Characterization of the TCP Gene Family in *Chrysanthemum nankingense* and the Role of *CnTCP4* in Cold Tolerance

Chang Tian 1,2, Lisheng Zhai 1, Wenjing Zhu 1, Xiangyu Qi 1, Zhongyu Yu 1, Haibin Wang 1, Fadi Chen 1, Likai Wang 1,* and Sumei Chen 1,*

1 State Key Laboratory of Crop Genetics and Germplasm Enhancement, Key Laboratory of Flower Biology and Germplasm Innovation, Ministry of Agriculture and Rural Affairs, Key Laboratory of Biology of Ornamental Plants in East China, National Forestry and Grassland Administration, College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China; 2012040318@njau.edu.cn (C.T.); 2019204048@njau.edu.cn (L.Z.); 201704094@njau.edu.cn (W.Z.); 2012204028@njau.edu.cn (X.Q.); 2019204044@njau.edu.cn (Z.Y.); hb@njau.edu.cn (H.W.); chenfd@njau.edu.cn (F.C.)

2 Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Genome Analysis Laboratory of the Ministry of Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518120, China

* Correspondence: wlk@njau.edu.cn (L.W.); chensm@njau.edu.cn (S.C.)

Abstract: Plant-specific TCP transcription factors play a key role in plant development and stress responses. *Chrysanthemum nankingense* shows higher cold tolerance than its ornamental polyplody counterpart. However, whether the TCP gene family plays a role in conferring cold tolerance upon *C. nankingense* remains unknown. Here, we identified 23 *CnTCP* genes in *C. nankingense*, systematically analyzed their phylogenetic relationships and synteny with TCPs from other species, and evaluated their expression profiles at low temperature. Phylogenetic analysis of the protein sequences suggested that *CnTCP* proteins fell into two classes and three clades, with a typical bHLH domain. However, differences between *C. nankingense* and *Arabidopsis* in predicted protein structure and binding sites suggested a unique function of *CnTCP* in *C. nankingense*. Furthermore, expression profiles showed that expression of most *CnTCPs* were downregulated under cold conditions, suggesting their importance in plant responses to cold stress. Notably, expression of *miR319* and of its predicted target genes, *CnTCP2/4/14*, led to fast responses to cold. Overexpression of *Arabidopsis* *CnTCP4* led to hypersensitivity to cold, suggesting that *CnTCP4* might play a negative role in *C. nankingense* responses to cold stress. Our results provide a foundation for future functional genomic studies on this gene family in chrysanthemum.

Keywords: TCP transcription factors; phylogeny analysis; expression profiles; cold tolerance

1. Introduction

The TCP gene family is a plant-specific group of transcription factors (TFs) that was first described in 1999 [1,2]. The family name is derived from four proteins, namely, TB1 (teosinte branched 1) in maize [3], CYC (CYCLOIDEA) in Antirrhinum [4], and PCF1 and PCF2 (Proliferating cell factors) in rice [2]. Furthermore, *TCP* genes have been found in various plant species, for instance, model plants such as *Arabidopsis* [1,5–7]; crop plants such as soybeans [8], maize [9] and rice [7]; and pluricellular green algae [10,11] such as cosmarium and chara, moss *Physcomitrium patens*, ferns and lycophyte *selaginella*, poplar (*Populus*) [12], and grapevine [13]. Furthermore, members of this gene family play numerous roles in plant development, including in embryonic growth [14], cell cycle regulation [15,16], pollen development [17], germination [14,18], senescence [19], circadian rhythms [20,21], leaf development [12,22–25], branching [26–28], floral organ morphogenesis and flowering [29–34], and hormone signaling [8,14,31,35–37].

Class II CIN-type TCPs, as *miR319* targets, have been widely analyzed [5,19]. TCPs regulated by *miR319* reportedly coordinate plant physiological characteristics such leaf
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2. Results

2.1. Classification and Phylogenetic Analysis of TCP Proteins

A total of 171 TCP proteins were selected for systematic analysis of evolutionary relationships, including 55 from *Glycine max* [8], 22 from *Oryza sativa* [5], 44 from *Zea mays* [38], flowering time [33], and cold tolerance [39]. TCP proteins harbor a bHLH motif that allows DNA binding and protein–protein interactions [5,6]. Many studies have shown that TCP transcription factors in Arabidopsis, tomato, and rice [5,6,23,40–45] exist as homodimers or heterodimers; furthermore, interaction usually occurs among TCP transcription factors of the same class. Additionally, some studies have reported that TCPs are more likely to form heterodimers than homodimers and that the former bind DNA more efficiently than the latter [2,40,41]. For instance, PCFl and PCF2 bind the rice meristematic tissue-specific expressed *PCNA* gene through homodimers or heterodimers, but the heterodimeric interaction between PCFl and PCF2 is considerably stronger than between the homodimers [2]. Consistently, AtTCP11 and AtTCP15 form heterodimers that show greater efficiency in binding DNA and have a different sequence preference than their corresponding homodimer counterparts [41]. Furthermore, TCP proteins might interact with other non-TCP proteins, thereby affecting the expression of downstream genes and regulating the plant development. For example, Chrysanthemum CmTCP20 interacts with CmAZ1 and downregulates *CmBPE2* expression, which regulates the petal size [46]. AtTCP5/13/17 directly interact with AtFD to activate *AtAPI1* expression and promote the photoperiodic flowering response [30]. These results suggest that the specific roles played by TCPs may be partially dependent on the proteins with which they interact.

Cold stress is one of the main abiotic stress conditions that severely reduce crop productivity, quality, and post-harvest longevity [47]. Cold stress induces transcriptional, post-transcriptional, and post-translational regulation of gene expression [39,48–50]. Evidence has shown that the inducer of the C-repeat binding factor (CBF) expression 1 (ICE1), CBF/DREB1 transcriptional cascade, and CBF-independent regulons are involved in transcriptional regulation during cold acclimation [50,51]. Furthermore, *ICE1* is regulated by an ubiquitin-mediated mechanism of translational control upon cold induction [52]; moreover, pre-mRNA splicing, mRNA export, and small RNA-directed mRNA degradation play important roles in cold stress responses by post-transcriptional regulatory mechanisms [39,53–56].

