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Root-Growth-Related MaTCP Transcription Factors Responsive to Drought Stress in Mulberry

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Abstract: Root growth regulation plays a crucial role in the acclimatization of plants to their surroundings, but the molecular mechanisms underlying this process remain largely uncertain. Teosinte branched1/cycloidea/proliferating cell factor (TCP) transcription factors are crucial elements linking together plant growth and development, phytohormone signaling, and stress response. In this study, 15 TCP transcription factors were identified in the mulberry (*Morus alba*) genome. Gene structure, conserved motif, and phylogenetic analyses revealed the conservation and divergence of these MaTCPs, thus providing insights into their functions. A promoter analysis uncovered distinct numbers and compositions of *cis*-elements in *MaTCP* gene promoter regions that may be connected to reproductive growth and phytohormone and stress responses. An expression pattern analysis of the 15 *MaTCP* genes in mulberry roots indicated that transcriptional levels of *MaTCP2*, *MaTCP4-1*, *MaTCP8*, *MaTCP9-1*, and *MaTCP20-2* are correlated with root development. As revealed by changes in their expressions after drought treatment, these five *MaTCP* genes are involved in root growth and may increase mulberry tolerance to drought. Our findings lay the foundation for future functional studies of these genes.

Keywords: mulberry; MaTCP transcription factor; drought tolerance; root development

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1. Introduction

Given their sessile nature, plants are constantly subjected to a range of adverse environmental conditions, including drought, heat, cold, and light. Future climate change will most likely intensify these environmental pressures. For example, many climate models anticipate that drought will increasingly threaten crop growth and productivity [1], and a deeper comprehension of the factors influencing drought tolerance is thus required. Plants have evolved a series of complex mechanisms to perceive and respond to environmental stimuli and can withstand drought using a variety of physiological, morphological, and biochemical processes [2,3]. Reduced transpiration, the development of a deep and robust root system, increased water intake, and maintenance of tissue water potential are the key methods used by plants to resist drought [4]. Nevertheless, the underlying physiological and molecular mechanisms are not fully understood.

Recent studies in diverse plant species have shown that teosinte branched1/cycloidea/proliferating cell factor (TCP) transcription factors are crucial for the development of plant roots [5–7]. Investigation into TCP in plant stress resistance has gained a lot of attention since it links together plant development, stress response, and hormone signaling [8–10]. TCP transcription factors constitute a plant-specific protein family with a conserved TCP domain containing a 59-amino-acid non-canonical basic helix–loop–helix (bHLH) structure that enables DNA binding and protein–protein interactions [11–14]. TCP proteins are categorized into two classes according to the characteristics of their TCP domains: class I (PCF or TCP-P class) and class II (TCP-C class) [12]. Class II TCP members are subdivided in turn into CIN and CYC/TB1 subclasses [15]. Genes encoding TCPs have been identified and analyzed in many plant species, and accumulating evidence indicates that the TCP family

plays important regulatory roles in plant growth and development, hormone signaling, and stress response [8,9,13,16]. AtTCP14 and AtTCP15 have been proposed to regulate cell proliferation and organ growth and promote gibberellin-induced seed germination in Arabidopsis [17,18]. In Arabidopsis embryonic root apical meristem, growth-repressor DELLA proteins bind and inhibit AtTCP14 and AtTCP15 activities during cell proliferation in the presence of low levels of gibberellic acid [7]. Moreover, *GbTCP*, the cotton homolog of *AtTCP15*, positively regulates jasmonic acid (JA) biosynthesis and response as well as other pathways. Silencing of *GbTCP* results in plants with lower JA levels and reduced cotton fiber elongation. When overexpressed in Arabidopsis, *GbTCP* also promotes the initiation and extension of root hair development [19]. In rice, OsTCP19 synthesis is triggered by exposure to water deficit and salt stress conditions. OsTCP19 induces the expression of ABI4, which encodes a transcription factor involved in ABA signal transduction, and directly interacts with the ABI4 protein to positively regulate its activity. Increased stress resistance in *OsTCP19*-overexpressing Arabidopsis is accompanied by decreased water loss, decreased production of reactive oxygen species, and lipid droplet hyperaccumulation [20]. In addition, *TCP* genes in Arabidopsis and tomato have been revealed to be targets of the microRNA319 (miR319) family [21,22], which is involved in plant stress response. Transgenic creeping bentgrass (*Agrostis stolonifera*) overexpressing *Osa-miR319a* exhibits improved drought and salt tolerance along with higher leaf wax content and increased water retention [23]. Taken together, these results demonstrate that the TCP family is essential for plant development and stress response.

Mulberry (*Morus* L.), an ecologically and economically important deciduous tree [24,25], has been used for thousands of years, both as a food source for domesticated silkworms and as a raw material for the production of juice, jam, wine, and traditional Chinese medicines. Because of its widespread distribution, rapid maturation cycle, well-developed root system, and capacity to resist a variety of environmental stresses, mulberry can be grown under many conditions that are unsuitable for other cash crops [26]. Mulberry is even a prospective species for the ecological rehabilitation of the drawdown zone of the Three Gorges Reservoir in China [27]. An understanding of the ability of mulberry to tolerate harsh settings is thus crucial for the selective breeding and cultivation of this species. Such knowledge would benefit the advancement of sericulture and the pharmaceutical industry and help preserve the ecological environment.

In the present study, we systematically identified and analyzed mulberry TCP transcription factors. We performed analyses of gene structures, expression patterns, subcellular localizations, and responses to drought stress, thereby generating new data for elucidating the roles of mulberry TCP transcription factors in future functional studies. This would aid in the selection and breeding of mulberry cultivars that are drought-tolerance.

2. Materials and Methods

2.1. Plant Materials and Culture Conditions

Seeds of mulberry (*Morus atropurpurea* 'Guisangyou12', abbreviated as GY12) were soaked in aseptic water for 48 h at 4 °C and then transferred to a 25 °C climate chamber under a 16-h light/8-h dark cycle. After germination, the mulberry seedlings were transplanted and raised on sterile soil under well-watered conditions. In subsequent experiments, 21- to 49-day-old mulberry seedlings were used to study root growth; the root lengths of 10 seedlings at each development stage were measured with a ruler with stamped millimeter graduation, while 1-month-old seedlings were used for drought treatment.

