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Identification of the CBF Gene Family in Wheat and *TaCBF14B* Could Enhance the Drought Tolerance of *Arabidopsis thaliana*

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Abstract: Drought stress is a devastating natural stress that threatens crop productivity and quality. Mitigating the adverse effects of drought stress on wheat is a key object in agriculture. C-repeat binding transcription factor/DROUGHT RESPONSE ELEMENT BINDING FACTOR 1 (CBF/DREB1) transcription factors are well known for their role in cold acclimation. However, the involvement of *CBF* genes in drought stress and the mechanisms underlying their function remain poorly understood. In this study, 81 *CBFs* were identified in wheat, which were further clustered into four distinct lineages based on phylogenetic analysis. Chromosomal localization indicated that most *CBF* genes were dispersed across chromosome 5. We identified three homoeologous genes (*TaCBF14A*, *TaCBF14B*, and *TaCBF14D*) that were simultaneously upregulated under drought stress based on RNA-seq analysis. According to the high expression after drought stress, *TaCBF14B* was selected for further functional analysis. Subcellular localization and transcriptional activation activity analysis indicated that *TaCBF14B* likely functions as a transcription factor involved in drought stress tolerance. Overexpression of *TaCBF14B* in *Arabidopsis* enhanced the primary root growth by 13.49% (OE1), 12.56% (OE2), and 19.53% (OE3) under 200 mM mannitol treatment, and 21.65% (OE1), 16.63% (OE2), and 28.13% (OE3) under 250 mM mannitol treatment compared to WT. Meanwhile, the water loss rate of transgenic lines was 56% in WT leaves, but only 44%, 50%, and 40% in OE1, OE2, and OE3 lines, respectively. Compared to the wild type, POD activities of OE1, OE2, and OE3 were significantly increased by 42.94%, 29.41%, and 62.52%, respectively. And the Pro activities in OE1, OE2, and OE3 were significantly increased by 16.33%, 5.18%, and 29.09%, respectively, compared to the wild type. Additionally, the MDA content in OE1, OE2, and OE3 was significantly reduced by 40.53%, 15.81%, and 54.36%, respectively. Further analysis showed that the transgenic lines were hypersensitive to abscisic acid (ABA), and exhibited increased expression of *AtABI3*. We speculate that *TaCBF14B* plays an important role in enhancing drought tolerance. In

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summary, our findings provide new insights into the functional roles of *CBF* genes in drought stress tolerance.

Keywords: wheat; CBFs; abscisic acid; gene expression; drought stress tolerance

1. Introduction

Plants are constantly exposed to dynamic environmental conditions, and various factors, such as drought, cold, heat, salinity, and biotic stresses, significantly affect their development and growth [1,2]. With the intensification of global climate change, drought stress has become one of the most severe challenges facing global agricultural production. These biotic and abiotic stresses represent major threats that lead to crop losses worldwide. Common wheat (*Triticum aestivum* L.) is a crucial food crop, feeding nearly 30% of the global population [3]. Drought stress, one of the most widespread environmental threats, can lead to a 5–10% reduction in crop yields [4,5]. Therefore, identifying genes associated with drought tolerance and dissecting the molecular mechanisms will help breeders to improve wheat's drought resistance and ensure global food security.

Many kinds of genes participating in multiple biological pathways are involved in response to drought stress. For example, *TaNHX2* could promote drought tolerance of wheat via modulating stomatal aperture in wheat [6]. *Ta4D.GSe* could enhance the drought tolerance of *Arabidopsis* by increasing the scavenging ability of reactive oxygen species (ROS) and osmotic adjustment [7]. The drought-response element binding (DREB) transcription factor family was also found to function in enhancing plant drought stress [8]. CBF (C-repeat binding transcription factor) transcription factors belong to the CBF/DREB1 subfamily of the AP2/ERF (APETALA2/ethylene-responsive factor) superfamily [9]. Unlike the other AP2/ERF proteins, the CBF protein family is characterized by the PKK/RPAGRxKFxETRHP and DSAWR motifs, which are located in the N- and C-terminal flanking region, respectively [10]. Numerous studies have reported the function of CBF genes in different plant species. Overexpression of the Arabidopsis *CBF1*, *CBF2*, or *CBF3* gene has been shown to improve the freezing tolerance of Brassica napus [10,11]. *CsCBF3* conferred cold tolerance in transgenic *Arabidopsis* via an ABA-independent pathway [12]. In addition to cold tolerance, CBF genes also participate in other abiotic stresses, such as drought, heat, and salinity [13,14]. Overexpression of *HvCBF4* could enhance drought, salinity, and low temperature tolerance in rice [9,15]. *GmDREB1* regulates the drought stress response in wheat through modulating the phytohormone melatonin biosynthetic pathway [16]. In *Arabidopsis*, CBF1/CBF2/CBF3 were induced by salt stress and repressed the expression of GALS1 by directly binding its promoter, leading to enhanced salt tolerance [17].

The phytohormone abscisic acid (ABA) plays a crucial role in regulating numerous aspects of plant development [18]. Plants respond to drought stress through two primary signaling pathways: ABA-independent and ABA-dependent pathways [18,19]. The ABA signaling pathway is by far the most well recognized in response to stress [20]. Upon drought stress, ABA accumulates and triggers a series of protective mechanisms. Firstly, PP2C releases the phosphorylation site of SnRK2, which is caused by the ABA receptor PYR/PYL/RCAR recognizing and binding to ABA. Then, SnRK2 phosphorylates downstream proteins, such as ABRE-binding protein/ABRE-binding factors (AREB/ABF), TFs, and the ABA-responsive element (ABRE) [21–23]. The transcript levels of *DBF1* are induced by salt, ABA, and desiccation in maize seedlings, acting as an activator of the *rab17* promoter [24]. AREB1, AREB2, and ABF3, important transcription factors, regulate

the expression of ABA-signaling-pathway-related genes, contributing to drought stress tolerance [25]. In wheat, ABA mediates drought tolerance by regulating the activities of glutathione reductase (GR), dehydroascorbate reductase (DHAR), and ascorbate peroxidase (APX) [26].

Although extensive studies have been conducted on the functions of CBF genes in various plant species, research on the CBF gene family in wheat remains limited, particularly regarding the role of *TaCBF14B* in drought tolerance, which has not been fully explored. In our studies, we performed a comprehensive analysis of the molecular characteristics, evolutionary relationships, gene structure, and expression patterns of the CBF gene family in wheat. Among these genes, the homoeologs of *TaCBF14* were simultaneously induced by drought stress. Furthermore, we investigated the role of *TaCBF14B* in drought tolerance, and the overexpression of *TaCBF14B* in Arabidopsis was used to assess its effectiveness in regulating drought stress tolerance. Our results also indicated that *TaCBF14B* contributes to drought tolerance in Arabidopsis by activating the expression of ABA-related genes.

2. Materials and Methods

2.1. Genome-Wide Identification of CBF Genes in Wheat

Based on the conserved domain (PKRxAGRxKFxETRHPV and DSAWR) characteristics of the CBF genes, 81 wheat CBF family genes were identified and screened from the wheat genome database by HMMER with the E-value set to 1×10^{-5}. The genome version used was IWGSC v 2.1. The corresponding genomic data files of the wheat CBF family were retrieved from Ensembl Plants (https://plants.ensembl.org/Triticum_aestivum/Info/Index) (accessed on 20 March 2024). Phylogenetic analysis of the wheat CBF family genes was conducted using MEGA 11.0, with the alignment method being MUSCLE and bootstraps run 1000 times, and the evolutionary tree, which was further refined and visualized with iTOL: Interactive Tree of Life (http://itol.embl.de/) (accessed on 20 March 2024). Subsequently, the published CBF family gene data for rice were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/protein) (accessed on 20 March 2024). Covariance analysis of the rice CBF family genes was performed using TBtools v2.008. The gene structure was visualized based on genome annotation information (GFF3 file), in which exon–intron organization was illustrated to reveal the structural features of each gene.