In addition to a great worldwide popularity as cut flowers and pot plants, chrysanthemums are highly appreciated as health foods and anti-inflammatory herbs of high commercial value in traditional Chinese medicine. During chrysanthemum growth, extremely low temperatures in early spring and winter, unusual freezing temperatures during late spring, and sudden frost during the fall often lead to growth arrest and flower bud or inflorescence inhibition, ultimately causing substantial yield and economic losses [48,57]. Therefore, improving cold tolerance of chrysanthemum is an important goal. The diploid species *Chrysanthemum nankingense* is closely related to the high market-value ornamental species, *C. morifolium*. Due to its simple diploid nature, *C. nankingense* has been selected as a convenient Asteraceae genomic model for rapid and effective exploration of gene function [22,57–61]. Additionally, *C. nankingense* shows greater cold tolerance than its ornamental polyploid counterpart [57,62]. Furthermore, a few *CnTCPs* found in *C. nankingense* are reportedly related to the plant response to low temperature [57]. However, to date, no attempt has been made to systematically describe the *CnTCP*-gene family members harbored by the *C. nankingense* genome. Therefore, this study was designed to systematically analyze and determine the phylogenetic relationships and synteny of *CnTCPs* present in *C. nankingense* with TCPs from other species. Here, we aimed to document the transcriptional behavior of *CnTCPs* under low temperature. Ectopic overexpression of *CnTCP4* in Arabidopsis led to reduced cold tolerance, suggesting that *CnTCP4* plays a negative role in the response of *C. nankingense* to cold.
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mays [9], 23 from C. nankingense, and 27 from Arabidopsis thaliana [5] (Figure 1). The 171 TCPs were classified into two main classes, i.e., class I and class II, each with three clades. The boundaries of these major clades illustrate the phylogenetic positions of several canonical TCP proteins, such as class I PCF proteins OsPCF1 and OsPCF2, CYC/TB1-like class II protein ZmTB1, and CIN-like class II protein AtTCP2-4. Furthermore, we found that TCP proteins from the three dicot plants used here, namely, Arabidopsis, G. max, and C. nankingense, seemingly clustered separately from those of the two monocots studied, namely, O. sativa and Z. mays (Figure 1). Furthermore, TCPs from G. max and C. nankingense TCPs showed a closer phylogenetic relationship among them than those from maize and rice did.

Figure 1. Phylogenetic relationships of CnTCP proteins. The phylogenetic tree was generated using the MEGA7.0 program; the bHLH domain amino acid sequences of TCP proteins from Glycine max (Gm), Oryza sativa (Os), Zea mays (Zm), Chrysanthemum nankingense (Cn), and Arabidopsis thaliana (At) were used.

All CnTCP proteins showed the basic helix loop helix (bHLH) domain (Figure 2). The distance matrix and homology matrix of the full-length and bHLH CnTCP protein sequences from five plant species are shown in Supplementary Tables S2 and S3. High homologies in full length and bHLH domain were noted among proteins (Figure 1). The conserved bHLH domain has a 59-amino acid bHLH structure that allows DNA binding and protein–protein interactions [1,2]. The most significant difference between the two protein classes is the absence of four amino acids in the basic domain of proteins in class I, compared with that of proteins in class II. Additional diagnostic residues for each class were found in the helices and the loop of the TCP bHLH domain (Figure 2). Importantly, our phylogenetic analysis and bHLH domain architecture support the classification of the C. nankingense and Arabidopsis TCP genes family. Our phylogenetic tree and bHLH domain analyses (Figures 1 and 2) showed that CnTCP14 and AtTCP2 clustered together.
into the same group and were classified into Class II CIN. In turn, CnTCP3 and CnTCP15 clustered with AtTCP18 and were classified into Class II CYC/TB1; meanwhile, CnTCP10 and CnTCP12 clustered with AtTCP16 and were classified into Class I PCF. Lastly, CnTCP18 clustered with AtTCP20 and was classified into Class I PCF.

Figure 2. Amino acid alignment among TCP proteins. Amino acids alignment using the bHLH domain amino acid sequences of TCP proteins from *Chrysanthemum nankingense* and *Arabidopsis thaliana*. The blue line shows PCF subfamily in class I; the yellow line shows CIN subfamily in class II; the green line shows CYC/TB1 subfamily in class II.

Compared with that of Arabidopsis, CnTCP proteins number occupies a lower proportion in the PCF subfamily, a higher proportion in the CIN subfamily, and a similar proportion in the CYC/TB1 subfamily. Specifically, among the five species studied herein, members in Class I PCF accounted for the largest proportion, with 47% in *G. max* (26/55), 39% in *Z. mays* (17/44), 48% in Arabidopsis (13/27), 45% in *O. sativa* (10/22), and 39% in *C. nankingense* (9/23); meanwhile, CYC/TB1 subfamily members accounted for 18% (10/55), 41% (4/22), 19% (5/26), 14% (4/21), and 17% (5/25), respectively. The proportion of CIN
members in the five species ranged between 20% and 43% (Figure 1), among which C. nankingense and Z. mays showed the highest and the lowest proportions, respectively.

Furthermore, we constructed an unrooted phylogenetic tree from alignments of the protein sequences of the bHLH domain from C. nankingense, Helianthus annuus, and Arabidopsis (Supplementary Figure S1). The Hidden Markov Model (HMM) profile of the TCP domain (PF03634) was used to identify the TCP proteins from H. annuus, one of the sequenced Asteraceae species [61,63]. A total of 29 TCP family proteins were identified in the sunflower genome, of which 14 clustered in the CYC/TB1 clades of class II, 9 in the CIN clades of class II, and 6 in the PCF clades of class I (Supplementary Figure S1).

