



Genome-Wide Identification and Characterization of TCP Family Genes in Pak-Choi [*Brassica campestris* (syn. *Brassica rapa*) ssp. *chinensis* var. *communis*]

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The TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP) gene family, a kind of plant specific transcription factor, is essential for stress response, cell growth, and cell proliferation. However, the characterization of TCP family is still not clear in Pak-choi [*Brassica campestris* (syn. *Brassica rapa*) ssp. *chinensis* var. *communis*]. In this study, genome-wide analysis of TCP gene family was performed and 26 TCP genes were identified in Pak-choi. Phylogenetic analysis demonstrated that the 26 BcTCPs were divided into two classes: Class I and Class II. Class II was further classified into two subclasses, CIN and CYC/TB1. The qPCR results suggested that most BcTCPs respond to abiotic stresses. The expressions of BcTCP3, BcTCP12, BcTCP21, and BcTCP22 were significantly changed under ABA and cold treatment. BcTCP3 and BcTCP12 were also up-regulated under osmotic treatment. Subcellular localization showed that BcTCP3 and BcTCP21 were located in the nucleus. Our results will facilitate revealing the functions and regulatory mechanisms of BcTCPs.

Keywords: abiotic stress, expression analysis, subcellular localization, TCP, Pak-choi

INTRODUCTION

The TCP gene family is a class of plant-specific transcription factors and plays important roles in plant growth and development. TCP was named from four genes members: TEOSINTE BRANCHED 1 (TB1) from maize (*Zea mays*) (Doebley et al., 1995), CYCLOIDEA (CYC) from snapdragon (*Antirrhinum majus*) (Luo et al., 1996), and the PROLIFERATING CELL FACTORS 1 (PCF1) and PCF2 from rice (*Oryza sativa*) (Kosugi and Ohashi, 1997). TB1 is a key factor to control apical dominance in maize (Doebley et al., 1997). CYC involves in the regulation of floral asymmetry in snapdragons. PCF1 and PCF2 bind to the promoter of PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) gene in rice, which involves in DNA replication and repair, maintenance of chromatin structure, chromosome segregation, and cell-cycle progression (Luo et al., 1996; Kosugi and Ohashi, 1997). TCP proteins contain a 59-amino-acid domain with a bHLH motif that involved in DNA binding and protein-protein interaction (Martin-Trillo and Cubas, 2010). According to the

conserved domain, TCP proteins can be divided into two classes: Class I (TCP-P Class) and Class II (TCP-C Class) (Kosugi and Ohashi, 2002; Navaud et al., 2007). Class II can be further divided into two subclades: CIN and CYC/TB1 (Martin-Trillo and Cubas, 2010). Class I includes *PCF1* and *PCF2* in rice, *TCP8*, *TCP9*, *TCP14*, *TCP20*, etc. in *Arabidopsis*. Class II contains *TB1* in maize, *TCP1*, *TCP2*, *TCP4*, *TCP10*, *BCR1*, *BCR2*, etc. in *Arabidopsis*.

