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Identification of the *bZIP* Gene Family and Investigation of Their Response to Drought Stress in *Dendrobium catenatum*

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Abstract: *Dendrobium catenatum* is a valuable Chinese herbal medicine that naturally grows on cliffs and tree trunks and is often threatened by adverse environmental conditions. The *bZIP* transcription factor is known to play a critical role in the response of plant to stress. However, the functions of the *bZIP* gene family in *D. catenatum* are poorly understood. In this study, 62 *bZIP* genes were identified from *D. catenatum*, which encoded proteins with an amino acid number of 130~692, a molecular weight of 15.24 to 74.94 kDa, and an isoelectric point of 5.13 to 11.58. The *bZIP* family can be divided into 10 subgroups by evolutionary tree analysis, and the conserved motifs of each protein subgroup were similar. The exon number of *bZIP* genes ranged from 1 to 12 as shown by gene structure analysis. *DcbZIP* promoter prediction analysis identified 21 *cis*-acting elements. The expression of *DcbZIP* genes under drought treatment was analyzed using the public RNA-seq data, and 33 upregulated genes were further screened. A co-expression network analysis revealed that 17 core genes were closely correlated with other genes and their expression was measured using RT-qPCR. The results showed that *DcbZIP6*, *DcbZIP34*, *DcbZIP42* and *DcbZIP47* are the main contributors to drought tolerance in *D. catenatum*. In summary, we identified candidate *bZIP* genes in *D. catenatum* with a apotential contribution to drought stress response, and this study lays the foundation for exploring the functions of *bZIP* and provides a theoretical basis for improving the drought tolerance of *D. catenatum*.

Keywords: *bZIP* gene family; drought stress; expression analysis; *Dendrobium catenatum*

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1. Introduction

Plants are subjected to a series of stresses during their life cycle. Transcription factors (TFs) play key roles in regulating biological processes in plant cells. When plants are stimulated by cold, heat, salt or drought stresses, a series of signal transductions can induce TFs to specifically bind to *cis*-acting elements and regulate the transcription expression of stress responsive genes, thereby improve plant stress resistance [1,2]. More than 60 TF families have been identified in plants [3]. As one of the largest TF families in plants [4,5], basic leucine zipper (bZIP) plays a critical regulatory role in plant development and growth, pest defense, abiotic stress and other physiological processes [6–8].

The bZIP transcription factor contains two conserved domains consisting of about 60~80 amino acid residues: one is the highly conserved alkaline amino acid region at the N-terminal, consisting of about 16~20 amino acid residues, which acts as a nuclear localization signal by binding to specific DNA sequences via a N-X7-R/K structure [9]; the other is the low conservative leucine zipper region at the C-terminal [10], which is composed of several repeated heptapeptides or hydrophobic amino acid residues and is

mainly responsible for the heterogenic or homologous dimerization of bZIP protein before binding to DNA [11,12].

Many eukaryotes have the *bZIP* TF family, for example, 89 *bZIP* TFs have been identified in rice [13], 75 in *Arabidopsis thaliana* [5], 55 in grape [14], 64 in cucumber [15], 160 in soybean [16], 66 in *Iponoea trifida* [17], 97 in oil palm [18] and 84 in pear [19]. Different numbers of *bZIP* gene families lead to functional changes in response to different kinds of biotic and abiotic stresses [20]. Previous studies have shown that the rice OsbZIP23 functions in the ABA signaling pathway, and the transgenic plants exhibited enhanced tolerance to drought stress [21]. OsbZIP71 was also found to enhance drought tolerance in an ABA-mediated pathway in rice [22]. Furthermore, OsbZIP71 formed heterodimers with OsbZIP73, and increased cold tolerance in the rice reproductive stage [23]. *Arabidopsis* and soybean overexpression of *GmbZIP2* improved plant resistance to drought and salt stresses [24], while GmbZIP15 negatively regulated salt and drought tolerance in soybean [25]. Under drought stress, 16 *MdbZIP* genes were differentially expressed in apple roots and leaves, indicating that these *MdbZIP* genes were associated with drought stress response [26]. The *Vigna radiata bZIP* transcription factor *VrbZIP52* was significantly upregulated after polyethylene glycol (PEG) treatment, indicative of the involvement of *VrbZIP52* in drought stress response [27]. After treatment with PEG, 8627 and 9281 differentially expressed genes (DEGs) were detected in the *Boehmeria nivea* roots and leaves, respectively, among which the *bZIP* transcription factors *Comp28477* and *Comp58004* may be related to drought stress [28]. After PEG treatment, the expression of 10 *CsbZIPs* were upregulated in cucumber roots and downregulated in leaves, indicative of tissue-specific expression of the *bZIP* gene [15].

Dendrobium catenatum is a perennial herb of the Orchid family, and has a long history in edible flowers. It is usually eaten fresh, as a flower dish or dried and dehydrated into a tea drink. Importantly, *D. catenatum* contains many active ingredients such as Dendrobium polysaccharide, which can scavenge free radicals in the body and improve immunity. However, wild *D. catenatum* is mostly attached to cliffs and rock crevices in high mountains; therefore, it often faces an adversity threat, especially of periodic drought [29,30], making wild resources scarce. Bionic cultivations are the main modes of *D. catenatum* artificial cultivation and these methods can affect the yield and quality of *D. catenatum*. The yield of the dry bark cultivation method was found to be highest, while the contents of dendrobium and polysaccharide were highest in the living trunk cultivation mode [31]. Therefore, identifying drought stress-related genes in the *D. catenatum* genome and investigating their functions is necessary and helpful. The genome-wide identification of some TF families in *D. catenatum* has been conducted and reported, such as *MYB* [32], *WRKY* [33] and *NAC* [34]. However, the *bZIP* gene family in the plant has not been investigated. In this study, based on the whole genome sequence of *D. catenatum* [35], we identified the *bZIP* gene family and analyzed their characteristics using bioinformatics methods. The expression of *bZIP* gene members under drought stress was further analyzed using RT-qPCR based on RNA-seq data. This study provides a theoretical basis for further improving the drought resistance of *D. catenatum*.

2. Materials and Methods

2.1. Identification of the bZIP Family Members of D. catenatum

The protein sequence, genome sequence, coding sequence (CDS) and GFF annotation files of *D. catenatum* were obtained from the NCBI GenBank database (PRJNA262478) [35]. To identify the *bZIP* gene family of *D. catenatum*, the bZIP domain (PF00170) hidden Markov model was acquired from the Pfam database (http://pfam.xfam.org/, assessed on 16 September 2021) and the database of *D. catenatum* proteins was queried through the Bio-linux bioinformatics documentation system. The conservative bZIP domains were detected by screening and submitting all the output proteins with E values $\leq 1 \times 10^{-10}$ [36] to the Simple Modular Architecture Research Tool (SMART, v9; http://smart.embl.de/smart/batch.pl, assessed on 16 September 2021) database and the Con-

served Domain Database (CDD, v3.19; https://www.ncbi.nlm.nih.gov/cdd/, assessed on 16 September 2021). The DcbZIP proteins were obtained after removal of incomplete and redundant sequences. Characteristics including the molecular weight (MW), the isoelectric point (pI) and amino acid number of the proteins were determined using the tool ExPASy (https://web.expasy.org/protparam/, assessed on 22 April 2022). The PSORT tool (https://www.genscript.com/psort.html, assessed on 22 April 2022) was applied to predict the subcellular localizations of the proteins.

2.2. Phylogenetic Analysis of bZIP Proteins

The bZIP proteins in *A. thaliana* and rice were identified as described previously [5,13]. To explore the phylogenetic relationships between the bZIP proteins, the protein sequences from *A. thaliana*, rice and *D. catenatum* (Gene IDs are shown in Supplementary Table S1) were aligned with clustalw 2.0, and the maximum likelihood (ML) phylogenetic tree was constructed using MEGA 6.0 software with default parameters, and was modified and visualized as described previously [34].

2.3. Analysis of Gene Structure, Conserved Motifs, and Promoter Cis-Regulatory Elements

The MEME (https://meme-suite.org/meme/doc/meme.html, assessed on 26 September 2022) online tool was used to predict the conserved motifs of the *D. catenatum* bZIP proteins with parameters of optimum motif width of 6~50 amino acid residues and maximum number of motifs of 20 [37]. TBtools software [38] was used to extract the *bZIP* gene structure file from the gff annotation file of *D. catenatum* genome, to extract the 2000 bp upstream sequence of the start codon (ATG) of *D. catenatum bZIP* gene as promoter sequence and to visualize the conserved motifs, gene structures and *cis*-elements following detection of *cis*-elements using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, assessed on 22 September 2022) software. Finally, the conserved motifs, gene structures and the *cis*-elements were visualized by TBtools.

2.4. Spatial Expression Profile Analysis Using RNA-Seq Data

The raw RNA-seq reads of different tissues (SRP091756) were obtained from the sequence read archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra, assessed on 12 April 2022). The gene expression data of different tissues (stem, root, root tip, white root, leaf, lip, sepal, column, flower bud and pollinia) were calculated and normalized to the transcripts per million (TPM) values by kallisto tools [34,39]. In this study, we screened the expression data of *bZIP* genes using their gene IDs from the normalized TPM values. A heatmap of differential expression of *bZIP* genes was constructed using TBtools.

