



# Genome-Wide Characterization of the C-repeat Binding Factor (CBF) Gene Family Involved in the Response to Abiotic Stresses in Tea Plant (*Camellia sinensis*)

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C-repeat (CRT)/dehydration responsive element (DRE)-binding factor CBFs, a small family of genes encoding transcriptional activators, play important roles in plant cold tolerance. In this study, a comprehensive genome-wide analysis was carried out to identify and characterize the functional dynamics of CsCBFs in tea plant (*Camellia sinensis*). A total of 6 CBF genes were obtained from the tea plant genome and named CBF1-6. All of the CsCBFs had an AP2/ERF DNA-binding domain and nuclear localization signal (NLS) sequence. CsCBF-eGFP fusion and DAPI staining analysis confirmed the nuclear localization of the CsCBFs. Transactivation assays showed that the CsCBFs, except CsCBF1, had transcriptional activity. CsCBF expression was differentially induced by cold, heat, PEG, salinity, ABA, GA, MeJA, and SA stresses. In particular, the CsCBF genes were significantly induced by cold treatments. To further characterize the functions of CsCBF genes, we overexpressed the CsCBF3 gene in *Arabidopsis thaliana* plants. The resulting transgenic plants showed increased cold tolerance compared with the wild-type *Arabidopsis* plant. The enhanced cold tolerance of the transgenic plants was potentially achieved through an ABA-independent pathway. This study will help to increase our understanding of CsCBF genes and their contributions to stress tolerance in tea plants.

**Keywords:** *Camellia sinensis*, CBF gene family, cold stress, functional genomics, transgenic *Arabidopsis thaliana*

## INTRODUCTION

Abiotic stresses in the natural environment, including low temperature, drought, and high salinity, seriously affect the growth, development, distribution, and productivity of plants (Kulik et al., 2011). Plants have evolved a complex set of adaptive mechanisms to survive these adverse environmental conditions, and these mechanisms involve a number of biochemical and physiological changes (Ramanjulu and Bartels, 2002). Different studies have suggested that transcription factors can activate the expression of related genes to protect plants from adversity (Agarwal and Jha, 2010). CBFs/DREBs (C-repeat binding factors/dehydration responsive element-binding factors) belong to the AP2/ERF (APETALA2/ethylene-responsive element-binding factor)

transcription factor family (Riechmann and Meyerowitz, 1998) and have been reported to play pivotal roles in freezing tolerance and cold acclimation (Stockinger et al., 1997). The AP2/ERF family contains a highly conserved AP2/ERF domain that harbors a DNA-binding motif of ~60 amino acids (Sakuma et al., 2002). When plants suffer from low temperatures, related regulatory proteins are modified to regulate the expression of the *CBF* gene. For example, the inducers of *CBF* expression 1 (*ICE1*), calmodulin-binding transcription activator 3 (*CAMTA3*) and brassinazole-resistant 1 (*BZR1*), positively regulate *CBF* expression, whereas MYB15, ethylene-insensitive 3 (*EIN3*), and 14-3-3 negatively regulate *CBF* expression (Liu et al., 2017). Subsequently, *CBF/DREB1* proteins bind to the cold- and dehydration-responsive DNA regulatory element designated the CRT (C-repeat)/DRE (dehydration response element), which is present in the promoters of *COR* (cold-regulated) genes and contains the core motif of *G/ACCGAC* (Yamaguchi-Shinozaki and Shinozaki, 1994; Huang et al., 2012), and to the promoters of other cold responsive genes, such as *COR15A*, *RD29A*, and *COR78* (Liu et al., 1998; Lucas et al., 2011; Akhtar et al., 2012), and stimulates their transcription (Baker et al., 1994; Jaglo et al., 2001; Zhao T. et al., 2012). Previous studies have identified a number of *CBF/DREB1* genes and verified their functions in *Arabidopsis thaliana* (Novillo et al., 2007) and other plant species, such as cotton (*Gossypium hirsutum*) (Shan et al., 2007), wheat (*Triticum aestivum*) (Shen et al., 2003), rice (*Oryza sativa*) (Wang et al., 2008), maize (*Zea mays*) (Qin et al., 2004), soybean (*Glycine max*) (Kidokoro et al., 2015), and tomato (*Lycopersicon esculentum*) (Zhang et al., 2004). These findings suggested the conserved roles of *CBF/DREB1* genes in the regulation of freezing tolerance across diverse plant species. Nevertheless, *CBFs* play different roles in responses to stress in different plant species (Ebrahimi et al., 2015). In addition to the cold response, *CBFs* could also respond to other abiotic stresses and hormones, such as heat, drought, salt, and abscisic acid (*ABA*) (Dubouzet et al., 2003; Xiao et al., 2006; Nada and Abogadallah, 2015).

Tea plants are the world's most important nonalcoholic beverage crop with a wealth of health benefits (Mukhopadhyay et al., 2016). The growth of tea plants is seriously affected by abiotic stresses (Wang et al., 2014), particularly extreme temperature and drought (Das et al., 2016; Liu et al., 2016; Hou et al., 2018; Zhou et al., 2018). Although several recent studies have demonstrated that *CsCBF1* enables cold stress in tea plants (Wang et al., 2012), overexpression of *CsDREB* increases salt and drought tolerance in transgenic *Arabidopsis thaliana* (Wang et al., 2017). However, given that the *CBF* transcription factor is a polygenic family, it is still unclear whether there are other new *CBF* genes in tea plants that respond to low temperature. In the present study, we thoroughly investigated the *CBF* genes in tea plant using tea plant genome and transcriptome datasets, with the aim of providing novel insights into the functional dynamics of *CBF* genes in tea plant. The overall obtained results provide a foundation for additional studies of the biological functions of *CsCBFs* under abiotic stresses and a new perspective for resistance breeding in tea plants.

## MATERIALS AND METHODS

### Plant Materials and Stress Treatments

One-year-old tea cutting seedlings of the Shuchazao cultivar were planted in a pot and grown with a natural photoperiod in a greenhouse (12 h light and 12 h dark photoperiod, 25°C temperature and 70% relative humidity) (Li et al., 2016) at the State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University (Hefei, China). For low and high temperature stresses, tea plants were grown at 4 and 38°C in a plant growth chamber. For the GA, ABA, SA, and MeJA treatments, a working solution of 100 μM was foliar sprayed on plants (Pan et al., 2017). For drought and salinity, the plants were transferred to a 20% PEG6000 and 200 mM NaCl solution (Li et al., 2010; Yue et al., 2014). The second or third mature leaves were harvested for gene analysis 0, 4, 12, and 24 h (Agarwal M. et al., 2006) after treatments. After collection, the samples were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Three biological replicates were conducted.

### Identification of *CsCBF* Genes and Phylogenetic Construction

The protein sequences of *AtCBFs* were downloaded from the NCBI (<https://www.ncbi.nlm.nih.gov/>) database and used as queries to search homologous sequences against the published genomes of tea plant (Wei et al., 2018; Xia E. et al., 2019) with the BlastP program at an *e*-value of 10<sup>-3</sup> (Pan et al., 2017). The biophysical properties of the *CsCBFs* were computed using the online ProtParam tool (<https://web.expasy.org/protparam/>). The protein sequences of *Camellia sinensis* and *Arabidopsis thaliana* were aligned by DNAMAN6.0. Multiple sequence alignments of *Camellia sinensis* and *Arabidopsis thaliana* were analyzed using MEGA 6.0 (<http://www.megasoftware.net/>). The phylogenetic tree was constructed using the neighbor-joining algorithm with 1,000 replicates.