Abiotic stress has an important effect on plant growth and productivity. Cold stress slows down the metabolic processes of plants, inhibits auxin transport and root elongation (Shibasaki et al., 2009). Salt stress leads to the accumulation of toxic ions in cells, which influences plant absorption of nutrients and water (Boudsocq and Laurière, 2005). Plants are subjected to dehydration under low temperature, high salinity, and drought environmental conditions, of which cause osmotic stress, inducing Abscisic acid (ABA) biosynthesis. ABA functions in multiple stresses, regulating genes related to dehydration and cold stress and controlling osmotic stress tolerance (Yamaguchi-Shinozaki and Shinozaki, 2006; Fujita et al., 2011). Moreover, ABA can reduce water loss by governing stomatal closure (Kim et al., 2010). The growth and development of plants will be severely affected when plants suffer from abiotic stresses. Hence, the study of response mechanisms during abiotic stresses is vital for species. At present, it is reported that many genes of the TCP family are related to various stresses. *TCP20* responds to nitrate availability by interacting with NIN-like protein 6 (NLP6) and NLP9, controlling plants root growth (Guan et al., 2017). *OsTCP19* is up-regulated under drought, salt, and cold stress, indicating *OsTCP19* may involve in the stress tolerance (Mukhopadhyay and Tyagi, 2015). In *Cicer arietinum*, five TCP genes (*CaTCP3/13/15/20/21*), which contained the MYB *cis*-elements, were strongly induced under drought conditions, and similar results were found in other legumes (Ling et al., 2020). *Oryza sativa miR319* (*Osa-miR319*), an upstream gene of *OsPCF5*, *OsPCF6*, *OsPCF8*, and *OsTCP21*, is decreased in the cold condition in rice. Meanwhile, overexpressing *Osa-miR319* results in down-regulation of its downstream target genes and enhances cold resistance, implying that *TCP21* could involve in cold stress (Yang et al., 2013; Wang et al., 2014). In creeping bentgrass (*Agrostis stolonifera*), overexpressing *Osa-miR319* also reduces the expression of its target genes (*AsPCF5*, *AsPCF6*, *AsPCF8*, and *AsTCP14*), enhancing salt and drought tolerance (Zhou et al., 2013). *GmTCP8* interacts with *GmPYL10* and involves in the ABA signal pathway in soybean (Feng et al., 2018). *ZmTCP42* is associated with ABA and drought stress, which plays an active role in drought tolerance (Ding et al., 2019). *AtTCP14* represses *ABA1* (*ABA DEFICIENT 1*) and other ABA-related stress genes in *Arabidopsis* seeds (Rueda-Romero et al., 2012).

Until now, the study of the TCP family has been more and more comprehensive, which have been identified in many species including *Arabidopsis thaliana* (Li, 2015), rice (*Oryza sativa*) (Yao et al., 2007), turnips (*Brassica rapa ssp. rapa*) (Du et al., 2017), poplar (*Populus trichocarpa*) (Ma et al., 2016), tomato (*Solanum lycopersicum*) (Parapunova et al., 2014), Switchgrass (*Panicum virgatum* L.) (Huo et al., 2019), *Brassica rapa* and *Brassica*

oleracea (Liu et al., 2019). Pak-choi [*Brassica campestris* (syn. *Brassica rapa*) ssp. *chinensis* var. *communis*] has gradually become worldwide vegetable crops. However, Pak-choi TCP genes and their regulatory mechanisms are still not clear.

In this study, genome-wide analysis of TCP family genes in Pak-choi was performed. 26 *BcTCP* genes were identified in Pak-choi. Phylogenetic relationship, subcellular localization, and conserved motifs were analyzed. QPCR was employed to illustrate the expression patterns of *BcTCPs* under salt, osmotic, cold, and ABA treatment. The results showed that *BcTCPs* may involve in multiple abiotic stresses. Our findings provide a theoretical basis for further research on the potential functions and regulatory mechanisms of *BcTCP* genes.

MATERIALS AND METHODS

Cloning and Identification of *BcTCP* Gene Family Members

Total RNA was extracted from Pak-choi using the RNeasy Mini Kit (Tiangen, Beijing, China). The CDSs of the *BcTCPs* were amplified with gene specific primers based on the sequences of *AtTCPs* by homology cloning (Huang et al., 2019 and **Supplementary Table 1**). The PCR products were cloned into the pMD18-T vector before sequencing. Then, the obtained plasmids were sequenced by TSINGKE (Nanjing, China). All putative *BcTCP* proteins sequences were analyzed using the Pfam database¹. Additionally, protein molecular weight, isoelectric point, and amino acid length of *BcTCPs* were computed by the ExpASy ProtParam tool².