2.5. Stress Treatments

D. catenatum seeds (*D. catenatum* 'Guangnan') were collected from Guangnan country, Yunnan province, China. The seeds were sterilized and used as explants to induce protocorm. After proliferation and regeneration, the complete plants were induced. The *D. catenatum* tissue culture seedlings were obtained through protocorm induction and plant regeneration from the mother plants, and then used in this study. The experiment was carried out in completely randomized design and conducted in the greenhouse in Hainan University. In the present study, the three-month-old tissue culture seedlings were grown in 1/2 MS medium in a growth chamber under the conditions as follows: light for 12 h, 70% humidity and temperature of 22–25 °C [32]. The uniform and robust growing seedlings were selected and treated with 20% PEG8000 for drought stress [32]. After treatment for 3, 6, 9, 12, 24 or 48 h, the stems, leaves and roots were randomly collected from five seedlings, mixed, quickly frozen in liquid nitrogen and stored at −80 °C for RNA extraction and gene expression analysis [32].

2.6. Gene Expression Analysis under Drought Treatment

The RNA seq data (SRP132541) of the *D. catenatum* leaves collected from plants treated with different drought stresses with a base matrix water content of 0%, 10–15% and 30–35% were downloaded from the public SRA database according to a previous study [40] and their expression profiles of *DcbZIP* genes were analyzed. Then, the TPM values were used for heatmap construction with TBtools. The differential genes were aggregated according to T1 and T2 values (T1 and T2 equal to the TPM of 10–15% and 0% water content, respectively, divided by a TPM of 30–35% water content).

RNA extraction and cDNA synthesis were performed according to a previous study [32]. Real-time fluorescence quantitative PCR (RT-qPCR) was used for analysis of samples treated with PEG as described by Zhang et al. [32] with the primers shown in Supplemental Table S2.

2.7. Analysis of Co-Expression and Protein–Protein Interaction Networks of DcbZIP Proteins

According to the TPM value of RNA-seq data, the *DcbZIP* gene co-expression relationships were analyzed using Origin software (version 2022b) [41]. The co-expression network analysis was performed using Cytoscape (version 3.9.1) based on their correlation coefficients [42]. The data used for correlation analysis were the means of four replications. The interactions between bZIP homolog proteins and other proteins were analyzed using STRING software (https://string-db.org/, assessed on 20 October 2022) [43].

3. Results

3.1. Identification and Physicochemical Properties of bZIP Family in D. catenatum

In total, 62 *bZIP* genes were identified from the *D. catenatum* genome by the bioinformatics method. The *bZIP* family members were named as *DcbZIP1* to *DcbZIP62* based on their relationships with rice and *A. thaliana* bZIP proteins (Table 1). The proteins encoded by the *bZIP* gene had an amino acid number ranging from 130 to 692, a molecular weight (MW) ranging from 15.24 to 74.94 kDa and a theoretical isoelectric point (pI) ranging from 5.13 (DcbZIP60) to 11.58 (DcbZIP5). DcbZIP8 and DcbZIP58 encoded proteins with the fewest amino acids and smallest MWs, and DcbZIP28 encoded a protein with the most amino acids with the largest MW. Most DcbZIP proteins were predicted to localize in the cell nucleus, while DcbZIP15 was localized in cytoplasm, and DcbZIP11 and DcbZIP14 were localized in mitochondria.

3.2. Phylogenetic Analysis of bZIP Proteins

An ML phylogenetic tree was constructed to study the evolutionary relationships of bZIP proteins using the bZIP family protein sequences from *D. catenatum* (Dc), *A. thaliana* (At) and rice (Os) (Supplementary Table S1). The bZIP proteins could be divided into 10 subgroups, as shown in Figure 1, consistent with those in rice and *Arabidopsis* [5,44]. The *D. catenatum* bZIP family members were distributed unequally in the subgroups (Figure 1 and Table 1). The subgroup S contained the most members, with fifteen *bZIP* genes (24.20% of the whole gene family), followed by subgroup A with eleven *bZIP* genes (17.74%), subgroup D with ten *bZIP* genes (16.13%), subgroup I with nine *bZIP* genes (14.51%), subgroup G with four *bZIP* genes (6.45%), subgroups B, C, E and H, which contained three *bZIP* genes (4.83%) and subgroup F, which only contained one *bZIP* gene (1.61%). The gene distribution of each subgroup in *D. catenatum* was similar to that of rice and *A. thaliana* (Supplementary Figure S1), suggesting that in these three species, the evolution trajectory of the *bZIP* gene was similar.

Table 1. Physicochemical properties of *D. catenatum* bZIP proteins.

Gene Name	Gene ID	Locus	Length	MW(kDa)	pI	Subcellular Localization	Group
DcbZIP1	LOC110102063	NW_021318745.1:471271 … 486572	153	16.97	9.98	nuclear	H
DcbZIP2	LOC110094784	NW_021319121.1:1711930 … 1713378	254	28.36	6.6	nuclear	E
DcbZIP3	LOC110115573	NW_021320155.1:215 … 1462	155	17.66	9.03	nuclear	S
DcbZIP4	LOC110112903	NW_021359921.1: 258 … 816	138	15.59	11.53	nuclear	S
DcbZIP5	LOC110095238	NW_021319911.1:476590 … 477012	140	15.67	11.58	nuclear	S
DcbZIP6	LOC110113945	NW_021318751.1:434550 … 435351	146	16.76	10.47	nuclear	S
DcbZIP7	LOC110114248	NW_021318952.1:1141827 … 1142966	149	17.16	11.09	nuclear	S
DcbZIP8	LOC110109372	NW_021318890.1:45595 … 46690	130	15.24	8.49	nuclear	S
DcbZIP9	LOC110095100	NW_021319641.1:962641 … 966712	356	38.76	5.18	nuclear	C
DcbZIP10	LOC110099409	NW_021319484.1:1588342 … 1604864	311	34.37	5.57	nuclear	C
DcbZIP11	LOC110116565	NW_021394673.1:450792 … 454576	317	35.95	6.49	mitochondrial	S
DcbZIP12	LOC110098756	NW_021319202.1:1038678 … 1046470	338	38.28	9.30	nuclear	A
DcbZIP13	LOC110100055	NW_021319455.1:997836 … 1000675	222	24.82	5.91	nuclear	A
DcbZIP14	LOC110112185	NW_021318640.1:185732 … 187053	265	28.71	9.30	mitochondrial	A
DcbZIP15	LOC110115326	NW_021319048.1:847890 … 872251	406	42.69	9.13	cytoplasmic	A
DcbZIP16	LOC110096541	NW_021319431.1:31825 … 50187	409	43.33	6.33	nuclear	G
DcbZIP17	LOC110099255	NW_021319682.1:13603178 … 13607863	683	74.16	6.38	nuclear	B
DcbZIP18	LOC110104466	NW_021319842.1:67813 … 111270	398	43.50	6.16	nuclear	I
DcbZIP19	LOC110116528	NW_021319455.1:2008465 … 2012390	259	28.90	6.41	nuclear	E
DcbZIP20	LOC110113515	NW_021320183.1:270501 … 301718	466	51.39	6.36	nuclear	D
DcbZIP21	LOC110096667	NW_021319983.1:403141 … 412564	489	54.63	5.71	nuclear	D
DcbZIP22	LOC110113543	NW_021320019.1:9020891 … 9022087	167	18.71	8.66	nuclear	S
DcbZIP23	LOC110102116	NW_021318705.1:1911115 … 1924234	273	29.49	5.94	nuclear	D
DcbZIP24	LOC110110500	NW_021319360.1:1021557 … 1023609	321	36.95	5.29	nuclear	F
DcbZIP25	LOC110109220	NW_021319982.1:676693 … 690196	362	39.53	5.89	nuclear	C
DcbZIP26	LOC110091873	NW_021319588.1:431917 … 450124	427	47.22	6.82	nuclear	D
DcbZIP27	LOC110102106	NW_021318705.1:1988364 … 1989735	162	18.00	9.76	nuclear	A
DcbZIP28	LOC110096161	NW_021318623.1:23442 … 27784	692	74.94	6.08	nuclear	B
DcbZIP29	LOC110116680	NW_021319255.1:1051643 … 1056686	542	59.41	6.21	nuclear	I
DcbZIP30	LOC110099156	NW_021318726.1:1712729 … 1719118	522	56.71	6.21	nuclear	I
DcbZIP31	LOC110102661	NW_021319579.1:559884 … 583214	350	38.19	6.88	nuclear	I
DcbZIP32	LOC110106152	NW_021318595.1:5943723 … 5946939	226	25.65	11.36	nuclear	S
DcbZIP33	LOC110096611	NW_021319126.1:383344 … 398145	339	37.15	6.13	nuclear	I
DcbZIP34	LOC110103109	NW_021320146.1:675175 … 678827	319	34.65	5.90	nuclear	E
DcbZIP35	LOC110098225	NW_021318839.1:350918 … 367871	311	34.83	9.50	nuclear	A
DcbZIP36	LOC110092531	NW_021318711.1:1796647 … 1806328	396	42.68	9.19	nuclear	A
DcbZIP37	LOC110109339	NW_021318998.1:251776 … 260296	407	43.50	7.70	nuclear	A
DcbZIP38	LOC110097322	NW_021319772.1:217773 … 219884	141	15.69	7.65	nuclear	S
DcbZIP39	LOC110114883	NW_021318595.1:12862132 … 12864684	357	38.96	7.60	nuclear	A
DcbZIP40	LOC110107968	NW_021319536.1:408463 … 445084	277	30.19	5.19	nuclear	A
DcbZIP41	LOC110095320	NW_021318963.1:1120306 … 1130172	359	38.07	7.11	nuclear	G
DcbZIP42	LOC110094070	NW_021318618.1:1794449 … 1795054	159	18.31	7.07	nuclear	S
DcbZIP43	LOC110109278	NW_021416702.1:86653 … 87395	183	21.41	6.72	nuclear	S
DcbZIP44	LOC110099470	NW_021318619.1:750999 … 751924	132	15.65	10.09	nuclear	S
DcbZIP45	LOC110110747	NW_021318796.1:2352740 … 2370581	427	47.26	8.46	nuclear	D
DcbZIP46	LOC110094737	NW_021319121.1:686641 … 695563	365	40.63	7.06	nuclear	D
DcbZIP47	LOC110110388	NW_021319315.1:2104944 … 2125602	381	42.91	7.68	nuclear	D
DcbZIP48	LOC110094083	NW_021318700.1:245031 … 246643	146	16.34	8.71	nuclear	H
DcbZIP49	LOC110091793	NW_021318565.1:363970 … 370844	447	50.01	7.32	nuclear	D
DcbZIP50	LOC110112805	NW_021318937.1:66248 … 73259	433	48.47	7.75	nuclear	D
DcbZIP51	LOC110105751	NW_021318677.1:122368 … 135356	341	37.24	6.22	nuclear	I
DcbZIP52	LOC110114853	NW_021319083.1:8027963 … 8050443	394	42.69	5.44	nuclear	I
DcbZIP53	LOC110099371	NW_021319442.1:514414 … 515591	143	16.69	8.85	nuclear	S
DcbZIP54	LOC110109693	NW_021378092.1:460796 … 469115	386	41.58	5.14	nuclear	G
DcbZIP55	LOC110094315	NW_021319161.1:846164 … 858315	269	30.97	8.75	nuclear	G
DcbZIP56	LOC110101858	NW_021319436.1:2509122 … 2519085	161	18.07	9.44	nuclear	H
DcbZIP57	LOC110111774	NW_021318920.1:351221 … 358735	369	41.59	5.68	nuclear	D
DcbZIP58	LOC110100891	NW_021318787.1:716526 … 717410	130	15.24	8.49	nuclear	S
DcbZIP59	LOC110095296	NW_021319910.1:646885 … 655651	401	44.12	5.79	nuclear	I
DcbZIP60	LOC110113665	NW_021324968.1:31584 … 34398	335	37.95	5.13	nuclear	B
DcbZIP61	LOC110101931	NW_021319436.1:1660963 … 1677531	361	39.45	8.47	nuclear	I
DcbZIP62	LOC110106828	NW_021319013.1:318529 … 320289	273	29.15	9.73	nuclear	A