2.2. Phylogenetic and Comparative Sequence Analyses of the TCP Family

Annotated sequences of *Arabidopsis thaliana* and *Populus euphratica* [28] TCP transcription factors and CDS regions were downloaded from the PlantTFDB (http://planttfdb.gao-lab.org/, accessed on 12 July 2022) and NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 12 July 2022) databases (Table S1). After performing tblastn searches (E-value < 10^{-10}) to identify candidate TCP members in the *M. alba* genome [29], we applied Interpro

(https://www.ebi.ac.uk/interpro/, accessed on 25 July 2022) to confirm the presence of the conserved TCP domain in each candidate [30]. A phylogenetic tree based on full-length amino acid sequences from *A. thaliana*, *P. euphratica*, and *M. alba* was then constructed in MEGA11 by the neighbor-joining method with 1000 bootstrap repetitions, with all other parameters kept at default settings [31]. Multiple alignments of the MaTCP proteins were performed by using CLUSTALW (https://www.genome.jp/tools-bin/clustalw, accessed on 6 August 2022). Conserved motifs of MaTCP proteins were determined using MEME (https://meme-suite.org/meme/, accessed on 5 August 2022).

2.3. Promoter Element Analyses

The PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 10 November 2022) was used to determine the number and composition of stress- and development-related elements in the 2-kb promoter of *MaTCP* genes. The data analysis and mapping were carried out with TBtools [32].

2.4. RNA Extraction, Gene Cloning, and Quantitative Real-Time PCR (RT-qPCR) Analysis

RNA was extracted from GY12 roots using an RNAprep Pure Plant Plus kit (Tiangen, Beijing, China). Next, 1 μg of RNA extracted from GY12 was synthesized into cDNA by using a PrimeScript RT Reagent kit with gDNA Eraser (Takara, Beijing, China). We used the NCBI Primer-BLAST online tool to design RT-qPCR primer pairs specific to the *MaTCP* genes shown in Table S2. *RPL15* was used as an internal reference gene [33]. RT-qPCR amplifications were conducted using SuperReal PreMix Plus (Tiangen, Beijing, China) on Applied Biosystems StepOne and StepOnePlus Real-Time PCR systems (Thermo Fisher Scientific, Waltham, MA, USA). Each experiment was performed with three technical replicates.

2.5. Subcellular Localization

The subcellular location of each MaTCP protein was predicted using Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/, accessed on 14 August 2022). For the subcellular localization experiment, the ORFs of *MaTCP2*, *MaTCP4-1*, *MaTCP8*, *MaTCP9-1*, and *MaTCP20-2* were inserted into a pZYGC plant expression vector containing a GFP reporter gene to generate *35S:MaTCP:GFP* fusion expression vectors. Transient transformation of onion epidermal cells was performed by the *Agrobacterium*-mediated method. Firstly, 1 cm^2 of the onions' inner epidermis was soaked in Agrobacterium solution for 20 min, then transferred to MS solid medium and incubated at 25 °C in dark for 8 h, followed by another 24 h under a photoperiod of 16 h light/8 h dark. The transformed cells were placed on a glass slide, stained with DAPI, and observed under an Olympus IX73 inverted fluorescent microscope (Olympus, Tokyo, Japan).

2.6. Stress Treatment

For the stress treatment, 1-month-old GY12 seedlings were treated with 10% PEG6000 solution for 0, 1, 3, 5, and 7 days. The initial day was determined to be day 0 of treatment. The 0-day plants were the control. The roots of the control and the treated plants were then sampled, frozen in liquid nitrogen, and stored at −80 °C for RNA extraction.

2.7. Data Processing and Statistical Analysis

Relative levels of *MaTCP* transcripts were determined by the $2^{-\Delta\Delta CT}$ method [34]. A time series analysis was performed online using BioLadder (https://www.bioladder.cn/web/, accessed on 8 October 2022). Correlation coefficients between GY12 root lengths and *MaTCP* gene expressions were calculated using Office 2010. Statistical comparisons of samples were performed by Student's t test for one-way ANOVA followed by the Student–Newman–Keuls (*SNK*) post-hoc test for multiple comparisons (p-values < 0.05).

3. Results

3.1. Characteristics of MaTCP Transcription Factors in Mulberry

A total of 15 *MaTCPs* were identified in the mulberry genome. As shown in Dataset S1 and Table 1, the highly conserved TCP domain (Interpro accession number PF03634) was present in each MaTCP protein. Eight and seven of the identified mulberry TCPs belonged to classes I and II, respectively. Seven class II MaTCPs were further subdivided into one CYC/TB1 MaTCP12 and six CIN-type MaTCPs.

Table 1. Information on MaTCP proteins identified in this study.

TFID	Common Name	Type	Conserved Domain (aa)	Chromosome Location	CDS Length (bp)	Protein Length (aa)	Predicted Protein Localization
M.alba_G0010963	MaTCP2	CIN	78–236	Chr04: 10504440..10506373	1491	496	Nucleus
M.alba_G0019145	MaTCP4-1	CIN	55–170	Chr09: 2738157..2740500	1332	443	Nucleus
M.alba_G0002273	MaTCP4-2	CIN	24–117	Chr10: 10539871..10541516	1077	358	Nucleus
M.alba_G0018610	MaTCP5	CIN	54–147	Chr08: 11690488..11691912	1083	360	Nucleus
M.alba_G0008688	MaTCP7	PCF	51–128	Chr02: 11919887..11922133	831	276	Nucleus
M.alba_G0015857	MaTCP8	PCF	172–336	Chr06: 21186236..21188526	1665	554	Nucleus
M.alba_G0005407	MaTCP9-1	PCF	113–190	Chr12: 15460670..15462253	1215	404	Nucleus
M.alba_G0015368	MaTCP9-2	PCF	17–144	Chr06: 17004501..17004995	495	164	Nucleus
M.alba_G0010736	MaTCP10	CIN	135–219	Chr04: 6280140..6281898	1083	360	Nucleus
M.alba_G0007510	MaTCP12	CYC/TB1	126–257	Chr14: 7692279..7693628	1350	449	Nucleus
M.alba_G0001832	MaTCP13	CIN	61–207	Chr10: 4961787..4963406	1125	374	Nucleus
M.alba_G0018434	MaTCP14	PCF	107–282	Chr08: 7338007..7339840	1308	435	Nucleus
M.alba_G0003268	MaTCP19	PCF	91–157	Chr11: 3914056..3915332	1102	366	Nucleus
M.alba_G0006782	MaTCP20-1	PCF	37–125	Chr14: 728387..729849	810	269	Nucleus
M.alba_G0001316	MaTCP20-2	PCF	69–156	Chr10: 593920..595287	1005	334	Nucleus

To investigate the relationships of MaTCPs to other TCPs and to obtain insights into their potential functions, we constructed a phylogenetic tree based on full-length amino acid sequences of TCPs from *M. alba*, *A. thaliana*, and *P. euphratica*. As shown in Figure 1A, all of the sequences were classified into two clades. Clade I was named the PCF clade and contained 39 TCP proteins. Clade II was subdivided into 10 TCPs with CYC/TB1 and 23 TCPs with CIN. MaTCPs were more closely related to the TCPs in *Populus* than to those in Arabidopsis.

