Genome-Wide Identification and Characterization of BrrTCP Transcription Factors in Brassica rapa ssp. rapa

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The teosinte branched1/cycloidea/proliferating cell factor (TCP) gene family is a plant-specific transcription factor that participates in the control of plant development by regulating cell proliferation. However, no report is currently available about this gene family in turnips (Brassica rapa ssp. rapa). In this study, a genome-wide analysis of TCP genes was performed in turnips. Thirty-nine TCP genes in turnip genome were identified and distributed on 10 chromosomes. Phylogenetic analysis clearly showed that the family was classified as two clades: class I and class II. Gene structure and conserved motif analysis showed that the same clade genes have similar gene structures and conserved motifs. The expression profiles of 39 TCP genes were determined through quantitative real-time PCR. Most CIN-type BrrTCP genes were highly expressed in leaf. The members of CYC/TB1 subclade are highly expressed in flower bud and weakly expressed in root. By contrast, class I clade showed more widespread but less tissue-specific expression patterns. Yeast two-hybrid data show that BrrTCP proteins preferentially formed heterodimers. The function of BrrTCP2 was confirmed through ectopic expression of BrrTCP2 in wild-type and loss-of-function ortholog mutant of Arabidopsis. Overexpression of BrrTCP2 in wild-type Arabidopsis resulted in the diminished leaf size. Overexpression of BrrTCP2 in triple mutants of tcp2/4/10 restored the leaf phenotype of tcp2/4/10 to the phenotype of wild type. The comprehensive analysis of turnip TCP gene family provided the foundation to further study the roles of TCP genes in turnips.

Keywords: TCP, transcription factors, expression analysis, turnip, cell proliferation

INTRODUCTION

Teosinte branched1/cycloidea/proliferating cell factor (TCP) gene family is a plant-specific transcription factor that regulates plant growth by controlling cell proliferation. TCP gene family has been identified in many plant species. For instance, Arabidopsis has 24 TCP genes, Oryza sativa has 28 TCP genes, tomato has 30 TCP genes, Populus euphratica has 33 TCP genes, Populus trichocarpa has 36 TCP genes, Citrullus lanatus has 27 TCP genes, and Prunus mume has 19 TCP genes...
genes (Martin-Trillo and Cubas, 2010; Parapunova et al., 2014; Ma X. et al., 2016; Shi et al., 2016; Zhou et al., 2016). The TCP domain contains a 59-amino-acid basic helix–loop–helix (bHLH) motif involved in DNA binding and protein–protein interaction (Martin-Trillo and Cubas, 2010). On the basis of the TCP domains, the members of the TCP family can be grouped into two subfamilies: class I (PCF or TCP-P class) and class II (TCP-C class) (Kosugi and Ohashi, 2002; Navaud et al., 2007; Martin-Trillo and Cubas, 2010). The difference between the two is a four-amino-acid deletion in the TCP domain in class I compared with class II.

Class I TCP genes are assumed to stimulate cell proliferation and leaf development, based mainly on the expression of rice PCF1/PCF2 and ArTCP20 in meristematic tissues (Kosugi and Ohashi, 1997; Li et al., 2005). In Arabidopsis, loss-of-function tcp15 mutant did not show any significant differences in comparison with wild-type plants. TCP15 fusion with SRDX repression domain elucidated that TCP15 regulated plant development via auxin response (Uberti-Manassero et al., 2012). tcp14 tcp15 double mutants displayed shortened internode length, altered leaf shape, and severe reduction in seed germination capability compared with wild type (Kieffer et al., 2011; Resentini et al., 2015). Moreover, AtTCP9 acts repeatedly with AtTCP20 in regulating leaf senescence via the jasmonate signaling pathway (Danisman et al., 2012). However, pentuple mutant tcp8 tcp15 tcp21 tcp22 tcp23 exhibited upregulated expression levels of SHOOT-MERISTEMLESS, BP, and CYCA1:1 and resulted in large leaf blades (Aguilar Martinez and Sinha, 2013).

