CBF transcription factors involved in the cold response of *Camellia japonica* (Naidong)

Menglong Fan [1], **Rui Zhou** [1], **Qinghua Liu** [1], **Yingkun Sun** [Corresp. 1]

[1] College of Landscape Architecture and Forestry, Qingdao Agricultural University, Qingdao, shandong, china

Corresponding Author: Yingkun Sun
Email address: sunyk678@qau.edu.cn

CBFs belong to the ERF subfamily of the AP2 supergene family and often play an important role in the cold acclimation of temperate plants. However, the role of CBFs in *Camellia japonica* (Naidong), the only*Camelliajaponica* population found in the temperate zones of China, remains unclear. It is very important to study the genetic composition of *C.japonica* (Naidong) to adapt to low temperature for Camellia species. Using full-length transcriptome data, we identified four CjCBF genes that respond to cold stress and analyzed their evolutionary relationships, domains, and expression patterns. The phylogeny of CBFs of 19 angiosperms divided the genes into three categories, and the four CjCBFs belong to a small subcluster. The strong response of *CjCBF1* to cold treatment and its sustained high level of expression indicated that it plays an important role in the process of cold acclimation. A yeast two-hybrid assay revealed an interaction between *CjCBF1*, *CjCBF2*, and *CjCBF5*, and subcellular localization confirmed this finding. The expression of CjCBFs was tissue-specific: CBF1 was mainly expressed in leaves, and CBF3 was mainly expressed in stem. The responses of the four CjCBFs to drought and high temperature and the effect of light were also characterized. Our study provides new insight into the role of CBFs in the cold response in *C. japonica* (Naidong).

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MengLong Fan[1], Rui Zhou[1], QingHua Liu[1], YingKun Sun[1*]

1College of Landscape Architecture and Forestry, Qingdao Agricultural University

Corresponding authors: YingKun Sun[1]

Street Address,College of Landscape Architecture and Forestry, Qingdao Agricultural University,

Qingdao 266109, Shandong, People's Republic of China

Email address:sunyk678@qau.edu.cn

Abstract

CBFs belong to the ERF subfamily of the AP2 supergene family and often play an important role in the cold acclimation of temperate plants. However, the role of CBFs in *Camellia japonica* (Naidong), the only *Camellia japonica* population found in the temperate zones of China, remains unclear. It is very important to study the genetic composition of *C.japonica* (Naidong) to adapt to low temperature for Camellia species. Using full-length transcriptome data, we identified four CjCBF genes that respond to cold stress and analyzed their evolutionary relationships, domains, and expression patterns. The phylogeny of CBFs of 19 angiosperms divided the genes into three categories, and the four CjCBFs belong to a small subcluster. The strong response of *CjCBF1* to cold treatment and its sustained high level of expression suggested that it plays an important role in the process of cold acclimation. A yeast two-hybrid assay revealed an interaction between *CjCBF1*, *CjCBF2*, and *CjCBF5*, and subcellular localization confirmed this finding. The expression of CjCBFs was tissue-specific: CBF1 was mainly expressed in leaves, and CBF3 was mainly expressed in stem. The responses of the four CjCBFs to drought and high temperature and the effect of light were also characterized. Our study provides new insight into the role of CBFs in the cold response in *C. japonica* (Naidong).

Introduction

Low temperature stress is an important abiotic stress that greatly affects the distribution of plants and social and economic development worldwide. The study of cold resistance mechanisms is becoming increasingly important as threats to the ecological environment grow. Plants can

29   reduce the damage caused by low temperature through complex mechanisms, such as
30   physiological metabolic pathways and molecular regulation (Agarwal et al, 2006a). Temperate
31   plants can improve their cold tolerance by experiencing low, non-freezing temperatures (i.e.,
32   cold acclimation). This process involves stress perception, signal transduction, and the regulation
33   of protein-coding genes, in which transcription factors act as important regulatory hubs that
34   control a large number of genes to mediate the response to different stresses.

35   C-repeat binding factors (CBFs) belong to the ERF family of the *AP2* supergene family, which
36   has a common DNA-binding domain and two conserved motifs. They are distinguished by two
37   signature sequences (PKK/RPAGRxKFxETRHP and DSAWR) (Haake et al, 2002). In some
38   plants, CBFs have been shown to be key regulators in the cold acclimation pathway (Stockinger
39   et al,1997;Medina et al,1999;Tayeh et al,2013;Francia et al,2016;Würschum et al,2017) and are
40   specifically associated with the C-repeat/dehydration-responsive motif (CRT/DRE;
41   G/ACCGAC) of the promoter region of the *COR* gene(Lata&Prasad,2011), which can mobilize a
42   large number of genes involved in cold acclimation independent of the ABA pathway(Kodama et
43   al,2000;Graham et al;1997;Ashraf et al,2007;Kornyeyev et al,2011). CBF transcription factors
44   have been shown to improve cold resistance in maize, potato, tomato, Arabidopsis, tobacco, and
45   other plants through overexpression verification(Ito et al,2006;Gilmour et al;2000;Liu et
46   al,2011;Dubouzet et al,2003;Mei et al,2009;Al-Abed et al,2007;Tang et al,2011;Hsieh et
47   al,2002;Hsieh et al,2002). In addition, previous studies have shown that CBF (DREB) not only
48   participates in low temperature stress but also responds to drought and saline-alkali stress.

49   However, the overexpression of CBF transcription factors can inhibit plant growth and
50   development and even lead to sterility (Tillett et al, 2012). This problem can be solved by
51   incorporating different promoters into overexpression vectors with *CBF* genes of different
52   species. For example, the incorporation of *Arabidopsis* DREB1A and rd29A into an expression
53   vector was not observed to significantly inhibit growth and sterility (Kasuga M et ak, 1999).
54   *AtCBF1*, *AtCBF2*, and *AtCBF3* in *A.thaliana* are not only involved in the regulation of cold
55   acclimation but also basic cold tolerance; however, *AtCBF2* appears to be more important than
56   *AtCBF1* and *AtCBF3* in the process of cold acclimation (Zhao et al,2016). In *Arabidopsis*
57   *thaliana*, CBF2 negatively regulates CBF1 and CBF3, but CBF1 and CBF3 do not directly
58   regulate the expression of other CBFs; instead, they coordinate the regulation of some
59   downstream target genes (Novillo, Medina&Salinas,2007). Thus, studying the combined
60   expression of multiple genes is essential for improving plant cold resistance.

61   *Camellia* plants are mostly distributed in the subtropics and are not tolerant of low temperatures.
62   Temperature greatly affects the economic value of *Camellia* plants. *C. japonica* (Naidong),
63   which is the most northerly distributed *Camellia* species in China, has excellent cold resistance,
64   but molecular research has been limited because of a lack of genomic sequences. Transcriptomic
65   study of *C. japonica* (Naidong) has shown that α-jasmonic acid may play an upstream signaling
66   role in the response to cold stress(Li et al, 2016). However, no studies have identified *CBF* genes
67   in *C. japonica* (Naidong) nor have characterized their expression patterns in the response to cold
68   stress.

69   Here, we identified and isolated four low temperature-responsive CjCBFs transcription factors
70   from low temperature treatment full-length transcriptome data (PacBio). Structure analysis,
71   phylogenetic analysis, Gene Ontology (GO) annotation, and the expression patterns of genes
72   were clarified by bioinformatics technology, and the role of CjCBFs in the response to low
73   temperature stress was characterized in detail. Overall, this study provides valuable information
74   that could be used to improve the cold tolerance of *Camellia* plants.

Materials and Methods

76   Identification of new CjCBFs genes in *C.japonica* (Naidong) involved in cold

77   tolerance

78   We used our *C.japonica* (Naidong) low temperature and drought full-length transcriptome data
79   based on PacBio and Illumina sequencing (PRJNA689105) as the local database. The protein
80   sequences of CBF family genes (i.e., *AtCBF1*, *AtCBF2*, *AtCBF3*, *AtCBF4*) in
81   Arabidopsis thaliana were queried to search the local databases using the BLASTP
82   method with an E-value threshold of <1e-20. Then, HMMER3.0 (Potter, S. C. et al, 2018)
83   was used to further screen sequences using default parameters (E<1e-5), the AP2 domain was
84   based on Pfam database (Pfam accession, PF00847). The longest protein was selected as a
85   representative for the gene. Sequences that do not contain the complete AP2 domain and
86   signature sequences were eliminated.