2.2. Gene Structure and Conserved Protein Motifs

Based on the GFF3 file of the wheat genome, the exon and intron numbers and length of TaCBF genes were analyzed using TBtools v2.008. The conserved motif of TaCBF proteins was analyzed using the MEME tool (http://meme-suite.org/) (accessed on 20 March 2024), with the number of motifs set to 8 and the width of motifs set to 6–50. The gene structure and conserved motif of *TaCBFs* were visualized using TBtools v2.008.

2.3. Plant Materials and Growth Conditions

Four drought-tolerant wheat cultivars—Shanrong 3, Jinan 17, Dekang 961, and Xiaoyan 60 [26–29]—and one drought-sensitive wheat variety (Chinese Spring) [30] were hydroponically cultivated. Three biological replicates were established for each cultivar, with each replicate consisting of five plants. All materials were grown in a greenhouse at 22/18 °C and a 16/8 h day/night temperature cycle with a light intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$. One-leaf seedlings were treated with 20% (w/m) polyethylene glycol (PEG) 6000 for 0, 1, 6, 12, 24, and 48 h, and leaves at the same stage under normal conditions were also collected for expression analysis.

2.4. Gene Expression Analysis of TaCBF

Based on the gene expression profiles of Giza168 before and after 20% PEG-6000 treatment, which were downloaded from Wheat Omics 1.0 (http://wheatomics.sdau.edu.cn/) (accessed on 20 November 2024) [31], a heatmap was created using TBtools v2.008 according to the TPM values of *TaCBF* genes. The expression data of *TaCBF* genes induced by PEG-6000 treatment were then analyzed separately, and the three homoeolog genes (*TaCBF14A*, *TaCBF14B*, and *TaCBF14D*), which simultaneously showed high expression levels, were selected for further analysis.

To validate the *TaCBF14B*, which showed the highest expression level among the three homoeolog genes after PEG-6000 treatment, we further checked the expression in Qingmai 6 leaves at the seedling stage with quantitative real-time PCR (qRT-PCR). The tissue-specific expression of *TaCBF14B* was also performed using a qRT-PCR on leaves and roots of Qingmai 6 at the seedling stage. Based on the expression of *TaCBF14*, it exhibited higher expression levels in leaves than roots, and *TaCBF14B* displayed the highest expression after PEG-6000 treatment in Qingmai 6. In order to verify the expression level of *TaCBF14B* between drought-resistant and drought-sensitive wheat varieties, the leaves before and after PEG-6000 treatment of drought-resistant wheat cultivars (Shanrong 3, Xiaoyan 60, Jinan 17, and Dekang 961) and one drought-sensitive wheat cultivar (Chinese Spring) were used for comparison, with the drought-sensitive cultivar (Chinese Spring) serving as the control.

Total RNA was extracted using the Total RNA Extraction Kit (Vazyme, Nanjing, China), and first-strand cDNA synthesis was carried out using a PrimeScript™ RT Reagent Kit (Perfect Real Time, Takara, Shiga, Japan). In the qRT-PCR, we used the SYBR Green system (Roche, Basel, Switzerland), and the *β-actin* gene was used for internal control. Each sample was performed with three biological replicates, and each biological replication had three technique replicates. The relative expression values of the gene were determined with the $2^{-\Delta\Delta Ct}$ method. All primers are listed in Table S1.

2.5. Subcellular Localization Assays of TaCBF14B

The full-length open reading frame (ORF) of *TaCBF14B* was obtained from the Chinese Spring genome; the non-terminator coding sequence was fused to the pCAMBIA1300-GFP vector with CaMV 35S promoter, and Xba1 was used as the cloning site. The Agrobacterium tumefaciens strain harboring the pCAMBIA1300:TaCBF14B construct was cultured overnight in liquid LB medium supplemented with appropriate antibiotics at 28 °C with shaking at 220 rpm. On the following day, the overnight culture was inoculated into fresh LB medium and grown until the optical density at 600 nm (OD_{600}) reached approximately 0.8. The bacterial cells were then harvested by centrifugation at 6000 rpm for 10 min and resuspended in infiltration buffer (100 mM $MgCl_2$, 100 mM MES, and 200 μM Acetosyringone), adjusting the OD_{600} to 0.8. The bacterial suspension was gently infiltrated into the abaxial side of Nicotiana benthamiana leaves using a needleless 1 mL syringe to ensure penetration into the mesophyll tissue. The expression of TaCBF14B-GFP and GFP in Nicotiana benthamiana leaf cells was analyzed after infiltration for 48 h. The samples were incubated with DAPI (4′,6-Diamidino-2′-phenylindole) staining solution for 10–15 min, followed by the sealing and preparation of slides. Dual-channel fluorescence detection was performed using a laser scanning confocal microscope (TCSsp5II; Agilent, Santa Clara, CA, USA); GFP fluorescence was detected at an excitation wavelength of 488 nm and emission wavelength of 507 nm, while nuclear fluorescence signals from DAPI were observed at an excitation wavelength of 364 nm and emission wavelength of 454 nm.

2.6. Transcriptional Activity Assay

The coding region of *TaCBF14B* was cloned into the pGBKT7 vector and co-transformed with pGADT7 into Y2H Gold yeast cells. The transformed yeast cells were cultured on SD/-Trp/-Leu and SD/-Trp/-Leu/-His/-Ade agar plates, and the expression of the *MEL1* reporter gene was detected using X-α-Gal solution based on the growth pattern. The co-transformation of pGBKT7–53 with pGADT7-T served as a positive control, while the co-transformation of pGBKT7-Lam with pGADT7-T served as a negative control.

2.7. Arabidopsis Transformation

The ORF of *TaCBF14B* was cloned into pCAMBIA1300, which was driven by CaMV 35S promoter and then transformed into Agrobacterium tumefaciens strain GV3101. The Agrobacterium tumefaciens containing the target vector was transferred into Arabidopsis. The positive transgenic lines were screened on Hygromycin plates first and then detected using reverse-transcription PCR. *Arabidopsis thaliana* were planted in a growth chamber at 22 °C with 16 h light/8 h dark and 70% relative humidity.

2.8. Drought Tolerance Assessment

The seeds of wild-type Arabidopsis (WT) and transgenic lines were surface-sterilized in 0.745% sodium hypochlorite with 0.004% Triton X-100 for 15 min and then washed five to six times with sterilized water. After vernalizing in darkness for 3 days under 4 °C, the seeds were plated on Murashige and Skoog medium (MS). To assess root length, Arabidopsis seedlings were grown on standard MS medium for 7 days. Uniformly developed seedlings were then transferred to MS medium supplemented with 0 mM (control), 200 mM, or 250 mM mannitol and cultured for an additional 10 days in a growth chamber. Root length was subsequently measured. In a separate experiment, 7-day-old seedlings were transplanted into a substrate composed of vermiculite and nutrient soil mixed at a 1:3 (v/v) ratio and grown for another two weeks in a growth chamber. Drought stress was imposed by completely withholding irrigation for 12 days. At the end of the drought treatment, plant height, fresh weight, dry weight, and relative electrolyte leakage were measured. All experiments were conducted under controlled environmental conditions: 22 °C, 70% relative humidity, a light intensity of 135 $\mu mol \cdot m^{-2} \cdot s^{-2}$, and a 16/8 h light/dark photoperiod.