2.2. Structural Analysis and Protein–Protein Interactions of CnTCP Genes

Family pair-wise analysis of the full-length CnTCP protein sequences indicated that the identities ranged from 9.40% to 68.10% in non-homologous gene pairs (Table 1). Relatively high identities were observed between CnTCP10 and CnTCP12 (68.10%), CnTCP2 and CnTCP14 (approximately 57.00%), CnTCP1 and CnTCP11 (52.30%), CnTCP5 and CnTCP17 (52.10%), and among CnTCP16, CnTCP10 and CnTCP12 (approximately 50.00%). Additionally, TCP-protein family pair-wise analysis among the sequences of the bHLH domain showed much higher homologies, e.g., 98.20% between CnTCP10 and CnTCP12 (Supplementary Table S4). Such a high degree of homology suggests that these two proteins may have similar structures and function. The structure of each AtTCP and CnTCP proteins are shown in Figure 3. The MEME motif search tool was used to identify the conserved motifs of TCPs domains in C. nankingense and Arabidopsis. Among the four distinct motifs identified, motif 1 was located in the bHLH domain (Figure 4), whereas motifs 2 and 3 were located at the upstream and downstream parts of motif 1, respectively. The amino acids in the front and back of motifs 2 and 3 were relatively conserved and located in the bHLH domain, while the other parts showed larger variation (Figures 3 and 4). The highly divergent, fast-evolving sequences outside the bHLH domain are essential for protein functional specificity. Outside the bHLH domain, an 18–20 amino acid residue, arginine-rich motif is also conserved in Arabidopsis and is specific to a subset of class II proteins (the R domain, motif 4) (Figures 3 and 4); this motif was predicted to form a coil that may mediate protein–protein interactions [5]. For instance, AtAP1 interacts with AtTCP24 but not with the closely related AtTCP3/5/13/17 proteins that also harbor almost identical TCP domains [64]. The R domain in Arabidopsis differed from its counterpart in C. nankingense in the number of amino acid residues and, consequently, related characteristics (Figures 3 and 4). Consistent with phylogenetic analysis and domain architecture, CnTCP3 and CnTCP14 harboring the R domain clustered together into the Class II subfamily (Figures 1 and 2). However, CnTCP18 harboring a predicted R domain clustered into Class I PCFs but lacked four amino acids in the basic bHLH domain. The differences in protein structure and motif prediction between C. nankingense and Arabidopsis might entail a unique function of CnTCP in C. nankingense.

Untranslated regions (UTRs) and introns present in genes may play regulatory roles, such as modulating mRNA stability or localization and translational efficiency [5,65–67]. Furthermore, introns can increase transcript levels by affecting the rate of transcription, nuclear export, and transcript stability, and by increasing the efficiency of mRNA translation [68]. Some AtTCP genes such as AtTCP1/8/12/13/18 have introns, while AtTCP12 and AtTCP24 have a long 3' UTR and 5' UTR. Although the annotation file of C. nankingense is not complete, several CnTCPs, such as CnTCP14/17/18, harbor long introns (Supplementary Figure S2).
Table 1. Percentage homology among full-length CnTCPs proteins.

| CnTCPs  | CnTCP4-1 | CnTCP4-3 | CnTCP4-2 | CnTCP14 | CnTCP2-3 | CnTCP2-2 | CnTCP2-1 | CnTCP13 | CnTCP5 | CnTCP17 | CnTCP3 | CnTCP15 |
|---------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| CnTCP10 | 10.70%   | 11.40%   | 11.00%   | 11.60%   | 11.80%   | 12.10%   | 12.10%   | 10.30%   | 12.90%   | 16.30%   | 12.30%   | 11.20%   |
| CnTCP12 | 11.20%   | 11.90%   | 11.20%   | 12.40%   | 11.30%   | 11.40%   | 11.30%   | 10.60%   | 14.20%   | 15.50%   | 12.00%   | 11.20%   |
| CnTCP16 | 14.10%   | 14.20%   | 14.10%   | 13.10%   | 15.50%   | 15.80%   | 12.90%   | 13.80%   | 16.10%   | 15.50%   | 15.20%   |
| CnTCP9  | 15.80%   | 15.70%   | 15.70%   | 11.80%   | 12.60%   | 12.70%   | 12.70%   | 13.20%   | 18.80%   | 17.60%   | 15.10%   | 14.40%   |
| CnTCP7  | 15.30%   | 14.80%   | 14.80%   | 14.70%   | 14.80%   | 15.00%   | 14.80%   | 12.00%   | 14.20%   | 13.80%   | 15.50%   | 16.80%   |
| CnTCP8  | 14.00%   | 13.40%   | 13.70%   | 9.40%    | 11.00%   | 11.00%   | 11.00%   | 12.20%   | 12.90%   | 12.80%   | 17.40%   | 14.10%   |
| CnTCP12-1| 11.80%   | 11.90%   | 11.80%   | 13.40%   | 13.60%   | 13.60%   | 13.80%   | 14.20%   | 15.50%   | 18.10%   | 16.40%   | 16.10%   |
| CnTCP12-2| 12.30%   | 12.40%   | 12.30%   | 14.00%   | 13.70%   | 14.00%   | 14.70%   | 16.00%   | 17.80%   | 16.50%   | 16.20%   |
| CnTCP6  | 16.40%   | 16.10%   | 16.40%   | 15.70%   | 14.90%   | 14.70%   | 14.60%   | 15.80%   | 17.60%   | 17.40%   | 14.80%   | 15.80%   |
| CnTCP1  | 19.80%   | 20.60%   | 20.60%   | 19.10%   | 17.60%   | 17.60%   | 17.60%   | 17.50%   | 19.70%   | 22.50%   | 31.10%   | 29.20%   |
| CnTCP11 | 20.70%   | 21.50%   | 20.80%   | 15.70%   | 17.40%   | 17.40%   | 17.30%   | 17.30%   | 19.10%   | 27.00%   | 25.90%   |
| CnTCP15 | 16.00%   | 16.50%   | 16.00%   | 17.60%   | 18.80%   | 18.80%   | 18.70%   | 16.00%   | 19.90%   | 46.20%   | 100%     |
| CnTCP3  | 16.50%   | 15.70%   | 16.10%   | 15.40%   | 17.00%   | 17.00%   | 16.90%   | 16.70%   | 17.80%   | 17.90%   | 100%     |
| CnTCP17 | 20.10%   | 20.30%   | 20.40%   | 25.30%   | 26.20%   | 26.10%   | 26.00%   | 37.20%   | 52.10%   | 100%     |
| CnTCP5  | 19.50%   | 20.10%   | 19.50%   | 23.90%   | 23.70%   | 23.70%   | 23.60%   | 34.70%   | 100%     |
| CnTCP13 | 19.90%   | 21.00%   | 21.00%   | 26.60%   | 24.60%   | 24.60%   | 24.80%   | 100%     |
| CnTCP2-1| 20.20%   | 19.40%   | 20.00%   | 57.40%   | 99.50%   | 99.80%   | 100%     |
| CnTCP2-2| 20.30%   | 19.50%   | 20.10%   | 57.10%   | 99.80%   | 100%     |
| CnTCP2-3| 20.40%   | 19.60%   | 20.10%   | 57.10%   | 100%     |
| CnTCP14 | 22.50%   | 21.90%   | 22.40%   | 100%     |
| CnTCP4-2| 93.80%   | 96.00%   | 100%     |
| CnTCP4-3| 90.10%   | 100%     |
| CnTCP4-1| 100%     |