### Characterization of *CsCBF* Genes and Proteins

The promoter sequences ~2 kb upstream of the transcription start site of each *CsCBF* gene were identified, and the cis-elements were analyzed by the online tool Plant CARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The full-length amino acid sequences of *CsCBF* were entered into the MEME (<http://meme-suite.org/tools/meme>) analysis tool to find their conserved motifs. Parameters of MEME are following: number of different motifs: 10, Minimum/Maximum motif width: 6/100.

### Subcellular Localization of *CsCBFs*

The full-length ORFs of *CsCBF* sequences were cloned into the pK7WGF2.0 vector containing the enhanced green fluorescent protein (eGFP) reporter gene by the gateway method. The isolated DNA was transformed into *Agrobacterium* strain GV3101. Six 35S::eGFP-*CsCBF* constructs and the control plasmids without *CsCBF* coding sequences were separately infiltrated into tobacco (*Nicotiana tabacum*) leaves by the

Agrobacterium-mediated genetic transformation method. DAPI (4',6-diamidino-2-phenylindole dihydrochloride) was used to identify nuclei. The cells of transformed tobacco leaves were observed by an Olympus IX81 fluorescence microscope (Olympus, Japan).

### Transactivation Assay Analysis

Each *CsCBF* gene was amplified and cloned into the pGBKT7 vector. Six pGBKT7-*CsCBF* vectors, the pGBKT7-*AtCBF2* vector (positive control) and the pGBKT7 vector (negative control) were separately transformed into the Y2HGOLD yeast strain. The transformed yeast cells were incubated on SD/-Trp, SD/-Trp/-His-Ade, and SD/-Trp-His-Ade-x-gal plates at 30°C for 3 d.

### RNA Isolation and Quantitative RT-PCR

Total RNA was extracted using the RNAPrep Pure Plant Kit (Tiangen, Beijing, China). A Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) was used to measure the concentration of isolated RNA, and the quality was assessed using 1.2% formaldehyde-agarose gel electrophoresis. cDNA was synthesized for qRT-PCR by the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Tokyo, Japan) and diluted 10-fold for PCR amplification.

The specific primers for qRT-PCR were designed by Primer Premier 5 software and synthesized by Sangon Biotech Co. (Shanghai, China). The qRT-PCR reaction program was performed under the following conditions: 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 30 s. The reaction volume was 25 μL, which contained 4 μL of diluted cDNA, 6.5 μL of deionized water, 12.5 μL of SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; TaKaRa), 1 μL of forward primer, and 1 μL of reverse primer. *CsGAPDH* was used as the reference gene (Pan et al., 2017). The relative gene expression levels were calculated using the comparative  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). Regarding the heatmaps, the  $2^{-\Delta\Delta C_t}$  values of the transcripts of the *CsCBF* genes were normalized as the log<sub>2</sub>-fold change. In the stress-treated plant samples, the values were normalized to plant samples of the 0 h treatment and expressed as a log<sub>2</sub>-fold change. qRT-PCR experiments were conducted with three independent total RNA samples.

### Functional Analysis of *CsCBF3*-Overexpressing Transgenic *Arabidopsis thaliana*

Expression of *CsCBF3* shows significant changes during cold treatment and we selected it for overexpression. The ORF of *CsCBF3* was cloned into the pBI121 vector. The construct was transformed into Agrobacterium strain GV3101 by electroporation, and the *Arabidopsis* plants were transformed using the floral dip method. *Arabidopsis* ecotype Columbia-0 (col-0) was used as the wild type in this study. Transformed plants were selected on the basis of their resistance to kanamycin, and 4-week-old homozygous T3 plants were used for further experiments. Two transgenic lines were treated at -4 and -8°C for 12 h and then grown under 25°C conditions for analysis of survival rate.

## RESULTS

### Identification and Characterization of CBF Genes in Tea Plant

We initially identified 10 *CBF* genes from genome and transcriptome databases of tea plant. Six of them were successfully cloned and deposited into the NCBI GenBank database under the accession numbers *CsCBF1* (EU563238.1), *CsCBF2* (KC702795.1), *CsCBF3* (MH017428.1), *CsCBF4* (KF988866.1), *CsCBF5* (MH165878.1), and *CsCBF6* (MN544638.1). Alignment of the sequences obtained from cDNA and genomic DNA indicated that the *CsCBF* genes were intronless. The CDS length of the *CsCBFs* ranged from 720 to 879 bp, and the genes encoded proteins with lengths varying from 239 to 292 amino acids. The molecular weights were between 26.43 and 32.86 kDa, and the *pI*-values ranged from 4.84 to 8.09. Most of the *CsCBF* proteins presented grand average hydropathicity (GRAVY) values of <0, implying their hydrophilic nature (Table 1).

### Sequence Alignment and Phylogenetic Analysis

We investigated the amino acid characteristics of CBF proteins of *C. sinensis* and *A. thaliana*. Alignment of the amino acid sequences of the *CsCBFs* and *AtCBFs* revealed that the *CsCBFs* contained one highly conserved DNA-binding domain (AP2 domain) consisting of 59 amino acids. A putative nuclear localization signal (NLS) sequence (PKKRRGRKKFK) was detected in the N-terminal region. In addition, the C-terminal regions of the *CsCBF* proteins were highly diverged, particularly *CsCBF6* (Figure 1).

A phylogenetic tree was constructed using the neighbor-joining method with the MEGA program to explore the evolutionary relationships among CBF homologs in *C. sinensis* and *A. thaliana* (Figure 2). The 60 collected DREBs from *C. sinensis* and *A. thaliana* were clustered into six groups. All 10 *CsCBF* proteins were clustered together with *AtDDF1-2* and *AtCBF1-4* in Group A1. The result showed that *CsCBFs* belongs to DREB-A1 subfamily.

### Motif Analysis

To explore the diversification of *CBF* genes in tea plants, we examined the conserved motifs within the *CsCBFs* proteins using the MEME program (Figure 3). Ten motifs were identified. Multilevel consensus sequences and the *E*-value of each motif are shown. The results revealed conserved motif distribution among *CsCBF* proteins. Motif 1 and motif 2 were found in all *CBFs*. Motifs 3–6 could be found in *CsCBF1*, 2, 3, and 5. Motif 7 was mainly identified in the C-terminal regions of *CBF1*, 2, and 3. Motif 8 was unique to *CsCBF6* and motif 9 was unique to *CsCBF4*. Motif 10 was distributed in *CBF1*, 2, and 3. Motif analysis supports the results of the phylogenetic analysis.