87   Sequence analysis

88   ExPASy (Gasteiger, E et al, 2003) was used to analyze the relative molecular weight, isoelectric
89   point, hydrophilicity, and other basic information of CjCBFs according to default parameters.

90   Motif and phylogenetic analysis

91   MEME (http://meme-suite.org/tools/meme) was used for the conservedmotif analysis of CjCBFs
92   and AtCBFs proteins, with the maximum number of motifs set at ten. The parameters were as
93   follows: minimum motif width, 6; maximum motif width, 50; Selected the motif classic mode
94   and 0-order model of sequences. MAFFT was used for sequence alignment according to default
95   parameters (FASTA format, auto). The R package ggmsa was used to visualize the sequence
96   alignment. In this study, *C. japonica* (Naidong) and 18 other plant species were selected from
97   dicotyledons and monocotyledons, including *Brachypodium distachyon, Oryza sativa* and three
98   additional monocotyledons, as well as *Camellia japonica, Arabidopsis thaliana, Cucumis sativus*
99   and 19 other dicotyledons. The CBF-like protein data of the 19 species (with the exception of *C.*
100 *japonica*) were downloaded from the NCBI Database. The protein sequences were selected by
101 HMMER3.0. If the AP2 domain was truncated, or the AP2 domain match E-value exceeded 1e-

102 5, the protein sequences were excluded. Based on this, 424 CBF-like genes were identified from
103 the 19 plant species in order to construct the phylogeny tree. The phylogeny was constructed
104 using the maximum likelihood method with 1000 bootstrapping replicates in MEGA (v. 6.0)
105 (Tamura, K, 2013). The R package ggtree (v2.0.4) was used to visualize the phylogenetic tree.

106 ## GO and interaction analysis

107 KOBAS(Xie et al, 2010) (http://kobas.cbi.pku.edu.cn/) and *A.thaliana* (thale cress) were used for
108 the GO enrichment analysis. The sequences of four *CjCBF*s were inserted separately into
109 pGADT7 Vector and pGBKT7 Vector; pGADT7-T+pGBKT7-Lam was the negative control and
110 pGBKT7-53+pGADT7-T was the positive control. In order to verify self-activation, the
111 pGADT7+ pGBKT7 - *CjCBF1/ CjCBF2/ CjCBF3/ CjCBF5* plasmid was transformed into
112 Y2HGold yeast competent cells, with 30 mg/ml α-gal employed to detect the self-activation.
113 Furthermore, in order to determine the suitable concentration of AbA, the inhibitory self-
114 activated AbA concentrations of 0.20 µl/ml, 0.30 µl/ml, 0.50 µl/ml, 0.70 µl/ml and 0.90 µl/ml
115 were adopted for the five working solutions. The pGBKT7 and pGADT7 + CjCBF1, pGBKT7 and
116 pGADT7 + CjCBF2, pGBKT7 and pGADT7 + CjCBF3, and pGBKT7 and pGADT7 + CjCBF5 plasmids were
117 transformed into Y2HGold yeast competent cells, respectively, to exclude false positives. Moreover, the
118 pGADT7+ *CjCBFs* and pGBKT7 + *CjCBFs* plasmid was transformed into Y2HGold yeast
119 competent cells to verify the interaction between CjCBFs.

120 ## RNA-seq data

121 In order to explore the transcriptional expression of CjCBFs at low temperature
122 stress across time, we evaluated our low temperature stress transcriptome
123 (PRJNA689105) under a 4°C treatment lasting 0 h, 12 h, 24 h, and 72 h, with 4
124 groups of transcriptome data. The expression of the CjCBFs gene was analyzed and
125 the results were visualized by the R package pheatmap. qRT-PCR technology was
126 employed to verify the transcriptome data and the co-expression method was used
127 to predict the potential lncRNA regulating CjCBFs (Li J et al, 2015).

128 ## Subcellular localization

129 Four DNA sequences encoding CBF proteins were inserted into the super1300-GFP
130 overexpression vector, and *Agrobacterium tumefaciens* GV3101 containing super1300-CjCBF1,
131 super1300-CjCBF2, super1300-CjCBF3, and super1300-CjCBF5 was injected into the Abaxial of
132 tobacco (*Nicotiana benthamiana*) leaves (Li, Z.Q, 2013). A Leica DM2500-DM2500 LED
133 fluorescence microscope was used to obtain fluorescence information.

134   Promoter cloning and Cis-Element analysis

135   *C. japonica* (Naidong) DNA was extracted via the CTAB method (Doyle JJ and Doyle JL,
136   1987) to clone the promoter. The *CjCBF1* upstream 5'UTR sequence was obtained
137   from the Takara Genome Walking Kit (Takara, Japan) following the manufacturer's
138   instructions. Design of nested PCR specific primers with primer 5.0 (Supplementary
139   Table S7). The obtained sequence was connected to a T vector for sanger sequencing
140   and the Cis-Elements in the sequence were analyzed based on the PlantCARE database
141   (Lescot M et al, 2002).

142   Plant materials and low temperature treatment

143   *C. japonica* (Naidong) seeds were obtained from the Qingdao Botanical Garden (36°05'N,
144   120°08'E), Qingdao Agricultural University Cooperative, China, in September 2018. The seeds
145   were sown in sand in the Qingdao Agricultural University culture room (20±2°C) during the
146   winter until the fourth true leaf appeared. Following germination, the plants were transferred into
147   the artificial climate room of the university (36°31'N, 120°39'E) under normal culturing
148   conditions (plastic pots: top/bottom diameter 20/12 cm, height 26 cm; temperature: 25±2°C;
149   relative humidity: 60%; soil: peat soil 80% + river sand 20%; natural light) for 3 months.

150   For the tissue specific expression experiment, we selected seedlings of a similar growth status
151   and divided them into two groups, with three plants per group. Each group was treated at 4°C for
152   either 0 h or 24 h in a low temperature incubator. Tender roots, stems, leaves, buds, and flowers
153   were collected at 0 h and 24 h. For the heat, cold and light treatments, seedlings were separated
154   into several groups. The first group was placed at 40°C (from 25°C to 40°C with a 1°C increment
155   per hour) and maintained for 24 h (2000lx light intensity and 60% humidity). The second
156   group was placed at 40°C (from 25°C to 40°C with a 1°C increment per hour) and maintained for
157   24 h (60% humidity). The plants were wrapped with foil to avoid light. The third group was
158   placed at 4°C (from 25°C to 40°C with a 1°C increment per hour) and maintained for 24 h (2000lx
159   light intensity). The fourth group was placed at 4°C (from 25°C to 40°C with a 1°C increment per
160   hour) and maintained for 24 h. (1 degree per hour from 25°C to 4°C). The plants were wrapped
161   with foil to avoid light. The drought treatment involved stopping watering for 20 days and
162   measuring the relative soil water content to determine the drought degree
163   (Supplementary Table S8). Unstressed seedlings were used as the control samples (CK). Three
164   biological replicates were collected from the same position at each collection time for RNA
165   isolation.

166   RNA extraction and Expression analysis of qRT-PCR

167   The total RNA extraction was performed using the SPARKeasy A0305 kit (QingDao, China),
168   following the manufacturer's instructions. Each treatment comprised three replications. The

169 integrity and concentration were detected by 1% agarose gel electrophoresis and a
170 NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was
171 prepared using the Takara PrimerScriptTMRT reagent kit with gDNA Eraser, and RT-qPCR
172 primers were designed by Primer 5.0 (Supplementary Table S7). Takara TB Green was used to
173 set up the Premix Ex Taq qGreTli RNaseH Plus for the qRT-PCR experiment. Per the
174 manufacturer's instructions, *18S* was used as an internal reference gene, and there were three
175 biological replicates and three technical replicates in each group. The relative expression was
176 determined by a three-step method (95°C for 5 s, 55°C for [15 s], and 72°C for 30 s, repeated 40
177 times) on an Applied Biosystems StepOnePlus. The gene expression level was calculated
178 according to the quantitative Ct method. The R package pwr was used to conduct Student's *t*-
179 tests. The R packages corrplot (0.84) and circlize (version 0.4.11) were used to conduct
180 correlation analysis.