Phylogenetic Tree and *Cis*-Acting Elements Analysis

24 *AtTCP* genes were retrieved from TAIR³. Multiple sequence alignments of putative *BcTCPs* and *AtTCPs* were performed using Clustal X 2.1. Phylogenetic tree was completed by the Maximum Likelihood (ML) method using MEGA 7.0 software. The bootstrap values were performed with 1,000 replications. PlantCARE⁴ was used to analyze *cis*-acting elements in promoters of each *BcTCP* gene. After classifying and counting the elements on each promoter (**Supplementary Table 2**), the heat map was made by TBtools.

Conserved Domain Analysis

The conserved motifs were analyzed using MEME program⁵ with the default settings except the maximum width was set to 200, and the minimum and maximum numbers of motifs were defined as 2 and 10, respectively.

¹<http://pfam.xfam.org/>

²<https://web.expasy.org/protparam/>

³<https://www.arabidopsis.org/>

⁴<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>

⁵<http://meme-suite.org/>

Plant Materials and Growth Conditions

The Pak-choi cultivar '49caixin' was selected in our experiment, which has a short life cycle and may flower within two months after sowing under long-day conditions (Tian et al., 2004). Plants were cultivated in pots with medium (Soil matrix and vermiculite, 1:1). All seedlings were grown in a controlled artificial climatic chamber under the same conditions (16 h light at 22°C/8 h dark at 18°C, 60-70% relative humidity). One-month-old seedlings were treated with 100 μ M ABA, 250 mM NaCl and 20% PEG6000, respectively. For cold treatment, one-month-old seedlings were transferred to 4°C. The leaves were collected under each stress treatment in a continuous time course (0, 10, 20, 30, 40, 50 min, 1, 2, 3, 4, 8 h) and stored at -70°C immediately for RNA extraction.

Expression Analysis of *BcTCP* Genes by qPCR

Following the manufacturer's instructions, total RNA was isolated from leaves using the RNA easy mini kit (Tiangen, Beijing, China). Then first-strand cDNA was synthesized with a RevertAid First Strand cDNA Synthesis Kit (Thermo, Shanghai, China). Primers for qPCR were designed using Primer 5.0 (Supplementary Table 1). QPCR was performed using the SYBR® Premix Ex Taq kit (Takara, Dalian, China). *BcActin* was used as an internal reference gene. Results were calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Subcellular Localization Analysis

WOLF PSORT⁶ was used to predict the subcellular localization of the putative *BcTCP* proteins. To further confirm their subcellular localization, the protein-coding regions of *BcTCP3* and *21* without the termination codon were amplified and then cloned into pCambia 1,302 vector in fusion with GFP at C-terminal end (35S: *GFP*), generating the fusion constructs. The 35S: *BcTCP3-GFP* and 35S: *BcTCP21-GFP* were generated. Each plasmid was injected into the tobacco leaves by *Agrobacterium tumefaciens*-mediated transient transformation (strain GV3101). The tobacco was incubated for 12 h under darkness. 2-3 days after injection, the fluorescence of GFP was observed and photographed by confocal microscopy (Leica, TCS SP2, Wetzlar, Germany). DAPI (nucleus specific dye) was used as nuclei dye in the experiment.

RESULTS

Identification of *TCPs* in Pak-Choi

A total of 26 *TCP* genes in Pak-choi were obtained by homology cloning based on the *Arabidopsis thaliana* *TCP* genes. These 26 genes were named *BcTCP1*~*BcTCP26*. The CDS length of the 26 *BcTCPs* was ranged from 651 to 1,395 bp. The physical and chemical properties were further analyzed using the ExpASY ProtParam tool. The molecular weight of 26 putative *BcTCP* proteins varied from 23.12 to 56.74 kDa. The isoelectric point was

ranged from 5.49 to 9.99, and the amino acid length was ranged from 216 to 520 aa (Supplementary Table 3).