### Subcellular Localization of *CsCBF* Proteins

To investigate the subcellular localization of *CsCBF* proteins, six 35S::eGFP-*CsCBF* constructs, one for each *CsCBF*, and a 35S::eGFP (positive control) construct were generated and

**TABLE 1** | The characteristics of *CsCBFs*.

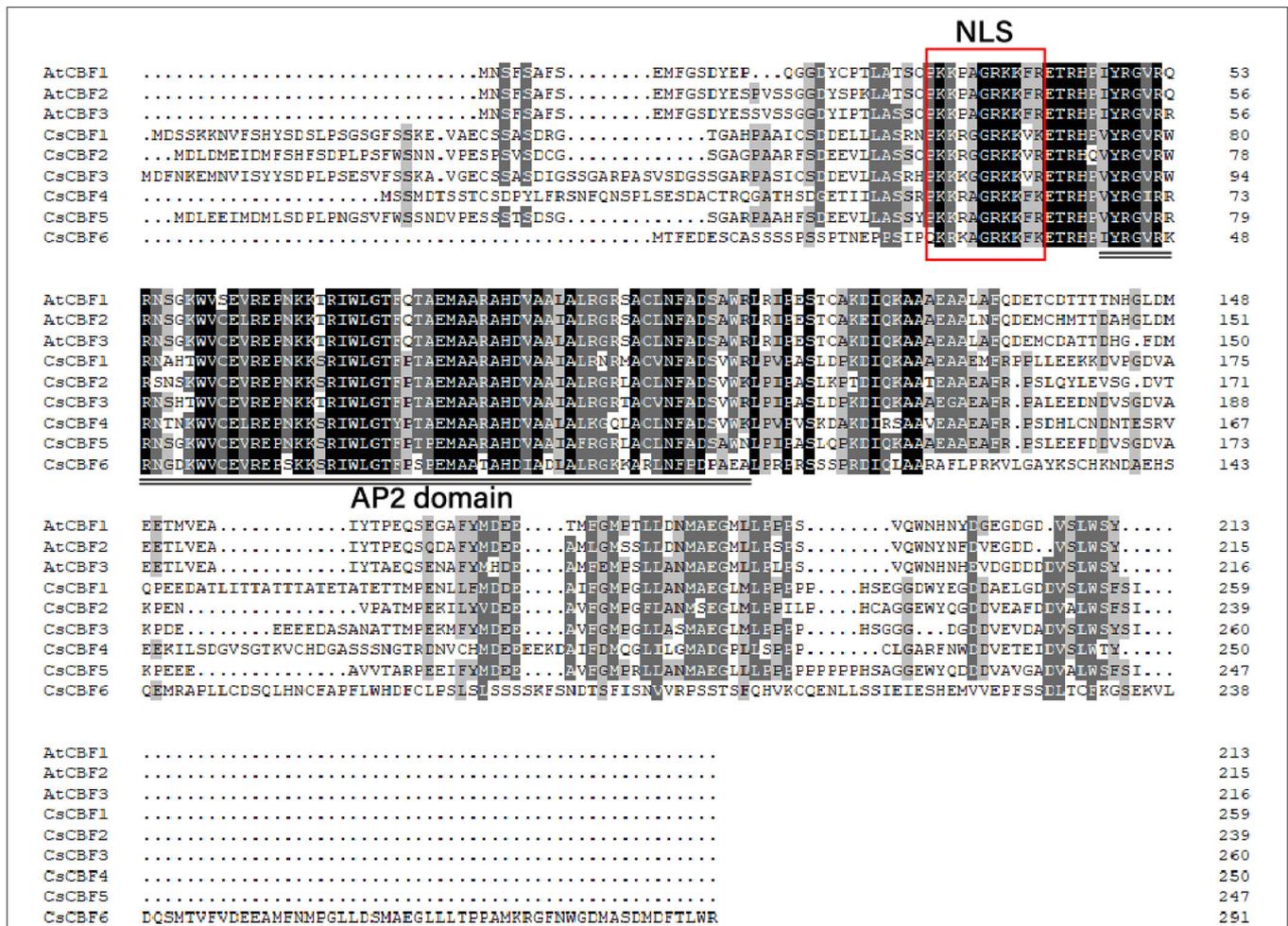
Gene symbol	Gene identifier <sup>a</sup>	CDS (bp)	Protein (aa)	MW (kDa) <sup>b</sup>	pI <sup>c</sup>	GRAVY <sup>d</sup>
<i>CsCBF1</i>	CSS023229.1	777	259	28.26	5.08	-0.48
<i>CsCBF2</i>	CSS018717.1	717	239	26.43	5.22	-0.28
<i>CsCBF3</i>	CSS002244.1	774	258	27.78	4.94	-0.47
<i>CsCBF4</i>	CSS022600.1	750	250	27.70	5.91	-0.6
<i>CsCBF5</i>	CSS001387.1	741	247	27.15	4.87	-0.44
<i>CsCBF6</i>	CSS038056.1	900	300	33.63	7.08	-0.52

<sup>a</sup>Gene IDs are from *C. sinensis* var. *sinensis* genome (marked with CSS) (Xia E. -H. et al., 2019).

<sup>b</sup>Molecular weight.

<sup>c</sup>Isoelectric point.