To detect the role of *TaCBF14B* in water loss regulation, WT and OE Arabidopsis lines were cultured under normal conditions for 12 days, and five leaves of uniform size were collected from each line, which were then placed in a culture chamber at 21 ± 1 °C. The weight was measured every hour [32,33]. For the survival rate detection, the five seedlings from each line were re-watered after 23 days of drought treatment, and the survival rate was calculated. Each experiment was performed at least three times.

2.9. Measurement of MDA and Proline Content and Analysis of POD Activity

Seedlings of wild-type (WT) Arabidopsis and transgenic lines (OE1, OE2, and OE3) with consistent growth were selected for drought treatment after 15 days of growth. A drought treatment group and a normal watering control group were established for each line, with four seedlings per treatment and three biological replicates. Leaf samples were collected after 12 days of treatment, ground in liquid nitrogen, and weighed to 0.1 g. The activity of peroxidase (POD), proline (Pro) content, and malondialdehyde (MDA) content were measured using commercial kits from Grace Biotechnology (Suzhou, China) following the manufacturer's instructions. For POD activity determination, 0.1 g leaf samples were homogenized in 1 mL of PBS extraction buffer on ice. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was collected. The supernatant was

mixed with Reagents 1–3, and the absorbance change at 470 nm was measured at 0 and 1 min to calculate POD activity. For proline content measurement, a standard curve for proline was first constructed. A 0.1 g leaf sample was homogenized in 1 mL of ninhydrin reagent and extracted by shaking in a 90 °C water bath for 10 min. After cooling to room temperature, the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant was collected. Then, 3-methyl-2-benzothiazolinone hydrazone (MBTH) and glacial acetic acid were added, followed by a 30 min incubation in a 95 °C water bath. After cooling to room temperature, 200 μL of the clear liquid was measured for absorbance at 520 nm, with a blank sample used for correction. For MDA content measurement, 0.1 g leaf samples were homogenized in 1 mL of PBS extraction buffer on ice, and the mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected, and thiobarbituric acid (Thiobarbituric Acid, TBA, Dorchester, UK) was added to induce the formation of a colored complex with MDA. The absorbance at 532 nm and 600 nm was measured using an ultraviolet–visible spectrophotometer (Cary 60, Agilent, Santa Clara, CA, USA) to calculate the MDA content.

2.10. ABA Treatment Analysis

For the detection of the germination rate after abscisic acid (ABA) treatment, the surface-sterilized seeds of WT and transgenic plants were plated on MS medium supplemented with different concentrations of ABA (0 μM, 0.5 μM, and 1 μM) (100 seeds per medium). After 7 days of incubation, the germination rate was calculated. For the analysis of ABA pathway-related gene expression, seedlings of WT and transgenic lines subjected to 12 days of drought treatment were used for the assays.

2.11. Statistical Analysis

Student's *t*-test and one-way analysis of variance (ANOVA) with the least significant difference (LSD) test were performed using SPSS 22.0. A value of $p < 0.05$ was used to determine significance. The significant differences are indicated in the figure legends. Error bars represent SDs.

3. Results

3.1. Genome-Wide Identification and Characterization of CBF Family Genes in Wheat

According to the conserved domains, PKRxAGRxKFxETRHPV, AP2/ERF, and DSAWR, 81 CBF genes were identified from the wheat genome (Table S2). The protein length of these CBF genes ranged from 175 to 290 (average 231.2 aa), and their molecular weights ranged from 19.08 kDa to 31.51 kDa (average 24.8 kDa). The theoretical isoelectric point (pI) was also investigated, and the results showed that approximately 74% of the proteins had a pI < 7, indicating that these proteins were acidic. In addition, all of these CBF proteins had negative grand average of hydropathicity (GRAVY) scores, except for four.

Chromosomal location analysis was performed using TBtools, and the results showed that CBF genes were distributed across 15 chromosomes, excluding 3A, 3B, 3D, 4B, 4D, and 7B (Figure 1A). The largest number of CBF genes were found on chromosome 5, while 20, 19, and 16 CBF genes were located on subgenomes A, B, and D, respectively.

Synteny analysis of the *TaCBF* genes illustrated that 16 homologous gene pairs existed between wheat and rice (Figure 1B). Meanwhile, six rice CBF genes had synteny with at least two wheat CBF genes, suggesting that these genes were highly conserved between wheat and rice.