Phylogenetic Analysis

An unrooted phylogenetic tree was constructed with the Maximum Likelihood (ML) method (Figure 1). 26 *BcTCPs* were divided into two classes, Class I and Class II. The Class II *TCPs* were further categorized into two subgroups, CIN and CYC/TB1. PCF Class (Class I) contained 13 genes, CYC/TB1 Class contained 7 genes, CIN only contained 6 genes. Sequence analysis showed that *AtTCP3*, 6, 11, 13, 16, 17, 20, 23 had no ortholog in Pak-choi. *AtTCP1*, 7, 15, 18, 21, and 24 had more than one ortholog in the Pak-choi.

Conserved Domain Analysis

The conserved motifs of *BcTCP* proteins were analyzed using MEME. A total of 7 motifs were found in *BcTCPs*. The motifs were more similar in the same class. Furthermore, similar conserved motifs arrangements can be observed in the same clade. Motif 1 was conserved in all *BcTCPs*. Motif 3 and motif 6 only existed in Class II except for *BcTCP5*, 6, 11, and 13 (Figure 1), suggesting that these *BcTCPs* may have similar functions. However, proteins among different groups shared diverse motifs. Motif 2 is presented in Class I (PCF) except for *BcTCP20*. Motif 7 was only existed in *BcTCP15*, 16, and 17. Motif 4 and motif 5 both existed in *BcTCP7*, *BcTCP8*, *BcTCP21*, *BcTCP22*, and *BcTCP23*. These results indicated that *TCP* genes may perform different functions in Pak-choi.

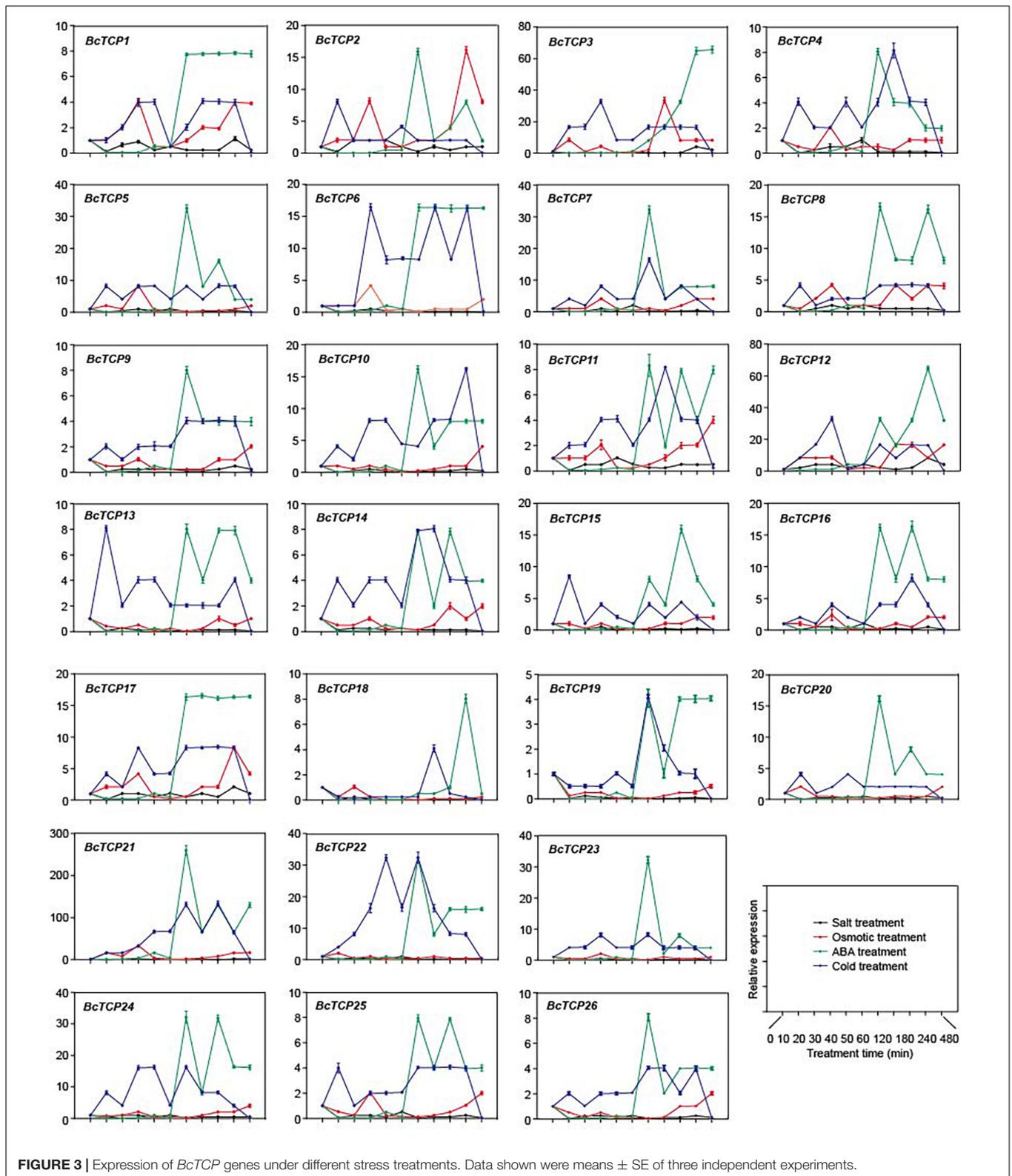
Analysis of *Cis*-Acting Elements in *BcTCPs* Promoters