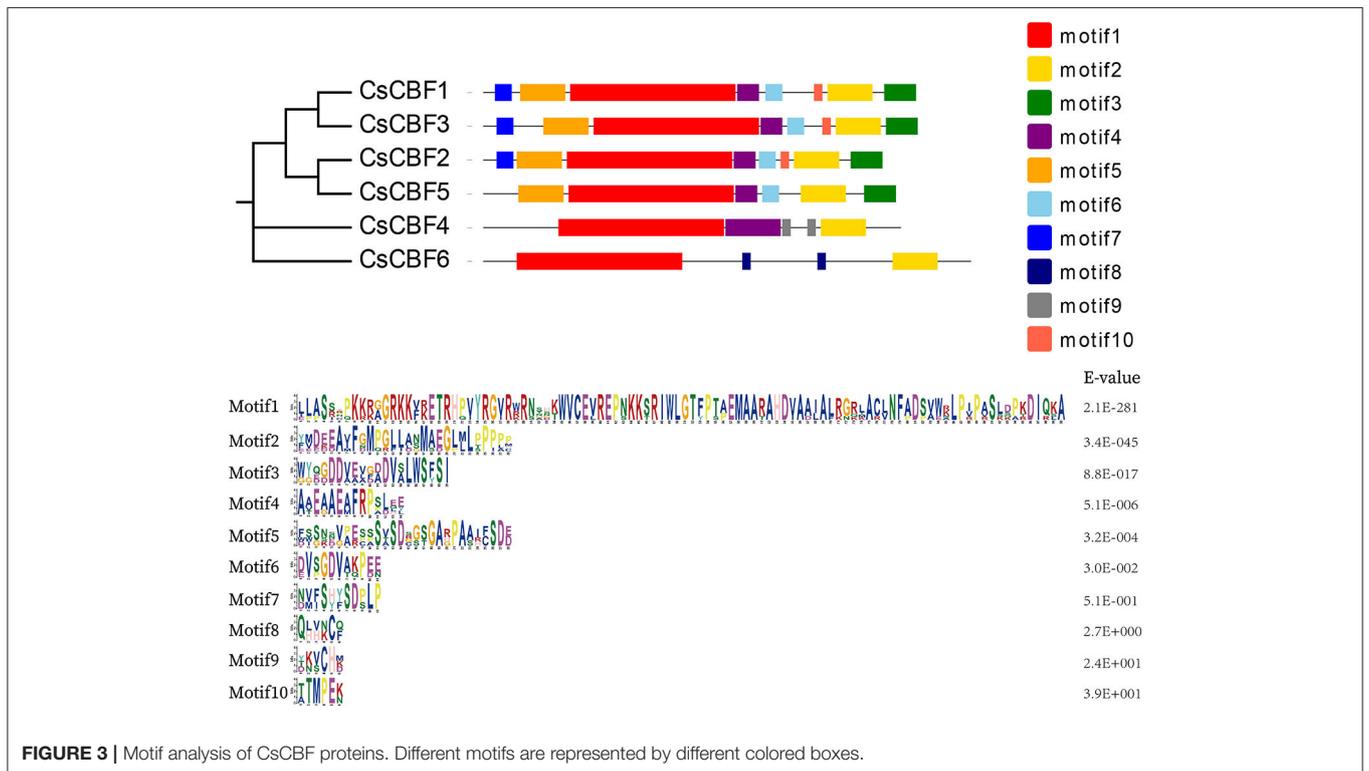
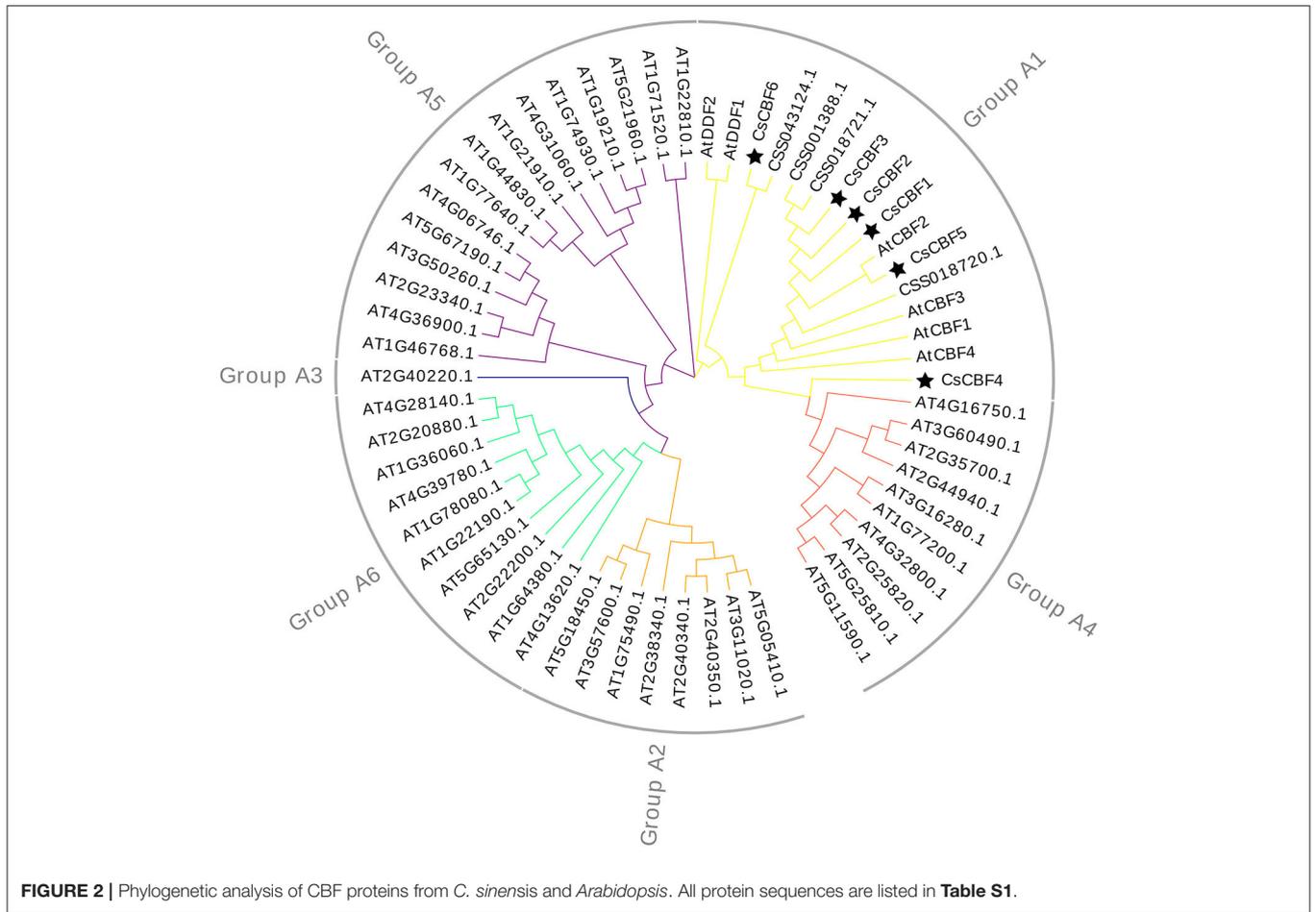
<sup>d</sup>Grand average of hydropathicity.

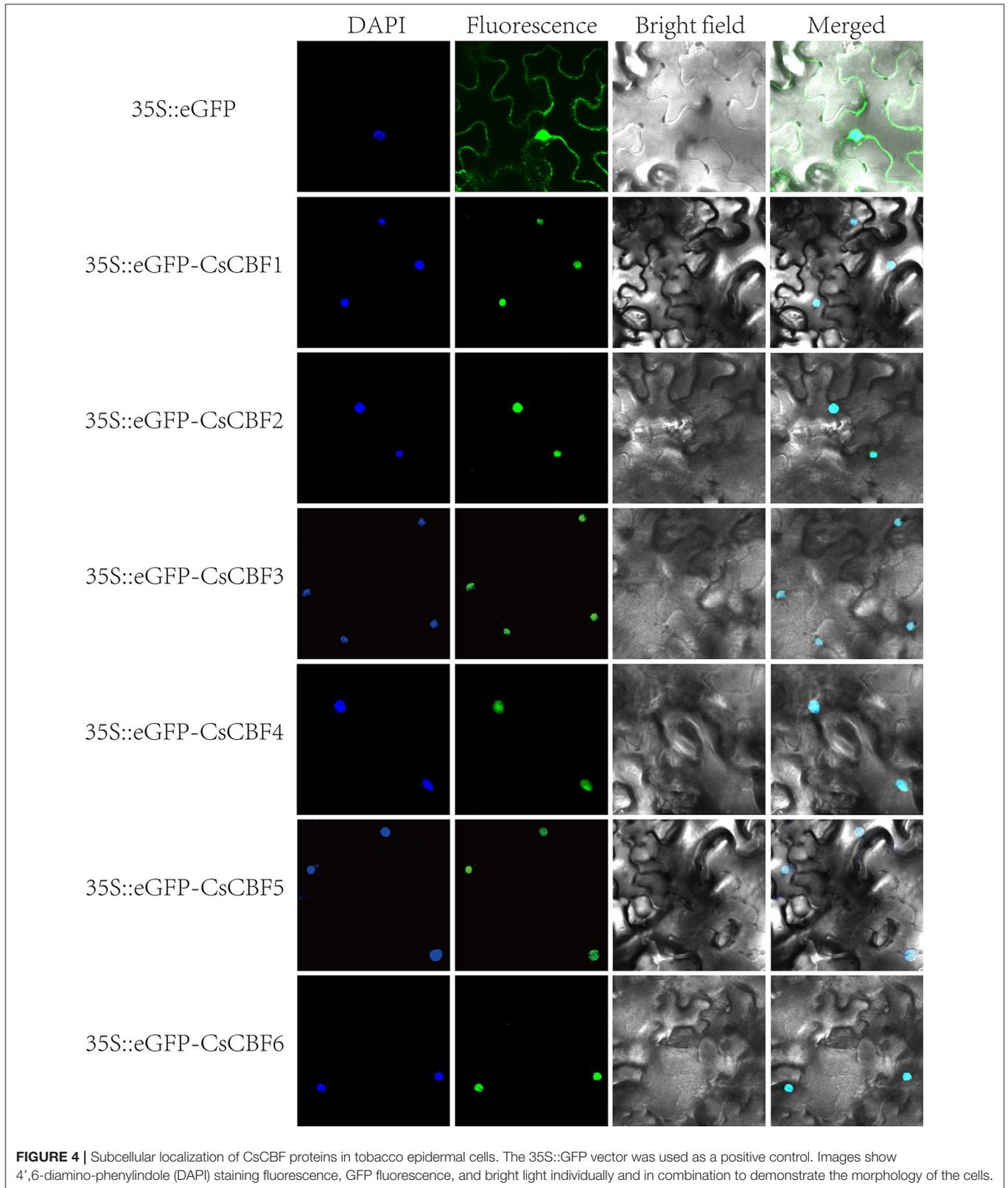


**FIGURE 1** | Sequence alignment of the amino acid sequence of CBFs in *C. sinensis* and *Arabidopsis*. Identical and conserved amino acids are shaded in black and gray, respectively. The conserved nuclear localization signal (NLS) and AP2 DNA-binding domain are shown. The NLS sequence is indicated by a red rectangle, and the conserved AP2 domain is underlined (double line).

transiently expressed in tobacco leaves. As shown in **Figure 4**, eGFP alone resulted in a diffuse distribution of green fluorescence throughout the entire cell. In contrast, *CsCBF*-eGFP proteins

localized predominantly to the nucleus, which was further confirmed by 4',6-diamidino-2-phenylindole (DAPI) staining. The results indicated that *CsCBFs* are nuclear localized proteins.