3.3. Conserved Motif and Gene Structure of bZIP Family Genes

The bZIP family of *D. catenatum* can be divided into 10 subgroups according to the evolutionary analysis (Figures 1 and 2A). The conserved motifs in the sequences of these proteins were then predicted using MEME software to elucidate their diversity and conservation (Figure 2B), and 20 motifs were identified (Supplementary Figure S2). Different subgroups contained different types and numbers of conserved motifs, while most of the DcbZIP proteins within the same subgroup displayed similar motif compositions (Supplementary Table S3). Motifs 8 and 17 were specific to subgroup I, whereas motif 20 was unique to subgroup C. Almost all the proteins contained motif 1, which was annotated as the bZIP domain, including two domains: a leucine zipper region and a basic region. The leucine zipper region consisted of 15 amino acid residues with an amino acid sequence model of L-X6-L-X6-L, and the basic region consisted of 18 amino acid residues with an amino acid sequence model of N-X7-R-X9. The leucine in the leucine zipper region was not conserved and could be replaced by methionine (M), valine (V), phenylalanine (F) and isoleucine (I), all of which are hydrophobic amino acids (Supplementary Figure S3).

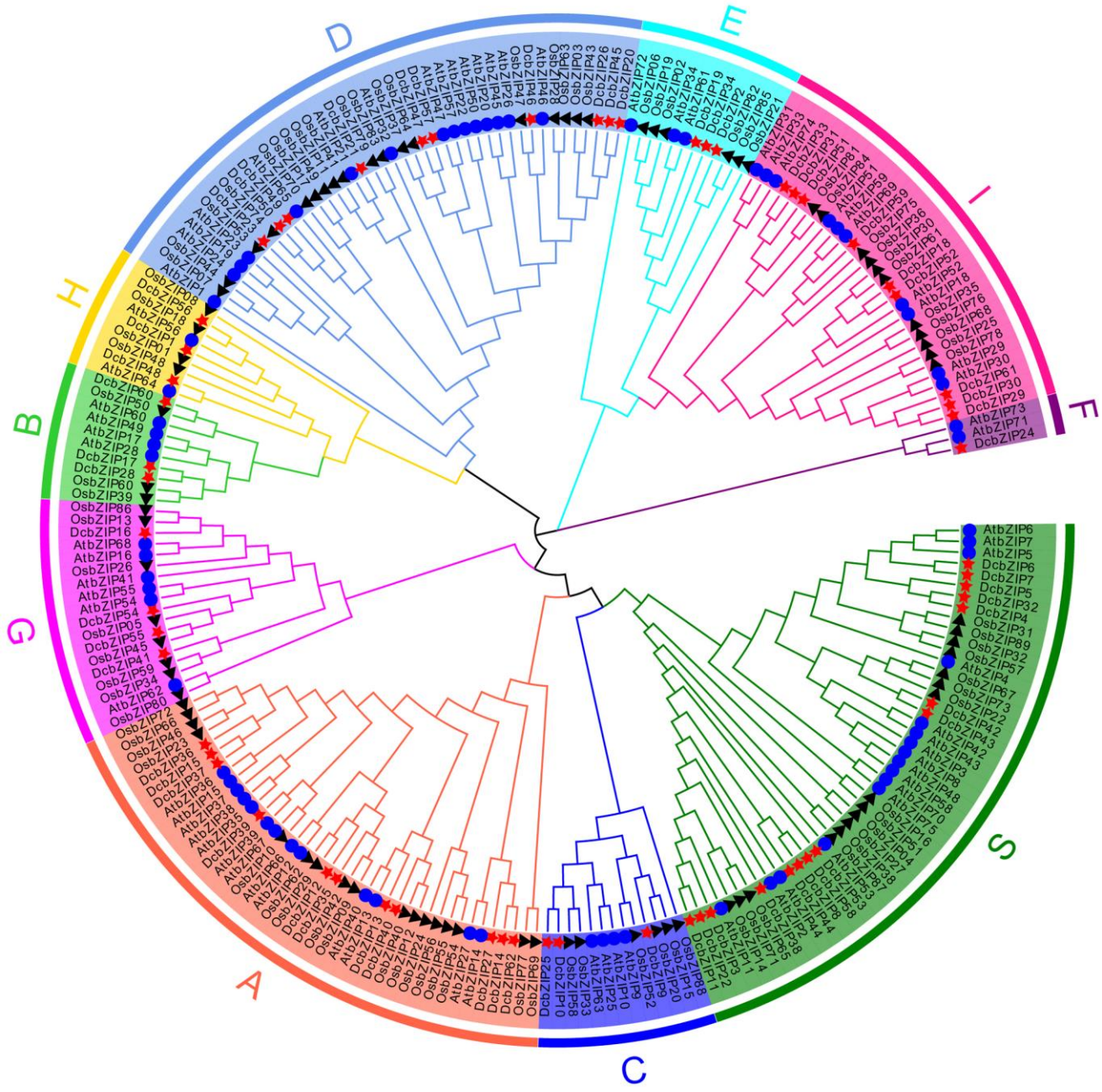


Figure 1. Phylogenetic analyses of bZIP proteins from *D. catenatum*, *Arabidopsis* and rice. A phylogenetic tree of bZIP proteins was constructed using MEGA 6.0 software. The 10 subgroups are indicated by different colors and different colored letters. The blue circles represent *A. thaliana* bZIPs (AtbZIPs), the red stars represent *D. catenatum* bZIPs (DcbZIPs) and the black triangles represent *O. sativa* bZIPs (OsbZIPs).

Gene structure analysis showed that the number of introns in the *DcbZIP* gene family ranged from 0 to 12 (Figure 2C and Supplementary Table S3). Approximately 80.65% of the *DcbZIP* genes contained fewer than five introns. The subgroup D members, except *DcbZIP23*, contained more than eight introns. *DcbZIP16*, *DcbZIP20* and *DcbZIP21* had the most numbers of introns (12 introns). Meanwhile, the same subgroup of *DcbZIP* genes had similar intron/exon structures. For instance, the subgroup S *DcbZIP* genes contained 0–3 introns, among which 73.33% (11/15) had no intron. Both subgroups H and E contained three introns.