181 Results

182 Identification and characterization of CjCBFs

183 First, we used the transcriptome data of *C. japonica* (Naidong) as the local blast resource bank to
184 obtain 11 candidate genes. Further screening incorrectly predicted CBF genes and redundant
185 sequences, and four CBF transcription factors responsive to low temperature were identified.
186 They were named according to their homology with CBF transcription factors in related plants
187 (*Camellia sinensis*). The nucleotide and amino acid sequences are provided in Supplementary
188 Table S1. The full lengths of the *CjCBF*s sequences are 1.82 kb, 1.25 kb, 1.42 kb, and 1.35 kb.
189 The CDSs are between 540 and 837 bp, and the number of amino acids are between 179 and 278.
190 The molecular weights of the four cjCBF proteins are 30282.9 kDa, 19449.25 kDa, 26655.03
191 kDa, and 26526.23 kDa. The isoelectric point ranges from 4.9 to 9.8. Hydrophilicity ranges from
192 3.02 to 1.8 (Table. 1).

193 Analysis of conserved motifs

194 A comparison of the CjCBFs sequences (Supplementary Fig. 1) revealed that the internal
195 similarity of CjCBFs was 73.96%, and the domain sequence was highly conserved. Comparison
196 of the domain sequences of *A. thaliana* and *C. japonica* (Naidong) (Fig. 1A) showed that the
197 domain of *C. japonica* (Naidong) consists of the AP2 family universal DNA-binding domain
198 (AP2 domain) and two signature sequences (DS-W-L), which were slightly different from the
199 conserved domain of *A. thaliana*. We then analyzed the conserved motifs of *A. thaliana* and *C.*
200 *japonica* (Naidong) and identified 10 motifs (Fig. 1B) with amino acid lengths between 6 and 50.
201 In particular, Motif10 (WYQGDD) was the shortest (6 residues), while Motif1
202 (KKVRETRHPIYRGVRQRNSGKWVCEVREPNKKSRIWLGTFPTAEM AARAH) and
203 motif2 (DVAAJALRGRSACLNFADSAWRLPIPESLCPKDIQKAA AEAAEAFRPELC) were
204 the longest (50 residues) and contained the AP2 domain. Only CjCBFs have motif8

205 (YLDVSGDVAKP), and only *A. thaliana* has motif7 (TTDHGLDMEETLVE). Only CjCBF1
206 and CjCBF5 have motif10. The functions of most of the motifs have not yet been elucidated. The
207 similarity of motifs within species indicates that CBFs within species are highly conserved. The
208 composition of different conserved motifs may be related to variation in the functional
209 differentiation of genes.

Analysis of the phylogenetic tree

211 To characterize the evolutionary relationships of *CBF*s among species, we constructed a genetic
212 tree (Fig. 2) using 424 *CBF*s from 19 species, including dicotyledons and monocotyledons.
213 These genes were generally divided into three categories. Monocotyledons and dicotyledons

214 were clearly separated, and dicotyledons were divided into two subclasses: Clade Ⅰ and Clade

215 Ⅱ. However, some monocotyledons (e.g., oil palm) were divided into A-Ⅱ subcluster in Clade

216 I, and rice was also partially distributed in this subcluster. This cluster is close to Clade III, with
217 groups containing only Monocotyledons. This may represent the region of functional overlap of
218 dicotyledon and monocotyledon CBFs. This also indicates that this cluster of *CBF* ancestral
219 genes had differentiated before the separation of monocotyledons and dicotyledons and thus that

220 the function and pedigree of *CBFs* became gradually enriched. A-Ⅰ and B-II were the largest

221 subclusters in Clade Ⅰ and Clade Ⅱ, respectively. *CjCBFs* were divided into A-Ⅰ subclusters

222 and clustered with *A. thaliana*. *CBFs* were highly conserved within species, and *CBFs* of the
223 same species often clustered together.

GO analysis

225 GO enrichment analysis of *CjCBFs* was conducted to better understand the biological function of
226 CjCBFs. CjCBFs were enriched in nucleus (GO:0005634), DNA-binding transcription factor
227 activity (GO:0003700), regulation of transcription, DNA-templated (GO:0006355), transcription
228 regulatory region sequence-specific DNA binding (GO:0000976), glucosinolate metabolic
229 process (GO:0019760), cold acclimation (GO:0009631), and response to cold (GO:0009409).
230 The results of the GO analysis confirmed that CjCBFs play a role in cold acclimation (4°C) (Fig.
231 3) (Xie et al, 2010).

Expression model of *CjCBFs* based on RNA-seq

233 Based on the data analysis of the full-length transcriptome under treatment with low temperature
234 stress, the standardized FPKM cluster analysis heat map (Fig. 4A) shows that *CjCBFs* responded
235 strongly to low temperature. Although *CjCBF1* played a role in all three gradient treatment

236 groups, it was significantly up-regulated at the 12h stage, and the strength of the effect was
237 significantly higher in the 12h stage than in the 24h and 72h stages. The response of *CjCBF3* to
238 low temperature was basically the same in the three treatment groups. *CjCBF2* responded in all
239 three treatment groups but was significantly up-regulated in 24h and then decreased in 72h. The
240 expression of *CjCBF3* and *CjCBF5* was similar to that of *CjCBF1*, except that *CjCBF5* was
241 significantly up-regulated in 24h but then decreased significantly and remained expressed at a
242 low level. qRT-PCR was used to verify the relative expression of *CjCBFs*. The pattern was
243 nearly identical, indicating that our transcriptome results were reliable (Fig.5C). The correlation
244 analysis also supports this finding (Fig. 4B). There is much evidence showing that lncRNA
245 participates in biological processes by regulating gene expression, including responses to stress
246 in plants. Our SMART and next-generation sequencing data were used to predict possible
247 lncRNA transcripts. A total of 147 possible *CjCBF*-containing transcripts (Fig. 4D) were
248 predicted by lncRNA.

249 Interactions of CjCBFs

250 To further study the post-transcriptional regulation mechanism of CjCBFs under cold stress, we
251 characterized the interactions among CjCBF1, CjCBF2, CjCBF3, and CjCBF5 using the
252 Y2Hgold yeast two-hybrid technique. All CjCBFs were completely inhibited when the
253 concentration of Aureobasidin A (AbA) reached 90 ng/µl, while the corresponding AbA
254 concentration for CjCBF2, CjCBF3, and CjCBF5 was 30 ng/µl (Fig. 5A), The results of self-
255 activation verification showed that the self-activation ability of CjCBF1 was significantly higher
256 than that of other CjCBFs, CjCBF2-PGAD, CjCBF3-PGAD, and CjCBF5-PGAD were
257 transformed into yeast Y2HGold cells in CjCBF1-PGBD; PGAD-T and PGBD-lam were
258 transformed into the negative control, and PGBD-T and PGBD-53 were transformed into the
259 positive control. The results of interaction verification showed that the yeast cells co-transformed
260 with CjCBF1 and CjCBF2, CjCBF1 and CjCBF5 could grow normally on the nutrient-deficient
261 medium, indicating that CjCBF1 could interact with CjCBF2 and CjCBF5 (Fig. 5B). To verify
262 the yeast two-hybrid results, we analyzed the subcellular localization of CjCBF1, CjCBF2, and
263 CjCBF5. CjCBF1 and CjCBF5 were located in the nucleus and cell membrane, and CjCBF2 was
264 located in the cell membrane (Fig. 5C). These results were consistent with the yeast two-hybrid
265 results. Several *CjCBFs* were expressed to aid the ability of *Camellia japonica* to cope with low
266 temperature stress.

267 Expression of CjCBF*s* in five *Camellia japonica* tissues

268 To more fully characterize the spatial expression characteristics of *CjCBFs* in *C. japonica*
269 (Naidong), the leaves, stems, flowers, buds, and roots of *C. japonica* (Naidong) were treated with
270 4℃ for 24h. The expression of *CjCBFs* was detected by qRT-PCR. the results showed, In the
271 normal cultured CK group, the expression of 4 CjCBFs was lower, but it was worth noting that the
272 expression of *CjCBF1*, *CjCBF3* in flowers was higher than that of *CjCBF2*, *CjCBF5*. This suggests

273   that *CjCBF1*, *CjCBF3* may be involved in flower development. after 24h of 4℃ treatment, 4
274   CjCBFs were Significant difference, but only *CjCBF1* was highly expressed in all five tissues,
275   especially in the petals, and *CjCBF2*, *CjCBF3*, and *CjCBF5* were expressed at low levels. The
276   expression of *CjCBF2* in roots and flowers was low, and the expression of *CjCBF3* and *CjCBF5*
277   in stems, leaves, and buds was significantly higher, and the expression of *CjCBF5* in buds was
278   higher (Fig. 6). These results suggest that *CjCBF1* may play an important role in the response to
279   cold stress in *C. japonica* (Naidong).