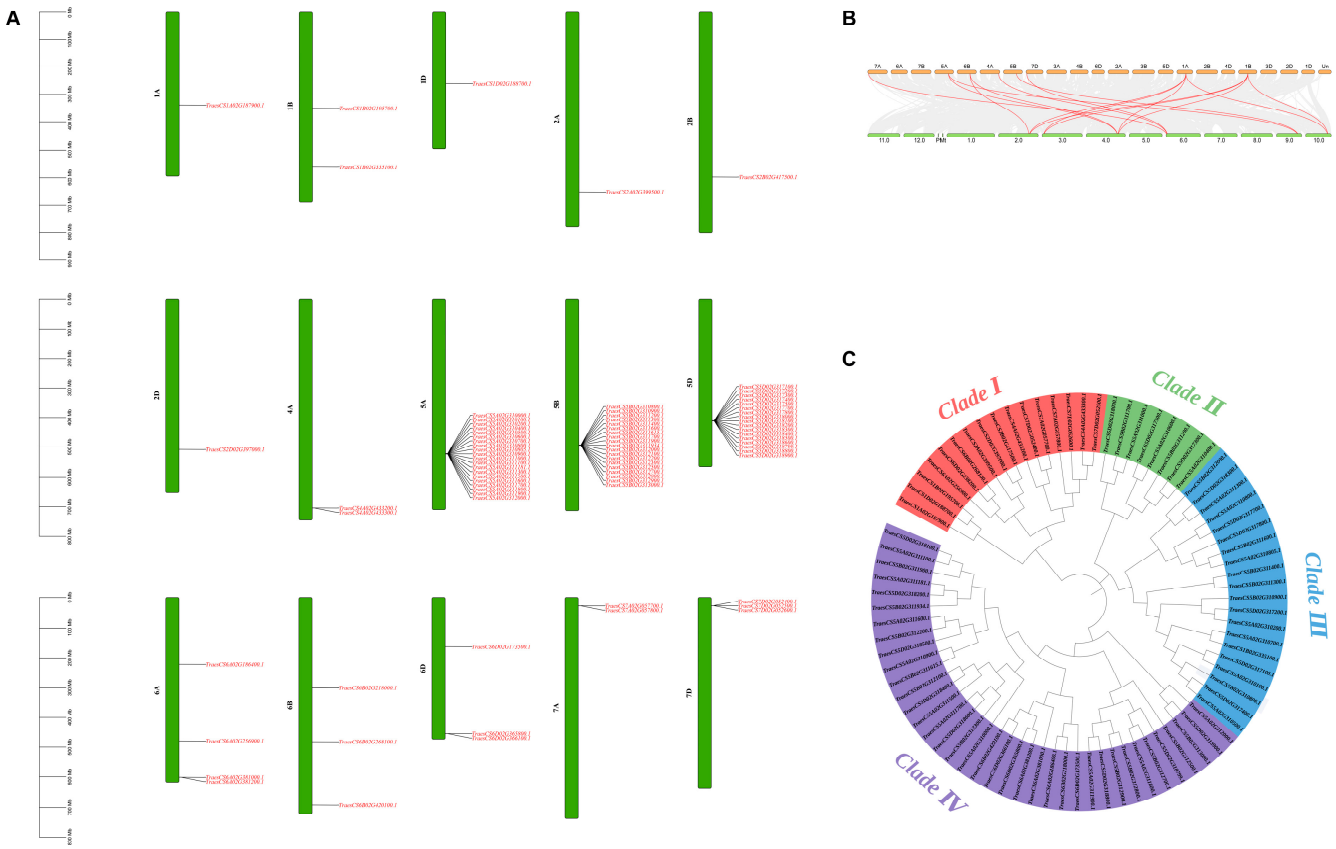


Figure 1. Genome identification of *CBF* genes in wheat. Chromosome distribution (**A**), synteny (**B**), and phylogenetic tree analyses (**C**) of *CBF* genes.

In order to investigate the evolutionary relationships of the 81 *TaCBF* genes, an unrooted phylogenetic tree was constructed using MEGA 11.0 (Figure 1C). According to their conserved structural features, the 81 *TaCBF* genes were divided into four major clades, with Clade IV containing the largest number of members.

3.2. Analysis of Conserved Motifs and Gene Structure of TaCBFs

Based on the evolutionary analysis (Figure 2A), we analyzed the conserved motifs (Figure 2B), functional domains (Figure 2C), and structural features of the genes (Figure 2D). Functional domain analysis revealed that all *TaCBF* genes contained the structural domain of CBFs (Figure 2C). Conserved motif analysis of TaCBF proteins using MEME identified 8 motifs, named Motif 1 to 8, with lengths ranging from 6 to 50 aa and most motifs exhibiting similar distribution patterns across different subfamilies (Figure 2B). For example, all subfamily members contained Motif 1, Motif 2, and Motif 3. This demonstrated that these motifs were highly conserved and potentially functionally significant in TaCBF proteins. Additionally, some members of the Clade IV subfamily contained the same motif composition as those in Clade I, while others contained the same motif composition as those in Clade II. This demonstrated that these motifs had a role in the specific functions of different subfamilies. Functional domain analysis revealed that all *TaCBF* genes contained a typical AP2/ERF domain, suggesting that TaCBF proteins may share similar biological functions and play important roles in the response to abiotic stress (Figure 2C). Gene structure analysis revealed that only *TraesCS1B02G335100.1*, *TraesCS5B02G310900.1*, and *TraesCS6A02G186400.1* contained a single intron, while all members of the Clade I and Clade II subfamilies were intronless (Figure 2D). Overall, members of the same subfamily

had similar conserved motifs and gene structural features. This demonstrated that the development evolution tree constructed in this study is accurate.

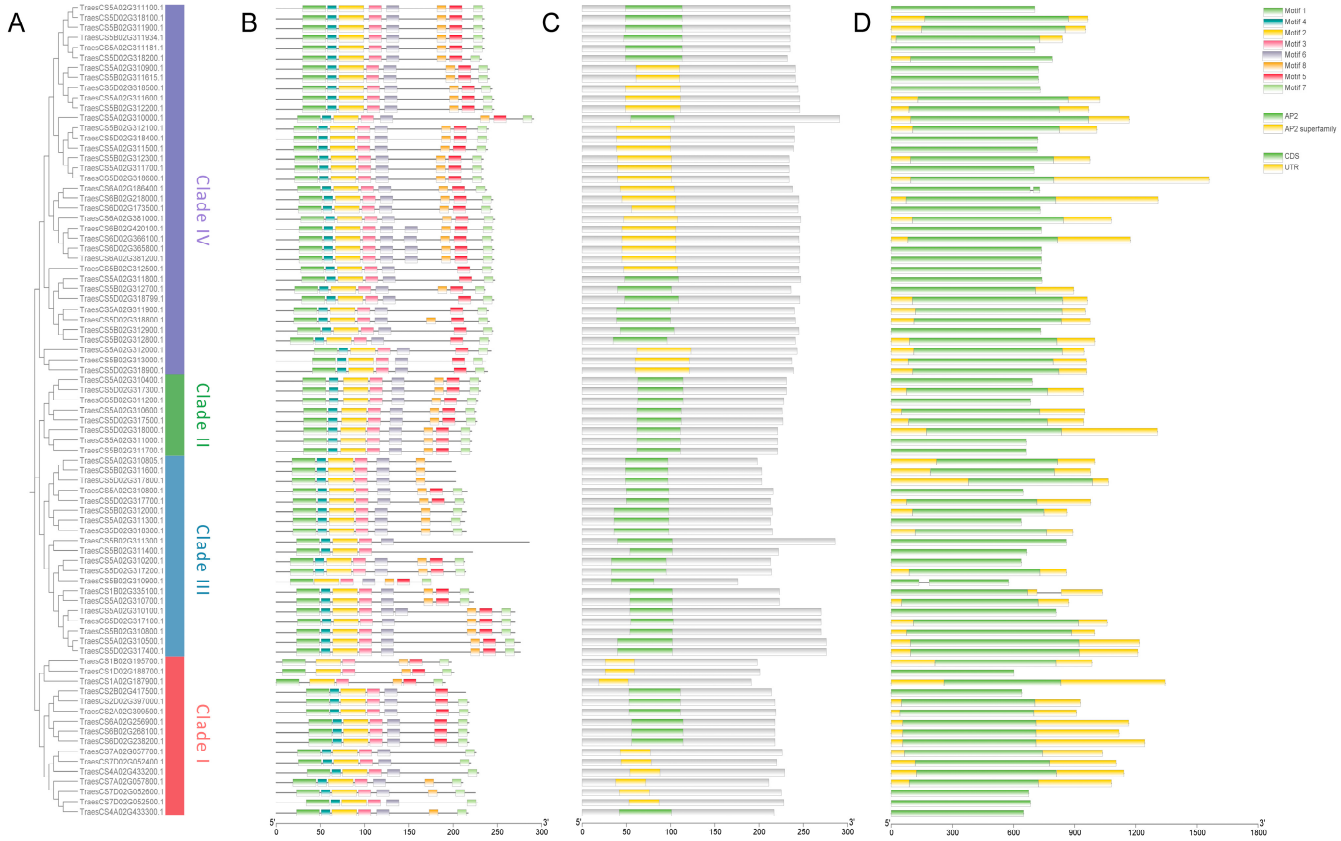


Figure 2. Motif and gene structure analysis of wheat *TaCBF* genes. (**A**) Phylogenetic tree of wheat *TaCBF* genes; (**B**) conserved motif composition of *TaCBF* genes; (**C**) functional domain organization of *TaCBF* genes; (**D**) exon–intron structure of *TaCBF* genes.