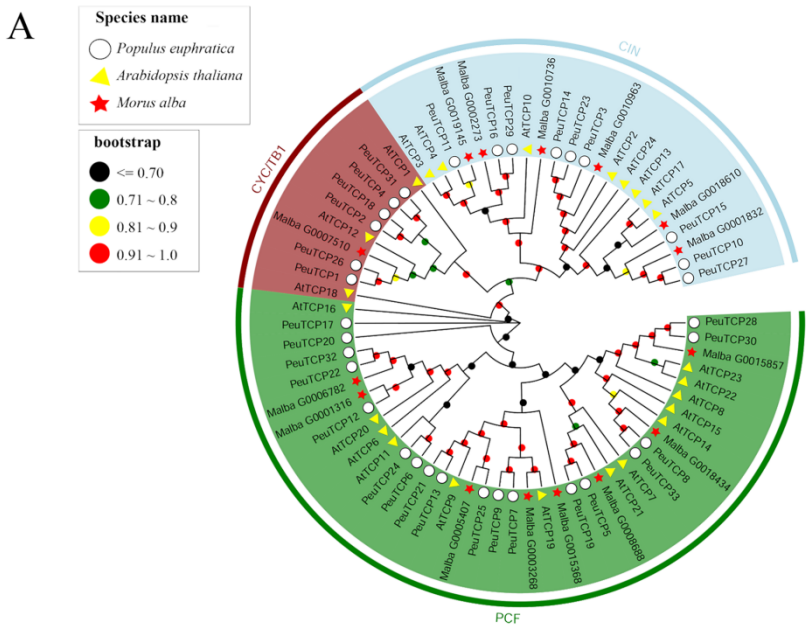


Figure 1. *Cont.*

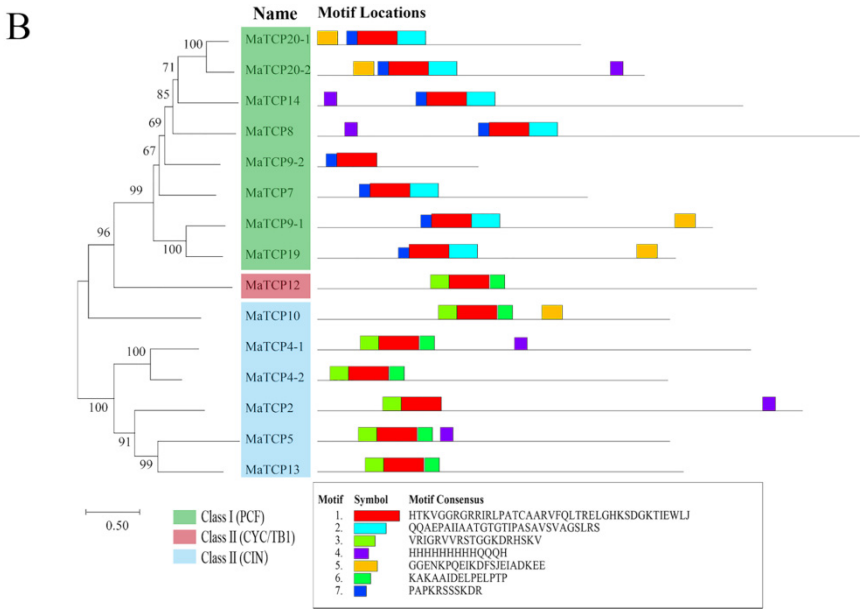


Figure 1. The phylogenetic and protein structure analyses of MaTCP family members. (**A**) Neighbor-joining phylogenetic tree of TCP transcription factors in *M. alba*, *P. euphratica*, and *A. thaliana.* Three different colors were designated as three subclades of PCF, CYC/TB1, and CIN. (**B**) Conserved motifs of MaTCP proteins. Different motifs were shown by different colors numbered 1–7.

To further examine the sequence features of MaTCP proteins, we looked for conserved motifs in the aligned set of TCP amino acid sequences (Figure S1). Seven conserved motifs were identified among MaTCP proteins. The N-terminal conserved core region (marked as motif 1) was present in all 15 MaTCP proteins. Within a given MaTCP subclass, the composition of motifs was similar, whereas compositions differed greatly between the groups. For instance, the majority of MaTCP proteins in the TCP-P class contained motif 2, whereas most TCP-C class MaTCP proteins possessed motifs 3 and 6 (Figure 1B).

3.2. The Promoter Analyses of MaTCP Genes

Cis-acting elements in the 2-kb upstream region of the *MaTCP* gene translational start site were also analyzed and classified into three functional categories: hormone responsiveness, abiotic stress responsiveness, and tissue-specific expression (Figure 2). Moreover, the *MaTCP2* gene promoter region contained three abiotic stress elements related to low-temperature responsiveness and drought inducibility and seven hormone elements involved in GA, MeJA, and SA responsiveness. *MaTCP4-1* possessed an abiotic stress element associated with defense and stress responsiveness and nine hormone elements related to ABA, GA, MeJA, and SA responsiveness. *MaTCP8* included two abiotic stress elements concerned with drought inducibility and defense and stress responsiveness and six hormone elements involved in ABA and MeJA responsiveness. *MaTCP9-1* harbored two abiotic stress elements associated with drought inducibility and 15 hormone elements with a function in ABA, GA, and MeJA responsiveness. *MaTCP20-2* contained a tissue-specific expression element related to meristem expression and six hormone elements involved in ABA, GA, SA, and MeJA responsiveness.