280   Bioinformatics Analysis of *CjCBF1* Cis-Elements

281   Promoter cis-elements function as transcription factor binding sites and are key for the
282   transcriptional regulation in response to abiotic stress (Moore M. J et al, 2010). As *CjCBF1* plays
283   an important role in CjCBFs, we cloned and analyzed the 1400 bp upstream sequences of
284   *CjCBF1*. Bioinformatics analysis revealed the sequence to contain 31 cis-acting elements (Fig. 7
285   and Supplementary Table S3), including 12 photosensitive responses and related cis-elements,
286   CGTCA and TGACG motifs (involved in methyl jasmonate responsiveness). The sequence was
287   also rich in TC-rich repeats related to stress, and MYB and MYC related cis elements. The
288   results suggest that *CjCBF1* has a wide response to abiotic stress and that light may play an
289   important role in the expression of CjCBFs.

290   Expression of CjCBF*s* under different stresses and the effect of light

291   *Camellia japonica* (Naidong) was treated with high temperature stress, drought stress, low
292   temperature stress, and light stress. Only *CjCBF1* and *CjCBF5* responded to high temperature
293   (40°C) stress(Fig. 8A), but the expression of *CjCBF1* was significantly lower under 40°C
294   treatment than under 4°C for 24 h and decreased slightly under joint high temperature and light
295   stress treatment. Under low temperature and shading stress(Fig. 8B), the expression of *CjCBFs*
296   decreased significantly compared with the non-shading treatment, which indicated that light may
297   not represent a signal that *CjCBFs* respond to at high temperature; however, the effect of light on
298   *CjCBFs* was more important at low temperature. The responses of *CjCBF1*, *CjCBF3*, and
299   *CjCBF5* to drought stress were weak; only *CjCBF2* showed a significant response to drought
300   stress, indicating that the responses of *CjCBFs* in *Camellia japonica* to drought stress are not
301   strong(Fig. 8C).

302   **Discussion**

303   When plants are subjected to abiotic stress, complex transcriptional and metabolic mechanisms
304   are mobilized. Previous studies have shown that CBF transcription factors are essential for
305   regulating responses to various stresses, and they can mobilize 35% of *COR* genes involved in
306   cold acclimation independent of the ABA pathway. The ICE-CBF-COR pathway is widely
307   known to be one of the core pathways in the response to low temperature stress in many plants,

308   including *A.thaliana*. *ICE1* and its homologous gene *ICE2* dynamically regulate the expression
309   of *CBFs* to ensure that they are expressed at appropriate levels in different periods. However, the
310   regulation of *CBFs* is complex; for example, the promoter has at least one CRT/DRE motif *COR*
311   gene, PHYTOCHROME-INTERACTING FACTOR4 (PIF4) and PIF7 (Barrero-Gil & Salinas,
312   2018) and the brassinosteroid signaling transcription factors BRASSIN AZOLE RESISTANT1
313   (BZR1) (Eremina,Rozhon& Poppenberger, 2016). In addition, the number of *CBFs* is often
314   related to the size of the genome (Wang et al, 2019). Five CBF transcription factors have been
315   identified in *Camellia sinensis* genomes, but the response patterns and characteristics of CBFs in
316   *C. japonica* (Naidong) remain unclear. As the northernmost *Camellia* in China, exploration of
317   the cold resistance mechanism of *C. japonica* (Naidong) is particularly important for enhancing
318   the cold tolerance of *Camellia*. However, its genome was not sequenced. Using data from the
319   PacBio and the Illumina full-length transcriptome, four *CjCBFs* were identified. These *CjCBFs*
320   participated in the response to cold stress at non-freezing temperatures, and their expression was
321   highest in the early stage of the stress response. They also responded to high temperature stress
322   and drought stress.

323   Plants have evolved several strategies to cope with environmental stress. The evolution of
324   multiple copies of genes can permit plants to quickly respond to stress. Multiple copies of *CBF*
325   genes have often been observed(Zhao et al, 2016). However, there are no reference genome
326   sequences of *C. japonica* (Naidong), precluding the possibility of determining the actual origin
327   of these gene copies. In our transcriptome data, we detected multiple CBF transcripts, which
328   have almost the same CDS region and significantly different UTR regions compared with
329   ontological annotations. However, the expression levels of these copies were not the same. We
330   speculate that the UTR region may also play an important role in the CBF pathway of cold-
331   resistant *Camellia japonica*. Comparison with the database revealed that lncRNAs are not
332   predicted in the UTR region of CjCBF; however, this does not mean that the UTR region is not
333   involved in the response to low temperature stress. In the phylogenetic tree, *CjCBF1* and
334   *CjCBF3* are clustered together, and *CjCBF2* and *CjCBF5* are clustered together. This, combined
335   with the results of our yeast experiment, indicates that *CjCBF1*, *CjCBF2*, and *CjCBF5* show
336   different patterns of expression and are regulated differently. *CjCBF3* appears to be a functional
337   redundancy of *CjCBF1* in the response to low temperature stress and does not directly participate
338   in the internal interaction. These results confirm the core role of *CjCBF1* in the response to low
339   temperature stress. Several studies have shown that *CBFs* are not only involved in the stress
340   response but are also involved in the inhibition of growth and development and delayed
341   flowering. Analysis of the expression in five *Camellia* tissues in the absence of cold treatment
342   revealed that *CjCBF1* was highly expressed in flowers, but there was no significant change after
343   cold treatment. Although *CBF* plays an important role in stress, *CBF* overexpression inhibits
344   plant growth (Li et al, 2016; Zhao et al, 2016; Gilmour et al, 2004); there is thus a need to find
345   ways to avoid the negative effects of overexpression of these genes.

346   We obtained the upstream 1400-bp sequence of *CjCBF1* by the walking method (Supplementary
347   table.S2) and identified 12 homeopathic elements related to light, 1 cis-acting element involved

348   in defense and stress responsiveness, and 8 cis-acting regulatory elements involved in MeJA
349   responsiveness (Supplementary table.S3). There are several light-response homeopathic elements
350   in CjCBFs; We analyzed the response of *CjCBFs* to light under stress in detail through a light
351   control experiment under low temperature. We found that the expression of *CjCBFs* decreased
352   significantly under shading, indicating that light promotes the low temperature response of
353   *Camellia japonica*.

354   However, light is not a necessary condition. Thus, the study of broad-spectrum and sensitive
355   stress genes is particularly important and is the reason for increased interest in studying the
356   effects of multiple types of stress. This suggests that the expression of *CjCBFs* is indeed different
357   between day and night in winter. Interestingly (Gilmour,Fowler&Thomashow,2004), our light
358   control experiment under high temperature stress revealed that light did not cause changes in the
359   expression of *CjCBFs*, and light intervention did not affect the response of *CjCBF1* and *CjCBF5*
360   to heat stress. We found that low temperature induces the strongest and most complex regulatory
361   response of *CjCBFs*. In addition, there are MeJA signal elements in the promoter sequence of
362   *CjCBF1*, indicating that it may be regulated by jasmonic acid and may be involved in the
363   metabolism of glucosinolates. *CBF* overexpression in *A.thaliana* promotes the accumulation of
364   glucose, sucrose, and proline, which aids the survival of plants at low temperatures.

365   *CBFs* are structurally expressed in *A. thaliana*. *CBF1* and *CBF3* are the main components
366   underlying the response to cold stress in plants(Gilmour et al,2000;Harmer et al,2000). *CBF2* is
367   involved in the regulation of *CBF3* and *CBF1*. We verified the relationship between *CjCBFs*.
368   *CjCBF1* plays a central regulatory role. *CjCBF1* may be expressed first and then regulate
369   *CjCBF5* and *CjCBF2*. The expression patterns indicated that the three *CjCBFs* were maintained
370   in a dynamic balance, and the rapid up-regulation of *CjCBF1* caused by low temperature breaks
371   this balance. *CjCBF2* and *CjCBF5* negatively regulate *CjCBF1* to maintain its level of
372   expression within a specific range.