| CnTCPs  | CnTCP11 | CnTCP1 | CnTCP6 | CnTCP18-2 | CnTCP18-1 | CnTCP8 | CnTCP7 | CnTCP9 | CnTCP16 | CnTCP12 | CnTCP10 |
|---------|---------|--------|--------|-----------|-----------|--------|--------|--------|---------|---------|---------|
| CnTCP10 | 12.40%  | 11.90% | 26.80% | 28.00%    | 28.30%    | 34.20% | 28.70% | 36.00% | 50.00%  | 68.10%  | 100%    |
| CnTCP12 | 11.60%  | 13.30% | 26.40% | 28.40%    | 28.60%    | 34.00% | 29.10% | 34.00% | 50.60%  | 100%    |
| CnTCP16 | 11.50%  | 13.80% | 27.40% | 31.30%    | 31.70%    | 38.10% | 35.70% | 39.30% | 100%    |
| CnTCP9  | 14.80%  | 18.20% | 28.70% | 31.50%    | 31.50%    | 28.90% | 33.10% | 100%   |
| CnTCP7  | 16.20%  | 16.20% | 28.80% | 30.20%    | 29.20%    | 38.50% | 100%   |
| CnTCP8  | 13.00%  | 14.50% | 28.80% | 29.70%    | 29.20%    | 100%   |
| CnTCP18-1| 14.10% | 16.20% | 41.70% | 97.30% | 100% |
| CnTCP18-2| 14.20% | 16.20% | 41.30% | 100% |
| CnTCP6  | 16.30%  | 13.90% | 100%   |
| CnTCP1  | 52.30%  | 100%   |
| CnTCP11 | 100%    |

Relatively high identities (>50%) were displayed in red font.
Figure 3. Predicted protein schematic structures of TCPs. The protein structure based on the TCP proteins from *Arabidopsis thaliana* (left) and *Chrysanthemum nankingense* (right); other additional domains are as identified by SMART. The bottom box indicates the motif number, symbol, and the motif consensus sequence of the corresponding domain. Actual motif length and order are presented for each protein.

Protein–protein interactions are the basis on which cellular structure and function are built, and interacting partners directly affect biological functions of each other. Here, the STRING online database (accessed on 28 October 2021, https://string-db.org/) and Cytoscape software were used to analyze the interactions between Arabidopsis TCPs and other proteins. A total of 123 proteins were filtered into a PPI network complex containing 835 edges (Supplementary Figure S3A). PPI networks revealed that cold stratification induced TCPs’ interactions with proteins GID1A and GID1B (Supplementary Figure S3A). TCP proteins were interacted with some circadian clock-related genes, such as TCP7/20/21/22 with TOC1, TCP18 and FT, and TCP7/11/22 and CCA1 (Supplementary Figure S3A). In this study, top one protein–protein interaction networks with the highest clustering score were established via MCODE analysis (Supplementary Figure S3B); furthermore, the top module comprised 19 proteins, including SPL/NZZ and most AtTCP proteins (Supplementary Figure S3B), which showed a complex interactive network of the TCP family.