## Transactivation Assay of CsCBFs

To examine the transcriptional activities of CsCBFs, full length sequences of six CsCBFs and AtCBF2 (positive control) were fused to the vector pGBKT7 containing the GAL4 DNA-binding domain and subsequently transformed into yeast. The yeast cells harboring pGBKT7-CsCBF2, pGBKT7-CsCBF3, pGBKT7-CsCBF4, pGBKT7-CsCBF5, pGBKT7-CsCBF6, and pGBKT7-AtCBF2 grew well on the selection media without Trp, His or adenine (SD/-Trp-His-Ade) and were positive for  $\alpha$ -galactosidase activity. The yeast cells with the empty vector pGBKT7 (negative control) and pGBKT7-CsCBF1, which is missing the transcriptional activation domain GAL4 AD, were unable to grow on the same medium. The results indicated that CsCBF2, CsCBF3, CsCBF4, CsCBF5, and CsCBF6 have transcriptional activity, while CsCBF1 has no transcriptional activity (Figure 5).

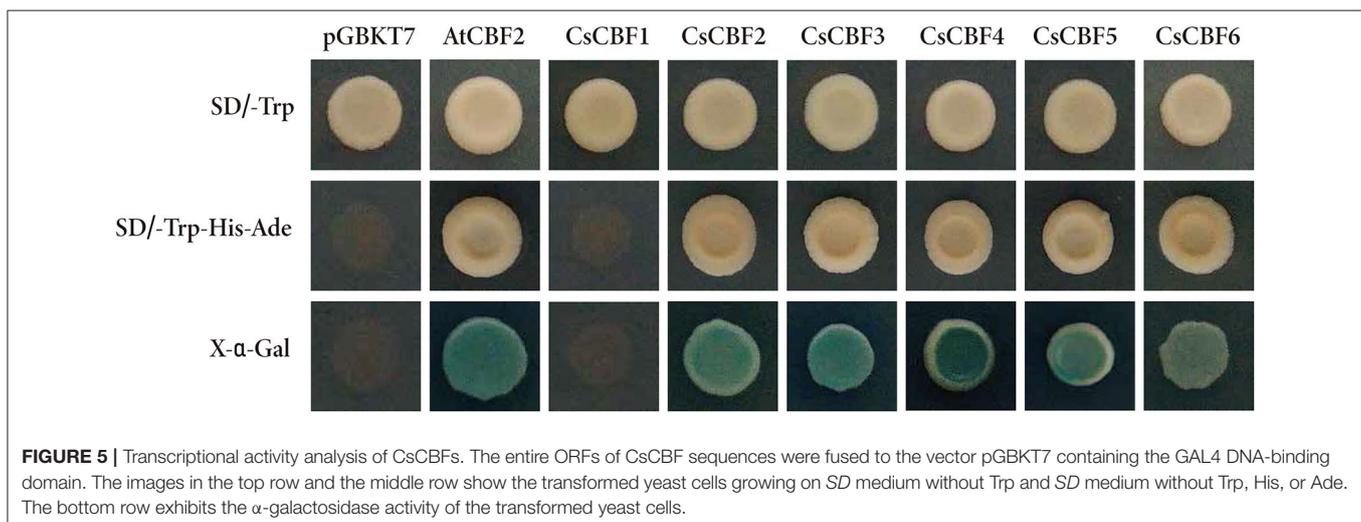
## Cis-Element Analysis of the Promoter Regions of CsCBF Genes

To further investigate the regulatory mechanism of CsCBFs, sequences 2,000 bp upstream of the start codon of the CsCBF genes were extracted from the tea plant genome database (Xia E. et al., 2019). As shown in Table 2, all CsCBF proteins contained the MeJA-responsive element (CGTCA), stress responsiveness element (MYB), and water stress and dehydration responsiveness element (MYC). The abscisic acid-responsive element (ABRE) was found in CsCBF1-pro, CsCBF3-pro, CsCBF4-pro, CsCBF5-pro, and CsCBF6-pro. Ethylene-responsive elements (EREs) were distributed in CsCBF1-pro, CsCBF2-pro, CsCBF3-pro, and CsCBF5-pro. The gibberellin-responsive element (GARE) was unique to CsCBF4-pro. The light-responsive element (G-box) was found in CsCBF1-pro, CsCBF3-pro, CsCBF4-pro, CsCBF5-pro, and CsCBF6-pro. The low-temperature-responsive element (LTR) was shown in CsCBF2-pro, CsCBF4-pro, CsCBF5-pro, and CsCBF6-pro. The Myb-binding site (MBS) was distributed in CsCBF2-pro, CsCBF3-pro, and CsCBF6-pro. Salicylic acid-responsive elements (TCA-element) were

unique to CsCBF4-pro and CsCBF5-pro. Defense and stress-responsive elements (TC-rich repeat) were found in CsCBF1-pro, CsCBF5-pro, and CsCBF6-pro. Auxin-responsive elements (TGA elements) were found in CsCBF2-pro and CsCBF4-pro (Table 2).