373   Conclusion

374   Four CjCBFs involved in the cold response of *C. japonica* (Naidong) were studied. An analysis
375   of the motifs and evolution of these proteins provided information that could be useful for
376   studying other *Camellia* species. The interaction mode of *CjCBF1*, *CjCBF2*, and *CjCBF5*
377   provided new insight into the biological function of CBF transcription factors in *Camellia*. The
378   exploration of various stresses and responses to light expanded the known functions of CjCBF
379   transcription factors and will aid future studies of the mechanism of stress adaptation in *Camellia*
380   and other plants.

381

382   Supplementary Materials

383   Supplementary Figure1. Sequence similarity analysis of CjCBFs

384   Supplementary Table S1. Sequencing of CjCBFs.

385   Supplementary Table S2. Sequencing of CjCBF1 promoter.

386   Supplementary Table S3. Analysis of the homeopathic elements of promoters.

387   Supplementary Table S4. Fig. 4c qRT-PCR.Raw data.

388   Supplementary Table S5. Fig. 6 qRT-PCR.Raw data.

389   Supplementary Table S6. Fig. 7 qRT-PCR.Raw data.

390   Supplementary Table S7. The primer of qRT-PCR and promoter clon.

391   Supplementary Table S8. The relative water content of soil.

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Table 1. The physicochemical properties for the 4 CBF gene family members in *C. japonica*.

Gene name	Full length(kb)	CDS(bp)	Number of amino acids	Molecular Weight (kDa)	Theoretical pl	Grand average of hydropathicity	Aliphatic index	Instability index
CjCBF1	1. 82	837	278	30282. 91	4. 92	−0. 533	66. 08	53. 31
CjCBF2	1. 25	540	179	19449. 25	9. 18	−0. 411	74. 19	51. 14
CjCBF3	1. 42	741	246	26655. 03	5. 25	−0. 407	66. 71	46. 86
CjCBF4	1. 35	720	239	26526. 23	5. 50	−0. 328	74. 35	48. 40

Figure 1

Fig. 1. Multiple sequence alignment of CBFs gene and Conservative motif analysis

(A) The AP2 domain is represented in the yellow box. (B) The architecture of the 10 conserved protein motifs predicted via MEME analysis. Each motif is represented in a different color (Motif 1–10).
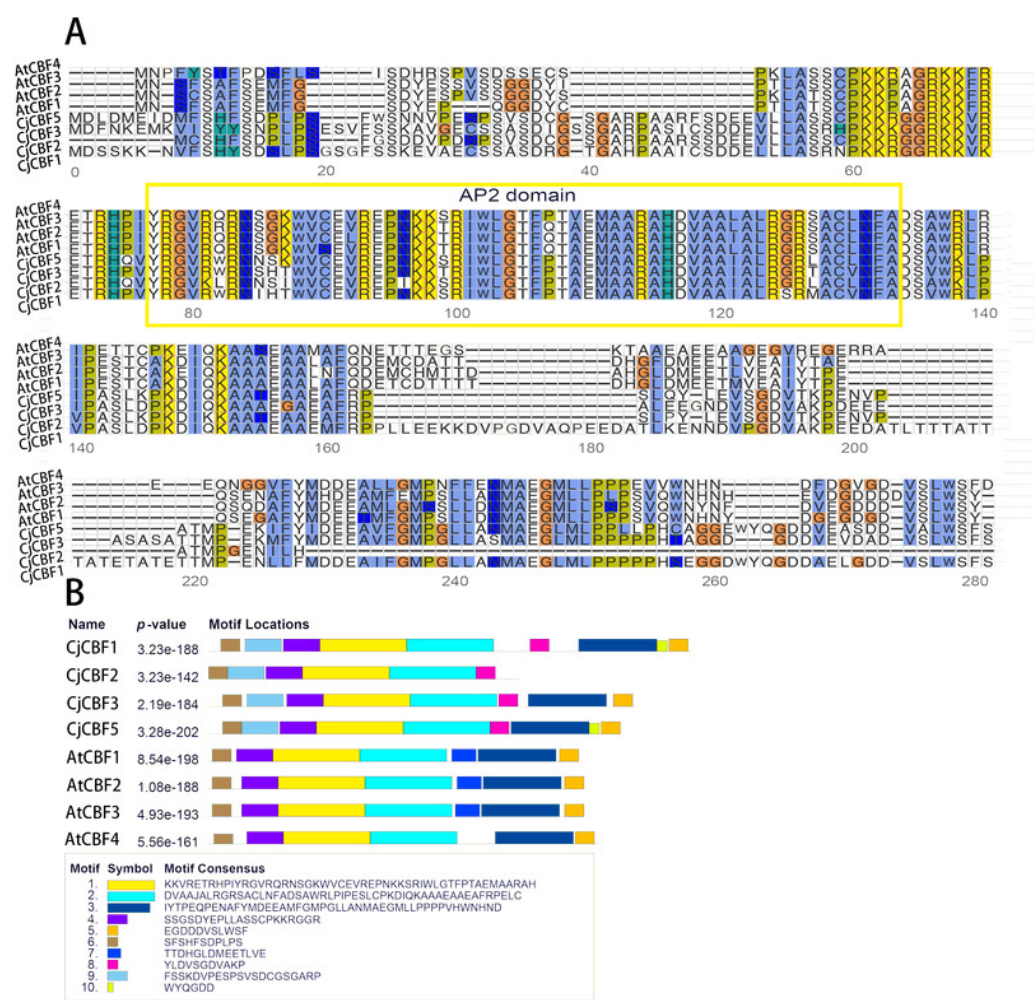
Figure 2

Fig. 2. Phylogenetic analysis of CBFs

Maximum Likelihood phylogenetic trees of the protein sequences of 424 genes encoding CBFs from 19 angiosperms. camellia (Cj: *Camellia japonica*) are marked red in the evolutionary tree, and Arabidopsis (At: *Arabidopsis thaliana*) are marked blue in the evolutionary tree. The other species are: Eucalyptus (Eug: *Eucalyptus grandis*); soybean (Gm: *Glycine max*); Cucumber (Cs: *Cucumis sativus*); Chili Peppers (Ca: *Capsicum annuum*); Cotton (Gh: *Gossypium hirsutum*); potato (St: *Solanum tuberosum*); western balsam poplar (Pt: *Populus trichocarpa*); Kiwi fruit (Ac: *Actinidia Chinensis*); Morning glory (Pn: *Pharbitis nil*); Sunflower (Ha: *Helianthus annuus*); Rubber Tree (Hb: *Hevea brasiliensis*); sesame (Si: *Sesamum indicum*); Carrot (Dc: *Daucus carotavar.sativa*); Medicago (Mt: *Medicago truncatula*); Brachypodium distachyon (Bd: *Brachypodium distachyon*); rice (Os: *Oryza sativa*); oil palm (Eg: *Elaeis guineensis*).

Clade I

Clade III

A-I

A-II

B-I

B-II

C.japonica(Naidong)

