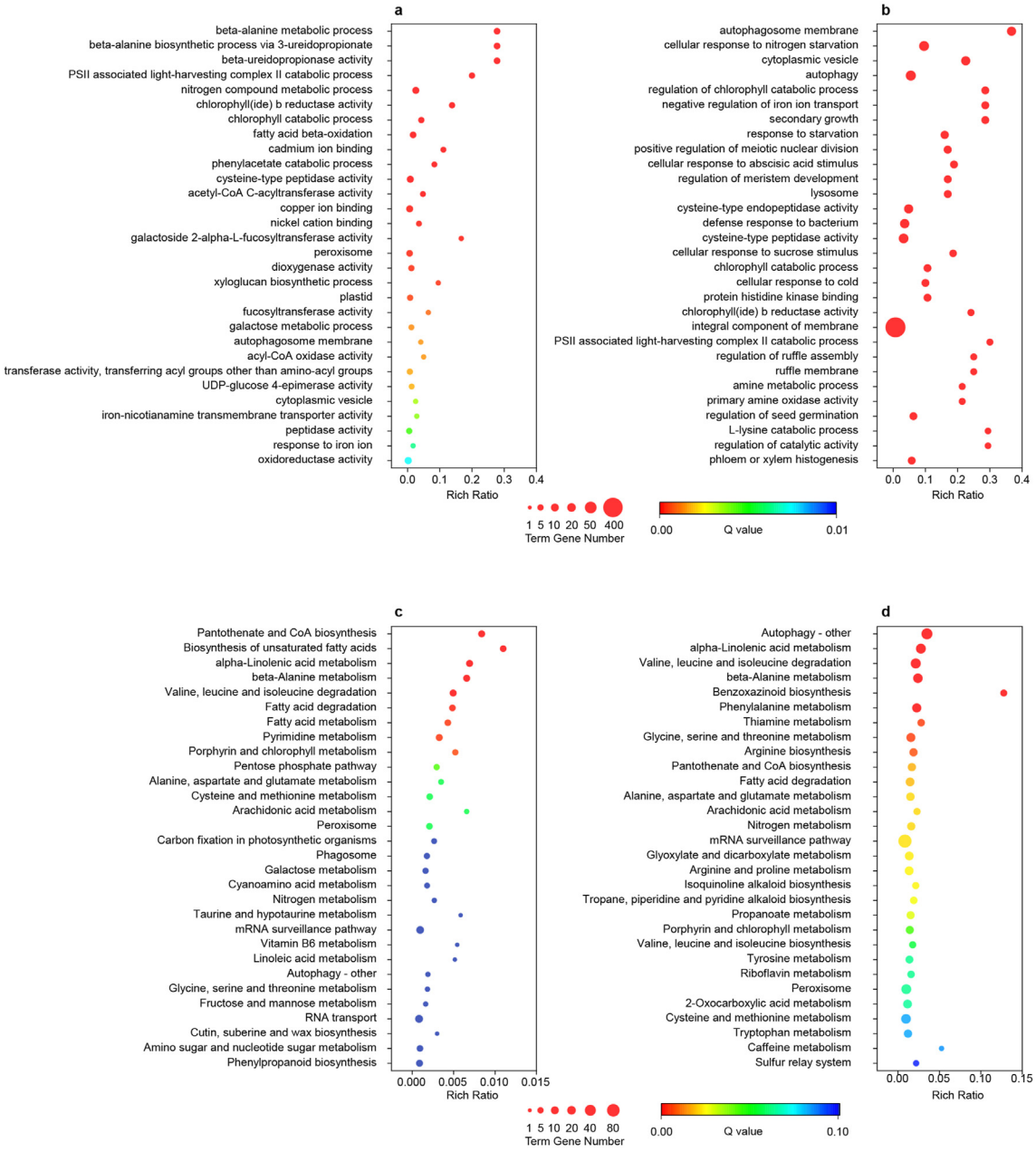

Figure 4. GO and KEGG enrichment analysis of unigenes related to putative TFs in the co-expression network of *D. sinicus*. (a) GO enrichment analysis of unigenes in Cluster A. (b) GO enrichment analysis of unigenes in Cluster B. (c) KEGG enrichment analysis of unigenes in Cluster A. (d) KEGG enrichment analysis of unigenes in Cluster A.

coiled-coil domain-containing protein 94 homolog) and 6 related TFs (2 in each of ARF, C3H, and MYB) were finally selected for qRT-PCR verification. All of them might be critical for the culm development and variation of *D. sinicus*. The qRT-PCR results showed that the expression patterns of most genes were similar to those from the RNA-seq data (Figure 6), indicating that our RNA-seq results were reliable.