Class II can be further divided into subclades: CIN and CYC/TB1 (Martin-Trillo and Cubas, 2010). Class II usually prevented cell proliferation and differentiation during the development of leaf blades. In Arabidopsis, CIN-type genes TCP2, TCP3, TCP4, TCP10, and TCP24 are targets of miR319a. jaw-D (overexpression of miR319a) plants resulted in large and wrinkled leaves (Palatnik et al., 2003). Single loss-of-function miR319a-targeted TCPs had slight developmental phenotypes. Double mutants (tcp2 tcp4) and triple mutants (tcp2 tcp4 tcp10) showed less increase in leaf size with some wrinkled signs than jaw-D plants. miR319a-targeted TCP transcription factors negatively regulated leaf growth and positively regulated leaf senescence via mediating LOX2 gene expression (Schommer et al., 2008). miR319a-targeted TCP4 is required for proper petal growth and development (Nag et al., 2009). miR319a-targeted TCPs interact with ASYMMETRIC LEAVES2 and ensure normal leaf development by repressing the expression of BP and KNAT2 by binding their promoter (Li Z. et al., 2012).

Turnip (Brassica rapa ssp. rapa) is one of the most economically important vegetable crops in the Tibet Plateau. However, no report on the turnip (Brassica rapa ssp. rapa) TCP family exists. The turnip genome has been sequenced and assembled (Lin et al., 2014), providing the basis for determining the turnip family. In this study, genome-wide identification of TCP transcription factors in turnips is performed. Thirty-nine BrrTCP genes were identified in the turnip genome, and their phylogenetic relationship, gene structure, protein motifs, chromosome location, transcript levels in different tissue, and forms of homo- and heterodimer interaction were analyzed. Furthermore, a CIN-type gene, BrrTCP2, was functionally characterized in transgenic wild-type and loss-of-function mutant Arabidopsis. Our findings indicate that the BrrTCP2 plays a vital function in leaf development by modulating cell division.

MATERIALS AND METHODS

Identification of the TCP Genes in Turnips

The genome sequence of turnips was downloaded from www.bioinformatics.nl/brassica/turnip. To find all TCP genes in turnips, NCBI BLASTn searches against a local database built using nucleic acid sequences were performed using sequences from all 24 known TCPs from Arabidopsis. Subsequently, the Pfam database was used to determine if each candidate TCP sequence was a member of the TCP gene family. To exclude overlapping genes, all candidate TCP genes were aligned using DNAMAN 4.0 (Lynnon Biosoft) and checked manually. All nonoverlapping TCP genes were used for further analysis.

Analysis of Conserved Motifs

Conserved motifs of BrrTCP proteins were analyzed using MEME (http://meme-suite.org/tools/meme) with the following parameters: (1) the optimum motif width was set from 6 to 200, and (2) the maximum number of motifs was set to identify 20 motifs.

Gene Structure, Genomic Distribution, and Divergence Time Estimation of BrrTCP Genes

BrrTCP genes were mapped on chromosomes by confirming their detailed chromosomal positions supplied by the Turnip Genome Database. To illustrate the structure of introns and exons of BrrTCP genes, full-length genome and coding sequences of BrrTCP genes were subjected to online GSDS analysis (http://gds.cbi.pku.edu.cn/). To determine their physical location, the starting positions of all BrrTCP genes on each chromosome were confirmed based on a local database of the complete sequence of the turnip genome through BLASTn searching. The segmental and tandem duplication regions were obtained from MCscanX. For synteny analysis, synteny block of the turnip gene was visualized using Circos (http://circos.ca/). Synonymous (Ks) and nonsynonymous (Ka) substitution rates were estimated by the codeml program of PAML4 (Yang, 2007). The divergence time (T) of turnip gene pairs was calculated using the formula $T = \frac{K_s}{2\lambda}$, where $\lambda$ represents the divergence rate of $1.5 \times 10^{-8}$ for Arabidopsis (Gaut et al., 1996).

Plant Material and Growth Conditions

Brassica rapa ssp. rapa “KTRG-B48a” from Xianggelila City, Yunnan Province, China, was used. For root collection, seedlings were grown on a Whatman filter paper and watered with 1/2 MS medium for 2 weeks. For other tissues, plants were grown in the greenhouse (22°C) under long-day conditions (16 h light/day).
Quantitative RT-PCR

Total RNA was extracted using Eastep™ Universal RNA Extraction Kit (Promega, Shanghai, China) from roots of 1-week-old plants, leaves and stems of 8-week-old plants, and floral buds of 10-week-old plants. RNA quality and concentration were assessed using electrophoresis and ND-1000 Spectrophotometer (Nanodrop Technologies, Delaware, USA). Two micrograms of total RNA were reverse transcribed using GoScript™ Reverse Transcription System (Promega). Quantitative RT-PCR (qRT-PCR) was performed with ABI7500 Real-Time PCR System using TransStart® Top Green qPCR SuperMix (TransGen, Beijing, China). BrrACT2 was used as reference gene. The primers are listed in Table S1. Triplet biological replicates were analyzed.