Arabidopsis

Clade II
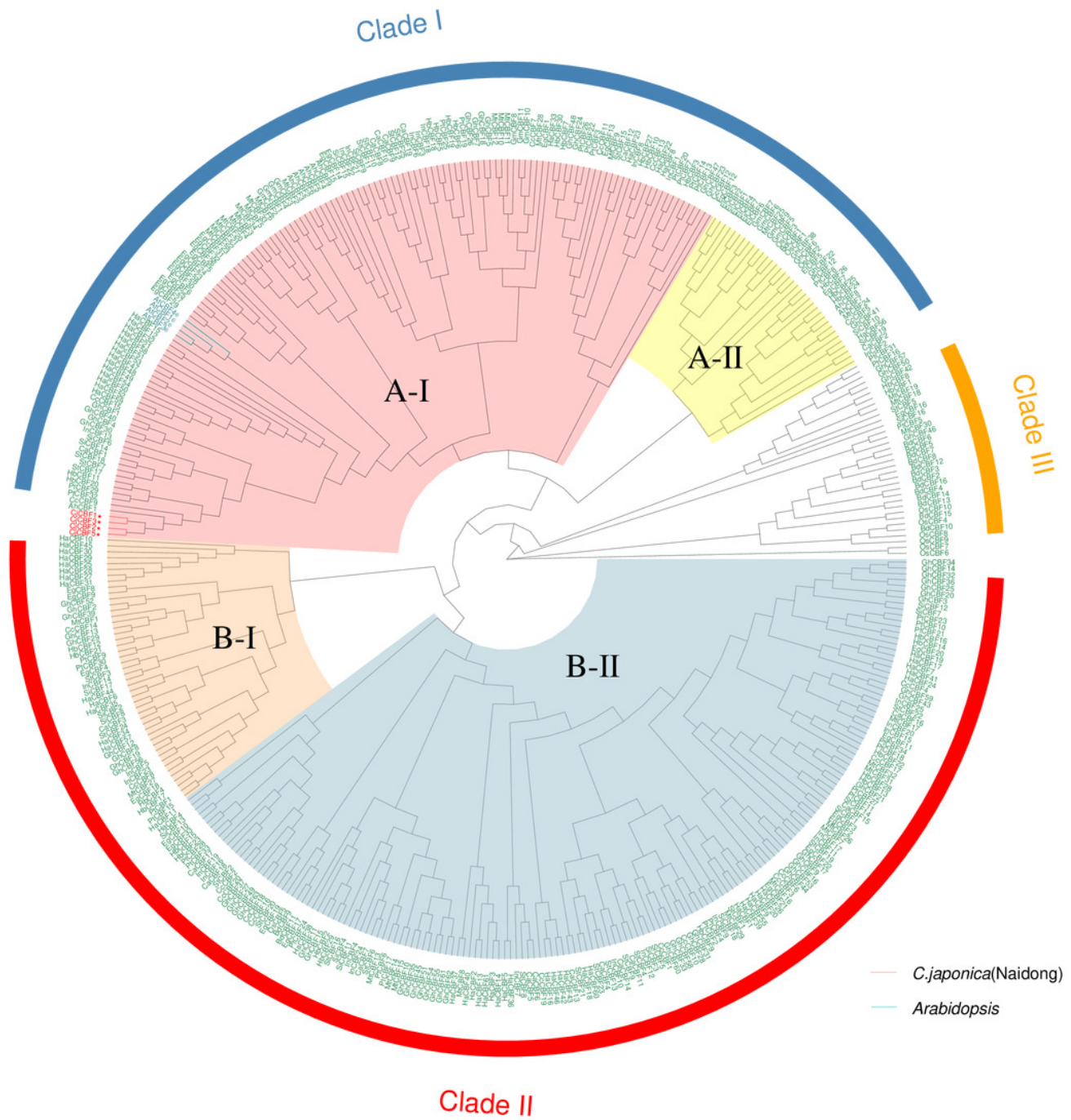
Figure 3

Fig. 3. GO analysis

The GO enrichment results of CjCBFs based on KOBAs. The enrichment degree increases with the -log10 (pvalue) value.
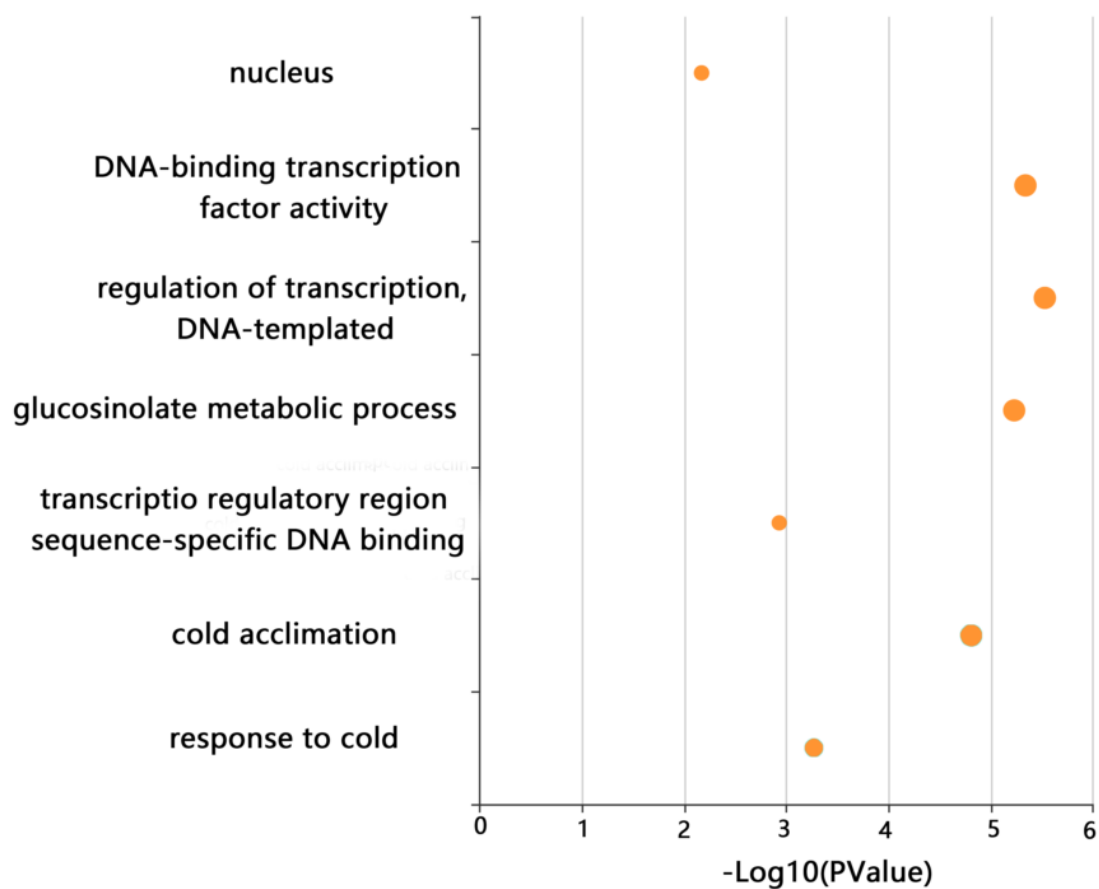
Figure 4

Fig. 4. Expression analysis based on the transcript group data

(A) Expression pattern of CjCBFs in response to cold 0h(CK), 12h(T1), 24h(T2), 72h(T3) treatments, The RNA-seq data were normalized based on the mean expression value (log2fold change, log2FC) of each gene, green and blue boxes indicate high and low expression levels, respectively. (B) Correlation analysis of four CjCBFs with different low temperature treatment groups (cyan region connects *CjCBF1* and four treatment groups, yellow region connects *CjCBF5* and four treatment groups, brown region connects *CjCBF3* and four treatment groups, purple region connects *CjCBF2* and four treatment groups), The wider the region, the higher the correlation. (C) Verification of the CjCBFs expression in the transcriptome via qRT-PCR. (D) Differential expression heat map of the lncRNAs that regulate CjCBFs based on the full-length transcriptome data. The data were normalized based on the mean expression value (log2fold change, log2FC) of each lncRNAs, green and blue boxes indicate high and low expression levels, respectively. Yellow, green and blue groups represent the regulation of *CjCBF2*, *CjCBF1*, and *CjCBF5*, respectively.