4. Discussion

TFs, which play important roles in plant development and stress responses, control gene expression by binding to specific cis elements [4]. Systematic identification of TFs, regulatory factors, and the genes with which they interact will therefore greatly promote elucidation of their mechanisms. Woody bamboo is the fastest growing plant known in the world, and it is an excellent model system for studying plant growth [16, 18]. Because there are two variants in *D. sinicus*, the transcriptional regulatory network can not only provide a reference for plant development, but also for mechanism of culm morphological variation in woody bamboo.

Through transcriptome analysis of two variants at different developmental stages, 10,236 unigenes from 57 TF families were annotated in *D. sinicus*. MYB, a TF family with multiple members and diverse functions in all eukaryotes, plays key regulatory roles in plant development, metabolism, and stress responses [37]. In *A. thaliana* and *Populus trichocarpa*, MYB TFs comprise nearly 200 members [37], while in *Phyllostachys edulis*, there are 114 R2R3 MYBs [38]. In this study, 1173 unigenes of the MYB family in *D. sinicus* were annotated. A large number of unigenes can be related to difficulty in obtaining the full length of unigenes without reference genome assembly. Beyond that, hexaploid *D. sinicus* aggravated this difficulty, so the number of TF members

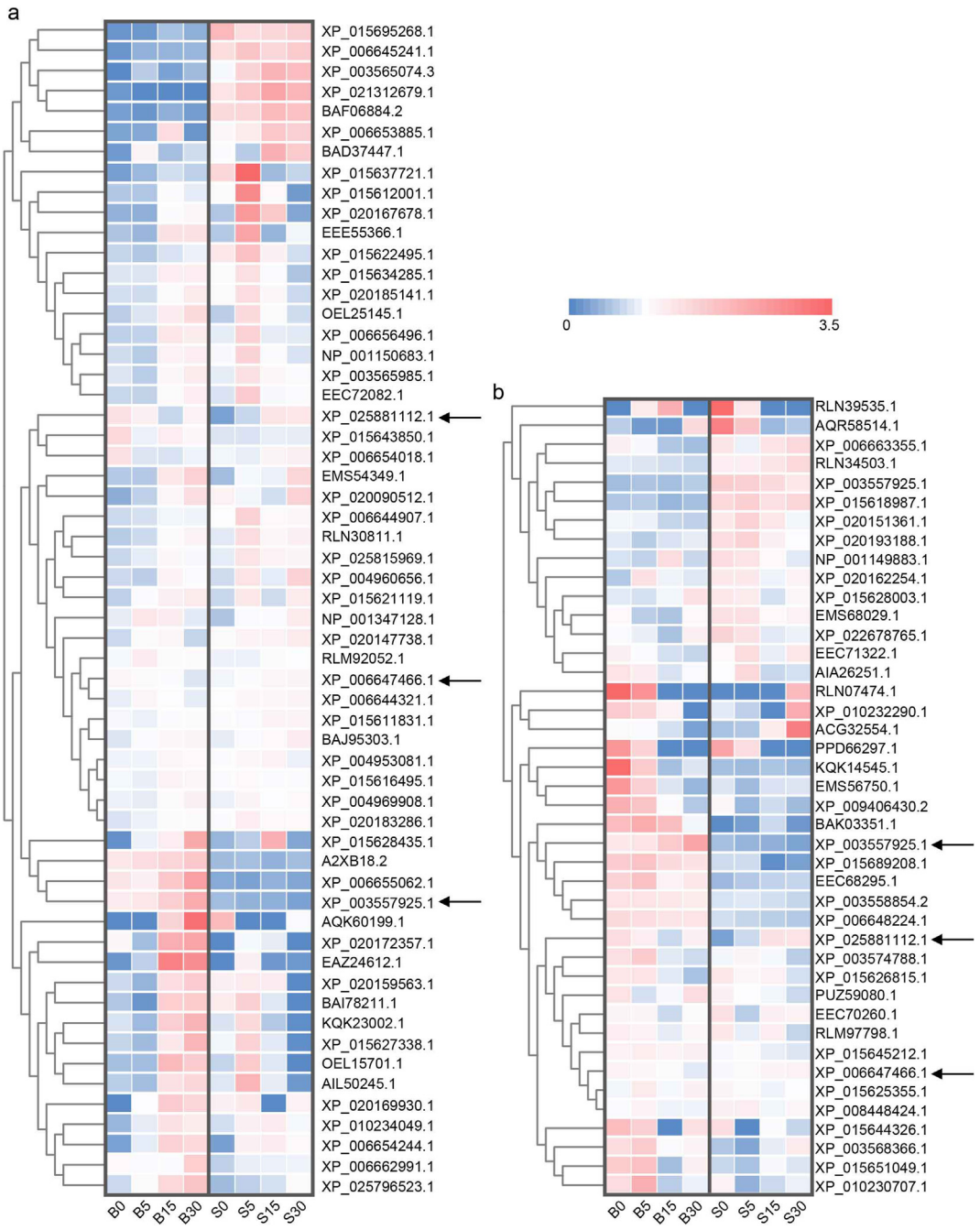

Figure 5. The expression profiles of predicted target genes with continuous increase (a) and decrease (b) in the development of at least one type of *D. sinicus*. The colour scale is shown at the top of figure b. Higher expression levels colour in red while lower expression levels colour in blue. The genes indicated by the arrow had the opposite expression trend with the development between the straight and bend culm types of *D. sinicus*.