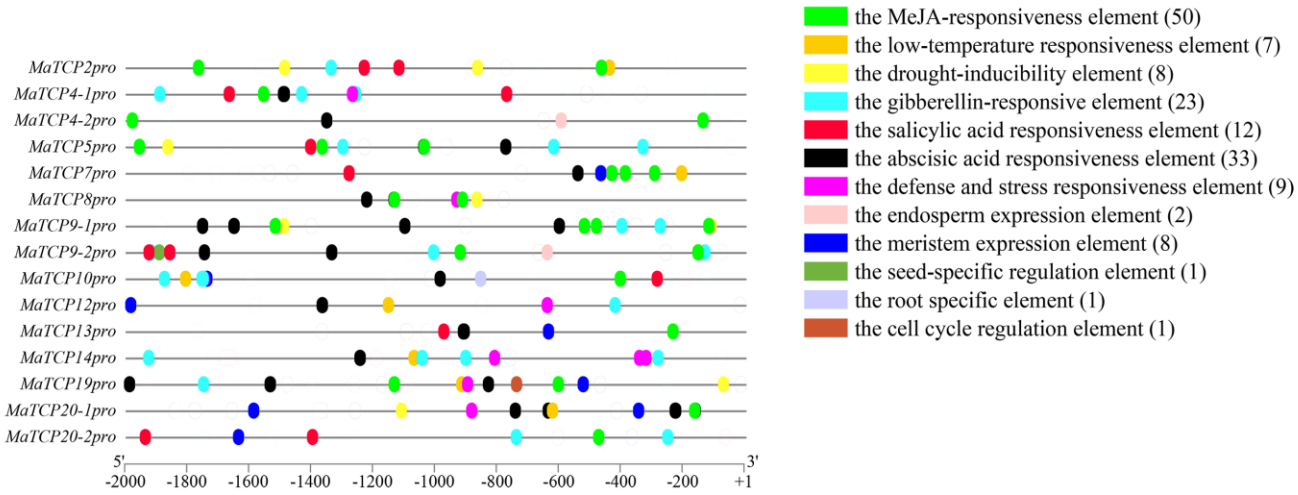


Figure 2. The promoter analyses of *MaTCP* genes. The number in parentheses indicated the number of *cis*-acting elements.

3.3. Expression Patterns of MaTCP Genes during Mulberry Seedling Growth

To analyze *MaTCP* expression patterns during mulberry seedling growth, transcript levels of 15 *MaTCP* genes were measured in GY12 seedlings at five developmental stages. Meanwhile, the roots of ten individuals for each stage were collected and their lengths were measured (Figure 3A,B). As shown in Figure 3C,D, the 15 *MaTCP* genes were grouped according to their expression profiles into three clusters. Clusters 1 and 2 contained four and five *MaTCP* genes, respectively, whereas six *MaTCP* genes were grouped into cluster 3. The expressions of *MaTCP* genes in cluster 3 gradually increased over time, with the highest transcript levels observed in 49-day-old GY seedlings (Figure 3C); this trend was the same as the increases in root length detailed in Table S3. A correlation analysis was also performed between the expressions of the 15 *MaTCP* genes and root lengths at five developmental stages. As shown in Table 2, the expressions of *MaTCP2*, *MaTCP4-1*, *MaTCP8*, *MaTCP9-1*, *MaTCP14*, *MaTCP19*, and *MaTCP20-2* were significantly correlated with root length, with all of them having correlation coefficients higher than 0.8.

3.4. Association of Five MaTCP Genes with Root Development

The expression trend of genes in cluster 3 was similar to that of root growth (Figure 3), and the relative expressions of *MaTCP2*, *MaTCP4-1*, *MaTCP8*, *MaTCP9-1*, and *MaTCP20-2* genes in cluster 3 were significantly correlated with root length (Table 2). These five genes were considered to be highly associated with root development and were thus subjected to further analysis (Figure 4).

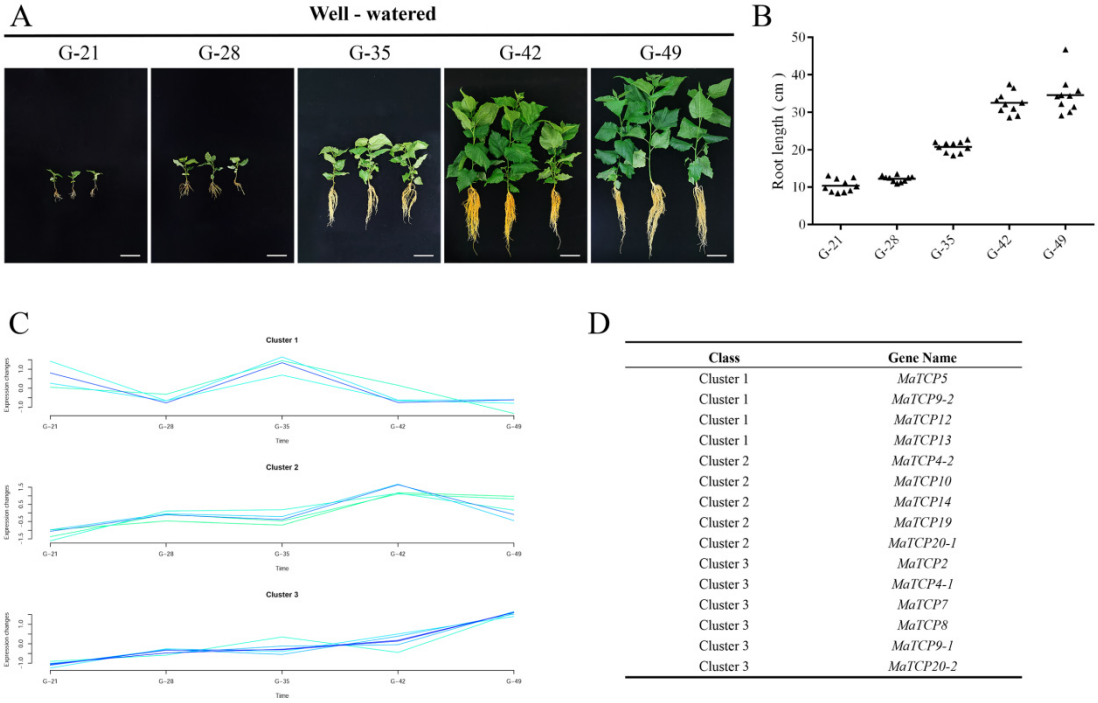
3.5. Subcellular Localization Analyses of Five MaTCP Proteins

To confirm the predicted nuclear locations of the five abovementioned MaTCP proteins, we carried out a subcellular localization analysis. As shown in Figure 5, green fluorescence was only detected in the nuclei of the onion epidermal cells transformed with *35S:MaTCP:GFP*, compared with those transformed with *35S:GFP*. MaTCP2, MaTCP4-1, MaTCP8, MaTCP9-1, and MaTCP20-2 proteins were thus localized to the nucleus.