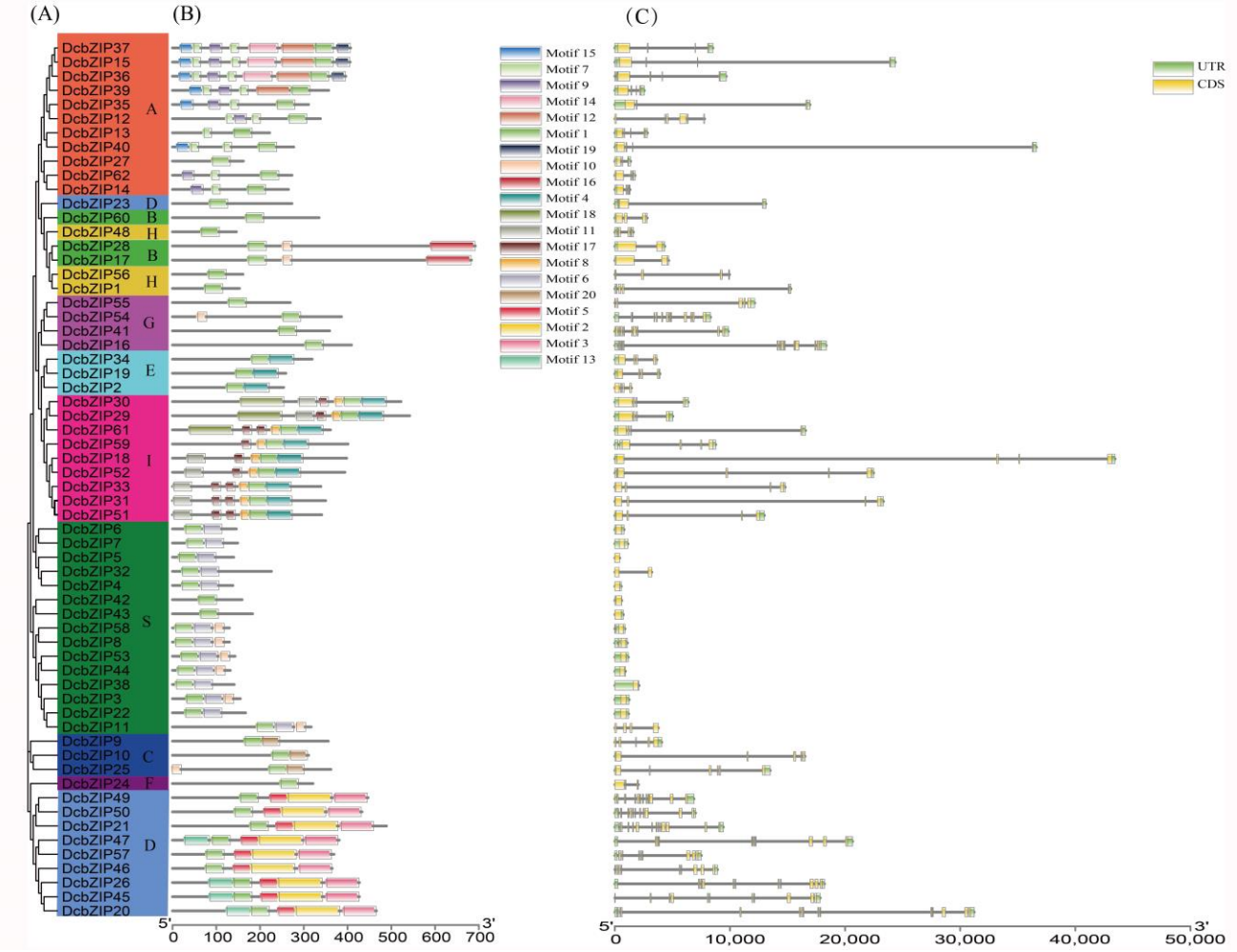


Figure 2. Motifs, structures and phylogenetic relationships of DcbZIP family members. (**A**) A phylogenetic tree of 62 DcbZIP proteins constructed using the maximum likelihood method. The different subgroups are indicated by different background colors and letters. (**B**) Conserved motifs of DcbZIP proteins. Different motifs are represented by different colored boxes. (**C**) Exon/intron structures of *DcbZIP* genes with UTR(s), exon(s) and intron(s) indicated with green boxes, yellow boxes and black lines, respectively. The phylogenetic tree, conserved motifs and gene structures were predicted with TBtools.

3.4. Cis-Element Analysis of bZIP Gene Promoters in D. catenatum

The upstream 2000 bp sequence of the *DcbZIP* gene start codon was extracted by TBtools software as promoters, and the *cis*-acting elements were then analyzed using PlantCARE online software (Figure 3 and Supplementary Table S4). Results revealed that the 21 *cis*-acting elements were mainly divided into four categories: stress-related elements, light-responsive elements, plant growth and development-related elements and hormone response elements (Figure 3B,C). Plant hormone response elements mainly included ABRE elements involved in abscisic acid regulation, salicylic acid-responsive elements (SARE and TCA element), gibberellin-responsive elements (TATC-box, GARE-motif and P-box) and auxin-responsive elements (TGA element and AuxRR-core). Among these elements, SARE only existed in the *DcbZIP40* and *DcbZIP61* promoter regions, suggesting that these two genes might be involved in the SA signaling pathway. Plant growth and development related elements mainly included circadian elements, seed-specific regulatory element (RY-element), meristem expression-related element (CAT-box), endosperm expression element (GCN4_motif) and zein metabolic regulatory element (O2-site). Light responsive elements mainly included the GT1-motif and MRE elements. Stress-related elements included wound-responsive element (WUN-motif), low-temperature responsiveness (LTR), drought-

inducibility (MBS) and stress and defense responsiveness (TC-rich repeats). Among these elements, LTR was detected in 35 (56%) *DcbZIP* gene promoters, MBS was detected in 27 (44%) *DcbZIP* gene promoters, WUN-motif existed in 7 (11%) *DcbZIP* gene promoters, and TC-rich repeat elements existed in 24 (39%) *DcbZIP* gene promoters. In addition, 33 (53%) gene promoters were observed to contain more than two stress-related elements (Figure 3C), suggesting that *DcbZIP* may be widely involved in abiotic stress response.

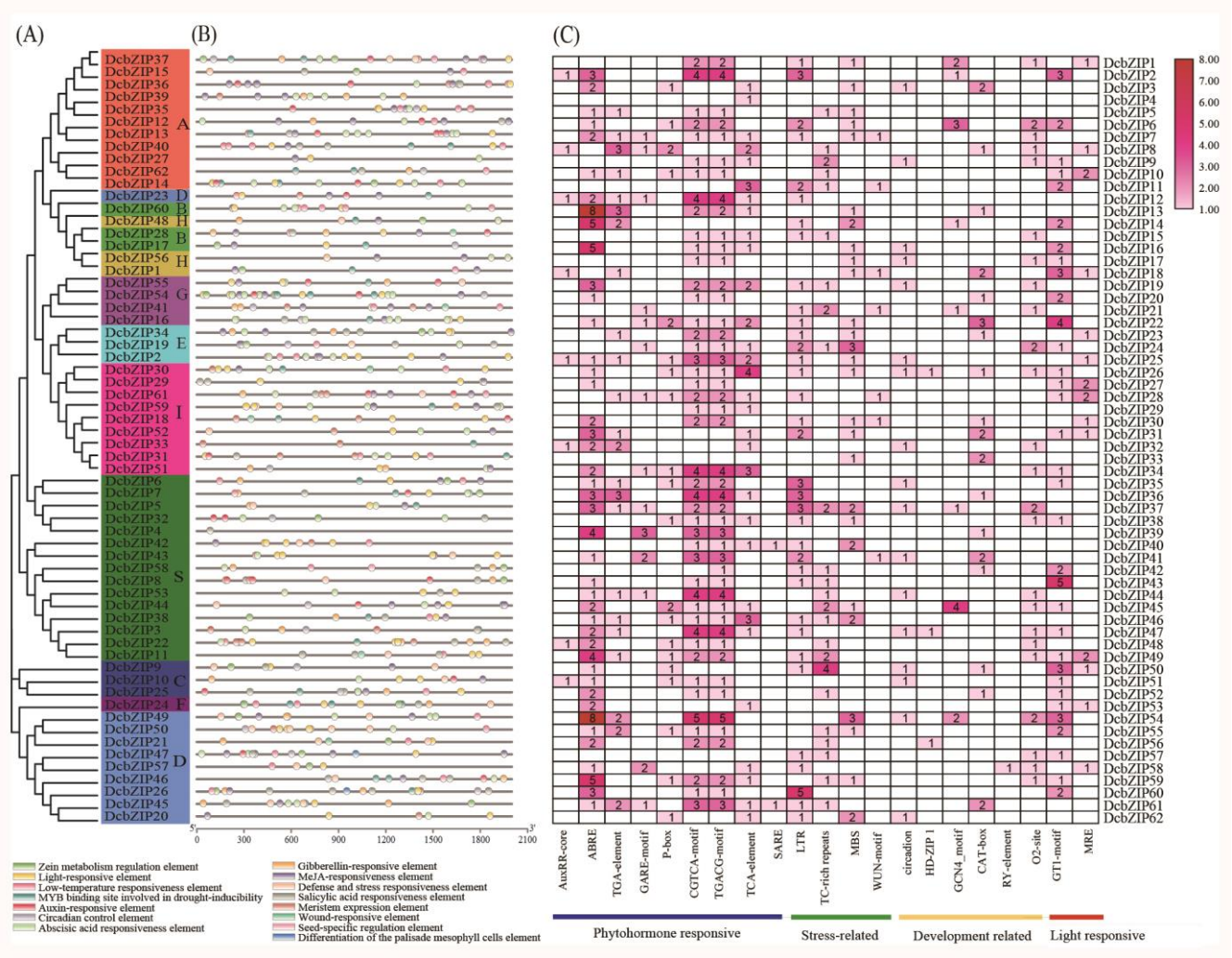


Figure 3. Analysis of *cis*-elements in the *DcbZIP* genes promoter regions. (**A**) A phylogenetic tree of 62 DcbZIP proteins was constructed with the ML method. The different subgroups are indicated by different background colors and letters. (**B**) The different colored blocks represent the different types of *cis*-elements and their locations in each *DcbZIP* gene. (**C**) The different colors and numbers in the grid indicate the numbers of different promoter elements in the *DcbZIP* genes. The types, numbers and locations of potential elements in the promoter regions 2-kb upstream of the *DcbZIP* genes were analyzed by PlantCARE.