3.3. The TaCBF14B Gene Was Identified as Being Induced by Drought Stress

To identify the expression characteristics of *TaCBF* genes under abiotic stress, a heatmap was generated using the expression data from PEG treatment based on Qingmai6 RNA-seq data (Figure 3A). Among these, 20 *TaCBF* genes were up-regulated, and three homoeologous genes (*TraesCS5A02G313000*, *TraesCS5B02G312000*, and *TraesCS5D02G318300*) were induced by PEG simultaneously (Figure 3B). Reciprocal BLAST 2.16.0+ analysis showed that *TraesCS5A02G313000*, *TraesCS5B02G312000*, and *TraesCS5D02G318300* were orthologs of *TaCBF14*. Accordingly, we identified these three genes as *TaCBF14A*, *TaCBF14B*, and *TaCBF14D*. Reverse transcription quantitative PCR (RT-qPCR) analysis showed that TaCBF14B exhibited a higher transcript level in the leaf compared to the root at the seedling stage (Figure 3C). Meanwhile, the transcript abundance of TaCBF14B was significantly increased in the leaf under PEG treatment in Qingmai 6 (Figure 3D). Compared to the salt-sensitive wheat cultivar Chinese Spring (CS), *TaCBF14B* transcript abundance was higher in the salt-tolerant wheat cultivars Shanrong 3, Xiaoyan 60, Jinan 17, and Dekang 961 (Figure S1). These results suggest that the higher expression of *TaCBF14B* may be associated with drought tolerance.

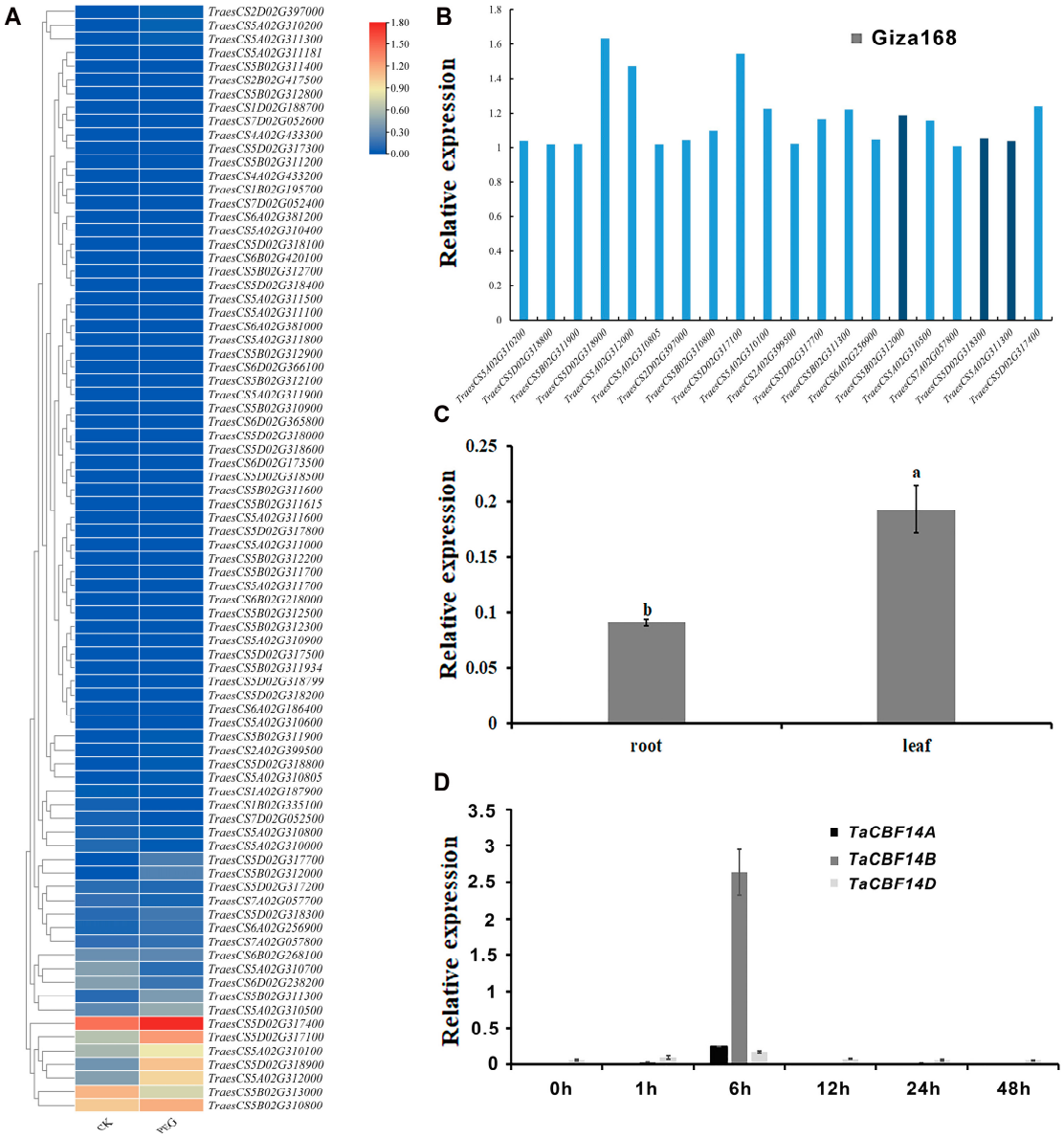


Figure 3. Characterized *CBF* genes induced by drought treatment. (**A**) Heat map of the expression profiling of *CBF* genes under PEG treatment. (**B**) Relative expression levels of *CBF* genes compared to normal condition based on the data of Giza 168 before and after 20% PEG-6000 treatment. (**C**) The expression level of *TaCBF14* in roots and leaves of Qingmai 6, respectively. (**D**) Expression level of *TaCBF14* in leaves of Qingmai 6 under PEG treatment. Different lowercase letters indicate statistically significant differences (least significant difference test; $p < 0.05$). Error bars indicate SDs (n = 3). The reference gene used is *β-actin*.

3.4. Subcellular Localization of the TaCBF14B Protein

In order to determine the subcellular localization of TaCBF14B protein, we examined its subcellular distributions in Nicotiana benthamiana leaves. The coding region of TaCBF14B was fused with GFP marker, and 35S:GFP was used as the control. The subcellular localization of the TaCBF14B-GFP fusion protein was observed using a confocal microscope. The GFP signal was observed both in the cytoplasm and nucleus under the control condition, but the TaCBF14B-GFP fusion protein was localized in the nucleus exclusively (Figure 4). This observation indicated that TaCBF14B is a nuclear localized protein, and may play a regulatory role.

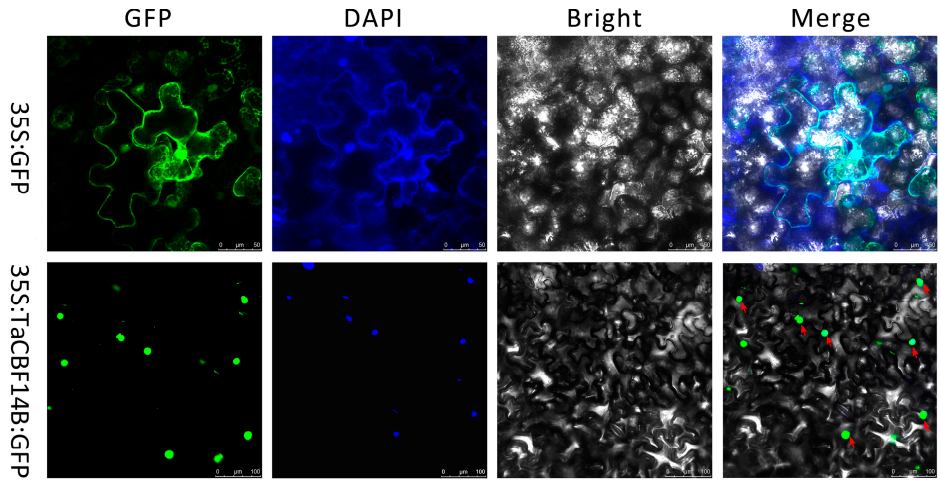


Figure 4. The subcellular localization of TaCBF14B. Scale bar: 10 μm (35S:GFP) and 20 μm (TaCBF14B:GFP).