Table 2. Correlation coefficients between the relative expression of MaTCP genes and root length.

	Root Length	***MaTCP2***	***MaTCP4-1***	***MaTCP4-2***	***MaTCP5***	***MaTCP7***	***MaTCP8***	***MaTCP9-1***	***MaTCP9-2***	***MaTCP10***	***MaTCP12***	***MaTCP13***	***MaTCP14***	***MaTCP19***	***MaTCP20-1***	***MaTCP20-2***
Root Length	1.0000															
MaTCP2	**0.8743**	1.0000														
MaTCP4-1	**0.8234**	0.9902	1.0000													
MaTCP4-2	0.6699	0.3515	0.2348	1.0000												
MaTCP5	−0.4257	−0.4922	−0.3966	−0.6194	1.0000											
MaTCP7	0.6955	0.8982	0.9492	0.0031	−0.1111	1.0000										
MaTCP8	**0.8761**	0.9808	0.9472	0.4683	−0.6480	0.7989	1.0000									
MaTCP9-1	**0.8481**	0.9968	0.9875	0.3488	−0.5323	0.8911	0.9839	1.0000								
MaTCP9-2	−0.2840	−0.3748	−0.2780	−0.4582	0.9480	0.0143	−0.5367	−0.4068	1.0000							
MaTCP10	0.5372	0.1679	0.0528	0.9789	−0.5054	−0.1547	0.2840	0.1647	−0.3416	1.0000						
MaTCP12	−0.6220	−0.6888	−0.6220	−0.6790	0.8925	−0.4070	−0.7914	−0.7299	0.7122	−0.5611	1.0000					
MaTCP13	−0.3205	−0.6062	−0.5719	−0.0533	0.7356	−0.3836	−0.6815	−0.6290	0.8311	0.1258	0.5628	1.0000				
MaTCP14	**0.8765**	0.7762	0.6946	0.8480	−0.7511	0.4828	0.8541	0.7840	−0.5664	0.7320	−0.8993	−0.4104	1.0000			
MaTCP19	**0.9249**	0.8014	0.7117	0.8032	−0.7134	0.4834	0.8760	0.7908	−0.6072	0.6673	−0.7799	−0.5066	0.9523	1.0000		
MaTCP20-1	0.7055	0.5086	0.4407	0.8764	−0.5261	0.3010	0.5691	0.5223	−0.2542	0.8447	−0.7812	0.0261	0.8811	0.7290	1.0000	
MaTCP20-2	**0.9031**	0.9684	0.9312	0.5514	−0.6460	0.7850	0.9895	0.9734	−0.4947	0.3818	−0.8315	−0.5890	0.9052	0.8945	0.6782	1.0000

Note: Significant positive correlations ($r > 0.8$) are indicated in bold.



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Class	Gene Name
Cluster 1	*MaTCP5*
Cluster 1	*MaTCP9-2*
Cluster 1	*MaTCP12*
Cluster 1	*MaTCP13*
Cluster 2	*MaTCP4-2*
Cluster 2	*MaTCP10*
Cluster 2	*MaTCP14*
Cluster 2	*MaTCP19*
Cluster 2	*MaTCP20-1*
Cluster 3	*MaTCP2*
Cluster 3	*MaTCP4-1*
Cluster 3	*MaTCP7*
Cluster 3	*MaTCP8*
Cluster 3	*MaTCP9-1*
Cluster 3	*MaTCP20-2*

Figure 3. Expressions of *MaTCP* genes in GY12 seedlings during root development. (**A**) Images of 21-, 28-, 35-, 42- and 49-day-old GY12 seedlings. Scale bars, 10 cm. (**B**) Corresponding root lengths. Each *black triangle* represents a GY12 seedling. (**C**) RT-qPCR-based time series analysis of the expressions of 15 *MaTCP* genes in GY12 seedlings at five developmental time points. Roots were collected from 10 individuals at each time point (21, 28, 35, 42, and 49 days after germination), and their lengths were measured. (**D**) Clustering of *MaTCP* genes based on the results of the time series analysis.

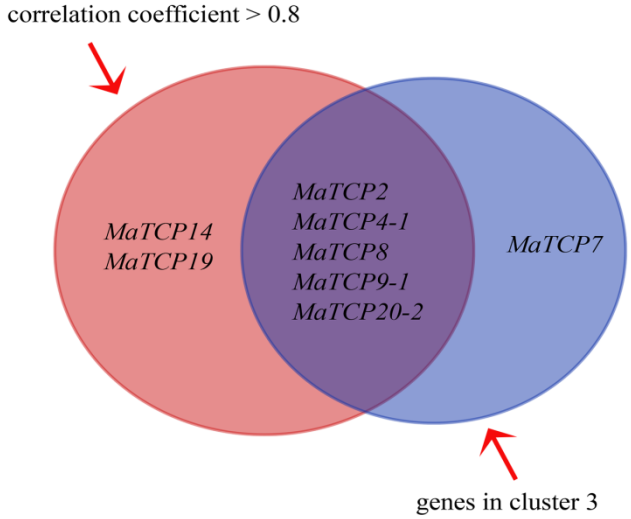

Figure 4. Venn diagram of *MaTCP* genes significantly associated with root length in a correlation analysis (red circle) and those exhibiting a similar trend in regard to root development in a time series analysis (blue circle).