| Name   | p-value     | Motif Locations | Name   | p-value     | Motif Locations |
|--------|-------------|-----------------|--------|-------------|-----------------|
| AtTCP1 | 7.11 X 10^{-49} | ![Motif](motif1.png) | CaTCP1 | 1.26 X 10^{-51} | ![Motif](motif2.png) |
| AtTCP2 | 8.94 X 10^{-70} | ![Motif](motif3.png) | CaTCP2 | 3.85 X 10^{-86} | ![Motif](motif4.png) |
| AtTCP3 | 1.65 X 10^{-53} | ![Motif](motif5.png) | CaTCP3 | 3.11 X 10^{-55} | ![Motif](motif6.png) |
| AtTCP4 | 1.50 X 10^{-54} | ![Motif](motif7.png) | CaTCP4 | 3.17 X 10^{-49} | ![Motif](motif8.png) |
| AtTCP5 | 2.76 X 10^{-52} | ![Motif](motif9.png) | CaTCP5 | 7.78 X 10^{-44} | ![Motif](motif10.png) |
| AtTCP6 | 4.93 X 10^{-44} | ![Motif](motif11.png) | CaTCP6 | 5.61 X 10^{-62} | ![Motif](motif12.png) |
| AtTCP7 | 4.19 X 10^{-64} | ![Motif](motif13.png) | CaTCP7 | 7.14 X 10^{-60} | ![Motif](motif14.png) |
| AtTCP8 | 2.10 X 10^{-71} | ![Motif](motif15.png) | CaTCP8 | 3.62 X 10^{-69} | ![Motif](motif16.png) |
| AtTCP9 | 2.51 X 10^{-62} | ![Motif](motif17.png) | CaTCP9 | 2.36 X 10^{-62} | ![Motif](motif18.png) |
| AtTCP10| 2.89 X 10^{-53} | ![Motif](motif19.png) | CaTCP10| 5.89 X 10^{-72} | ![Motif](motif20.png) |
| AtTCP11| 3.88 X 10^{-51} | ![Motif](motif21.png) | CaTCP11| 1.07 X 10^{-55} | ![Motif](motif22.png) |
| AtTCP12| 6.71 X 10^{-54} | ![Motif](motif23.png) | CaTCP12| 5.93 X 10^{-71} | ![Motif](motif24.png) |
| AtTCP13| 5.09 X 10^{-53} | ![Motif](motif25.png) | CaTCP13| 2.05 X 10^{-44} | ![Motif](motif26.png) |
| AtTCP14| 8.40 X 10^{-70} | ![Motif](motif27.png) | CaTCP14| 2.86 X 10^{-91} | ![Motif](motif28.png) |
| AtTCP15| 1.40 X 10^{-71} | ![Motif](motif29.png) | CaTCP15| 1.74 X 10^{-44} | ![Motif](motif30.png) |
| AtTCP16| 7.54 X 10^{-37} | ![Motif](motif31.png) | CaTCP16| 5.91 X 10^{-69} | ![Motif](motif32.png) |
| AtTCP17| 1.25 X 10^{-54} | ![Motif](motif33.png) | CaTCP17| 7.70 X 10^{-40} | ![Motif](motif34.png) |
| AtTCP18| 1.05 X 10^{-51} | ![Motif](motif35.png) | CaTCP18| 4.09 X 10^{-94} | ![Motif](motif36.png) |
| AtTCP19| 1.15 X 10^{-61} | ![Motif](motif37.png) |        |             |                 |
| AtTCP20| 2.74 X 10^{-59} | ![Motif](motif38.png) |        |             |                 |
| AtTCP21| 5.41 X 10^{-61} | ![Motif](motif39.png) |        |             |                 |
| AtTCP22| 1.60 X 10^{-72} | ![Motif](motif40.png) |        |             |                 |
| AtTCP23| 6.80 X 10^{-66} | ![Motif](motif41.png) |        |             |                 |
| AtTCP24| 1.43 X 10^{-68} | ![Motif](motif42.png) |        |             |                 |

| Motif Symbol | Motif Consensus |
|---------------|-----------------|
| 1. | DRRTKVGGRDRRRLPACARVFQTLRELGKSDGTFIEWLLQQAEPAI |
| 2. | IVEPRVRVSRTGGKDRHS |
| 3. | IAAATGTGTPANFSLNSLSSST |
| 4. | KELRAKARERARERATAEMKMR |
2.3. Expression Profiling of CnTCP Genes in Response to Cold Stress

Some TCP genes are involved in plant abiotic stress responses, such as in cold [39], salt [8], and drought stress [8,9]. At the molecular level, such changes induced by adverse conditions are mainly mediated by transcription factors binding to specific recognition sequences upstream from the specific stress response genes (cis-elements) for transcriptional regulation. In this study, to further elucidate the possible regulatory mechanisms underlying the expression of CnTCP genes during abiotic/biotic stress responses, the corresponding promoter sequences were analyzed using the PlantCARE online database to search cis-elements in the promoter region within 3000 bp upstream from the initiation codons of TCPs. At least 60 cis-regulatory elements were identified in CnTCP and AtTCP promoters (Supplementary Table S5). The upstream regions of most CnTCP and AtTCP genes contained at least one phytohormone-related element, such as abscisic acid-responsive element (ABRE), ethylene-responsive element (ERE), gibberellin-responsive element (P-box), MeJA-responsive element (CGTCA-motif and TGACG-motif), and salicylic acid-responsive element (TCA element) (Supplementary Table S6). Our results showed the same trend previously observed in five legume species [8]. Furthermore, stress-related elements were found in the promoters of CnTCPs and AtTCPs, such as low-temperature
responsive (LTR) elements, drought-inducibility (MBS), and defense and stress responsiveness TC-rich repeats (Supplementary Table S6). These results indicate that TCP genes might play critical roles in plant responses to a range of abiotic stress conditions [9,69]. Furthermore, most CnTCPs and AtTCPs promoters seemingly harbor the predicted binding sites of transcription factors such as AP2, ERF, bHLH, MYB, WRKY, and TCP (Table 2). There are several additional binding sites that contribute to cold stress mitigation, such as the ABA-responsive elements (ABRE), the G-box, and the MYC binding sites (Supplementary Table S6).

Table 2. Statistical analysis of TFs identified from CnTCP promoter sequences.

| Family          | AtTCP | CnTCP | Family          | AtTCP | CnTCP |
|-----------------|-------|-------|-----------------|-------|-------|
| AP2             | 24    | 18    | TCP             | 22    | 17    |
| B3              | 24    | 18    | ARF             | 24    | 16    |
| bHLH            | 24    | 18    | BBR-BPC         | 22    | 16    |
| bZIP            | 24    | 18    | WRKY            | 24    | 16    |
| C2H2            | 24    | 18    | ZF-HD           | 22    | 16    |
| Dof             | 24    | 18    | LBD             | 21    | 16    |
| ERF             | 24    | 18    | SBP             | 21    | 16    |
| G2-like         | 24    | 18    | C3H             | 20    | 16    |
| GATA            | 24    | 18    | WOX             | 22    | 15    |
| HD-ZIP          | 24    | 18    | CPP             | 21    | 15    |
| MIKC_MADS       | 24    | 18    | EIL             | 18    | 15    |
| MYB             | 24    | 18    | NF-YB           | 16    | 15    |
| MYB_related     | 24    | 18    | E2F/DP          | 23    | 14    |
| NAC             | 24    | 18    | Nin-like        | 22    | 14    |
| Trihelix        | 23    | 18    | RAV             | 22    | 14    |
| GRAS            | 23    | 17    | SRS             | 21    | 13    |
|                 |       |       | ARR-B           | 20    | 7     |

Data indicate the number of TCP genes with predicted TFs.