3.5. TaCBF14B Has Transactivation Activity in Yeast

To examine whether TaCBF14B has transcription activation activity, we used the yeast two-hybrid system for analysis. The fusion plasmids pGBKT7-TaCBF14B and pGBKT7 (control) were transformed into the yeast strain Y2H Gold. The results showed that yeast cells containing the pGBKT7-TaCBF14B fusion protein grew well on both SD/-Leu-Trp and SD/-Ade-His-Leu-Trp media, while cells containing pGBKT7 alone only grew on the SD/-Leu-Trp medium (Figure 5). Additionally, yeast cells could grow normally on SD/-Ade-His-Leu-Trp medium in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-α-Gal) (Figure 5), indicating activation of the *MEL1* reporter gene. These results demonstrate that TaCBF14B had transcriptional activation activity.

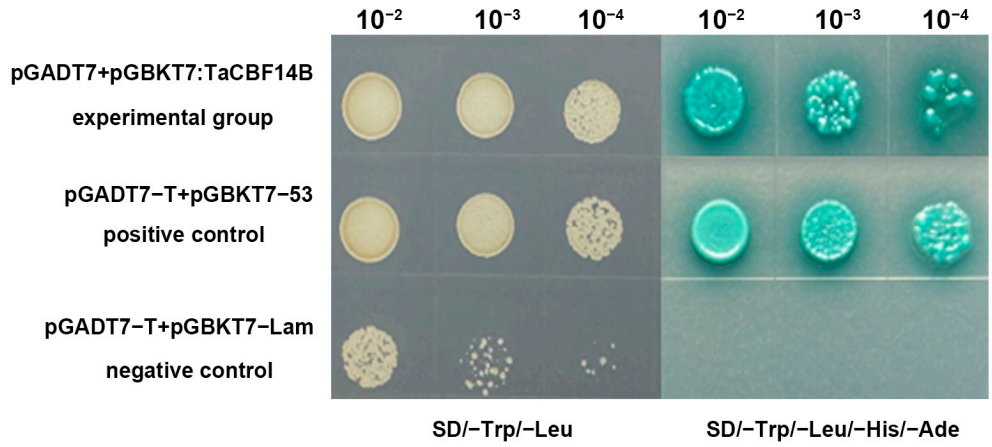


Figure 5. Transcription activation activity analysis of TaCBF14B. pGADT7-T + pGBKT7–53 was used as a positive control, pGADT7-T + pGBKT7-Lam was used as a negative control, and pGADT7 + pGBKT7:TaCBF14B was the experimental group.

3.6. TaCBF14B Overexpression in Arabidopsis Showed Enhanced Tolerance to Drought Tolerance

In order to investigate the function of *TaCBF14B* under drought stress, we introduced *TaCBF14B* into Arabidopsis. A total of eight transgenic lines were obtained after screening on selective media and confirmed using RT-PCR. Three homozygous transgenic lines (2, 3, and 6) with higher transcription levels of *TaCBF14B* than the wild type were selected for further analysis, and named OE1, OE2, and OE3, respectively (Figure 6A). The primary root growth was comparable between OE and wide-type (WT) Arabidopsis seedlings, which is a key indicator of various stress responses. All plants grew well under normal conditions,

and there were no significant differences in primary root growth between OE and WT plants, except for OE3, which exhibited a longer root length (Figure 6B and Figure S2). After mannitol-induced drought stress, OE seedlings had longer root length compared to WT. Furthermore, primary root growth was impaired in a dose-dependent manner (13.49%, 12.56%, and 19.53% for OE1, OE2, and OE3, respectively, compared to WT under 200 mM mannitol treatment, and 21.65%, 16.63%, and 28.13% for OE1, OE2, and OE3, respectively, under 250 mM mannitol treatment) (Figure 6B). These results indicate that the overexpression of *TaCBF14B* could enhance the drought tolerance of *Arabidopsis* at the seedling stage.