Yeast Two-Hybrid Assays

All BrrTCP ORFs were amplified from seedling cDNA of KTRG-B48a into pENTR vector used primers (Table S2). The entry vectors were subcloned into the pGADT7 prey vector (pDEST-GSDT7) and pGBK7 bait vector (pDEST-GBK7) using gateway method according to Bai et al. (2016). The prey vector was transformed into yeast strain Y187, and all bait vectors were transformed into yeast strain Y2H gold and selected on SD plates lacking Leu and Trp. After selection, the yeasts harboring prey and bait plasmids were spotted onto control medium (SD plates lacking Leu and Trp) and test medium (SD plates lacking Leu, Trp, and His). Yeast growth was observed daily in a growth chamber at 28°C for 2–5 days.

Scanning Electron Microscopy

In the present study, BLAST was carried out to search BrrTCP in the turnip genome. The obtained sequences were further verified through hidden Markov model. Finally, a total of 39 nonredundant BrrTCPs were identified from turnip genome. The BrrTCP genes were named following the nomenclature of Arabidopsis thaliana depending on protein sequence similarities (Figure 1). Sequence analysis revealed that AtTCP11 and 16 had no orthologs in turnip. AtTCP1, 4, 7, 9, 13, 15, 17, 18, 20, 21, and 24 had more than one ortholog in the turnip genome. Given the lack of standard annotations assigned to the 39 TCPs in turnips, the orthologs were designated as shown in Table 1 based on protein sequence similarities to their orthologs in Arabidopsis.

Identification of TCP Genes in Turnips

The release of the complete turnip genome sequences allowed the genome-wide identification of turnip genes (Lin et al., 2014). In the present study, BLAST was carried out to search BrrTCP in the turnip genome. The obtained sequences were further verified through hidden Markov model. Finally, a total of 39 nonredundant BrrTCPs were identified from turnip genome. The BrrTCP genes were named following the nomenclature of Arabidopsis thaliana depending on protein sequence similarities (Figure 1). Sequence analysis revealed that AtTCP11 and 16 had no orthologs in turnip. AtTCP1, 4, 7, 9, 13, 15, 17, 18, 20, 21, and 24 had more than one ortholog in the turnip genome. Given the lack of standard annotations assigned to the 39 TCPs in turnips, the orthologs were designated as shown in Table 1 based on protein sequence similarities to their orthologs in Arabidopsis.

Information of the BrrTCP gene family members is shown in Table 1. The ORF length of BrrTCP genes varied from 603 to 1,612 amino acids. Table 1 lists the BrrTCP genes with their orthologs in Arabidopsis and their sequence identities. The BrrTCP gene family members were classified into seven subgroups (A–G) based on their sequence identities to their Arabidopsis orthologs. The subgroup A contained the highest number of genes in both the turnip and Arabidopsis genomes, followed by subgroup E.

Figure 1

Phylogenetic tree of TCP proteins from turnips and Arabidopsis. The phylogenetic tree was generated using the neighbor-joining (NJ) method implemented in MEGA 7.0 software with JTT model and pairwise gap deletion option. Bootstrap analysis was conducted with 1,000 iterations.
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**TABLE 1 | TCP gene family in turnip.**