Gene expression patterns provide important information for gene function analysis. Downregulation of the expression of two TCP genes in rice, OsPCF5, and OsPCF8 results in enhanced cold tolerance [54]. We tested the transcriptional behavior of CnTCPs after a 7 d cold stress in C. nankingense. As shown in Figure 5, the expression level of CnTCP14 under 22 °C room temperature (RT) was comparable to that under 4 °C low temperature (LT); furthermore, when the expression level of CnTCP14 under RT was used as the normalized control, most of the TCP genes’ expression levels were downregulated under 4 °C low temperature, including CnTCP3/4/6/7/8/9/10/11/12/15/16/17/18 (i.e., LT treatment). The results have shown that CnTCP5/13/14 have no expression changed after cold tolerance, while CnTCP2 upregulated (Figure 5). Our phylogenetic tree and bHLH domain analyses (Figures 1 and 2) showed that CnTCP2/4/5/13/14/17 clustered together into the Class II CIN group. Although the CnTCP4/5/13/17 proteins have the same protein structure, as well as CnTCP2/14 (Figure 3), the bHLH motif sequence differences (Figure 2) may lead to differences in their gene functions.
A previous study reported the importance of timing and expression levels of miR319 and its target genes involved in cold tolerance [54,55]. The Arabidopsis miR319a and miR319b precursor sequences were used to search the miR319 sequences in the transcriptome of C. nankingense (Supplementary Table S1). Due to the two members of miR319 precursor in C. nankingense sharing the same mature sequences with the mature sequence of Arabidopsis miR319, 5′ TTGGACTGAAGGGAGCTCCC 3′, this sequence was selected for expression validation and further functional analysis. As shown for other species, mature miR319 was also predicted to target the expression of CnTCP2, CnTCP4, and CnTCP14. The critical miRNA-target pairing region spans the nucleotides 2–13, which were perfect matches (Figure 6A). Stem-loop qPCR analysis showed that the expression level of mature miR319 was rapidly upregulated after 6 h of LT treatment, but decreased to control (0 h) level after 24 h (Figure 6B). Some studies have shown the EF-1α gene was sufficient for accurate normalization in cold-treated samples, and the EF-1α gene was the frequently-used internal control in the genus chrysanthemum for normalizing transcription data [70–72]. When performing microRNA detection, because a better strategy is choosing a constitutively expressed small noncoding RNA as a reference, we often chose the U6 gene [73–75]. The U6 gene sequence of C. nankingense was retrieved using the AtU6 gene sequence from Arabidopsis (Supplementary Table S1). Interestingly, the expression levels of CnTCP2, CnTCP4, and CnTCP14 were significantly downregulated at 6 h at 4 °C, concomitantly
with a significant upregulation of miR319 expression. Similarly, the expression level of CnTCP4 tended to be upregulated, consistent with the downregulated expression of miR319 observed at 24 h (Figure 6B). As shown in Figure 6B, there is a significant difference between the expression of CnTCP2 or 14 with CnTCP4. Compared with the expression patterns of CnTCP2 and CnTCP14, which were first downregulated and then stabilized, the trend of first downregulation and then upregulation of CnTCP4 was more consistent with the expression pattern of miR319, indicating that CnTCP4 may be more dependent on the regulation of miR319. Moreover, after one week of cold acclimation, CnTCP4 was still downregulated compared with the control (Figure 5B), indicating that CnTCP4 may maintain a continuous function during cold acclimation compared to CnTCP14 and CnTCP2.

![Figure 6. Mature miR319 and predicted target CnTCP expression in response to cold stress. (A) Predicted CnTCP transcript targets of miR319. (B) Expression levels of mature miR319 and its predicted targets CnTCP2/4/14 in two-month-old Chrysanthemum nankingense plants treated at 4 °C for 0, 6, and 24 h. U6 and EF-1α were used as the internal control in miR319 and CnTCP expression detection, respectively. Values are means ± SD. The different capital letters represent significant differences at p < 0.01 according to the Duncan's test.

2.4. CnTCP4 Transcription Factors Are Involved in Cold Stress Response

Next, we selected CnTCP4 to test its potential role in cold acclimation using CnTCP4 overexpressing Arabidopsis lines previously generated by our group [60]. The expression levels of CnTCP4 in transgenic plants and wildtype are shown in Supplementary Figure S4A, which showed obviously higher expression levels in overexpressing CnTCP4 Arabidopsis plants. In Figure 7B and C, OE-CnTCP4 Arabidopsis showed more wilting and yellowing leaves than the wildtype (Figure 7A) after freezing treatment. Thus, CnTCP4 overexpressing lines showed higher sensitivity to freezing after cold acclimation. To further determine the mechanism of action of CnTCP4 on cold tolerance in Arabidopsis, we measured chlorophyll, soluble sugar, and malondialdehyde (MDA) contents in CnTCP4 overexpressing Arabidopsis after cold acclimation for two days. Compared to wildtype Arabidopsis thaliana, CnTCP4 overexpression decreased chlorophyll (Figure 7D) and soluble sugar contents (Figure 7E), while MDA content increased compared with that in control plants (Figure 7F). Previous studies have reported that expression of CBF genes was rapidly induced when Arabidopsis plants were exposed to low temperature (4 °C) [76,77]. Conversely, our findings show that CnTCP4 negatively regulated the expression of cold-induced genes AtCBF1/2/3 (Figure 7G) and the CBF-regulated COR genes AtCOR15A and AtKIN1 (Supplementary Figure S4B).
Figure 7. CnTCP4 negatively regulated cold acclimation. Sensitivity of cold acclimated plants to freezing. (A–C) show the wildtype Arabidopsis, CnTCP4-2, and CnTCP4-3 overexpressing seedlings after a 2-day recovery from a 20 min freezing treatment at −20 °C, respectively. The leaves with wilting and yellowing phenotypes were labeled by red arrows. (D–F) show the chlorophyll, soluble sugar, and malondialdehyde (MDA) contents in 4-week-old CnTCP4 overexpressing Arabidopsis seedlings exposed to 4 °C for two days, respectively. (G) 8-day-old seedlings of OECnTCP4 Arabidopsis were treated at 4 °C for 0, 6, and 24 h. Expression of cold-related genes AtCBF1/2/3 were assessed by performing quantitative real-time PCRs. ACTIN8 was used as the internal control. Values are means ± SD. One and two asterisks represent significant differences at p < 0.05 and p < 0.01, respectively, according to the Duncan’s test.