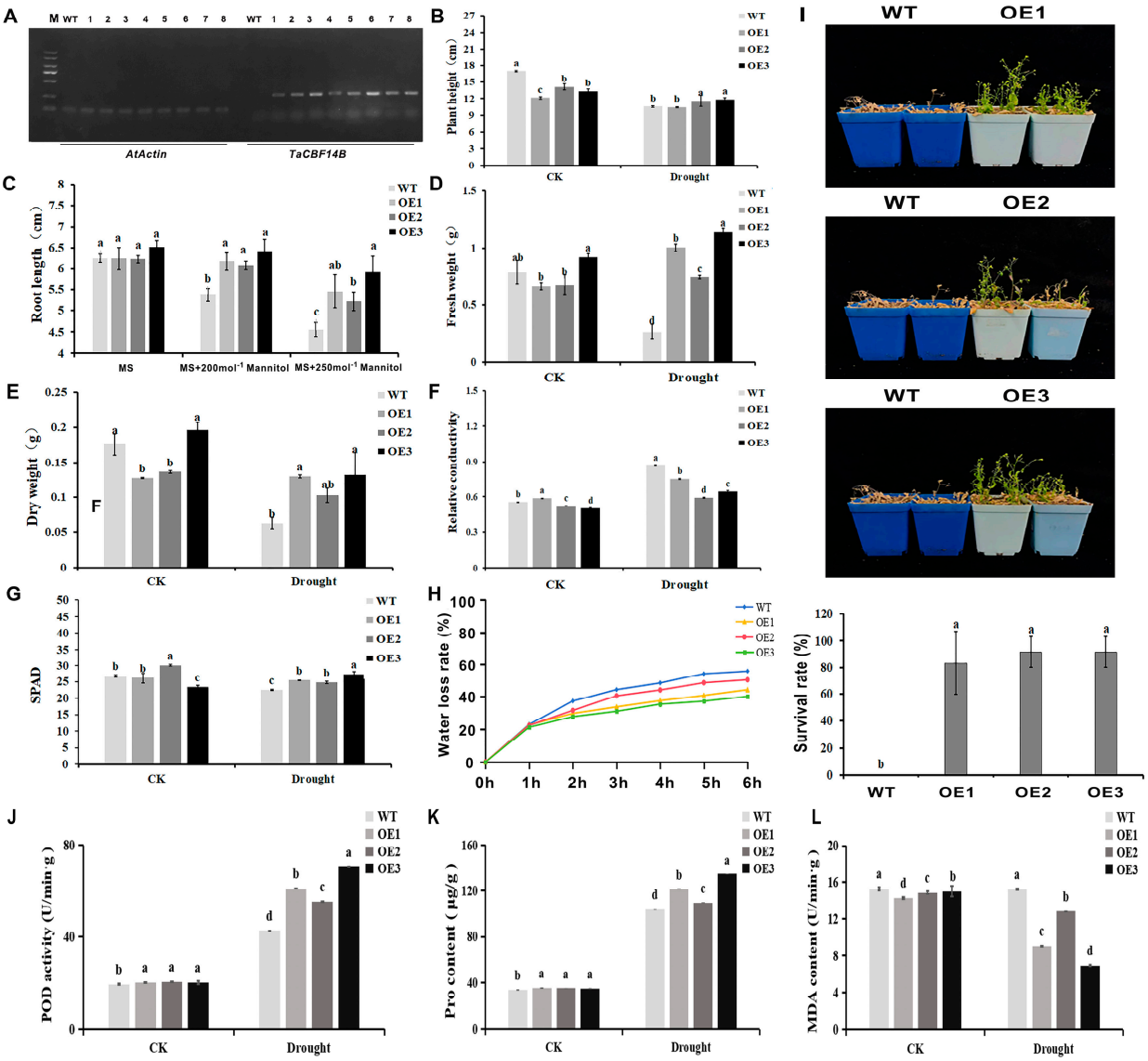


Figure 6. Overexpression of *TaCBF14B* enhances drought tolerance in Arabidopsis. (**A**) Identification of transgenic lines by RT-PCR. (**B**) plant height. C-G Root length of WT and transgenic lines before and after mannitol treatment (**C**), fresh weight (**D**), dry weight (**E**), relative conductivity (**F**), and SPAD before and after drought stress (**G**). (**H**) Water loss rates of the detached Arabidopsis leaves from WT and OE lines over time during dehydration. (**I**) Phenotype and survival rate of WT and homozygous transgenic lines under normal and rewatering conditions. J-K POD activity (**J**), Pro content (**K**), and MDA content (**L**). Different lowercase letters indicate statistically significant differences (least significant difference test; $p < 0.05$). Error bars indicate SDs (n = 3).

Moreover, we further investigated the role of *TaCBF14B* in drought tolerance at the vegetative stage. The 15-day-old OE and WT Arabidopsis seedlings were used for drought tolerance evaluation with or without water treatment. Under normal conditions, the OE lines displayed shorter plant height compared to WT. However, after drought treatment, the opposite trend was observed, with OE2 and OE3 displaying higher plant height (Figure 6C). Moreover, *TaCBF14B* overexpression improved the fresh weight, dry weight, and SPAD values, and significantly reduced the relative conductivity under drought stress (Figure 6D–H and Figure S3). Consistently, the water loss rate (WLR) of leaves from transgenic lines was markedly lower than that of WT plants. After 6 h of dehydration, WLR reached approximately 56% in WT leaves, but only 44%, 50%, and 40% in OE1, OE2, and OE3 lines, respectively (Figure 6H and Figure S3). Additionally, the survival rate of OE lines after rewatering was significantly higher than that of WT, following 23 days of drought treatment (Figure 6I). These results indicated that *TaCBF14B* overexpression could contribute to drought tolerance in Arabidopsis at the vegetative stage.

When plants were exposed to environmental stresses, MDA, Pro, and POD played important roles in their physiological responses. MDA was a product of lipid peroxidation in cell membranes and could serve as an indicator of oxidative damage, reflecting the degree of oxidative stress in plants under stress conditions. Pro, as an osmotic regulator, helped plants maintain water balance between the inside and outside of cells. POD played a key role in antioxidant defense by scavenging reactive oxygen species (ROS) generated by stress. To investigate the role of the *TaCBF14B* gene in drought resistance mechanisms, wild-type and transgenic lines subjected to normal and drought treatments were used as samples for the assessment of ROS-related traits, including POD activity, and Pro and MDA content (Figure 6J–L). Under normal conditions, no significant differences were observed between the transgenic lines (OE1, OE2, OE3) and the wild type. Under drought stress, the POD activity in OE1, OE2, and OE3 was significantly increased by 42.94%, 29.41%, and 62.52%, respectively, compared to the wild type. The Pro activity in OE1, OE2, and OE3 was significantly increased by 16.33%, 5.18%, and 29.09%, respectively, compared to the wild type. Additionally, the MDA content in OE1, OE2, and OE3 was significantly reduced by 40.53%, 15.81%, and 54.36%, respectively, compared to the wild type. The results indicated that the *TaCBF14B* gene enhanced drought resistance in transgenic lines by regulating osmotic regulators (such as Pro) and antioxidants (such as POD). At the same time, the MDA content in the transgenic lines was significantly reduced, further supporting the important role of this gene in the drought resistance mechanism of plants.

3.7. Overexpression of TaCBF14B in Arabidopsis Contributes to ABA Hypersensitivity

To determine whether *TaCBF14B* participates in drought stress through ABA-independent signaling, we investigated seed germination in OE and WT plants under ABA treatment. As shown in Figure 7A, the germination rate of OE seeds was higher than that of WT under normal conditions, but under ABA treatment, the OE seeds were more sensitive and displayed lower germination rates. Additionally, we examined the expression levels of ABA signaling and synthesis genes *AtABF4*, *AtABI3*, and *AtABI5* in WT and OE plants before and after drought stress treatment. As shown in Figure 7B, *AtABI3* expression was higher in OE plants than in WT plants after PEG treatment. These results suggest that the *TaCBF14B* gene may be involved in signaling pathways mediated by osmotic and drought stress, as well as ABA.

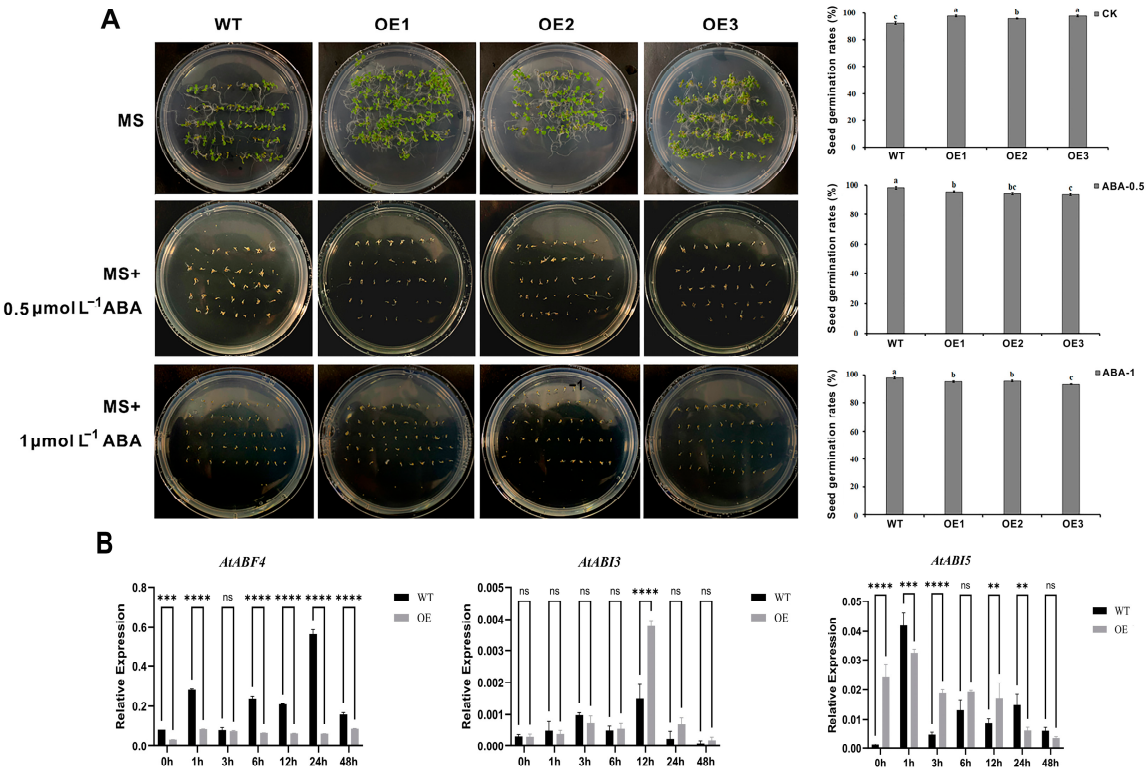


Figure 7. Overexpression of *TaCBF14B* in Arabidopsis causes hypersensitivity to abscisic acid (ABA). (**A**) Germination phenotypes of OE and WT Arabidopsis seedlings grown on MS medium with or without ABA treatment for 7 days. (**B**) Seed germination rates of OE and WT Arabidopsis seeds sown on MS medium with or without ABA after 7 days of treatment. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. ns, The difference was not significant. Different lowercase letters indicate statistically significant differences (least significant difference test; $p < 0.05$). Error bars indicate SDs (n = 3).