| Gene Name | Accession number | Length(aa) | MW(Da) | pI | Chr. Location |
|-----------|-----------------|------------|--------|----|---------------|
| BrrTCP1   | KY607998        | 351        | 39,826.39 | 6.47 | Chr07: 17379980–17381035 |
| BrrTCP1a  | KY607999        | 347        | 39,385.23  | 6.55 | Chr07: 16762673–16763813 |
| BrrTCP1b  | KY608000        | 346        | 39,314.74  | 5.54 |Chr02: 10171599–10172639 |
| BrrTCP2   | KY608001        | 384        | 42,403.17  | 7.47 |Chr03: 23210500–23211639 |
| BrrTCP5   | KY608002        | 341        | 37,222.15  | 7.34 |Chr06: 1024759–1025793 |
| BrrTCP4   | KY608003        | 406        | 44,173.60  | 7.13 |Chr05: 20022294–20023314 |
| BrrTCP4a  | KY608004        | 407        | 44,425.04  | 8.13 |Chr03: 17113530–17114753 |
| BrrTCP4b  | KY608005        | 348        | 38,161.10  | 7.06 |Chr01: 24280209–24281261 |
| BrrTCP5   | KY608006        | 366        | 40,706.24  | 6.52 |Chr02: 21107231–21108331 |
| BrrTCP6   | KY608007        | 224        | 24,641.60  | 8.02 |Chr04: 81058488–8106522 |
| BrrTCP7   | KY608008        | 252        | 27,180.36  | 9.25 |Chr01: 16403313–16407846 |
| BrrTCP7a  | KY608009        | 252        | 18,681.04  | 8.06 |Chr02: 25480706–25481263 |
| BrrTCP7b  | KY608100        | 219        | 23,770.77  | 8.21 |Chr01: 8925053–8929517 |
| BrrTCP8   | KY608101        | 394        | 41,385.39  | 6.38 |Chr05: 25002424–25002918 |
| BrrTCP9   | KY608102        | 325        | 33,838.78  | 8.98 |Chr03: 10874971–10875906 |
| BrrTCP9a  | KY608103        | 318        | 36,714.10  | 8.71 |Chr05: 7091828–7092874 |
| BrrTCP10  | KY608104        | 348        | 38,324.79  | 8.66 |Chr02: 11017245–11018356 |
| BrrTCP11  | KY608105        | 366        | 40,706.24  | 6.52 |Chr03: 14413642–14414472 |
| BrrTCP12  | KY608106        | 309        | 34,451.40  | 7.56 |Chr05: 23621188–23622581 |
| BrrTCP13  | KY608107        | 466        | 50,250.46  | 6.83 |Chr06: 10402184–10403578 |
| BrrTCP14  | KY608108        | 321        | 34,030.17  | 7.26 |Chr04: 81058488–8106522 |
| BrrTCP15  | KY608109        | 245        | 23,770.77  | 8.21 |Chr03: 14413642–14414472 |
| BrrTCP16  | KY608110        | 246        | 25,691.98  | 6.74 |Chr03: 14228322–14229372 |
| BrrTCP17  | KY608111        | 301        | 33,762.21  | 6.83 |Chr03: 1792188–17922942 |
| BrrTCP18  | KY608112        | 246        | 27,372.44  | 6.82 |Chr03: 1428184–1428909 |
| BrrTCP19  | KY608113        | 424        | 48,340.37  | 8.69 |Chr05: 23621188–23622581 |
| BrrTCP20  | KY608114        | 285        | 32,645.80  | 9.46 |Chr01: 22202115–222022887 |
| BrrTCP21  | KY608115        | 261        | 30,182.56  | 5.52 |Chr02: 8215556–8216431 |
| BrrTCP22  | KY608116        | 200        | 22,050.51  | 5.48 |Chr02: 22055944–22056191 |
| BrrTCP23  | KY608117        | 311        | 32,789.26  | 7.88 |Chr06: 22527874–22528797 |
| BrrTCP24  | KY608118        | 278        | 29,658.68  | 6.38 |Chr07: 1164212–1165048 |
| BrrTCP25  | KY608119        | 234        | 24,508.21  | 8.11 |Chr03: 1493264–1493988 |
| BrrTCP26  | KY608120        | 234        | 24,261.29  | 10.20 |Chr03: 1493264–1493988 |
| BrrTCP27  | KY608121        | 229        | 23,872.73  | 9.55 |Chr06: 2637716–2638405 |
| BrrTCP28  | KY608122        | 374        | 39,116.38  | 8.95 |Chr03: 12405545–12406669 |
| BrrTCP29  | KY608123        | 356        | 37,152.33  | 7.64 |Chr07: 19406027–19407097 |
| BrrTCP30  | KY608124        | 319        | 35,402.64  | 7.93 |Chr09: 22077911–22078867 |
| BrrTCP31  | KY608125        | 313        | 34,970.16  | 7.44 |Chr08: 16381466–16383191 |