3. Discussion

The plant-specific TCP family comprises a group of transcription factors that influence cell proliferation and differentiation and consequently regulate growth and developmental processes. Furthermore, TCP family members have been identified in a range of plant species. Although plant-specific TCP genes have been thoroughly analyzed in numerous species, systematical analysis of this gene family in C. nankingense had not been reported. In this study, phylogenetic and structural analyses of 23 CnTCP genes were conducted, and our results revealed the existence of fewer TCP genes in C. nankingense than in Arabidopsis [5], maize [9], or soybeans [8], suggesting that these few CnTCPs might perform multiple functions and be part of gene regulatory networks.

Furthermore, at least in some cases, interaction partners might reportedly alter TCP functional specificity [30]. Some interactions with circadian clock-related genes were identified, such as the interactions between TCP7/20/21/22 and TOC1, between TCP18...
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TCP with TCPs (Supplementary Figure S3), thus providing new insights into Osa-miR319b overexpressing lines to a significantly greater extent than that in the wildtype; AtCBF1 transcripts [81]. Additionally, PPI networks revealed GID1A and GID1B interactions with TCPs (Supplementary Figure S3), thus providing new insights into TCP-mediated cold response mechanisms. In rice, expression levels of Osa-MIR319a and Osa-MIR319b are downregulated after 24 h incubation at 4 °C [54]. However, miRNA319 expression in sugarcane reportedly increases after 24 h of cold stress and then returns to the baseline level at 48 h after treatment initiation [55]. Similarly, here, miRNA319 expression in C. nankingense increased after 6 h of cold stress and returned to the baseline level after 24 h of treatment initiation (Figure 6B). This finding suggests that the response of miRNA319 to cold stress is a dynamic and complex process. Importantly, the corresponding change in the expression of the predicted miR319-target gene CnTCP4 was observed in response to cold tolerance (Figure 6B), which allowed us to infer that CnTCP4 might be an important gene involved in cold acclimation. Indeed, the overexpression of CnTCP4 in Arabidopsis negatively regulated cold acclimation by downregulating cold-induced genes such as AtCBF1/2/3, AtCOR15A, and AtKIN1 (Figure 7G and Supplementary Figure S4B) and reducing physiological indicators such as chlorophyll and soluble sugar contents, while increasing MDA content (Figure 7). At the same time, we found that higher CnTCP4 expression (Supplementary Figure S4A) caused stronger inhibition of the expression of the CBF genes (Figure 7G) and higher sensitivity phenotype to cold treatment (Figure 7A–C), suggesting that there may be a dose effect of CnTCP4 during plant cold tolerance regulation. Cold stress-related marker genes, such as DREB1/2A, were induced in Osa-miR319b overexpressing lines to a significantly greater extent than that in the wildtype; moreover, a great increase in transcript levels of DREB1A and DREB2A was observed in the OsPCF6- and OsTCP21-RNAi lines, concomitant with a decrease in overexpressing lines [39]. Therefore, we hypothesized that TCP family genes may be involved in the regulation of plant cold tolerance by directly regulating the expression level of downstream cold response-related target genes. All 18 TCPs form a network and interact with each other (Supplementary Figure S3B), consistent with a previous report according to which TCPs form homo- and heterodimers [1,5]. DNA binding seemingly requires dimer formation [40]. Furthermore, homo- and heterodimer interactions show different DNA-binding efficiencies, or mutually modulate their activity, and possibly bind to slightly different cis-regulatory elements [82]. However, whether CnTCP4 or its interaction complex binds directly to the cold-related marker genes warrants further research.

Meanwhile, the target TCP genes of miRNA319 might also be regulated by other transcription factors, such as AP2, ERF, bHLH, MYB, WRKY, and TCP encoded TFs, which might bind to cis-regulatory elements in CnTCPs and AtTCPs promoters (Supplementary Table S5). Moreover, in Arabidopsis, TCP2 could interact with the cryptochrome 1 (CRY1) protein in yeast and plant cells and be a transcription activator which acts downstream of CRY1 [83]. CRY1 regulates both basal and acquired freezing tolerance in Arabidopsis [84]. The Class I TCP family members with which CnTCP14 was adjacent with CnTCP2 in the evolutionary tree (see Figure 1) also have the same sequence of bHLH domains (see Figure 2) and same protein structures (see Figure 3). Therefore, CnTCP14 may play the same function as CnTCP2. These results suggest that TCP2 and TCP14 are not only regulated
by miR319 during cold acclimation, but these members may also be regulated by other transcriptional regulators.

4. Materials and Methods
4.1. Phylogenetic Analysis of the CnTCP Family

To investigate the phylogenetic relationship of the TCP family, the conserved bHLH-domain amino-acid sequences of CnTCPs identified in C. nankingense, H. annuus, Arabidopsis, maize, rice, and soybeans were aligned using the multiple sequence alignment program ClustalX. A neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA 7.0 program with the bootstrap value set at 1000 [85]. The TCP gene sequences from C. nankingense used for analysis were chosen according to previous studies of our group [57–60,62,86]. The TCP gene sequences from Arabidopsis, maize, rice, and soybean used for analysis were chosen according to previous studies [5,8,9]. To identify all TCP proteins in H. annuus, all proteins were downloaded from the Sunflower Genome Database [https://www.sunflowergenome.org/, accessed on 18 February 2022] [63]. The Hidden Markov Model (HMM) profile of the TCP domain (PF03634) was downloaded from Protein family (Pfam) [http://pfam.sanger.ac.uk/, accessed on 18 February 2022] and used for identifying TCP proteins from the downloaded database of Sunflower Genome using the HMM search of TTools [87]. The percent identities among full-length CnTCP proteins from C. nankingense or the bHLH motif sequences of TCP proteins from other species were analyzed using the DNAMAN software.