3.5. Tissue-Specific Expression Profile of DcbZIP Gene Based on RNA-Seq

The *DcbZIP* gene expression levels in different tissues were detected according to the RNA-seq data from the public SRA database (SRP091756) (Supplementary Table S5). Based on the TPM values of *DcbZIP* genes in 10 different tissues including stem, root, leaf, lip, root tip, sepal, white root, flower bud, column and pollinia, their spatial expression patterns were displayed using a heatmap, as shown in Figure 4. The *DcbZIP* genes showed different expression patterns. For example, 10 genes (*DcbZIP10*, *DcbZIP11*, *DcbZIP14*, *DcbZIP15*, *DcbZIP16*, *DcbZIP17*, *DcbZIP26*, *DcbZIP28*, *DcbZIP30* and *DcbZIP55*) were highly

expressed in the roots, 13 genes (*DcbZIP3*, *DcbZIP7*, *DcbZIP8*, *DcbZIP20*, *DcbZIP27*, *DcbZIP33*, *DcbZIP38*, *DcbZIP41*, *DcbZIP42*, *DcbZIP47*, *DcbZIP51*, *DcbZIP57* and *DcbZIP62*) were highly expressed in stems, 3 genes (*DcbZIP25*, *DcbZIP38* and *DcbZIP58*) were highly expressed in leaves, 11 genes (*DcbZIP1*, *DcbZIP4*, *DcbZIP5*, *DcbZIP6*, *DcbZIP8*, *DcbZIP9*, *DcbZIP42*, *DcbZIP48*, *DcbZIP53*, *DcbZIP56* and *DcbZIP58*) showed high expression levels in sepals, 5 genes (*DcbZIP1*, *DcbZIP4*, *DcbZIP9*, *DcbZIP37* and *DcbZIP40*) were highly expressed in lips, 14 genes (*DcbZIP2*, *DcbZIP5*, *DcbZIP13*, *DcbZIP17*, *DcbZIP18*, *DcbZIP19*, *DcbZIP24*, *DcbZIP32*, *DcbZIP34*, *DcbZIP36*, *DcbZIP39*, *DcbZIP52*, *DcbZIP59* and *DcbZIP61*) showed high expression levels in pollinia, 5 genes (*DcbZIP16*, *DcbZIP28*, *DcbZIP46*, *DcbZIP59* and *DcbZIP61*) were highly expressed in flower buds and 7 genes (*DcbZIP6*, *DcbZIP29*, *DcbZIP30*, *DcbZIP42*, *DcbZIP44*, *DcbZIP48* and *DcbZIP54*) were highly expressed in columns. These results indicate tissue-specific expression of *DcbZIP* genes, suggesting that these genes might be involved in growth and development of different tissues.

3.6. DcbZIP Gene Expression under Drought Stress Based on RNA-Seq

The drought RNA-seq data of *D. catenatum* leaf samples (SRP132541) were obtained from the public SRA database according to a previous study [40], and the TPM values of *DcbZIP* genes were calculated to study the response of *DcbZIP* genes to drought stress (Supplementary Table S6). When the matrix water content decreased from 30–35% to 0%, different expression patterns were observed for different *DcbZIP* genes (Figure 5), indicating that these *DcbZIP* genes were highly sensitive to drought stress. The expression patterns of 62 *DcbZIP* genes in *D. catenatum* under drought stress were shown in a hierarchical clustering heat map. Based on their expression patterns, these genes were classified into four clusters. In cluster I, there were two genes with T1 and T2 values >2, suggesting they might function in drought stress response. Thirteen *DcbZIP* genes were grouped into cluster II with T1 and T2 values <1, indicating their decreased expression with increasing drought stress. The T1 and T2 values were both more than one for all members in cluster III, except for *DcbZIP58* (0.52), *DcbZIP28* (0.32), *DcbZIP34* (0.71), *DcbZIP16* (0.62), *DcbZIP30* (0.98) and *DcbZIP45* (0.98). The T2 value of most genes was greater than the T1 value, indicating that the expression levels of genes under 0% treatment were higher than those under 10–15% treatment, suggesting that most cluster III genes might play critical roles in drought stress response. In cluster IV, seven of the fourteen *DcbZIPs* had T1 and T2 values <1. In total, 33 *DcbZIP* genes in clusters I and III might be involved in positive regulation of *D. catenatum* drought tolerance, so they were selected for further study.

3.7. Analysis of Drought-Related DcbZIP Genes

Co-expression analysis has been broadly used to screen key genes from large gene members [45]. In order to explore the relationships of drought-related *bZIP* genes, the 33 genes in clusters I and III *DcbZIP* with T1 and T2 values >1 were subjected to correlation analysis using Origin software. Genes with absolute Pearson correlation coefficients of >0.95 were selected. The results revealed that 140 pairs of *DcbZIP* genes were positively correlated and 78 pairs of *DcbZIP* genes were negatively correlated (Figure 6A). A co-expression network was constructed using Cytoscape based on Pearson correlation coefficients (Figure 6B). A total of 17 core genes (*DcbZIP24*, *DcbZIP45*, *DcbZIP53*, *DcbZIP12*, *DcbZIP28*, *DcbZIP47*, *DcbZIP61*, *DcbZIP58*, *DcbZIP23*, *DcbZIP30*, *DcbZIP8*, *DcbZIP60*, *DcbZIP34*, *DcbZIP9*, *DcbZIP6*, *DcbZIP3* and *DcbZIP42*) were identified, among which the *DcbZIP12*, *DcbZIP24*, *DcbZIP45* and *DcbZIP53* genes dominated in the network due to their high connectivity with other genes.

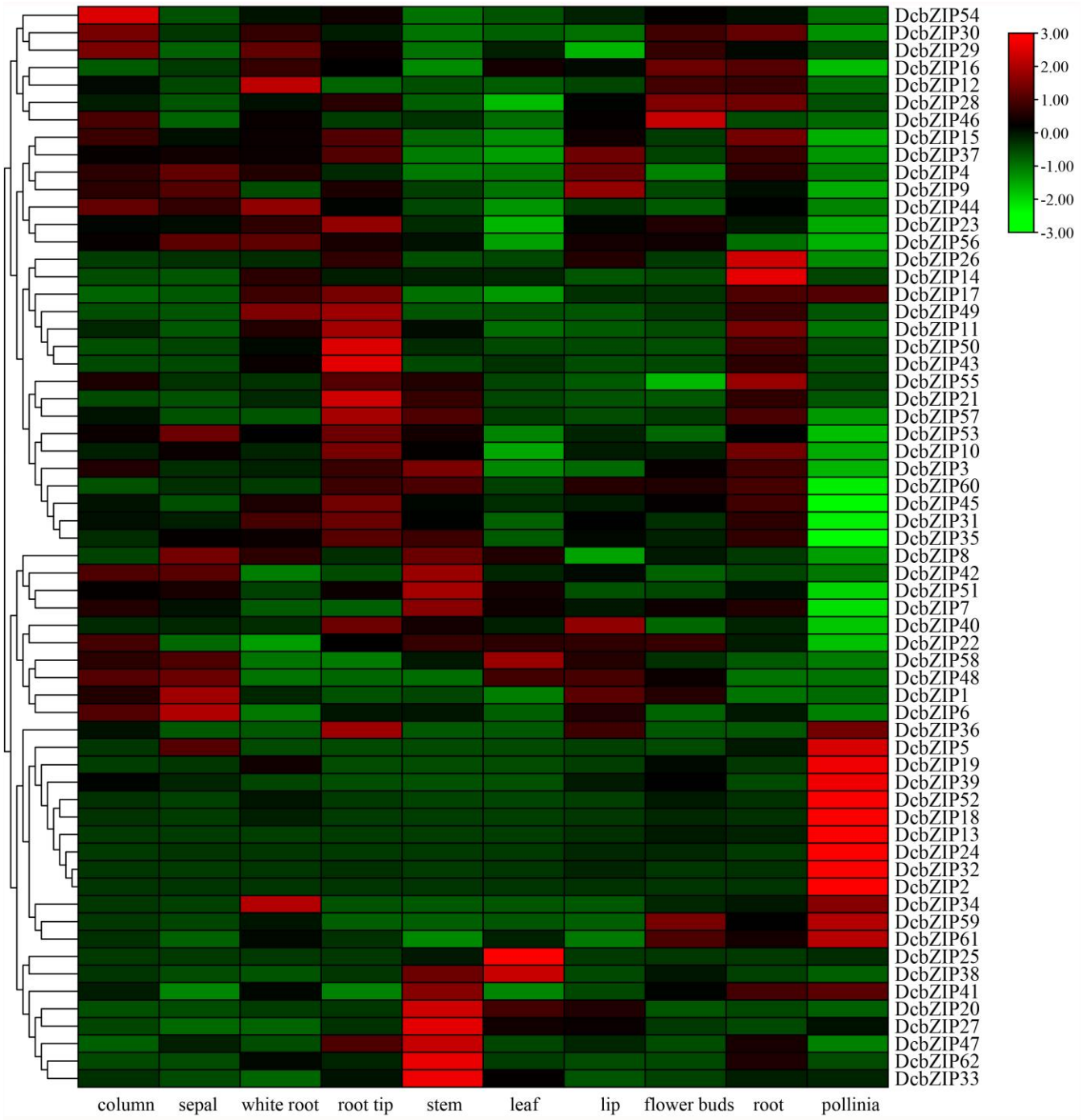


Figure 4. Hierarchical clustering of *DcbZIP* genes expression profiles across different *D. catenatum* tissues. Data were normalized relative to each gene's mean expression value across all tissues and log2−transformed. TPM (transcripts per million) values were used to create heat maps showing the expressions of *DcbZIP* genes in different tissues. The expression level ranges from low expression (green) to high expression (red).