Cis-acting elements existed in gene promoters can affect gene expressions and functions. In this study, we found that *BcTCPs* promoters contained not only basic core elements, like TATA-box and CAAT-box, but also a variety of *cis*-acting elements, such as light response elements, hormone response elements, environment response elements, development response elements, and other functional elements (Figure 2). All Class II *BcTCPs* promoters contained G-box elements and ABRE elements except for *BcTCP20* and *BcTCP21*. In Class I *BcTCPs*, the promoters of *BcTCP2*, 3, 11, 12, 13, 25, and 26 all contained the low temperature response element (LTR). AT~TATA box1 was identified in most all *BcTCP* promoters except *BcTCP6* and *BcTCP23*. The promoters of *BcTCP2*, 3, 4, 10, 11, 12, 14, 15, 20, 21, 22, 26 contained MYB, MYC, and MYB-like sequence elements at the same time. As a result, we hypothesized that these *BcTCP* genes may be involved in stress (Abe et al., 2003; Xu et al., 2021).

Expression Analysis of *BcTCP* Genes Under Multiple Abiotic Stresses

To investigate the functions of *BcTCPs*, qPCR was performed to detect the expressions of 26 *BcTCPs* under four treatments (salt, osmotic, cold and ABA) (Figure 3). The results showed that the expressions of most *BcTCPs*, except for *BcTCP12*, were

⁶<http://wolfsort.org/>



not significantly changed under salt treatment. *BcTCP2*, 3, 5, 6, 7, 8, 10, 12, 15, 16, 17, 20, 21, 22, 23, and 24 all up-regulated after 1 h of the ABA treatment. The expression levels

of *BcTCP2*, 5, 6, 7, 8, 10, 17, 20, 21, 22, 23, and 24 reached the maximum at 2 h, *BcTCP15* and 16 had the highest expression at 3h, the largest amount of *BcTCP12* expressed in 4 h, and the