4.2. Structural Analysis of the TCP Genes

Information about the gene structure for Arabidopsis TCP genes was obtained from the gff3 file GCF_000001735.4_TAIR10.1_genomic downloaded from the website [https://www.ncbi.nlm.nih.gov/genome/browse#!/overview/, accessed on 18 February 2022]. All 23 C. nankingense CnTCP gene sequences were selected to blast the most homologous genes in the C. nankingense genome scaffolds and protein files [http://www.amwayabrc.com/, accessed on 18 February 2022] for searching the accession numbers. For C. nankingense TCP genes, information about the gene structures was procured from the gff3 file Chrysanthemum_genome_gene_v2.0 using the searched accession numbers. The TBtools software was used to draw the gene structure diagram [87].

Protein sequences were analyzed in the MEME program [http://meme.sdsc.edu/meme/cgi-bin/meme.cgi, accessed on 18 February 2022] to confirm the conserved motifs. The MEME program was used under the following parameters: zero or one occurrence per sequence (zoops), maximum number of motifs—4, and optimum motif width set to >6 and <50. The psRNATarget website was used for miR319 target gene prediction [https://www.zhaolab.org/psRNATarget/, accessed on 18 February 2022]. The mature sequences of miR319 and all CnTCPs family sequences were used for target site analyses.

4.3. Protein–Protein Interaction Network Analysis

Protein–protein interaction networks of CnTCPs were analyzed using the search tool for Retrieval of Interacting Genes (STRING; [http://stringdb.org/, accessed on 18 February 2022] online). The screened networks were visualized by Cytoscape 3.9.0. The MCODE software was used to establish PPI network modules using parameters of degree cutoff 2, k-core 2, max. depth 100, and node score cutoff 0.2.

4.4. Putative Cis-Elements in the Promoter Regions

The 3000-bp upstream sequences from the translation start codon of all CnTCP family genes were obtained from the C. nankingense Genome scaffolds files [http://www.amwayabrc.com/, accessed on 18 February 2022]. The cis-elements of promoters were identified using the PlantRegMap: Binding Site Prediction [http://plantregmap.gao-lab.org/, accessed on 18 February 2022] [88] and PlantCARE: Search for CARE [89].
4.5. Plant Growth Conditions and Cold Treatment

Diploid *C. nankingense* was obtained from the Chrysanthemum Germplasm Resource Preserving Center at Nanjing Agricultural University, China. Plants were grown under a 22/15 °C day/night temperature conditions, at a relative humidity of 70–75%, and a 16/8 h (day/night) photoperiod. For cold stress, plants were subjected to 4 °C during the day/night and a 16/8 h photoperiod environment. CnTCP4 overexpressing Arabidopsis was previously generated by our group [22,60]. For the freezing test, four-week-old plants were cold-acclimated (at 4 °C) for two days and then subjected to freezing at −20 °C for 20 min; finally, they were observed after a 2 d recovery at 23°C. Chlorophyll, soluble sugar, and malondialdehyde (MDA) contents in OECnTCP4 Arabidopsis were measured after cold stress according to the methods previously described [90–92]. Samples were harvested without any additional treatment. For each treatment, aboveground plant biomass was harvested. For cold treatment shown in Figures 6 and S4B, the plant part of two-month old plants of *C. nankingense* from the apical bud to the third fully expanded leaf were harvested.

4.6. Verification of CnTCP Expression

Total RNA was extracted from each sample. After digestion with RNase-free DNase I (TaKaRa; Tokyo, Japan) and reverse transcription with Reverse Transcription M-MLV (TaKaRa), about 100 ng cDNA product was used for each reaction (10 μL 2 × SYBR® Premix Ex TaqTM II and 0.5 μL of 10 μM primer pairs mixed in a 20 μL reaction system). DNA was extracted from the OECnTCP4 and wildtype Arabidopsis seedlings using the CTAB method. The chrysanthemum EF-1α gene (Genbank accession number KF305681) and Arabidopsis ACTIN8 gene were used as reference genes, respectively. Primers used in this study are listed in Supplementary Table S1.

5. Conclusions

Here, we identified 23 CnTCP genes in *C. nankingense*, systematically analyzed their phylogenetic relationships and synteny with TCPs from other species, and evaluated their expression profiles at low temperature. A total of 23 CnTCP proteins fall into two classes and three clades, with a typical bHLH domain. Predicted protein structure and binding sites analysis suggested a unique function of CnTCPs in *C. nankingense*. Expression of most CnTCPs were downregulated under cold conditions, suggesting their importance in plant responses to cold stress. Notably, expression of miR319 and of its predicted target genes, CnTCP2/4/14, led to fast responses to cold. Overexpression of CnTCP4 in Arabidopsis led to hypersensitivity to cold, suggesting that CnTCP4 might play a negative role in *C. nankingense* responses to cold stress. Our results provide a foundation for future functional genomic studies on this gene family in chrysanthemum.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11070936/s1, Figure S1. Evolutionary tree of TCP proteins; Figure S2. Gene structure of TCPs; Figure S3. Co-expression network analysis of TCP proteins and correlated proteins; Figure S4. Expression of cold-related genes after cold stress and identification of OECnTCP4; Figure S5. Overexpression of CnTCP4 and CnTCP13 delayed bolting in Arabidopsis thaliana; Table S1. Primers and sequences used in this research; Table S2. Distance and homology matrices of 171 sequences were generated with the DNAMAN program using the full-length amino acid sequences from Glycine max, Oryza sativa, Zea mays, Chrysanthemum nankingense, and Arabidopsis thaliana; Table S3. The distance and homology matrix of 171 sequences were generated with the DNAMAN program using the bHLH amino acid sequences from Glycine max, Oryza sativa, Zea mays, Chrysanthemum nankingense, and Arabidopsis thaliana; Table S4. Distance and homology matrices of 23 sequences were generated with the DNAMAN program using the full-length and bHLH amino acid sequences from Chrysanthemum nankingense; Table S5. Binding site prediction results of TCP promoters from Chrysanthemum nankingense and Arabidopsis thaliana using the PlantRegMap website; Table S6. Cis-acting regulatory element prediction results of TCP promoters from Chrysanthemum nankingense and Arabidopsis thaliana using the PlantCARE website.
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