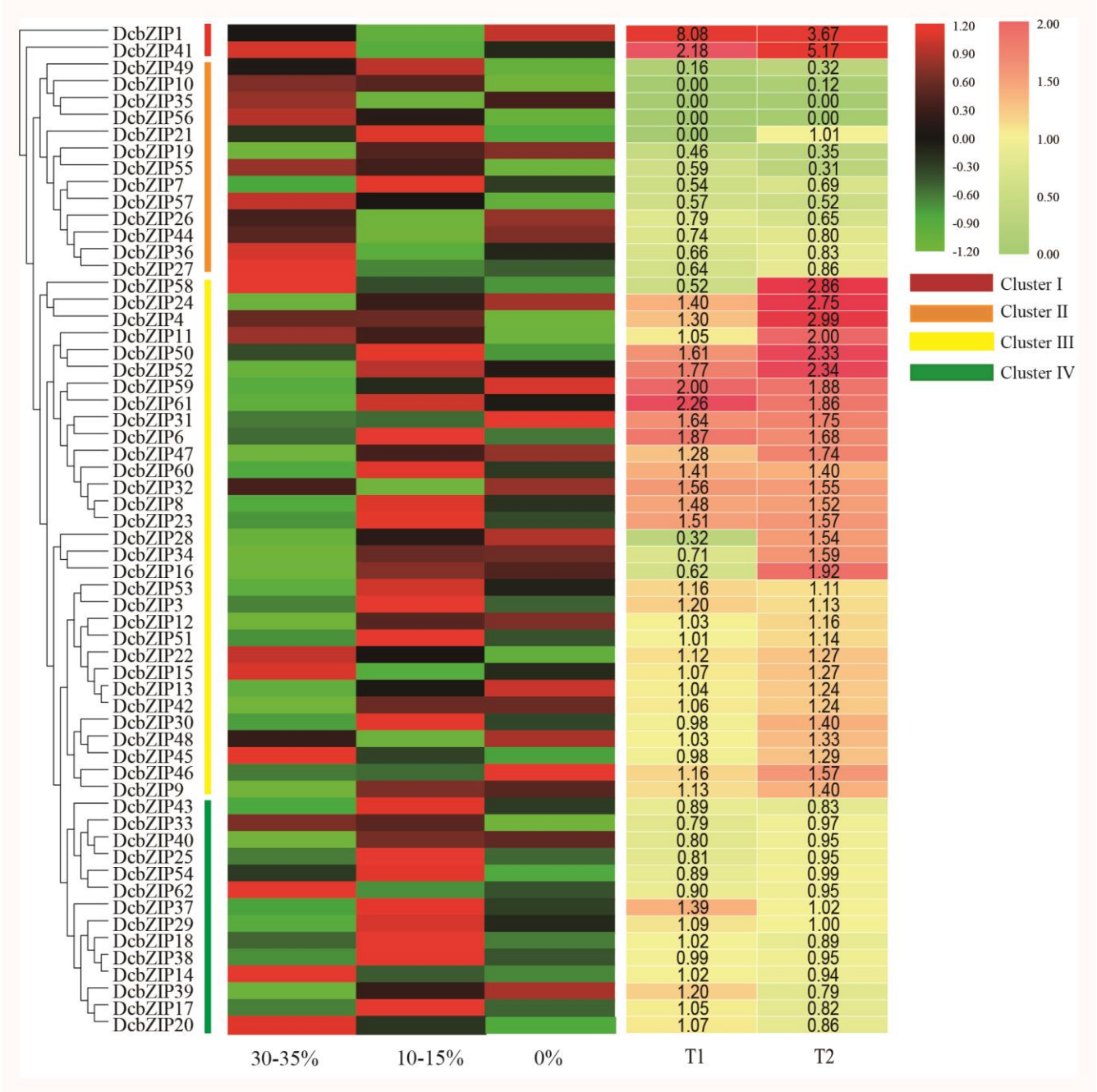


Figure 5. The expression patterns of *DcbZIP* genes evaluated by RNA-seq. The left heatmap shows the expressions of *DcbZIP* genes under different volumetric water contents of base material. Data were normalized relative to each gene's mean expression value across all treatments and log2-transformed. TPM (transcripts per million) values were used to create heat map showing the expression of *DcbZIP* genes. The expression levels range from low expression (green) to high expression (red). The right heatmap shows the TPM ratios, with high ratios in light orange and low ratios in cyan. T1 was equal to the TPM of 10–15% divided by the TPM of 30–35%, and T2 was equal to the TPM of 0% divided by the TPM of 30–35%. Ranges of 30–35%, 10–15% and 0% represent the volumetric water content of the base material declining to ~30–35%, ~10–15% and ~0%, respectively.

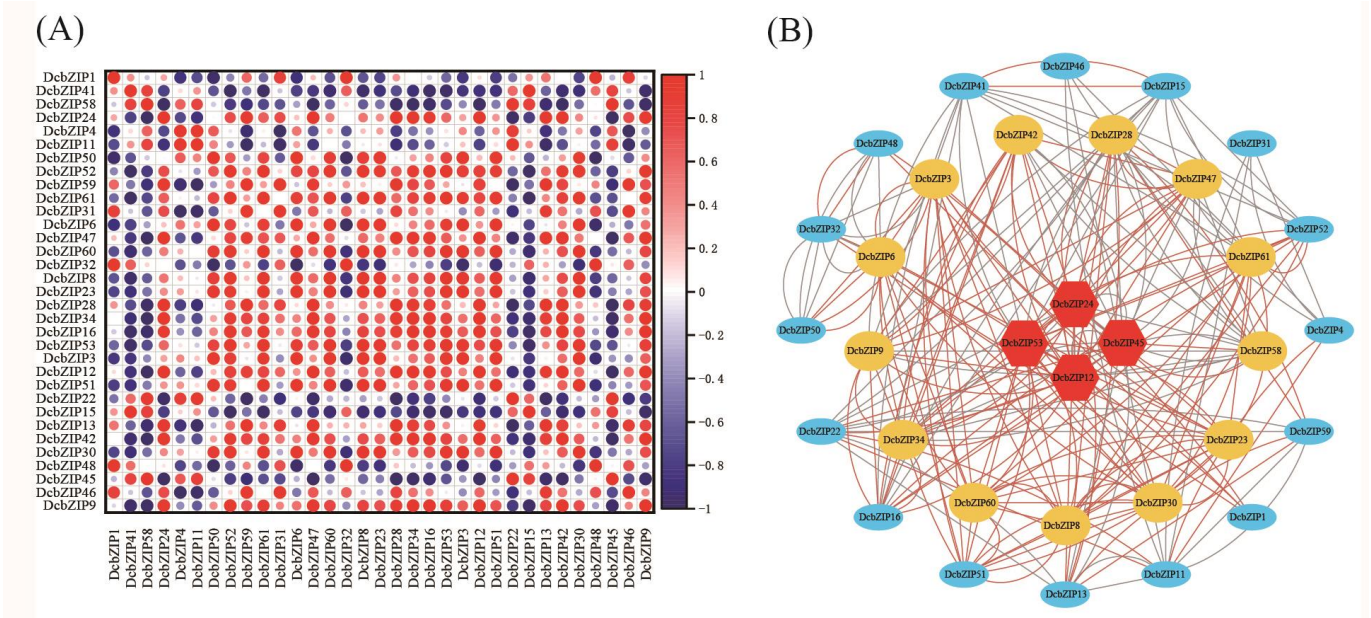


Figure 6. Comprehensive analysis of drought-related *DcbZIP* genes. (**A**) Correlation analysis of 33 up-regulated *DcbZIP* genes. Red indicates a positive correlation and blue indicates a negative correlation. The circle size indicates the absolute value of the correlation coefficient. (**B**) Co-expression network analysis. The red lines represent a positive correlation. The size represents the degree calculated by the Cytoscape. The four polygons situated in the center represent core genes with high connectivity.