## Expression Analysis of CsCBFs Under Various Abiotic Stresses

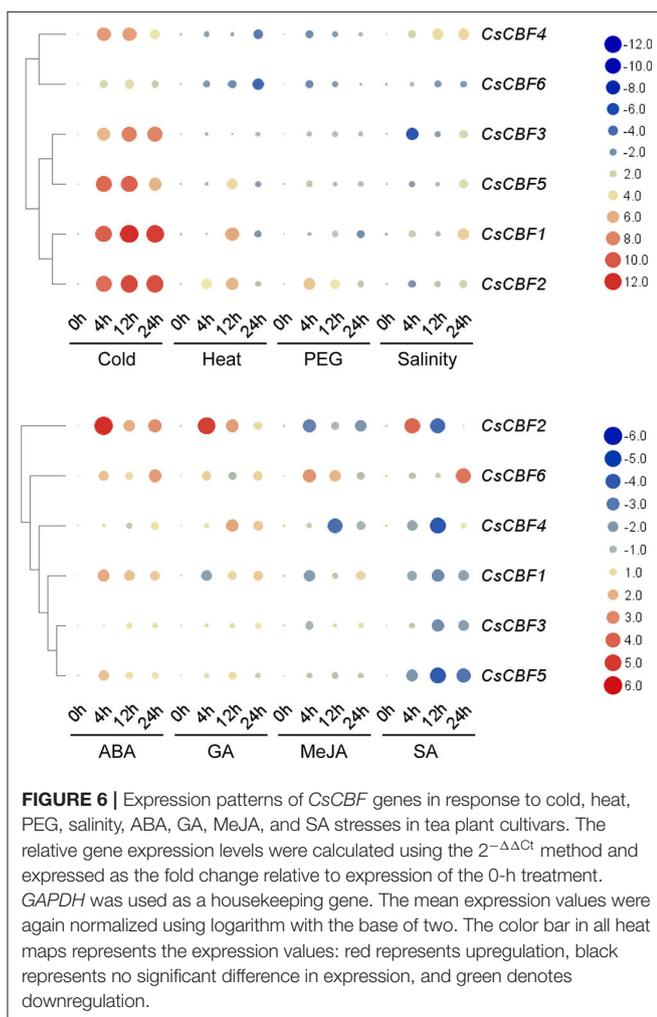
We investigated the expression profile of CsCBF genes in response to eight stress treatments (cold, heat, PEG, salinity, ABA, GA, MeJA, and SA) by qRT-PCR (Figure 6). Under cold treatment, all CsCBFs showed high expression levels during 24 h. Notably, the expression of CsCBF1,2,3,4, and 5 was much higher than that of CsCBF6. The expression of CsCBF1, 2, and 3 increased a 100-fold or even 1000-fold throughout the entire incubation time, while the expression of CsCBF4, 5, and 6 increased with processing time but reached a maximum at 12 h. Under heat stress, CsCBF1, 2, and 5 presented an expression trend of rising first and then falling, while the expression of CsCBF4 and 6 declined during processing, and the expression of CsCBF3 fell to 12 h and then returned to normal. Under PEG stress, CsCBF2 had a strong increasing response. CsCBF3 and 5 showed no response to the treatment. The expression of CsCBF4 and 6 was downregulated and reached a minimum at 12 h, and then the expression level returned to the control level. CsCBF1 showed a decrease over time. Under salt stress, CsCBF1 and 4 showed a trend of increasing expression over time. The expression of CsCBF2, 3, and 5 first dropped and reached the lowest level at 4 h, then began to rise, and finally was overall positively expressed. CsCBF6 expression decreased. The results of the treatment of four plant hormones were as follows: under ABA treatment, the expression of CsCBF1 and 2 increased and peaked at 4 h. CsCBF3 and 5 had almost no response. CsCBF4 was downregulated and reached its minimum at 12 h, then it returned to the control level. CsCBF6 slowly increased with the treatment process. Under GA treatment, CsCBF1 and 6 both decreased and reached a minimum at 4



**FIGURE 5 |** Transcriptional activity analysis of CsCBFs. The entire ORFs of CsCBF sequences were fused to the vector pGBKT7 containing the GAL4 DNA-binding domain. The images in the top row and the middle row show the transformed yeast cells growing on SD medium without Trp and SD medium without Trp, His, or Ade. The bottom row exhibits the  $\alpha$ -galactosidase activity of the transformed yeast cells.

**TABLE 2** | The cis-element analysis of *CsCBFs* promoter regions.

cis-element	Putative function	CsCBF1	CsCBF2	CsCBF3	CsCBF4	CsCBF5	CsCBF6
ABRE	Abscisic acid responsiveness element	1	0	7	15	2	5
CGTCA	MeJA-responsiveness	3	1	3	9	3	1
ERE	Ethylene-responsive element	1	1	6	0	4	0
GARE	Gibberellin-responsive element	0	0	0	1	0	0
G-box	Light responsiveness	1	0	8	18	3	5
LTR	Low-temperature responsiveness	0	1	0	1	2	1
MBS	Myb-binding site involved in drought inducibility	0	1	1	0	0	2
MYB	Stress responsiveness	3	3	2	2	1	2
MYC	Water stress and dehydration responsiveness	3	3	4	3	3	2
TCA-element	Salicylic acid responsiveness	0	0	0	1	1	0
TC-rich repeats	Defense and stress responsiveness	2	0	0	0	1	1
TGA-element	Auxin-responsive element	0	1	0	2	0	0



and 12 h, respectively, and finally returned to the control level. *CsCBF2* and 4 both increased and reached a maximum at 4 and 12 h, respectively. *CsCBF3* showed no response to GA. *CsCBF5*

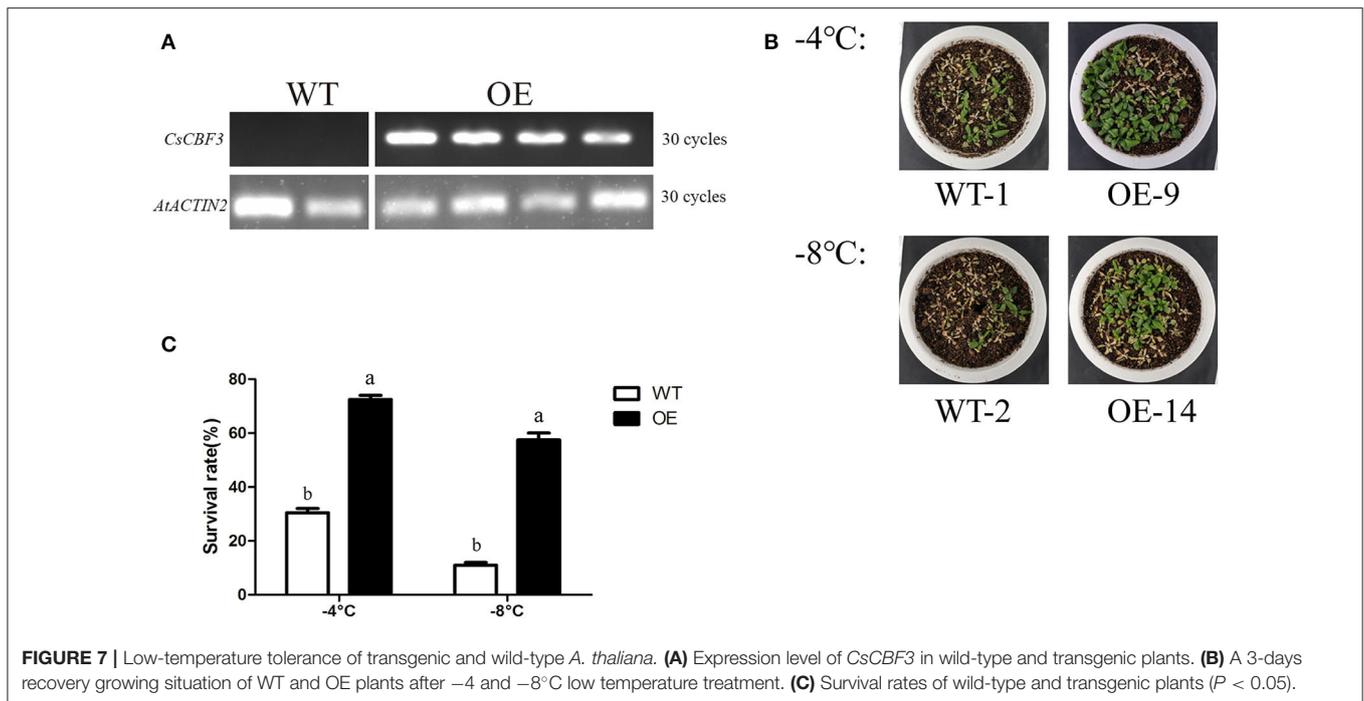
showed a downward adjustment in the later stages of processing. Under MeJA treatment, the expression of *CsCBF1*, 2, 3, 4, and 5 was downregulated. *CsCBF6* rose in 4 h and then decreased with time. Under SA treatment, *CsCBF1*, 3, and 5 were downregulated and reached a minimum at 12 h. *CsCBF2* was positive in the first 4 h and then decreased to negative over time. *CsCBF4* was downregulated and reached its minimum at 12 h, then it returned to the control level. *CsCBF6* was negatively regulated and then positively regulated.