1,401 bp, encoding polypeptides of 200–466 amino acids, with a predicted molecular mass of 22.05–50.25 kDa. The theoretical pI ranged from 5.48 to 10.20. Genomic localization of each BrrTCP in turnips is shown in Figure 2. These genes were distributed in all 10 turnip chromosomes. The maximum number of BrrTCP genes per chromosome was found for chromosome A02 with 9 TCP genes. Eight genes were located at chromosome 3, and five and four genes were located at chromosomes 7 and 5, respectively. Chromosomes 4 and 10 had the minimum BrrTCP genes, only one each. Three genes each were located on chromosomes 1, 6, and 9. Chromosome 8 contained two BrrTCP genes. A total of seven pairs of putative BrrTCP paralog proteins were produced by segmental duplication, which were distributed in different chromosomes. These results indicated the large-scale segmental duplication events involved in the expansion of BrrTCP gene family in turnips.

The divergence time (T) of seven pairs of BrrTCP paralog proteins was estimated by measuring the synonymous (Ks) and nonsynonymous (Ka) mutation rates (Table 2). The estimated divergence time (T) for the BrrTCP paralogs was from 10.3067 to 27.8600 million years ago (MYA), with an average duplication time of approximately 18.7862 MYA. Schranz et al. (2006)
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FIGURE 2 | BrTCPs’ chromosome distributions, synteny block, and the turnip genome duplication event caused paralogous relationships. Chromosomes are shown in different colors and in the outer circle, where the numbers represent the chromosome length in 100 Kb. The BrTCP genes are marked on the approximate positions with specific color lines on the circle. Filled blocks in different colors denote the syntenic relationships of turnip TCP genes.

TABLE 2 | Dates of duplication of duplicated gene pairs.

| Seq1         | Seq2         | Identity (%) | Ks     | Ka      | to    | T (MYA)  |
|--------------|--------------|--------------|--------|---------|-------|----------|
| BrTCP1a      | BrTCP1b      | 73.24        | 0.3092 | 0.1173  | 0.379 | 10.306   |
| BrTCP4       | BrTCP4a      | 79.24        | 0.416  | 0.0443  | 0.106 | 13.867   |
| BrTCP9       | BrTCP9a      | 70.76        | 0.6658 | 0.1743  | 0.261 | 22.193   |
| BrTCP15a     | BrTCP15b     | 88.10        | 0.3792 | 0.0404  | 0.106 | 23.886   |
| BrTCP21      | BrTCP21a     | 86.50        | 0.544  | 0.0441  | 0.081 | 18.133   |
| BrTCP22      | BrTCP23      | 71.76        | 0.4577 | 0.1117  | 0.244 | 15.256   |
| BrTCP24      | BrTCP24a     | 68.13        | 0.8358 | 0.3047  | 0.364 | 27.860   |

estimated the time of very early radiation of Brassicaceae at 34 MYA. Comparative physical mapping studies have confirmed genome triplication in a common ancestor of *B. oleracea* (O’Neill and Bancroft, 2000) and *B. rapa* (Park et al., 2005) since its divergence from the *A. thaliana* lineage at least 13–17 MYA (Yang et al., 1999; Town et al., 2006; Beilstein et al., 2010). The divergence time of three pairs of BrTCP paralogs (*BrTCP9/9a, BrTCP22/23, and BrTCP24/24a*) occurred precedent to the period of the origin of the *B. rapa*. The *ω* values for the BrTCP paralogs were less than one. However, two pairs of BrTCPs (*BrTCP1a/1b, ω = 0.3794; BrTCP24/24a, ω = 0.3646*) achieved relatively large *ω* values, which suggested that they may have evolved rapidly from the last common ancestor.