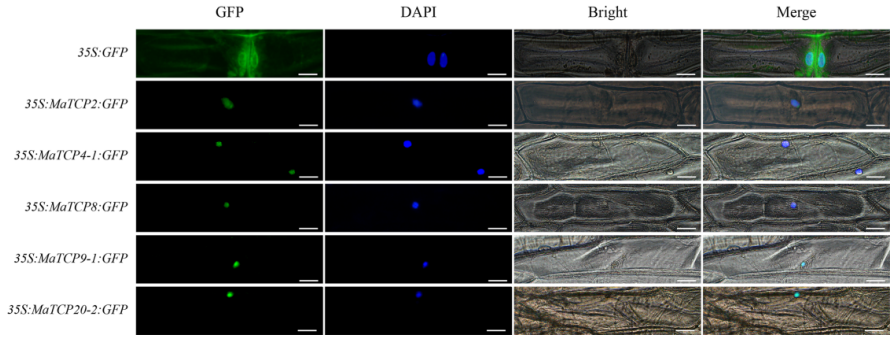

Figure 5. Subcellular localization of MaTCP2, MaTCP4-1, MaTCP8, MaTCP9-1, and MaTCP20-2 transcription factors. *35S:GFP* is an empty vector control, and DAPI was used for nucleic acid staining. Scale bars, 50 μm.

3.6. Expression of Five MaTCP Genes under Drought Treatment

To investigate whether the five *MaTCP* genes are involved in plant responses to drought stress, we examined the expression levels of these genes in GY12 seedlings treated with a 10% PEG6000 solution. RT-qPCR analysis revealed a rapid decrease in *MaTCP2*, *MaTCP4-1*, *MaTCP8*, and *MaTCP9-1* transcripts following 1 day of drought stress. Transcript levels of *MaTCP2*, *MaTCP8*, and *MaTCP9-1* gradually recovered as the treatment time was extended, and *MaTCP20-2* expression significantly increased after 7 days of treatment (Figure 6).

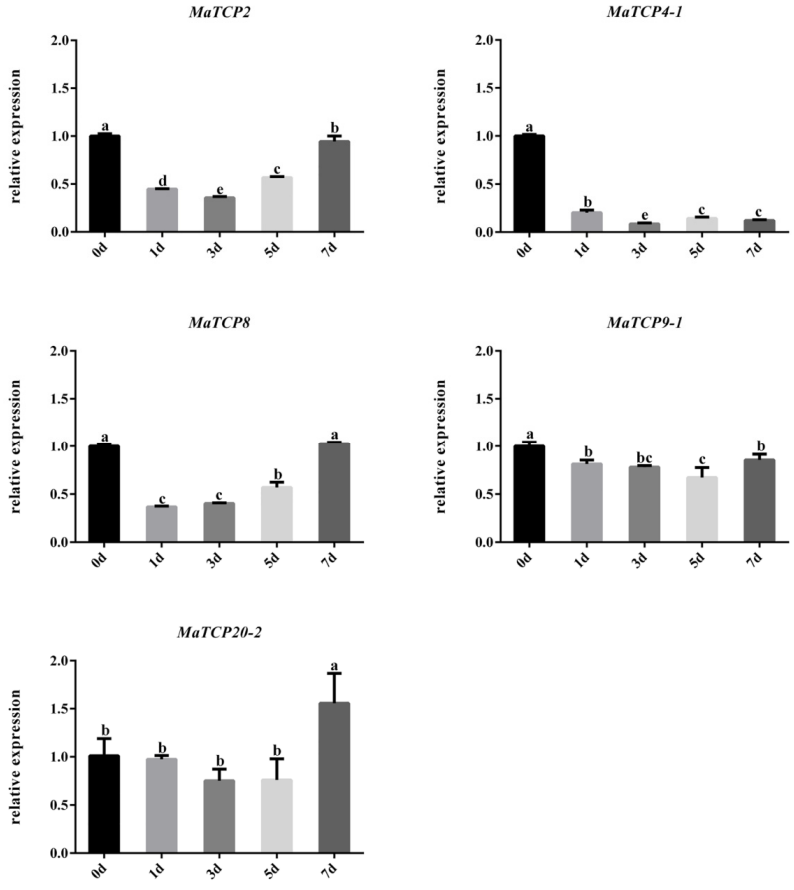

Figure 6. Expressions of *MaTCP* genes during drought stress. The plants were treated with 10% PEG6000 solution for 0, 1, 3, 5, and 7 days. The 0-day plants were the control. Error bars indicate SE. Different lowercase letters indicate statistically significant differences among treatments ($p < 0.05$).

4. Discussion

As the only food source for cultivated silkworms, mulberry trees are a crucial component of sericulture. In light of global warming and increasing water scarcity, the cultivation of mulberry species adapted to various agroclimatic conditions is vital for sustainable sericulture. Consequently, a top research objective for sericulture is the expansion and exploitation of the natural capacity of mulberry to withstand stress and adversity. Mulberry trees are characterized by their extensive root systems, fast growth, and high biomass, and are quite tolerant to their surroundings [35]. According to a previous study, mulberry seedlings can retain high root activities under long-term drought stress by increasing their root absorptive area and improving their capacity for water retention [36]. Moreover, drought tolerance can be improved by drought hardening during the seedling stage [37].

Although attention has focused on mulberry as a plant suitable for ecological restoration, little is known about the molecular mechanisms underlying its tolerance to drought. In particular, studies on the root system and drought tolerance of mulberry seedlings are lacking. Sequencing of the mulberry genome has recently been completed [24,29], and transcriptional data have been obtained from the roots of several mulberry species [38]. A foundation has thus been laid for the investigation of the mulberry root system.

In the present study, we identified 15 MaTCP transcription factors. A protein multiple sequence analysis revealed that MaTCP proteins of the same subclass share a similar motif makeup, but considerable differences exist between subclasses. For instance, the majority of TCP-P MaTCP-class proteins contain motif 2, whereas most TCP-C MaTCP-class proteins possess motifs 3 and 6 (Figure 1B). The TCP family is important for plant growth and development, hormone signaling, and stress response. Prolonged moderate drought also promotes root growth, which boosts a plant's drought resilience. We therefore hypothesized that increased expression of a previously undiscovered *MaTCP* gene cluster enhances root development in mulberry and thereby improves plant drought tolerance. In a time series analysis, *MaTCP2*, *MaTCP4-1*, *MaTCP7*, *MaTCP8*, *MaTCP9-1*, and *MaTCP20-2* gene expression patterns mirrored those of GY12 root development (Figure 3). In addition, the correlation analysis indicated that *MaTCP2*, *MaTCP4-1*, *MaTCP8*, *MaTCP9-1*, *MaTCP14*, *MaTCP19*, and *MaTCP20-2* expressions were significantly correlated with root length (Table 2). Taking into account the results of the two experiments, we thus identified *MaTCP2*, *MaTCP4-1*, *MaTCP8*, *MaTCP9-1*, and *MaTCP20-2* as the key candidate genes involved in GY12 root development. A promoter analysis revealed that *cis*-acting elements involved in MeJA, ABA, SA, GA, and abiotic stress responsiveness are abundant in the promoter regions of the five *MaTCP* genes. Finally, a gene expression analysis indicated that the five *MaTCP* genes are involved in the response of mulberry to drought stress.