In general, the results illustrated that cold, ABA and GA could induce high expression of most of the *CsCBF* genes.

### Overexpression of *CsCBF3* in *Arabidopsis thaliana* Improves Tolerance to Cold Stress

To confirm the *in vivo* functions of the *CsCBF3* gene during low-temperature stress in plants, we transferred *CsCBF3* into *A. thaliana*. The expression of *CsCBF3* was detected using real-time PCR assay in overexpressed (OE) plants but not in wild-type (WT) plants (Figure 7A). Two overexpressed lines (OE-9 and OE-14) were treated in low temperature conditions. Thirty seeds of wild-type and overexpressed plants were selected for treatment. Under normal growth conditions (25°C), there were no obvious differences in survival rate between the WT and transgenic plants. A 3-days recovery after low-temperature treatment at  $-4$  and  $-8^{\circ}\text{C}$  for 12 h, the survival rates of WT plants significantly decreased compared to those of transgenic plants (Figure 7B). Recovery for 3 d after  $-4^{\circ}\text{C}$  treatment, ~31% of wild-type plants survived, while 73% of OE-9 survived. Recovery for 3 d after  $-8^{\circ}\text{C}$  treatment, the survival rates of wild-type plants and OE-14 were 11 and 58%, respectively (Figure 7C).

In addition, we detected the expression of downstream target ABA-dependent stress-induced genes (*AtRD29B*, *AtRAB18*, *AtABII*, and *AtABI2*) and ABA-independent stress-induced genes (*AtCOR15a* and *AtRD29A*) to explore the potential *CsCBF3*-associated regulatory pathway. Under unstressed conditions, the expression of *AtCOR15a* and *AtRD29A* in



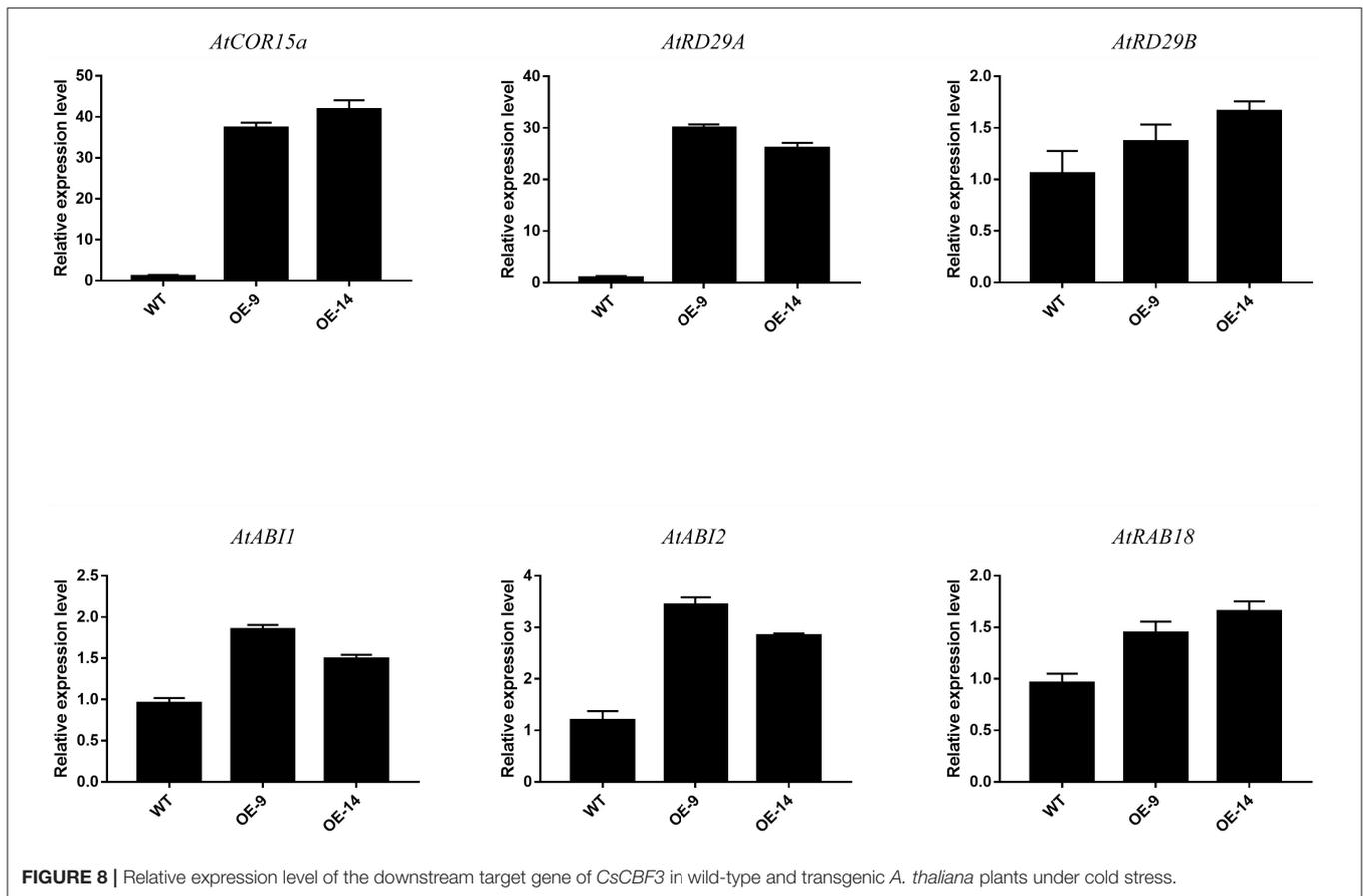
transgenic plants was significantly higher than that in wild-type plants. The expression of *AtRD29B*, *AtRAB18*, *AtABI1*, and *AtABI2* showed slight differences in WT and OE plants (Figure 8). These results indicated that *CsCBF3* may affect the expression of ABA-independent stress-induced genes to increase plant tolerance to cold stress.

## DISCUSSION

Low temperature is a major abiotic factor that limits crop productivity. When plants suffer from cold stress, CBF/DREB transcription factors are triggered and regulate ~12% of the cold responsive transcriptome, showing important roles in cold tolerance (Sun et al., 2014). To date, two *CsCBF* genes' biological function have been reported in tea plant. Wang et al. (2012) and Ban et al. (2017) found that *CsCBF1* was not expressed at normal temperature ( $20^{\circ}\text{C}$ ) but was significantly induced at low temperature ( $4^{\circ}\text{C}$ ). In addition, Wang et al. (2012) used a DNA-binding assay to demonstrate that *CsCBF1* can specifically bind to the CRT/DRE cis-element, suggesting that *CsCBF1* can regulate downstream genes containing CRT elements such as *COR15* and *RD29A*. Wang et al. (2017) showed that overexpression of a *CsDREB* gene in *Arabidopsis thaliana* plants could increase the salt and drought tolerance of transgenic plants. Wang et al. (2019) identified five *CsCBFs* from tea plant genome sequences (Wei et al., 2018), namely *CsCBF1-5*. However, we failed to clone *CsCBF2* (TEA010423), which might be caused by draft nature of the current genome assembly with low assembly quality. The *CsCBF1* identified by Wang et al. (2012) was consistent with that identified in this study but totally different from that identified by Wang et al. (2019) (Data Sheet S3). Similarly,

we were not only unable to find homologous gene of the *CsCBF3* identified by Yin et al. (2016) in the current tea plant genome assembly, but also failed to clone it in this study. We here successfully cloned a total of 6 *CsCBFs* (*CsCBF1-6*) based on the latest version of tea plant genome annotation (Xia E. et al., 2019). Of them, *CsCBF1-4* were in agreement with those identified by Wang et al. (2019), and *CsCBF5* and 6 were newly discovered (Data Sheet S3). Sequence analysis showed that *CsCBFs* contain an AP2 DNA-binding domain and two signature motifs of the CBF family (Figure 1). These results showed that *CsCBF1-6* typically belong to the CBF family. The C-terminus of *CsCBF6* is longer than those of the other *CsCBFs*, and it may cause functional divergence in plants under cold temperatures. Expression of *CsCBF6* is much less than other *CsCBFs* verified this (Figure 6).