To further investigate the functions of these 17 core genes, the interaction network between these DcbZIP proteins and their homologous AtbZIP proteins in *Arabidopsis* was predicted by STRING software. A total of 34 interacting proteins were predicted and their annotated information is shown in Supplemental Table S7. As shown in Figure 7, AFP3 (involved in ABA signaling) and DPBF2 (involved in ABA signaling) interacted with AREB3 (homologous protein of DcbZIP24). Furthermore, AREB3 also had associations with SNRK2.2/2.3/2.4/2.10, OST1 and BZIP34/6. *Arabidopsis* AHBP-1B, homologous to DcbZIP45, could interact with NPR1. EEL (homologue of DcbZIP12) had associations with OST1, bZIP1, BZIP34, BZIP60 and TGA10. BZIP53, homologous to DcbZIP53, DcbZIP58 and DcbZIP8, could interact with BZIP25, bZIP1, bZIP44, BZIP17, NAC089 and TGA1. AT4G38900 (homologue of DcbZIP61 and DcbZIP30) had an association with TGA10. bZIP19 (homologue of DcbZIP23) could interact with BZIP34 and bZIP68. The proteins interacting with bZIP6 (homologous to DcbZIP6) had associations with bZIP1, bZIP44 and bZIP68. BZIP60 (homologous to DcbZIP60) had associations with EEL, BZIP17, IRE1-1, BZIP53 and bZIP1. BZIP9 (homologous to DcbZIP9) could interact with bZIP1, BZIP53, BZIP34 and BZIP60. bZIP44, the homologue of DcbZIP3, had associations with BZIP25, AT4G38900, TGA10, BZIP60, BZIP53, bZIP1, ERD5 and BZIP17. BZIP17 (homologue of DcbZIP28) had associations with BZIP60, NAC089, IRE1-1, BZIP34, bZIP1 and BZIP53. TGA1 (homologue of DcbZIP47) could interact with NPR1, NPR3 and NPR4. BZIP34, the homologue of DcbZIP34, had associations with EEL, TGA10 and bZIP1.

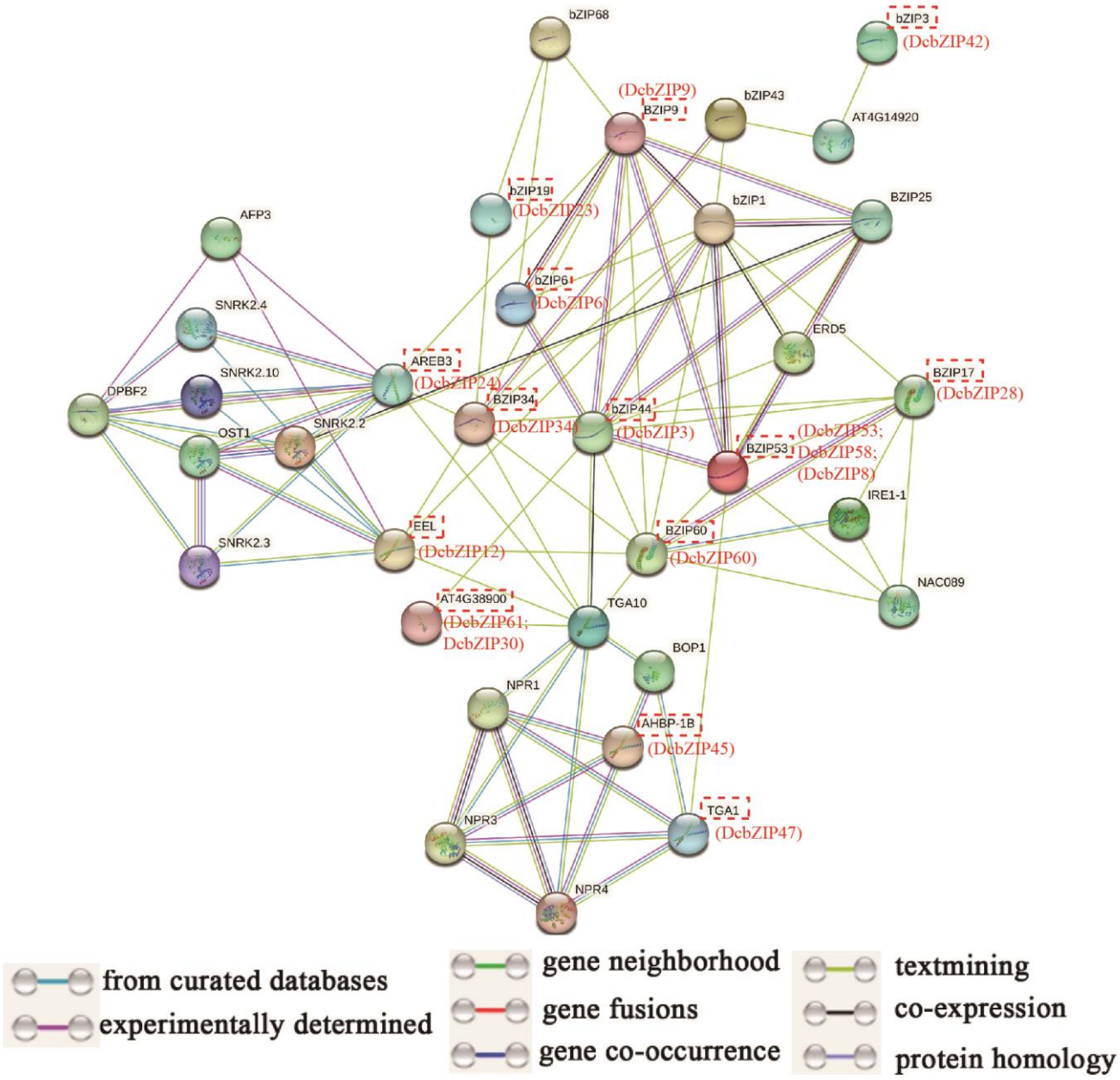


Figure 7. Interaction networks of the DcbZIPs in *D. catenatum* and their orthologs in *Arabidopsis*. The network was predicted by STRING. The DcbZIP proteins were shown in the red font below with the *Arabidopsis* orthologs in the red dashed box. Empty nodes represent proteins with unknown 3D structures. Filled nodes represent known or predicted 3D structures. Light blue lines and rose lines represent known interactions. Green, orange and blue lines represent predicted interactions. Cyan lines represent text mining, black lines represent co-expression and light purple lines represent protein homology.

3.8. Expression Profile Analysis of 17 Core DcbZIP Genes under Drought Stress Using RT-qPCR

The expression levels of 17 core *DcbZIP* genes under 20% PEG8000 treatment were analyzed using RT-qPCR to investigate their response to drought stress. As shown in Figure 8, consistent with the RNA-seq data, after PEG treatment, the expression levels of most *DcbZIP* genes increased. The *DcbZIP34* and *DcbZIP42* genes in roots were significantly increased and showed the highest expression level after 3-h and 48-h treatments, with a 15-fold and 18-fold increase, respectively, compared to 0 h. The *DcbZIP6* and *DcbZIP47* genes in stems were highly induced after drought stress treatment, with 133-fold and

130-fold higher expression levels compared with the control, respectively. However, the increasing trend in *DcbZIP* genes was not obvious in leaves.

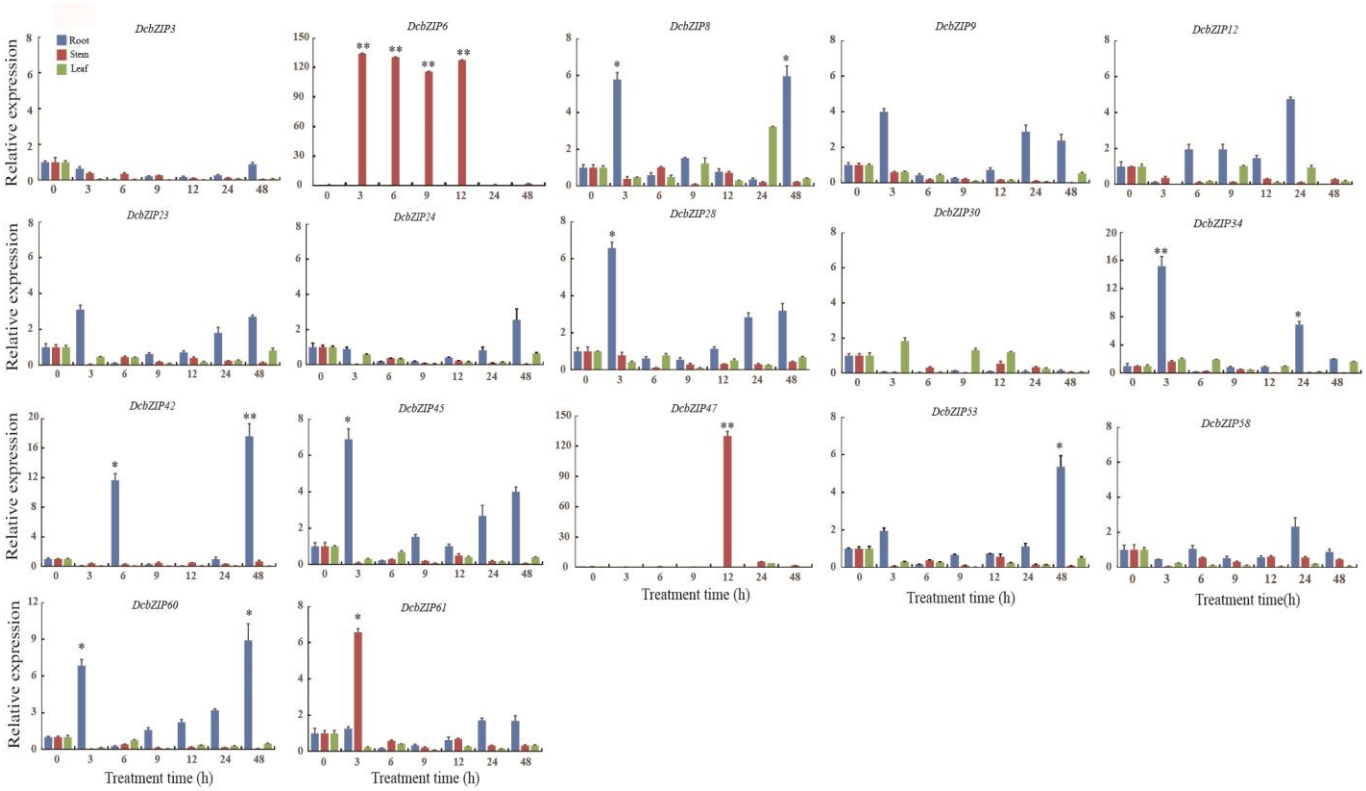


Figure 8. Real-time quantitative PCR analyses of the 17 core *DcbZIP* genes under drought stress in roots, stems and leaves. The mean expression value was calculated from three replicates. Vertical bars indicate the standard deviation. Values of 0, 3, 6, 9, 12, 24 and 48 indicate hours after treatment. Mean values and standard deviations were calculated according to the data. The unstressed level (0 h) was used as a control. Asterisks (* or **) indicate a significant difference at $p < 0.05$ or $p < 0.01$, respectively.