obtained in this study may be higher than the actual numbers. Similarly, in the medicinal plant *Saussurea lappa*, 24,610 unigenes from 58 TF families were obtained by analysing comparative transcriptomics [39]. Even so, the results of this study, combined with GO and KEGG functional classifications, WGCNA co-expression networks, and protein interaction networks, can still provide valuable information for revealing the transcriptional regulation network on the growth and development of woody bamboos.

4.1. TF roles in the culm development of D. sinicus

Analysis of the TFs profiled in the development of *D. sinicus* showed that most were related to basic biological functions. Among 57 TF families, 49 and 34 were involved in the regulation of nucleus and DNA binding, respectively, but the focus on TFs involved in regulation of plant development was slightly different in our study. The MYB family regulates a variety of biological processes, such as differentiation, cellular morphogenesis, and secondary-cell-wall biosynthesis and meristem formation [38]. An *MYB* gene from *Betula platyphylla* (*BplMYB46*) increases lignin precipitation and secondary-cell-wall thickening by activating gene expression during secondary-cell-wall synthesis, while in Eucalyptus, *EgMYB1* inhibits lignin synthesis by combining with histone variant EgH1.3 [38, 40, 41]. Here, the results of GO enrichment indicate that the MYB family plays a significant role in regulating cell differentiation. The family may integrate developmental signals to prevent premature or inappropriate lignification of secondary cell walls, providing a