A

CBF1 CBF2 CBF3 CBF5
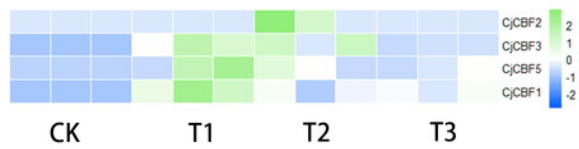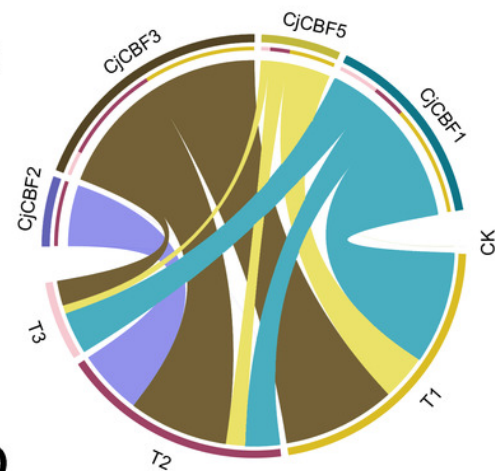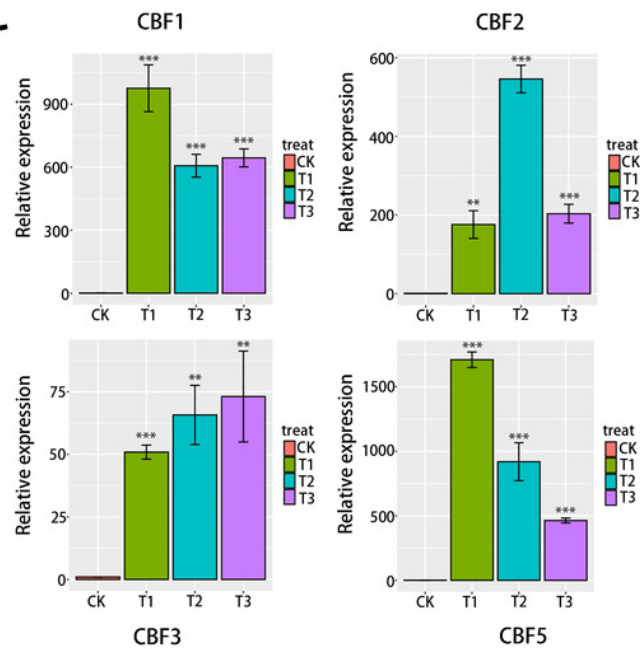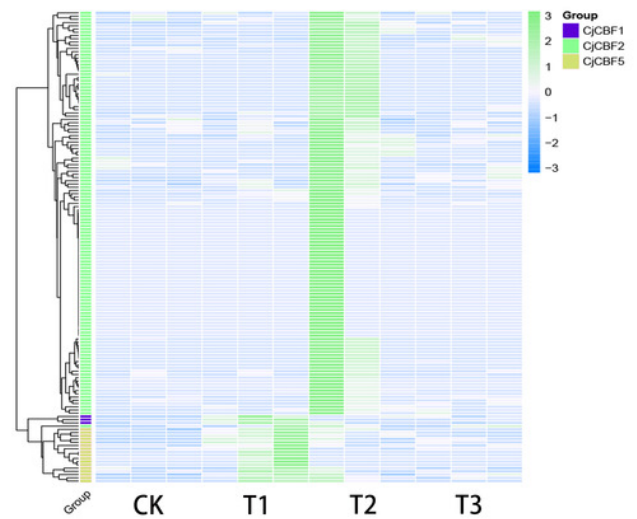
Figure 5

Fig. 5. Protein interaction identification and subcellular localization

(A)Self-activation verification. White colony indicates the ability of yeast to grow on the deficient medium, blue color indicates that the self-activating activity of CjCBFs was not able to grow on the deficient medium supplemented with AbA. This points towards the inhibition of the self-activating activity. (B)Yeast two-hybrid. Yeast cells exhibit normal growth on the nutrient-deficient medium, indicating the interaction between CjCBF1, CjCBF2 and CjCBF5. (C)Subcellular localization of Super1300, CjCBF1, CjCBF2 and CjCBF5 in Nicotiana benthamiana. In bright, the black line is the outline of the cell. For eGFP, the green region is the expression site of the plasmid with GFP fluorescent protein and the CjCBFs gene.

A

	-L/T (X-gal)	-L/T	-L/T (AbA) (30ng/ml)	-A/H/L/T (AbA) (30ng/ml)	-L/T (AbA) (90ng/ml)	-A/H/L/T (AbA) (90ng/ml)
PGAD+BDcjCBF1						
PGAD+BDcjCBF2						
PGAD+BDcjCBF3						
PGAD+BDcjCBF5						

B

	-L/T	-L/T (AbA)	-A/H/L/T (AbA)
PGAD+BD-1am			
PGAD+BD-53			
ADcjCBF2+BDcjCBF1			
ADcjCBF5+BDcjCBF1			

C

	Bright	eGFP	Merged		Bright	eGFP	Merged
1300-GFP				CjCBF2			
CjCBF1				CjCBF5			
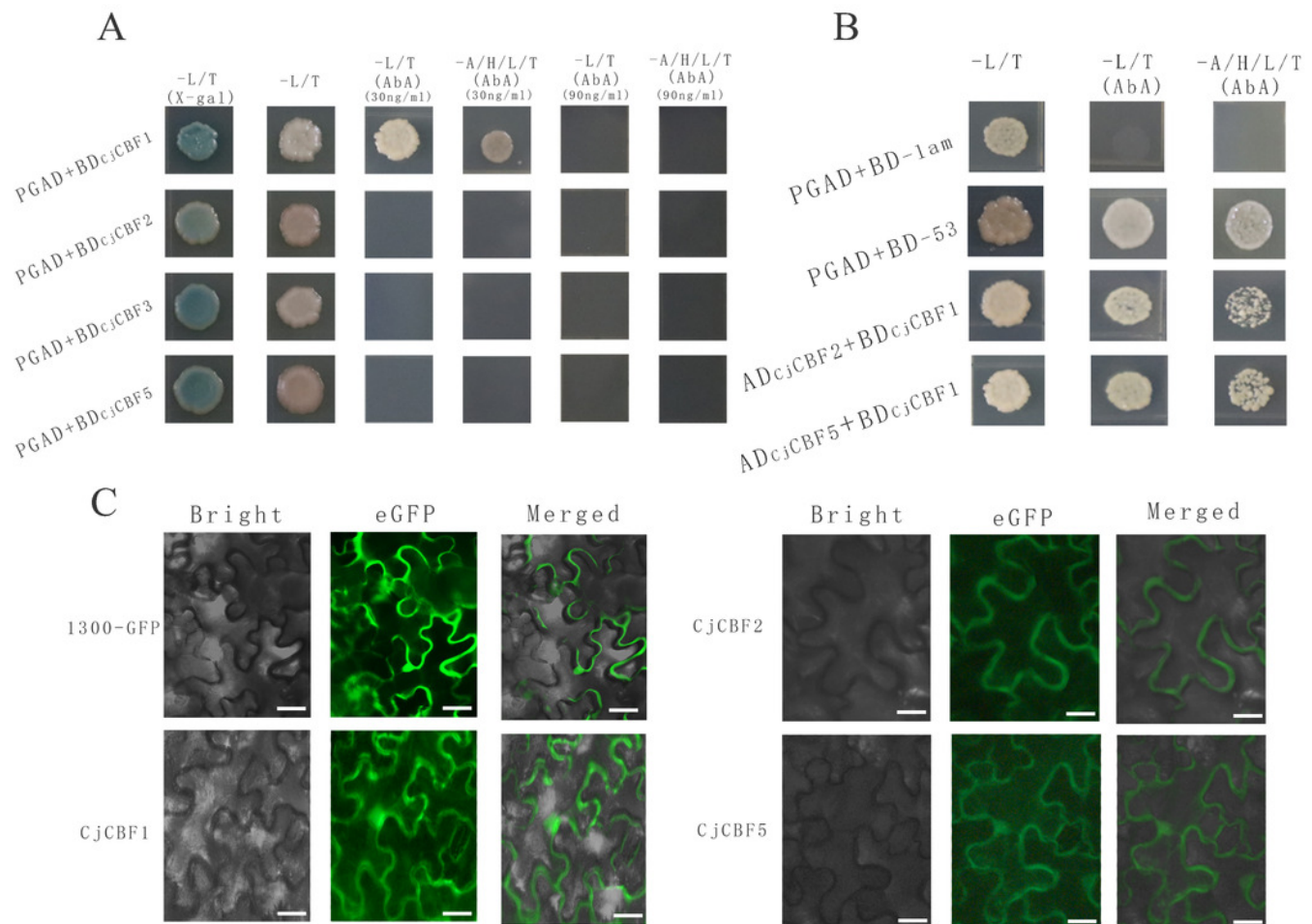
Figure 6