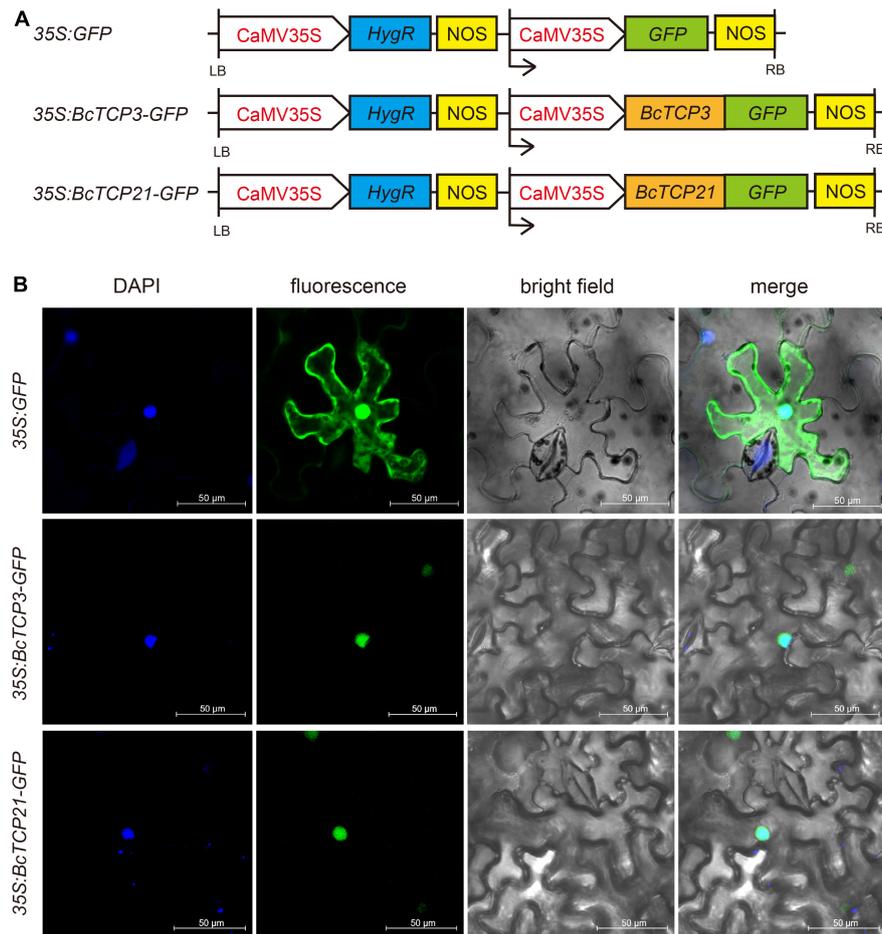


FIGURE 4 | Subcellular localization of BcTCP3 protein and BcTCP21 protein: **(A)** 35S: GFP, 35S: BcTCP3, and 35S: BcTCP21 construct. **(B)** Transient expression of 35S:GFP, 35S: 35S: BcTCP3-GFP, and 35S: BcTCP21-GFP fusion protein in tobacco leaves (Scale bars = 50 μ m).

largest amount of *BcTCP3* expression quantity in 4 h. Under cold treatment, *BcTCP3*, 6, 7, 10, 12, 21, 22, and 24 showed a significant increase, indicating that these genes, especially *BcTCP21*, may involve in cold stress. Meanwhile, *BcTCP2*, 3, and 12 were notably up-regulated under osmotic treatment. *BcTCP2* reached its peak at 4 h, and *BcTCP3* and 12 reached their peak at 2 h. These suggested that most *BcTCP* genes were involved in abiotic stresses.

Subcellular Localization

WoLF PSORT predicted that BcTCP proteins were localized in the nucleus (**Supplementary Table 3**). To test this, 35S: GFP, 35S: BcTCP3-GFP, and 35S: BcTCP21-GFP constructs were transiently overexpressed in tobacco leaves (**Figure 4A**). As seen in **Figure 4B**, 35S: GFP was localized in the nucleus and cytoplasm. The GFP signals emitted by BcTCP3 -GFP and BcTCP21 -GFP fusion protein were detected in the nucleus and overlapped with the DAPI staining. This results indicated that BcTCP3 and BcTCP21 were nuclear-localized proteins and may function as transcription factors.