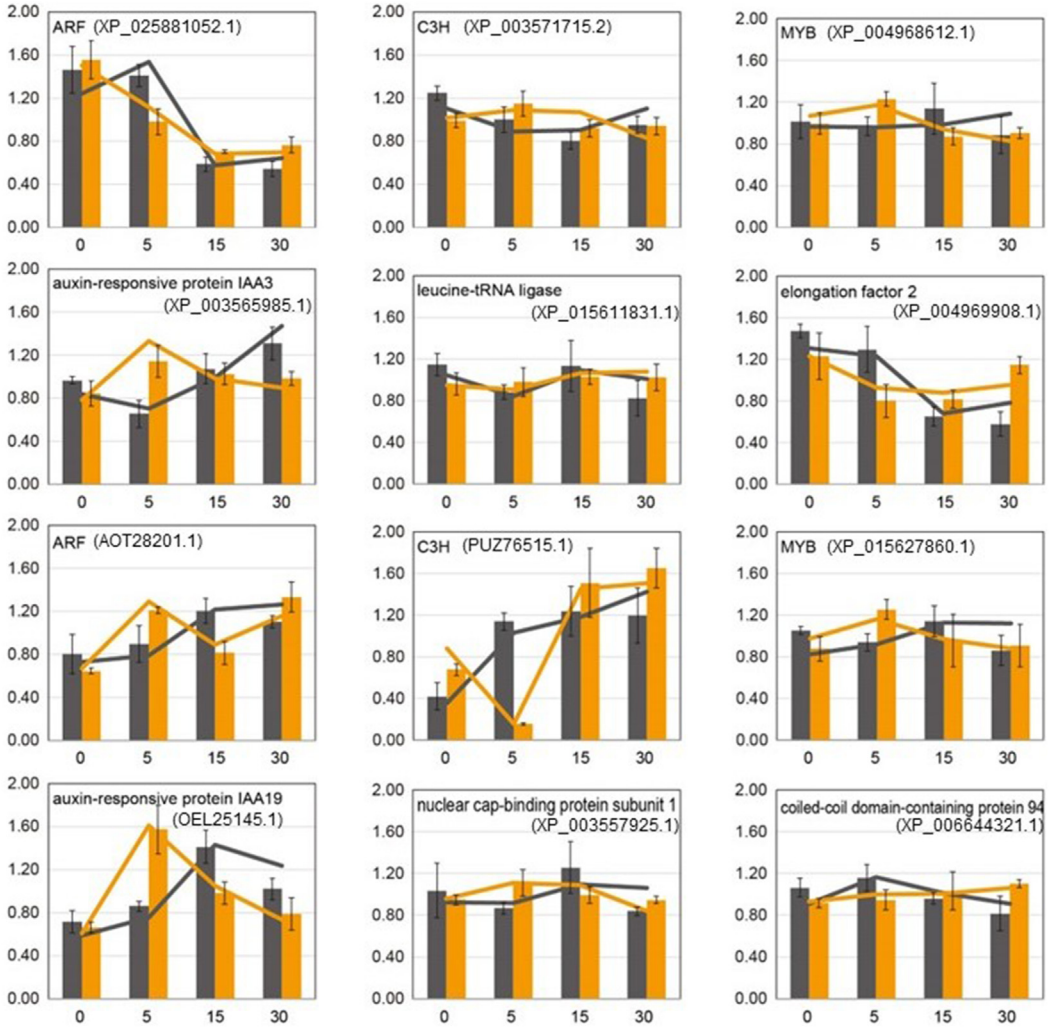

Figure 6. qRT-PCR validation profiles of 12 selected genes, including 2 in each of ARF, C3H, MYB and their target genes from different developmental stages of shoots at 0, 5, 15, and 30 days after shooting in different types of *D. sinicus*. The grey and orange represent bent and straight culm types of *D. sinicus*, respectively. The data was normalized by using *Actin* as an internal reference. The fold change values of relative expression patterns of different genes are shown on Y axis for qRT-PCR. The broken lines show the expression trend of RNA-Seq data.

mechanism to fine tune the differentiation of xylem cells in time and space [42]. This might be important for the development of *D. sinicus* and even be related to the formation of its straight or bent culm types. MYB, together with bHLH and C2C2-Dof, regulate the circadian rhythm in the development of *D. sinicus*. Recent studies have also shown that the circadian rhythm can integrate the sugar response and the strigolactones pathway to regulate rice plant architecture [42]. bHLH might regulate culm development by regulating cell expansion, as the main regulator of protein dimerization activity in the development of *D. sinicus* [43]. Previous studies have also confirmed that the production of flavonoids, the most important secondary metabolite in the phenylpropanoid pathway, is regulated by the MBW repeat transcription complex model [12]. Predicted results of TF and co-expressed genes during the development of *D. sinicus* (Figure 5) also showed a correlation between MYB and bHLH. In *D. sinicus*, differences in gene expression in the phenylpropanoid pathway are an important control factor in the formation and development of two culm variants [23]. These results indicate that the MYB and bHLH families play synergetic roles in the development of *D. sinicus*.