Fig. 6. Organ-specific expression of four *CjCBFs*

(A)-(E) We measured the relative expression amounts of 4 CjCBFs in each tissue type for A, B, C, D, E, and set the expression amounts of *CjCBF1* in the CK group to 1 for each tissue. This was then used to calculate the relative expression amounts of other CjCBFs for all tissue types. (F)-(I) We measured the relative expression of each CjCBFs in 5 tissues got F, G, H, I, and set the expression of each CjCBFs in the root of the CK group to 1. This was then used to calculate the relative expression of each CjCBFs in other tissues.
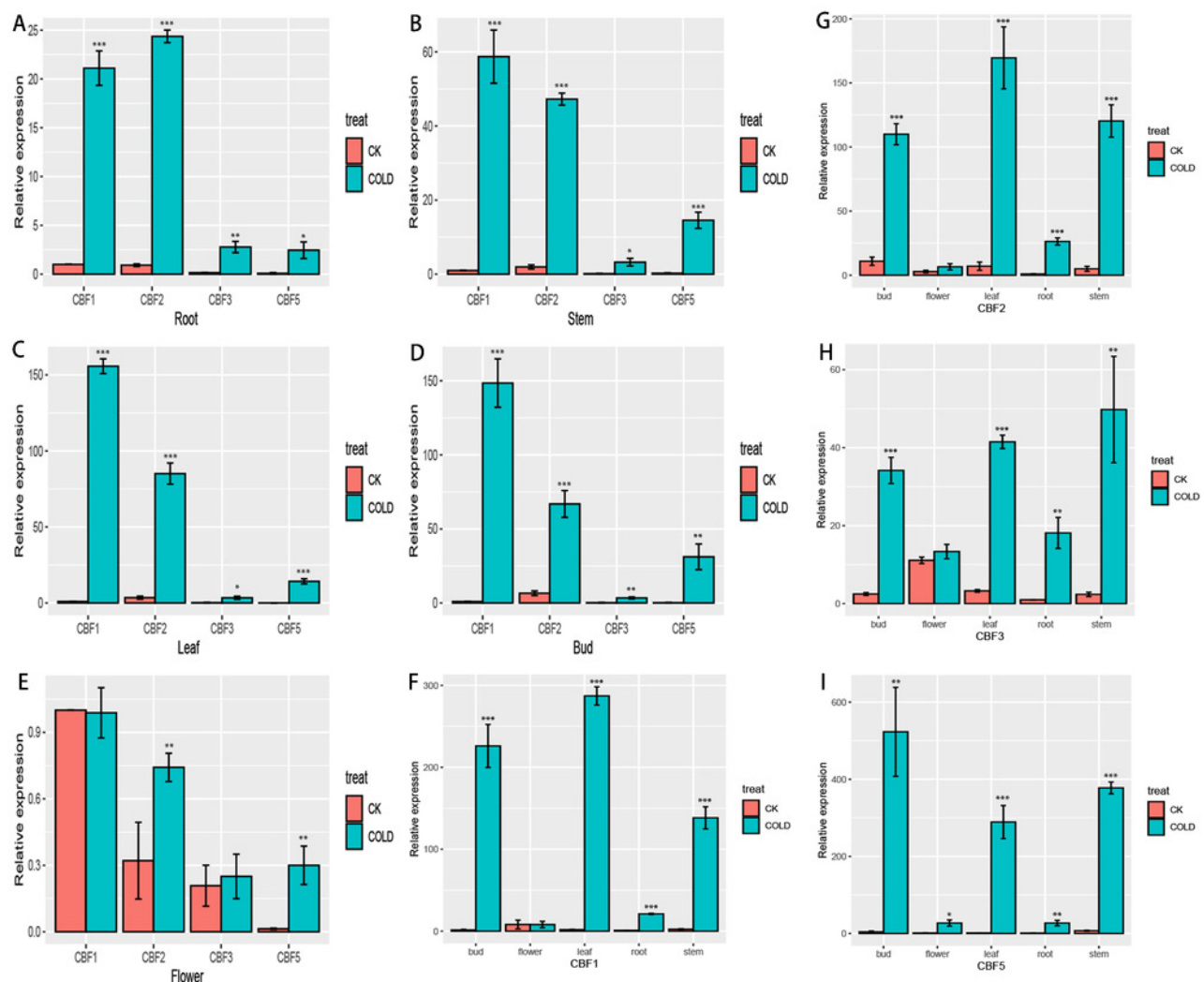
Figure 7

Fig. 7. Prediction of cis-elements in the *CjCBF1* promoters.
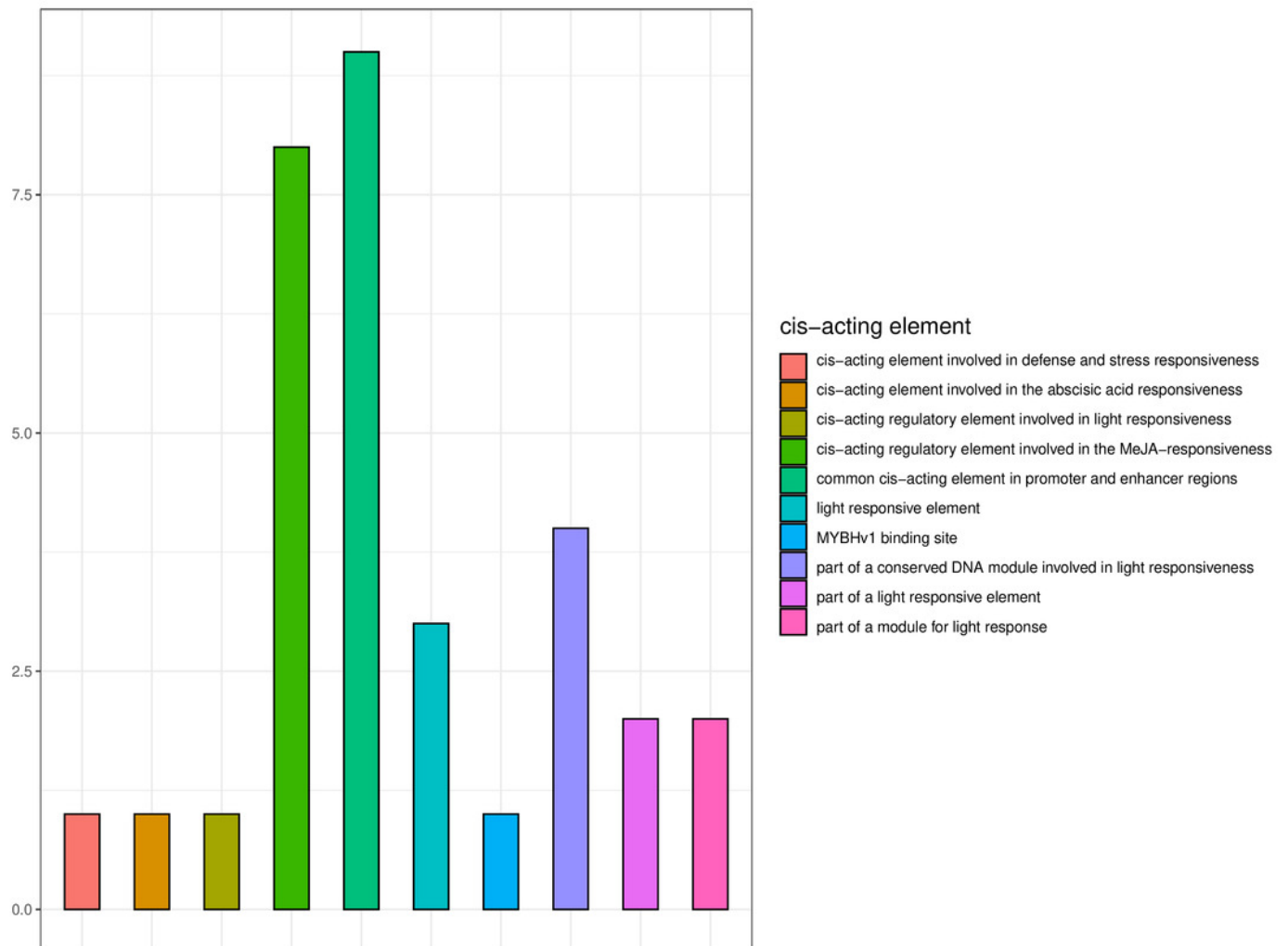
Figure 8

Fig. 8. Response of *CBFs* to multiple stresses

(A)Relative expression of CjCBFs under the heat and hot (no-light) treatments. The expression of CjCBFs in each CK group was set to 1. (B)Relative expression of CjCBFs under the cold and cold (no-light) treatments. The expression of CjCBFs in each CK group was set to 1. (C) Relative expression of CjCBFs in the drought treatment. The expression of CjCBFs in each CK group was set to 1.