DISCUSSION

TCP gene family involves in multiple biological processes, including the circadian clock, abiotic stresses, leaf sizes and shapes, flower development and flowering (Takeda et al., 2006; Pruneda-Paz et al., 2009; Mimida et al., 2011; Niwa et al., 2013; Liu et al., 2017; Feng et al., 2018). The roles of TCP genes under abiotic stresses have been reported in some model plants, such as rice, *Zea mays*, *Arabidopsis thaliana*, and soybean (Rueda-Romero et al., 2012; Mukhopadhyay and Tyagi, 2015; Feng et al., 2018; Ding et al., 2019). However, virtually less systematic and comprehensive information of the TCP gene family in Pak-choi was reported, which is a nutritious and economically important vegetable crop and widely cultivated in Asia. Unlike *A. thaliana*, *Brassicac* crops not only underwent this complex evolutionary history, but also the whole-genome triplication (WGT) event between 13 and 17 million years ago (MYA). These events were essential for evolution and resulted in differences among species (Wang et al., 2011). *A. thaliana* has been identified 24 TCP genes (Yao et al., 2007). In this study, 26 *BcTCP* genes were isolated and studied in Pak-choi. These *BcTCPs* were divided into Class I

and Class II, Class II was then divided into CIN and CYC/TB1, which was consistent with the previously described in *Arabidopsis*, rice, tomato, and strawberry (Yao et al., 2007; Parapunova et al., 2014; Wei et al., 2016). The phylogenetic analysis found that 16 *AtTCPs* had orthologs in Pak-choi. Among them, *AtTCP1*, 7, 15, 18, 21, and 24 had more than one ortholog. This indicated that BcTCP family genes and AtTCP family genes may have some similar functions. According to conserved domain, we found that the similarity of BcTCPs in the same class is higher than that of different classes of BcTCPs. For example, CYB/TB1 members all contained motif 1, motif 3, and motif 6. Analysis of promoter regions showed that some *BcTCPs* promoters contained MBS *cis*-regulatory elements, MYB *cis*-regulatory elements, ABRE elements, and G-box, suggesting that the *BcTCP* genes might play significant roles in stress responses (Abe et al., 2003; Xu et al., 2021).

QPCR analysis demonstrated that some *BcTCPs* were involved in multiple abiotic stress. For instance, *BcTCP3*, 12, 21, 22, and 24 all responded to cold and ABA treatment. *BcTCP3* had no response to salt stress but had relatively obvious responses under cold and ABA treatments. *BcTCP12* responded to salt, osmotic, ABA, and cold treatments. Under salt treatment, the expression of *BcTCP12* was significantly activated, indicating that *BcTCP12* may enhance salt tolerance, which is same as previous studies in *Oryza sativa* (Xiao et al., 2009; Almeida et al., 2017). The expressions of *BcTCP3*, 5, 7, 12, 21, 22, 23, and 24 displayed more than 30 fold up-regulation under ABA treatment. During drought treatment, *BcTCP2*, 3, and 12 were up-regulated by more than 15 fold. *BcTCP3*, 12, 21, and 22 were also up-regulated by more than 30 fold under cold treatment. These results were consistent with previous research results in rice (Yang et al., 2013; Wang et al., 2014). Subcellular localization analysis demonstrated BcTCP3 and BcTCP21 were located in the nucleus, indicating they may function as transcription factors.

These results revealed that *BcTCPs* were associated with multiple abiotic stresses. Our study may be helpful for improving plant stress tolerance. This will provide a piece of vital evidence

for detecting the molecular mechanisms of *TCPs* under abiotic stresses. The systematic characterization of *BcTCPs* in Pak-choi will provide a better foundation for further functional studies of this gene family in plant growth and development.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FH, CS, and YZ completed the experiments and wrote the manuscript. XH revised and approved the manuscript. All authors have read and agreed to the published version of 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.2022.854171/full#supplementary-material>

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