Plant hormone signals are involved in multiple vital plant processes, including growth, development, and senescence, and the differential expression of genes related to plant hormones is a key factor in the initiation of *D. sinicus* culm differentiation [24]. In this study, the TFs regulating plant hormone signal transduction were mainly members of the bHLH, ARF, SBP, G2-like, FAR1, and GRAS families. Together with WRKY, bHLH and FAR1 also regulated the MAPK signalling pathway. These two signalling pathways are closely related. In rice, MAPK signalling has major biological significance in plant hormones, especially SA, jasmonic acid (JA), ethylene (ET), and ABA responses [44]. Among the seven TF families involved in these two pathways, ARF and G2-like were specifically associated with auxin and cytokinin activated signalling pathways, respectively. GRAS was involved in the SA mediated signalling pathway, and WRKY was involved in the response of ABA and GA. As a plant-specific TF, GRAS also regulates asymmetric cell division, bundle sheath cell fate specification, and radial pattern formation, which are extremely important in bamboo development. In *A. thaliana*, many members of the WRKY family participate in the GA pathway to regulate plant development through interaction with the DELLA protein [10]. Expression of *P. edulis PheWRKY72-2* in Arabidopsis increases the drought resistance of transgenic plants by regulating stomatal closure via the ABA pathway [45], as GO enrichment predicted in this study. This study did not predict which hormone signal SBP was involved in. However, in Arabidopsis, some SBP genes like *AtSPL3* and *AtSPL8* are involved in the response of plant pollen development in GA signalling, while the apple (*Malus* × *domestica* Borkh.) *MdSBP* gene is also induced or inhibited by different plant hormones, indicating their roles in plant development or stress responses [46, 47]. However, due to limited

research on the role of the *SBP* gene in the plant hormone signalling pathway, direct evidence supporting these hypotheses is limited. Therefore, further study on the potential roles of the *SBP* gene in hormone signal transduction is of great significance.

4.2. Speculation of key TFs in the culm development of D. sinicus

It has been proven that co-expression networks are an effective way to discover the function of TFs in plants, animals, insects, and yeast [48]. Twenty TFs related to the development of *D. sinicus* were identified by constructing a co-expression network of the whole transcriptome.

However, according to the GO enrichment results, four transcription factors—NAC, MYB, WRKY and AP2-EREBP—were mainly related to responses to various signals and regulation of various biological processes. The results of co-expression network analysis in Arabidopsis, rice, and sugarcane show that these four TF families are related to cell wall synthesis [48, 49, 50, 51], implying that they may have similar functions in *D. sinicus*. In *P. edulis*, the four TF families also involved in shoot growth, but most of them were down regulated [44]. Limited studies have shown that RWP-RK, a class of plant-specific TF, is a key regulator of nitrogen responses and gametophyte development [52, 53]. Here, GO enrichment results also showed that RWP-RK relates to nitrogen responses. The co-expression network in this study implied that RWP-RK may have a similar status to that of NAC, MYB, WRKY and AP2-EREBP. In the plant kingdom, bamboo is famous for its rapid growth. The complex structure of bamboo cell walls is a key factor in the physical and mechanical properties of bamboo [54]. It is evident that these TFs involved in cell-wall synthesis play major roles in the development of *D. sinicus*. In the meantime, we identified other 15 TF families in our co-expression network, including members of the ARF, C3H, GRAS, bHLH, C2C2, etc., similar to the work of Ferreira and colleagues [48]. These genes identified in two types of *D. sinicus* may represent conserved modules in woody bamboo cell wall metabolism.

4.3. Selection of key interacting genes in the development of D. sinicus

The interacting proteins of TFs play important roles in the regulation of downstream gene expression by TFs. In the same biological process, the proportion of co-occurring regulatory pairs is usually used to evaluate network quality [3]. In this study, 477 genes from 29 TF families were predicted by protein-protein interaction network analysis, and 111 regulatory co-occurring pairs were found, implying that the regulatory network diagram is reliable.