All of these results suggest that *MaTCP*s play an essential role linking together mulberry growth, drought response, and phytohormone signaling. Further research on mulberry to elucidate the detailed functions and regulatory mechanisms of *MaTCP* genes would thus be valuable. The resulting findings should contribute to the selection and cultivation of mulberry varieties resistant to harsh settings, the advancement of sericulture, and the preservation of the ecological environment.

5. Conclusions

In conclusion, MaTCP2, MaTCP4-1, MaTCP8, MaTCP9-1, and MaTCP20-2 act as transcription factors and localize to the nucleus. The promoter regions of these five *MaTCP* genes contain a large number of *cis*-acting elements involved in MeJA, ABA, SA, GA, and abiotic stress responsiveness. Their transcriptional levels are highly correlated with root development and may increase mulberry tolerance to drought. Our results have increased the understanding of the function of the mulberry TCP protein and have provided candidate genes for the selective breeding and cultivation of mulberry.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f14010143/s1. Figure S1. Multiple alignments of the amino acid sequences of the MaTCP proteins. Table S1. TF IDs and common names of the TCP transcription factors used in phylogenetic analyses. Table S2. List of primers used in the RT-qPCR analyses of MaTCP genes. Table S3. The length of mulberry seedling roots at five developmental periods. Dataset S1. FASTA files of MaTCP proteins in mulberry.

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References

1. Mega, R.; Abe, F.; Kim, J.S.; Tsuboi, Y.; Tanaka, K.; Kobayashi, H.; Sakata, Y.; Hanada, K.; Tsujimoto, H.; Kikuchi, J.; et al. Tuning water-use efficiency and drought tolerance in wheat using abscisic acid receptors. *Nat. Plants* **2019**, *5*, 153–159. [CrossRef] [PubMed]
2. Yue, B.; Xue, W.Y.; Xiong, L.Z.; Yu, X.Q.; Luo, L.J.; Cui, K.H.; Jin, D.M.; Xing, Y.Z.; Zhang, Q.F. Genetic basis of drought resistance at reproductive stage in rice: Separation of drought tolerance from drought avoidance. *Genetics* **2006**, *172*, 1213–1228. [CrossRef] [PubMed]
3. Fang, Y.J.; Xiong, L.Z. General mechanisms of drought response and their application in drought resistance improvement in plants. *Cell. Mol. Life Sci.* **2015**, *72*, 673–689. [CrossRef] [PubMed]
4. Shavrukov, Y.; Kurishbayev, A.; Jatayev, S.; Shvidchenko, V.; Zotova, L.; Koekemoer, F.; de Groot, S.; Soole, K.; Langridge, P. Early flowering as a drought escape mechanism in plants: How can it aid wheat production? *Front. Plant Sci.* **2017**, *8*, 1950. [CrossRef]
5. Wang, Y.; Yu, Y.H.; Chen, Q.J.; Bai, G.H.; Gao, W.W.; Qu, Y.Y.; Ni, Z.Y. Heterologous expression of GbTCP4, a class II TCP transcription factor, regulates trichome formation and root hair development in *Arabidopsis*. *Genes* **2019**, *10*, 726. [CrossRef] [PubMed]
6. Wang, Y.; Yu, Y.H.; Wang, J.D.; Chen, Q.J.; Ni, Z.Y. Heterologous overexpression of the *GbTCP5* gene increased root hair length, root hair and stem trichome density, and lignin content in transgenic *Arabidopsis*. *Gene* **2020**, *758*, 144954. [CrossRef]
7. Resentini, F.; Felipo-Benavent, A.; Colombo, L.; Blazquez, M.A.; Alabadi, D.; Masiero, S. TCP14 and TCP15 mediate the promotion of seed germination by gibberellins in *Arabidopsis thaliana*. *Mol. Plant.* **2015**, *8*, 482–485. [CrossRef]
8. Lopez, J.A.; Sun, Y.; Blair, P.B.; Mukhtar, M.S. TCP three-way handshake: Linking developmental processes with plant immunity. *Trends Plant Sci.* **2015**, *20*, 238–245. [CrossRef]
9. Danisman, S. TCP transcription factors at the interface between environmental challenges and the plant's growth responses. *Front. Plant Sci.* **2016**, *7*, 1930. [CrossRef]
10. Fang, Y.J.; Zheng, Y.Q.; Lu, W.; Li, J.; Duan, Y.J.; Zhang, S.; Wang, Y.P. Roles of miR319-regulated TCPs in plant development and response to abiotic stress. *Crop J.* **2021**, *9*, 17–28. [CrossRef]
11. Cubas, P.; Lauter, N.; Doebley, J.; Coen, E. The TCP domain: A motif found in proteins regulating plant growth and development. *Plant J.* **1999**, *18*, 215–222. [CrossRef]
12. Martin-Trillo, M.; Cubas, P. *TCP* genes: A family snapshot ten years later. *Trends Plant Sci.* **2010**, *15*, 31–39. [CrossRef] [PubMed]
13. Manassero, N.G.U.; Viola, I.L.; Welchen, E.; Gonzalez, D.H. TCP transcription factors: Architectures of plant form. *Biomol. Concepts* **2013**, *4*, 111–127. [CrossRef] [PubMed]
14. Aggarwal, P.; Das Gupta, M.; Joseph, A.P.; Chatterjee, N.; Srinivasan, N.; Nath, U. Identification of specific DNA binding residues in the TCP family of transcription factors in *Arabidopsis*. *Plant Cell* **2010**, *22*, 1174–1189. [CrossRef]
15. Horn, S.; Pabon-Mora, N.; Theuss, V.S.; Busch, A.; Zachgo, S. Analysis of the CYC/TB1 class of TCP transcription factors in basal angiosperms and magnoliids. *Plant J.* **2015**, *81*, 559–571. [CrossRef] [PubMed]
16. Nicolas, M.; Cubas, P. TCP factors: New kids on the signaling block. *Curr. Opin. Plant Biol.* **2016**, *33*, 33–41. [CrossRef]
17. Kieffer, M.; Master, V.; Waites, R.; Davies, B. TCP14 and TCP15 affect internode length and leaf shape in *Arabidopsis*. *Plant J.* **2011**, *68*, 147–158. [CrossRef]