**Gene Structure and Conserved Motifs**

To better understand the diversification of the TCP genes in turnips, the exon/intron organization and conserved motifs of BrTCPs were analyzed. A new neighbor-joining phylogenetic tree was constructed using the protein sequences of BrTCPs (Figure 3A). The genome sequences and corresponding coding sequences of TCP genes in turnip analysis revealed that most BrTCP genes have similar gene structures in the same group (Figure 3B). A total of 32 members of *BrTCP* gene family have one exon (82%), 4 genes have two exons (10%), and 3 have four exons (8%). In *Arabidopsis*, the number of exons ranged from one to four, and 82% of the genes contained only one exon. The TCP genes in turnips exhibited similar gene structure. The MEME online tool was used to predict the conserved motif composition of BrTCPs (Figure 3C). The number of motifs varied from 2 to 11. The motifs were evaluated using InterProScan for annotation. The results revealed that motifs 1, 2, and 3 were identified as TCP domain. In addition, some motifs only presented at specific subclades, such as motifs 6, 7, 10, and 19 in *BrTCP1a, 1b, and 1b*; motif 9 in *BrTCP15, 1a, and 1b*; and motif 16 in *BrTCP4a, 4a, and 4b*, suggesting that they may have subclade-specific functions.

In *Arabidopsis*, miR319 controls jasmonate biosynthesis and senescence by cleaving TCP transcription factors (Schommer et al., 2008) and petal development (Nag et al., 2009). The AtmiR319-targeted TCP genes, namely, *AtTCP2, AtTCP3, AtTCP4, AtTCP10,* and *AtTCP24,* all belong to the CIN clade.
Similarly, four TCP genes in turnips contain miR319 binding sites (Figure 4), and all of them were CIN family members.

Subcellular Localization

The known members of the TCP gene family function as transcription factors. The GFP gene was fused with BrrTCPs as a reporter. The GFP signals of BrrTCP-GFPs were detected in the nucleus using laser confocal microscopy (Figure 5), which suggested that BrrTCPs functioned as transcription factors.

qRT-PCR Analysis of the Turnip TCP Genes

The expression pattern of a gene is always relative to its function (Xu et al., 2015). To probe possible functions of BrrTCP genes in turnips, qRT-PCR was performed to examine the expressions of BrrTCP genes in different organs of turnips (Figure 6). Interestingly, expression levels of most CIN-type BrrTCP genes were high in leaves and weak in the roots and stems. BrrTCP18 and BrrTCP18a, the members of CYC/TB1 subclade, are highly expressed in flower buds and weakly expressed in roots. In contrast, class I clade showed more widespread but less tissue-specific expression patterns; for example, BrrTCP7 and BrrTCP9 are highly expressed in roots, stems, leaves, and flower buds; BrrTCP8, 14, 18a, 20, 21b, 22, and 23 are highly expressed in the stems, leaves, and flower buds; and BrrTCP7b, 13a, 15, and 21 are highly expressed in leaves and flower buds. These results indicated that every clade possessed a characteristic expression profile.

Interactions between Turnip TCP Proteins

TCP proteins tend to form homodimers or heterodimers with other TCP proteins, and dimerization may be required for their DNA-binding activity and hence for their biological activity.

Yeast two-hybrid screening was carried out to investigate the interactions among the BrrTCP proteins, as shown in Figure 7, where the proteins are arranged according to their subclades except their autoactivation activity. A total of 8 out of 39 AD-fusion proteins tested had autoactivation activity in yeast. Meanwhile, out of the 39 BD-fusion proteins tested, 13 had autoactivation activity in yeast. Among them, five BrrTCP proteins had autoactivation activity in AD- and BD-fusion proteins. Although we selected 219 interactions, this number may not be accurate due to autoactivation. A total of 90 (45 pairs) proteins interacted in AD- and BD-orientations, including 9 homodimer formations. The class I BrrTCP transcription factors formed 91 homo- or heterodimers within class I members. Meanwhile, class II BrrTCP transcription factors formed 42 homo- or heterodimers within class II. The members of class I proteins more preferentially formed dimerization properties in the same class than class II.