4. Discussion

Drought tolerance involves a series of transcriptional events. Many transcription factors (TFs) have been reported to participate in drought tolerance through binding to the promoter regions of target genes, such as AP2/ERF (APETALA2/ETHYLENE RESPONSIVE FACTOR), NAC (NAM, ATAF and CUC), SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE), WRKY, bZIP, and MYB [1,34–38].

The role of CBF transcription factors in cold acclimation is well studied, and many plant CBF transcription factors have been identified [39,40]. However, their involvement in drought stress remains less understood. In this study, the CBF genes of wheat were identified in the whole genome, and a total of 81 *TaCBF* genes (Table S1) were characterized via bioinformatic and function analysis. Our results indicated that CBF family members are divided into four clades, with the majority of genes falling into Clade IV and the fewest genes belonging to Clade II (Figure 1), indicting that members of the same subfamily have similar functions. Further sequence analysis revealed that all *TaCBF* genes contain the conserved CBF domain, and most have identical motifs, particularly Motif 1, Motif 2, and Motif 3, suggesting their potential functional significance (Figure 2). Additionally, certain members of Clade IV exhibit motif compositions similar to those of Clade I or Clade II, indicating their possible involvement in subfamily-specific functions. Gene structure analysis showed that most *TaCBF* genes are intronless, with only a few containing a single intron, further supporting the reliability of the phylogenetic classification. These findings highlight the evolutionary conservation of *TaCBF* genes and provide a valuable foundation for further functional investigations. Although all TaCBF proteins possess the conserved CBF domain, their biological functions may differ due to variations in expression patterns,

domain context, or post-translational modifications. Members of the same subfamily often exhibit functional differentiation in response to specific environmental conditions, such as cold, drought, or salt stress [41]. Thus, while domain consistency supports functional similarity, the functional specificity among different TaCBF members requires further investigation. Based on its high expression levels under PEG-6000 treatment, *TaCBF14B* was selected for further analysis (Figure 3), suggesting that *TaCBF* genes may play an important role in drought stress response.

Previous research found that *TaCBF14*, *TaCBF15*, and *TaCBF16* were induced by cold in wheat [42]. Research has demonstrated that *TaCBF14* and *TaCBF15* could enhance frost tolerance of spring barley [9]. In Triticum monococcum, *TmCBF12*, *TmCBF14*, and *TmCBF15* were involved in cold tolerance, which was related to the high expression level of DHN5 and COR14b [43]. In this study, we focused on the drought stress tolerance function of *TaCBF14B*. *TaCBF14B* overexpression lines in Arabidopsis showed longer root length and higher fresh and dry weights under 200 mM of mannitol treatment (Figure 6). In addition, *TaCBF14B-OE* displayed a higher survival rate after repeated drought treatment and rehydration (Figure 6). Thus, the observed drought tolerance in *TaCBF14B*-overexpressing Arabidopsis suggests its potential application in wheat breeding for drought tolerance.

Transcription factors typically regulate downstream genes by repression or transcriptional activation. The CBF transcription factors have been shown to bind to the CRT/DRE motif and activate the expression of cold-responsive (COR) genes [32,33,37]. In our study, the subcellular localization of TaCBF14B revealed that it is a nuclear-localized protein (Figure 4), consistent with its role as a transcription factor. Additionally, transcriptional activation activity was confirmed through a yeast two-hybrid assay (Figure 5).

ABA, a stress-induced phytohormone, plays a key role in the response to multiple abiotic stresses, including drought stress [32,44–46]. Research has found that the high transcription level of 9-cis-epoxycarotenoid dioxygenases (*NCEDs*) and ABA biosynthesis could contribute to Arabidopsis drought resistance [46]. Overexpression of ABA-signaling-pathway-related genes such as ABI, SnRK, and PP2C could improve drought resistance [47,48]. In our study, higher transcription levels of ABA signaling and synthesis genes were observed in transgenic Arabidopsis plants overexpressing *TaCBF14B* after drought treatment (Figure 7). Consistent with previous research, it is well known that ABA could suppress seed germination [49]. In support of this notion, the overexpression lines showed lower germination rates under ABA treatment compared to WT (Figure 7), suggesting that *TaCBF14B* functions as a positive regulator in ABA signaling. Overall, these results indicated that *TaCBF14B* could enhance drought tolerance in *Arabidopsis*, which was of great significance for dryland wheat breeding. However, the detailed correlation between TaCBF14B and ABA-signaling-pathway-related genes will be studied in future.

5. Conclusions

In the study, 81 *TaCBFs* were identified in wheat, and they were grouped into four lineages based on the phylogenetic tree. Their chromosomal localization, synteny, gene structure, and conserved protein motifs were also analyzed to understand gene evolution. We identified that *TaCBF14A*, *TaCBF14B*, and *TaCBF14D* might be involved in the drought stress response. Due to its high expression level, *TaCBF14B* was selected for further functional analysis. The subcellular location and transcriptional activation activity of TaCBF14B indicated that it might function as a transcription factor in drought stress tolerance. Furthermore, the results of *TaCBF14B* overexpression in *Arabidopsis* indicated that *TaCBF14B* played a key role in drought tolerance through improving the scavenging of ROS and osmotic adjustment ability, as well as promoting the expression of *AtABI3*. Overall, this study is of great value for understanding the functional roles of CBF genes in drought stress tolerance.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15061265/s1: Figure S1: The expression pattern of *TaCBF14B* in different wheat varieties under PEG treatment. Drought-tolerant wheat varieties: Shanrong 3, Xiaoyan 60, Jinan 17, and Dekang 961. Drought-sensitive variety: Chinese Spring (control). Different lowercase letters indicate statistically significant differences (Student's *t*-test; $p < 0.05$). Figure S2: Representative images of WT and OE Arabidopsis seedlings grown on MS medium with or without mannitol for 10 days under long-day conditions; Figure S3. Representative images of detached Arabidopsis leaves from 12-day-old WT and OE lines over time during dehydration. Table S1: Primers used for gene mapping and vector construction. Table S2: Characteristics of CBFs/DREBs proteins in one wheat species.

Author Contributions: Y.Z. and W.G. designed the research; Z.A., Y.T., Y.X. (Yitu Xu), S.C. and C.L. conducted the research; Z.A., Y.T., Z.H., N.N., Y.X. (Yuxin Xie), X.L. (Xiangyu Liu), J.X., J.Y. and J.L. prepared the samples; W.G., Z.A. and Y.T. analyzed the data; W.G. wrote the draft; Y.Z., X.L. (Ximei Li), H.W., M.W. and N.G. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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