4. Discussion

The *bZIP* TF is widely involved in the regulation of plant development, growth and response to biotic and abiotic stresses [46–48], which has been identified and analyzed in a variety of plants such as *Arabidopsis* [5], sorghum [49], barley [50], maize [51], rice [44], soybean [16] and rape [52]. However, there are few reports on the *bZIP* family genes of *D. catenatum*. Sixty-two *bZIP* gene family members were identified using bioinformatics techniques from the *D. catenatum* genome in this study. Similar gene numbers have been reported in other plants. *Isatis indigotica* has 65 *bZIP* genes [53], *Pyrus bretschneideri* has 62 *PbbZIP* genes [54], cucumber has 64 *bZIP* genes [15] and watermelon has 62 *ClabZIP* genes [55]. The phylogenetic relationships of bZIP proteins and their response to drought stress, conserved motif, promoter region *cis*-acting elements and gene structure were analyzed.

The *bZIP* gene family can be classified into 10 subgroups based on phylogenetic analysis (Figure 1), with subgroup F containing the fewest members and subgroup S containing the most members. The subgroup proportions were similar to those in rice and *Arabidopsis* (Supplementary Figure S1), as well as in switchgrass [56] and mung bean [57], indicating evolutionary conservation of the *bZIP* gene in plants. Previous studies showed that the subgroup A proteins AtbZIP39, AtbZIP36, AtbZIP38 and AtbZIP35 played important roles in the response to stress and ABA treatment [58–60], suggesting the same subgroup of proteins has a similar function. In addition, AtbZIP10 and AtbZIP25 in subgroup C could regulate the expression of storage proteins in *A. thaliana* [5]. Subgroup D members functioned by preventing pathogen invasion and plant development [5]. Subgroup G proteins were involved in UV and blue light signal transduction [61]. AtbZIP56 in subgroup

H functioned in the development of photomorphology in seedlings [62]. Therefore, we could speculate that the *D. catenatum* genes in the same group may have similar functions. Although phylogenetic analysis provided important information for understanding the functions of candidate genes, their functions cannot be clearly characterized based on the phylogenetic analysis alone. To this end, we examined additional evidence including gene structures and conserved motifs to support the reliable subgroup classification.

Gene structure is a critical basis for gene evolution and gene amplification studies [56]. Analysis of structure features of the *bZIP* gene family in *D. catenatum*, showed that the presence of introns and exons in each subgroup exhibited similar rules. The *DcbZIP* genes in subgroup S contained 0–3 introns, among which 73.33% (11/15) of members had no introns. Both subgroups H and E of *DcbZIP* genes contained three introns (Figure 2C and Supplementary Table S3), similar to those found in Chinese jujube [63], soybean [16], switchgrass [56] and grape [14], suggesting the intron-exon structure was conserved. It should be noted that some *DcbZIP* genes, especially those in the subgroup S, generally lacked introns (Figure 2C and Supplementary Table S3), which may shorten the post-transcriptional process. Thus, they can immediately respond to abiotic stress [64]. Similarly, the subgroup S of *bZIP* genes in soybeans and watermelons also lacked introns [16,55]. Genes lacking introns evolve faster than intron acquisition after gene amplification, and most *bZIP* genes in subgroups D and G had more introns than those in other subgroups (Figure 3). Therefore, the subgroups D and G, members of *D. catenatum bZIP* gene family, may contain more original genes compared with other subgroups [65]. In addition, 20 motifs were predicted in DcbZIP proteins using MEME analysis. The different conserved motif number and composition in different subgroups indicated their functional differences, which is important for determining specific functions of bZIP proteins [66].

The promoter region *cis*-acting elements can bind to target genes and induce their expression under abiotic stresses [67,68]. *Arabidopsis* AtbZIP17 has a high homology with tobacco TGA1b protein, which belongs to the TGA/OBF subfamily of *bZIP* TFs. Both AtbZIP17 and TGA1b can specifically bind to TGACG elements to regulate downstream gene expression [69]. All the promoters of 51 tomato *HD-Zip* genes contain the low temperature-responsive element (LTR), indicating their involvement in plant response to low temperature stress [70]. The promoter of *D. catenatum, bZIP* transcription factor, contained multiple hormone signaling-related and stress response-related elements (Figure 3), such as ABRE, SARE, LTR and MBS. The results indicated that *DcbZIP* transcription factor was widely involved in the regulation of plant stress response. Among them, 35 *DcbZIP* gene promoters contained LTR elements and 27 *DcbZIP* gene promoters contained MBS elements, suggesting that most *bZIP* genes in *D. catenatum* were related to abiotic stress.

Wild *D. catenatum* grows in cliffs and rock crevices and is often threatened by adversity. Drought stress is the main affecting factor for its growth and development. The ABA signaling pathway plays an important role in plant response to abiotic stress [71,72]; AREB3 (homologous protein of DcbZIP24), as an ABA-responsive element-binding protein (AREB) [73], plays a key role in gene expression regulation involved in ABA signaling [68]. Meanwhile, the interaction network displayed that AREB3 could interact with BZIP34, a homologue protein of DcbZIP34. These results suggested that DcbZIP24 and DcbZIP34 might regulate drought resistance by affecting ABA signal transduction pathways. Overexpression of *AtbZIP1* improved plant tolerance to salt, osmotic and drought stress [74]. AtbZIP1 is an interacting protein of bZIP6 (homologue protein of DcbZIP6). The results indicated that DcbZIP6 may be involved in regulating plant drought tolerance. The above analysis suggested that many *DcbZIPs* might play key roles in the response to drought stress. Therefore, the expression of *DcbZIP* genes under drought stress was studied next. The *DcbZIP* gene expression under different drought conditions was first analyzed using public RNA-seq data, and these genes were divided into four clusters according to their expression levels. Among them, 33 *DcbZIP* genes belonging to clusters I and III were upregulated under different drought treatments, and correlation analysis showed that most of the upregulated genes were positively related (Figure 6A), indicating that they may have

a synergistic effect in development of drought resistance. In addition, 17 core genes that were closely correlated with other genes were screened out using a co-expression network (Figure 7B). These results suggest that these 17 key genes may play leading roles in the development of drought resistance. RT-qPCR analysis further revealed that *DcbZIP6* and *DcbZIP47* genes in stems and *DcbZIP34* and *DcbZIP42* genes in roots were significantly upregulated under drought stress (Figure 8), suggesting these four genes can significantly contribute to the development of drought resistance in *D. catenatum*, and more research on their functions might be performed by transgenic or CRISPR techniques in the future.

5. Conclusions

In this study, 62 *DcbZIP* TFs were identified in the *D. catenatum* genome, which were divided into 10 subgroups based on their correlations with rice and *Arabidopsis* bZIP proteins. Gene structures and conserved motifs indicated similarities in bZIP clustering in the phylogenetic tree. Promoter *cis*-element analysis indicated the potential multiple roles of bZIP in abiotic stresses. Using the public drought RNA-seq data, the *DcbZIP* genes were classified into four clusters based on their expression levels, among which 17 *DcbZIP* genes were further screened by RT-qPCR and four *DcbZIP* genes (*DcbZIP6*, *DcbZIP34*, *DcbZIP42* and *DcbZIP47*) were finally found to be highly induced by drought stress, which could be main contributors to drought tolerance in *D. catenatum*. For the first time, this study performed a genome-wide analysis of the *DcbZIP* gene family in *D. catenatum* and investigated their expression patterns under drought stress. This study provides a foundation for the molecular breeding of drought tolerance in *D. catenatum*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13010236/s1, Figure S1: The percentage of members in each bZIP subgroup in *D. catenatum* (A), *A. thaliana* (B) and *O. sativa* (C); Figure S2: The amino acid sequences of each motif identified in DcbZIP proteins; Figure S3: Sequence alignment of motif 1 in DcbZIP proteins; Table S1: The gene ID of bZIP transcription factors in *D. catenatum*, *A. thaliana* and *O. sativa*; Table S2: Primers used in this study; Table S3: Motifs and the number of introns of each DcbZIP gene; Table S4: Information on *cis*-elements of *DcbZIP* gene promoters; Table S5: Expression of *DcbZIP* genes in different tissues (TPM); Table S6: Expression of *DcbZIP* genes under drought stress (TPM); Table S7: Annotation information of DcbZIP homologous proteins.

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