Overexpression of BrrTCP2 Rescued the tcp2/4/10 Phenotype

BrrTCP2 is a member of the CIN subclade of TCP in turnips. Given the unavailability of tcp-related mutant in turnips,
we constructed transgenic Arabidopsis plants overexpressing BrrTCP2. As shown in Figure 8, the sixth rosette leaf of tcp2/4/10 triple mutants showed the most enhanced leaf size, with some signs of crinkling (Figures 8B,F). Meanwhile, the OXBrrTCP2 plants showed the most diminished leaf size (Figures 8C,G). OXBrrTCP2 line was crossed with tcp2/4/10, and the phenotype of the homozygote F2 plants restored the leaf phenotype of tcp2/4/10 to the phenotype of wild type (Figures 8D,H). Western blot analysis showed that OXBrrTCP2 and F2 plants had high expression levels, whereas no signal was detected in WT and tcp2/4/10 plants (Figure 8I). Arabidopsis leaf size is normally regulated by the cell size and number. To assess the cell size and number, we selected a site midway along the length of the lamina and between the margin and the midvein of the expanded sixth rosette leaf for analysis using scanning electron microscopy. The adaxial epidermal cell size of tcp2/4/10 was larger than WT (Figures 9A,B), and fewer cells were noted per unit area (Figure 9E). Meanwhile, the adaxial epidermal cell size of OXBrrTCP2 plants was smaller than WT (Figures 9A,C), and more cells were noted per unit area (Figure 9E). The F2 plant had similar cell size and cell number per unit area with WT. The abaxial epidermal cell size and cell number were similar to the adaxial epidermis. The tcp2/4/10 had larger cell size and fewer cell number per unit area than WT (Figures 9F,G,J), whereas OXBrrTCP2 plants had smaller cell size and more cell number per unit area than WT (Figures 9F,H,J). Overexpression of BrrTCP2 in tcp2/4/10 also restored the abaxial epidermal phenotype of tcp2/4/10 to WT (Figures 9D,I). BrrTCP2 may have a function in cell division and/or differentiation.

**DISCUSSION**

TCP proteins are plant-specific transcription factors involved in the regulation of multiple processes during plant development and growth, such as leaf morphogenesis and senescence, flower development, circadian clock, endoreduplication, branching, fiber development, and phytohormone biosynthesis (Schommer et al., 2008; Nag et al., 2009; Sugio et al., 2011; Danisman et al., 2012; Hao et al., 2012; Li Z. Y. et al., 2012; Wang et al., 2013; Ma J. et al., 2016; Zhou et al., 2016). Previous studies revealed that all Brassicaceae, including Arabidopsis and Brassica, underwent polyploidization events, such as γ triplication (135 MYA) and β (90–100 MYA) and α (24–40 MYA) duplications (Wang and Kole, 2015). B. rapa shares this complex history, with the addition of a whole-genome triplication (WGT) thought to have occurred between 13 and 17 million years ago (MYA) making “mesohexaploidy” a characteristic of the Brassicaceae tribe of the Brassicaceae (Lysak et al., 2005). The Brassica genomes diploidized after this triplication event through genome fractionation and rearrangements (Mun et al., 2009). Several studies revealed that the three subgenomes did not behave similar and that the dominat subgenome retained most genes; in addition, the genome fractionation was not a random process, as certain gene families retained more copies (Park et al., 2005; Wang et al., 2011; Chalhoub et al., 2014). The BrrTCP gene family in turnips may be caused by genome duplication processes, including multiple segmental duplications, tandem duplication, transposition events, and whole-genome duplication. Except gene duplication, differences in exon/intron organizations can also clarify the evolutionary history of the gene family. The gene structure of BrrTCPs compared with the same clade showed that TCP genes shared similar exon/intron distribution in terms of exon length and intron numbers; meanwhile, BrrTCPs with the same clade displayed similar motif distribution. Similar to tomato TCP proteins (Parapunova et al., 2014), more interactions were found for class I proteins than class II proteins (91 vs. 42), although the number of interactions for class I and class II may be underestimated because of the autoactivating members. The interactions obtained by a comprehensive yeast two-hybrid screen of turnip TCP transcription factors have not
yet been reported for TCP members from other species than tomato. Expression analysis and dimerization properties may help to identify TCP protein pairs that function together and explain observed functional redundancies in case of overlapping interaction maps of turnips in the future.

In *Arabidopsis*, *miR319*-targeted *AtTCP2, 3, 4, 10, and 24* regulate leaf development and petal growth (Palatnik et al., 2003; Ori et al., 2007; Nag et al., 2009). The three closest turnip *TCP* genes have a putative binding site for *miR319c*. Gene function is also related to its expression profile (Zhou et al., 2016). In this study, we detected the expression patterns of 39 *BrrTCP* genes in four organs using qRT-PCR. These genes vary widely among the turnip organs. Two CIN-type genes (*BrrTCP4a* and *BrrTCP4b*), which are *miR319c* targeted, exhibited high expression levels in leaves, particularly *BrrTCP4a*. Meanwhile, *BrrTCP4* exhibited low expression levels in all detected organs. This phenomenon
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FIGURE 7 | Interaction of BrTCP proteins in yeast two-hybrid assay. AD-fusion is listed in the left column. BrTCP protein names are ordered according to their subclades (CIN subclade is represented by black, TB1 by blue, and PCF by red).