The MYB family originated early in plant evolution, about 1000 million years ago. The essential involvement of MYB family members in plant growth and development, senescence, and stress response has been proven [26]. In *P. edulis*, its importance in shoot growth had also been mentioned [44], but how they work is unknown. In this study, PPI network and gene expression pattern showed 20 of 50 key interacting genes for regulating culm development with MYB. Although there is no evidence that MYB directly interact with these genes, their involvement in plant development has been reported. In *A. thaliana*, *CLUMSY VEIN* (*CUV*), a homologous gene of pre-mRNA-processing factor 19, facilitates auxin-meditated vascular development, while the splicing factor for phytochrome signalling (*SFPS*) can regulate genes involved in light signalling, photosynthesis, and the circadian clock [55, 56]. In this study, the results of TF metabolic pathway enrichment and co-expression network analysis showed that MYB is involved in circadian rhythm and photosystem regulation in *D. sinicus*, and may participate in the regulation of related pathways by interacting with pre-mRNA-processing factor. CCCH zinc finger genes are involved in post transcriptional regulation of gene expression through RNA-binding, which affects plant growth and development [57]. PaC3H17, a CCCH gene in *Populus* was found regulating xylem formation by interacting with MYB [58]. In co-expression network of TFs in *D. sinicus*, CCCH domain-containing proteins directly connected to MYB, were also predicted to interact with MYB (Figure 3). In addition, MYB may regulate the growth and development of *D. sinicus* by interacting with pre-mRNA-processing factor, elongation factor, actin-related protein, ATP-dependent helicase, and TATA-box-binding protein. Direct evidence about the roles of elongation factor and actin in cell wall formation has been confirmed in *Lilium formosanum* [59], but these hypotheses need to be confirmed in *D. sinicus*. In particular, expression of some interacting genes, such as pre-mRNA-processing factor 19 (XP_015614850.1), pre-mRNA-splicing factor SLU7 (XP_020147738.1), and zinc finger CCCH domain-containing protein 49-like (XP_020090512.1), increased during the development. These genes may be critical in the development of *D. sinicus*, which will be the focus of future research.

C3H zinc finger genes are closely related to cell wall biosynthesis and xylem formation [58]. In this study, 14 genes interacting with 8 C3H were screened as key candidate genes in culm development. Especially, 9 of 18 genes involved in the variation of culm shape in *D. sinicus* were regulated by C3H. In *D. sinicus*, C3H were co-expressed with glutamine-tRNA ligase (XP_006655062.1), nuclear cap-binding protein (XP_003557925.1 and XP_004953081.1), ATP-dependent DNA helicase DDX11 isoform X1 (XP_025881112.1), and luc7-like protein 3 (XP_015695268.1), which control plant development and stress responses through alternative splicing regulation [60]. In the present study, C3H and the above genes would work together to regulate culm development and variation in *D. sinicus*. The role of IAA3, IAA17 and IAA19 related to ARF in the culm development of *D. sinicus* has been reported [20, 24]. In this study, the result of co-expression network verified the previous result. The functions of these genes will be further studied to understand their mechanism.

Due to the lack of genomic data, it is impossible to establish the direct interaction relationship between genes and TFs through promoter characteristic sequences. However, in this study, we identified the TFs and profiled the co-expression of these TFs with other genes and their interacting protein genes. Under the interaction with the interacting genes, TFs directly or indirectly regulated the downstream genes that may exist in the co-expression network. In particular, MYB, C3H, and ARF might play core regulatory roles in the growth and development of *D. sinicus*. The three TF families might interact with 40 genes involved in culm development and 14 involved in shape variation in *D. sinicus*, respectively. The regulatory network constructed in this study can serve as a reference for further studies on the regulation mechanism of the TFs involved in the culm development and variation of *D. sinicus*.

Declarations

Author contribution statement

Lingna Chen, Yongkun Chen and Hanqi Yang: Conceived and designed the experiments.

Peitong Dou and Lushuang Li: Performed the experiments.

Lingna Chen and Yongkun Chen: Analyzed and interpreted the data.

Lingna Chen, Yongkun Chen, Peitong Dou, Lushuang Li, and Hanqi Yang: Wrote the paper.

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Data availability statement

Data associated with this study has been deposited at the China National GeneBank DataBase (CNGBdb) of the Chinese National Standard Agency (CNSA) (https://db.cngb.org/cnsa/) with accession number

CNP0000900, and accession number PRJNA610455 in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA).

Declaration of interest's statement

The authors declare no competing interests.

Additional information

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