It is widely accepted that transcription factors must be present in the nucleus to perform their functions (Wang et al., 2008; Yang et al., 2011). Bioinformatics analysis showed the presence of nuclear localization signal (NLS) sequences in *CsCBFs*. In our *in vivo* targeting experiment using a *CsCBFs*-fused GFP as a fluorescent marker demonstrated that the fusion protein was localized to the nucleus of tobacco leaf, suggesting that *CsCBFs* are nuclear proteins and functions as transcription factors. The results are consistent with the findings in cotton (Shan et al., 2007) and eggplant (Zhou et al., 2018). We designed a yeast single hybrid experiment to verify the transcriptional activities of *CsCBFs*. The results showed that *CsCBF2-6* had transcriptional activity in yeast Y2HGold cells, but not *CsCBF1*. This finding is similar to the findings of Sakuma and Zhao (Sakuma et al., 2006; Zhao X. et al., 2012). A plausible explanation is probably because the secondary structure of the *CsCBF1* protein itself



is abnormal or *CsCBF1* is required to be activated by a posttranslational modification.

CBF encodes a member of the DREB subfamily A-1 of ERF/AP2 transcription factor family. *CBF/DREB* genes from different plant species may have inconsistent expression profiles in response to various stresses (Zhou et al., 2016). There are six members in this subfamily, including *CBF1*, *CBF2*, *CBF3*, and *CBF4* in *Arabidopsis thaliana*. *AtCBF1-3* gene is involved in response to low temperature and abscisic acid. *AtCBF4* gene is involved in response to drought stress and abscisic acid treatment, but not to low temperature (Novillo et al., 2004). *EgCBF3* and *FeDREB1* can be upregulated not only by cold but also by osmotic and high-salt stresses (Ebrahimi et al., 2015; Fang et al., 2015). *CaDREBLP1* is not upregulated by low temperature but by dehydration and salt (Hong and Kim, 2005). We examined the expression patterns of *CsCBF* genes in relation to various environmental stresses. At normal growth temperatures, *CsCBF* genes is not transcribed, or is transcribed at lower levels, while *CsCBF* genes except *CsCBF4,6* are rapidly, transiently and strongly induced by cold stress. *CsCBF* genes were induced to varying degrees by other abiotic stress treatment including exposure to high temperature, drought, exogenous hormones, or salinity. *CsCBF4* had a strong response to salinity stress, which was similar to the study by Wang et al. (2017) in *CsDREB*. *AtCBF2* gene is involved in

a negative regulatory or feedback circuit of the CBF pathway (*CBF2/DREB1C* is a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A* expression and plays a central role in stress tolerance in *Arabidopsis*). Whether there is redundancy or feedback circuit function among *CsCBFs* genes needed to be further research.

Numerous studies have demonstrated that the expression of *CBFs* is regulated by GA, JA, ABA, ETH, and brassinosteroids (BRs) (Shan et al., 2007; Hu et al., 2013; Eremina et al., 2015; Barrero-Gil and Salinas, 2017). Different numbers plant hormone-responsive cis-elements were detected in the *CsCBF* promoters including abscisic acid, MeJA, ethylene, gibberellin, salicylic acid, auxin-responsive element. Results consistent with Wang et al. (2019). The molecular regulatory mechanisms of *CsCBFs* at the crossroads of plant hormone signaling in cold stress response need to be further elucidation. Light responsiveness motifs were also found in *CsCBF* promoters. Previous studies have showed that light is required for many cold-responsive genes, and there is a complex cross-talk between light and cold (Catalá et al., 2011). Large numbers of stress responsiveness cis-elements were found in *CsCBF* promoters, giving the reason tea plant can defend the cold stress. Sequence analysis of the *CsCBF* promoters revealed the existence of different numbers of MYB and MYC binding site, suggesting that induction of the *CsCBF* genes in response to low temperature is

involved in the regulation of transcription factors, such as ICE1 and MYB15 in *Arabidopsis* (Chinnusamy et al., 2003; Agarwal M. et al., 2006).

To confirm *in vivo* functions of the *CsCBF3* gene during low-temperature stress in plants, we ectopically expressed *CsCBF3* into *Arabidopsis*. The results showed that in the case of *CsCBF3* overexpression, transgenic plants showed enhanced resistance to cold damage. We also observed overexpression of *CsCBF3* resulting in delayed flowering and dwarfism. Under a cold environment, 4-weeks-old overexpression plants had a much higher survival rate than wild-type plants. Similar function was observed in *AtCBF1-3*, *GhDREB1*, *SmCBF*, *LpCBF3* (Xiong and Fei, 2006; Novillo et al., 2007; Shan et al., 2007; Zhou et al., 2018). To clarify how *CsCBF3*-overexpressing transgenic plants cope with low temperature stress, we examined the relative expression level of the downstream target gene of *CBF* identified in *Arabidopsis* plants. The results showed that the genes belonging to the ABA-independent pathway had a higher expression than the ABA-dependent genes when transgenic *Arabidopsis* plants were exposed to cold stress. Zhang et al. (2004) demonstrated the *LeCBF1* in a heterologous system could activate the *Arabidopsis* cold related (COR) genes involved in increasing freezing tolerance, but that *LeCBF1* in tomato plants did not up-regulate equivalent genes (Zhang et al., 2004). The function of *CsCBF3* overexpression in tea plant can't be studied because of genetic transformation system has not been established. We speculated that *CsCBF3* overexpression in transgenic plants improved cold tolerance mainly through the ABA-independent pathway, which was consistent with CBFs belonging to the ABA-independent pathway.

## CONCLUSION

CBF/DREB transcription factors were identified in tea plants. *CsCBF* proteins were localized to the nucleus. *CsCBFs* had transcriptional activity except *CsCBF1*. *CsCBF* gene expression could be affected by abiotic stress and plant hormones. Ectopic expression of *CsCBF3* in *Arabidopsis* induced cold tolerance, and the mechanism of *CsCBF3* regulation of downstream target genes was mainly the ABA-independent pathway.

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## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI, *CsCBF1* (EU563238.1), *CsCBF2* (KC702795.1), *CsCBF3* (MH017428.1), *CsCBF4* (KF988866.1), *CsCBF5* (MH165878.1), *CsCBF6* (MN544638.1).

## AUTHOR CONTRIBUTIONS

YL and EX designed the study. ZH, QB, JH, XZ, YC, JM, and ML conducted the experiments and analyzed the data. ZH and QB prepared the manuscript. All authors consent to the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.00921/full#supplementary-material>

**Table S1** | Primers used in this study.

**Table S2** | Name of genes in this study.

**Data Sheet S1** | The amino acid sequences of CBF genes used in phylogenetic tree construction.

**Data Sheet S2** | Expression level of *CsCBFs* under abiotic stresses.

**Data Sheet S3** | Expression level of downstream target genes of *CsCBF3* in *Arabidopsis*.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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