FIGURE 8 | Phenotype effects of constitutive expression of BrrTCP2 in transgenic Arabidopsis. (A,E) Phenotype of wild-type Arabidopsis. (B,F) Phenotype of tcp2/4/10 mutants. (C,G) Expression of BrrTCP2 in wild-type Arabidopsis. (D,H) Phenotype of the homozygote F2 plants alleviates the phenotype of the tcp2/4/10 mutants. (I) Western blot analysis of BrrTCP2 protein levels in transgenic plants. BrrTCP2 protein was analyzed through immunoblotting using an anti-GFP antibody. AtACT2 served as the control. Bar = 0.5 cm.

FIGURE 9 | Scanning electron micrographs of leaf epidermal cells. Adaxial epidermis in the sixth rosette leaf from WT (A), tcp2/4/10 (B), 35S:BrrTCP2 transgenic plants (C), and F2 (D). (E) Number of adaxial epidermis cells of sixth rosette leaves. Abaxial epidermis in the sixth rosette leaf from WT (F), tcp2/4/10 (G), 35S:BrrTCP2 transgenic plants (H), and F2 (I). (J) Number of abaxial epidermis cells of sixth rosette leaves. Bar = 50 μm.

was also found in other duplicated gene pairs, such as BrrTCP7, BrrTCP7a, and BrrTCP7b. BrrTCP7 showed high expression levels in roots, leaves, and flowers, whereas BrrTCP7a showed low expression levels in all detected organs. However, BrrTCP7b exhibited high expression levels in leaves and flowers. Gene duplication plays a vital role in the process of plant genomic
and organismal evolution and confers new gene functions and the evolution of gene networks (Flagel and Wendel, 2009). Gene duplication might confer new functions to the paralogous BrrTCP genes. The other CIN-type genes, such as BrrTCP2, BrrTCP10, BrrTCP13a, and BrrTCP24, exhibited high expression levels not only in leaves but also in flowers. Turnip CIN-type genes may have a function in leaf and flower development. Class I and class II have antagonistic functions based on similar putative binding sites (Danisman et al., 2012). Class I BrrTCP genes, such as BrrTCP7b and BrrTCP14, were also detected to have high expression levels in leaves and flowers. In Arabidopsis, TCP14, which is homologous to BrrTCP14, acts repeatedly with TCP15 in modulating cell proliferation in developing leaf blades and flowers (Kieffer et al., 2011). Some members of class I and class II competitively regulated the cell proliferation in leaf development.

In Arabidopsis, loss-of-function mutants tcp2, tcp4, and tcp10 caused slight phenotype defect. Meanwhile, tcp2/4 double mutants exhibited an increased phenotype defect, and tcp2/4/10 triple mutants showed the most significant phenotype defects, with increase in leaf size and signs of crinkling (Schommer et al., 2008). TCP2, 4, and 10 repeatedly regulated leaf development. BrrTCP2 overexpression in Arabidopsis exhibited as small leaves with few epidermal cells. Overexpression of BrrTCP2 in tcp2/4/10 triple mutants restored the defect leaf phenotype to mimic wild-type leaf phenotype. BrrTCP2 might function in leaf development via inhibiting cell proliferations.

CONCLUSION

In summary, we identified 39 TCP genes, which were distributed on 10 chromosomes with different densities, in the turnip genome. Y2H analysis showed that these transcription factors preferentially formed heterodimers. Expression analysis showed that these genes exhibited varied expression profiles. In addition, BrrTCP2 was involved in leaf development via regulating cell proliferations.

AUTHOR CONTRIBUTIONS

XS, YoY, and HS conceived and designed the study; JD, SH, QY, CW, and YuY performed the experiments and analyzed the data; XS, YoY, and JD wrote the paper; all authors have read and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01588/full#supplementary-material

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