18. Peng, Y.C.; Chen, L.L.; Lu, Y.R.; Wu, Y.B.; Dumenil, J.; Zhu, Z.G.; Bevan, M.W.; Li, Y.H. The ubiquitin receptors DA1, DAR1, and DAR2 redundantly regulate endoreduplication by modulating the stability of TCP14/15 in *Arabidopsis*. *Plant Cell* **2015**, *27*, 649–662. [CrossRef]
19. Hao, J.; Tu, L.L.; Hu, H.Y.; Tan, J.F.; Deng, F.L.; Tang, W.X.; Nie, Y.C.; Zhang, X.L. GbTCP, a cotton TCP transcription factor, confers fibre elongation and root hair development by a complex regulating system. *J. Exp. Bot.* **2012**, *63*, 6267–6281. [CrossRef]
20. Mukhopadhyay, P.; Tyagi, A.K. *OsTCP19* influences developmental and abiotic stress signaling by modulating ABI4-mediated pathways. *Sci. Rep.* **2015**, *5*, 9998. [CrossRef]
21. Ori, N.; Cohen, A.R.; Etzioni, A.; Brand, A.; Yanai, O.; Shleizer, S.; Menda, N.; Amsellem, Z.; Efroni, I.; Pekker, I.; et al. Regulation of *LANCEOLATE* by *miR319* is required for compound-leaf development in tomato. *Nat. Genet.* **2007**, *39*, 787–791. [CrossRef]
22. Nag, A.; King, S.; Jack, T. miR319a targeting of *TCP4* is critical for petal growth and development in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 22534–22539. [CrossRef] [PubMed]
23. Zhou, M.; Li, D.Y.; Li, Z.G.; Hu, Q.; Yang, C.H.; Zhu, L.H.; Luo, H. Constitutive expression of a *miR319* gene alters plant development and enhances salt and drought tolerance in transgenic creeping bentgrass. *Plant Physiol.* **2013**, *161*, 1375–1391. [CrossRef] [PubMed]
24. He, N.J.; Zhang, C.; Qi, X.W.; Zhao, S.C.; Tao, Y.; Yang, G.J.; Lee, T.H.; Wang, X.Y.; Cai, Q.L.; Li, D.; et al. Draft genome sequence of the mulberry tree *Morus notabilis*. *Nat. Commun.* **2013**, *4*, 2445. [CrossRef] [PubMed]
25. Huang, H.-P.; Ou, T.-T.; Wang, C.-J. Mulberry (sang shen zǐ) and its bioactive compounds, the chemoprevention effects and molecular mechanisms in vitro and in vivo. *J. Tradit. Complement. Med.* **2013**, *3*, 7–15. [CrossRef]
26. Rohela, G.K.; Shukla, P.; Muttanna; Kumar, R.; Chowdhury, S.R. Mulberry (*Morus* spp.): An ideal plant for sustainable development. *Trees For. People* **2020**, *2*, 100011. [CrossRef]
27. Liu, Y.; Willison, J.H.M. Prospects for cultivating white mulberry (*Morus alba*) in the drawdown zone of the Three Gorges Reservoir, China. *Environ. Sci. Pollut. Res.* **2013**, *20*, 7142–7151. [CrossRef]
28. Ma, X.D.; Ma, J.C.; Fan, D.; Li, C.F.; Jiang, Y.Z.; Luo, K.M. Genome-wide identification of TCP family transcription factors from *Populus euphratica* and their involvement in leaf shape regulation. *Sci. Rep.* **2016**, *6*, 32795. [CrossRef]
29. Jiao, F.; Luo, R.S.; Dai, X.L.; Liu, H.; Yu, G.; Han, S.H.; Lu, X.; Su, C.; Chen, Q.; Song, Q.X.; et al. Chromosome-level reference genome and population genomic analysis provide insights into the evolution and improvement of domesticated mulberry (*Morus alba*). *Mol. Plant.* **2020**, *13*, 1001–1012. [CrossRef]
30. Mulder, N.J.; Apweiler, R.; Attwood, T.K.; Bairoch, A.; Bateman, A.; Binns, D.; Bradley, P.; Bork, P.; Bucher, P.; Cerutti, L.; et al. InterPro, progress and status in 2005. *Nucleic Acids Res.* **2005**, *33*, D201–D205. [CrossRef]
31. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [CrossRef] [PubMed]
32. Chen, C.J.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.H.; Xia, R. TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant.* **2020**, *13*, 1194–1202. [CrossRef] [PubMed]
33. Qi, X.W.; Shuai, Q.; Chen, H.; Fan, L.; Zeng, Q.W.; He, N.J. Cloning and expression analyses of the anthocyanin biosynthetic genes in mulberry plants. *Mol. Genet. Genom.* **2014**, *289*, 783–793. [CrossRef] [PubMed]
34. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef]
35. Huang, R.Z.; Jiang, Y.B.; Jia, C.H.; Jiang, S.M.; Yan, X.P. Subcellular distribution and chemical forms of cadmium in *Morus alba* L. *Int. J. Phytoremediat.* **2018**, *20*, 448–453. [CrossRef]
36. Feng, D.L.; Huang, X.H.; Liu, Y.; Willison, J.H.M. Growth and changes of endogenous hormones of mulberry roots in a simulated rocky desertification area. *Environ. Sci. Pollut. Res.* **2016**, *23*, 11171–11180. [CrossRef]
37. Huang, X.H.; Liu, Y.; Li, J.X.; Xiong, X.Z.; Chen, Y.H.; Yin, X.H.; Feng, D.L. The response of mulberry trees after seedling hardening to summer drought in the hydro-fluctuation belt of Three Gorges Reservoir Areas. *Environ. Sci. Pollut. Res.* **2013**, *20*, 7103–7111. [CrossRef]
38. Checker, V.G.; Saeed, B.; Khurana, P. Analysis of expressed sequence tags from mulberry (*Morus indica*) roots and implications for comparative transcriptomics and marker identification. *Tree Genet. Genomes* **2012**, *8*, 1437–1450. [CrossRef]