Genome Wide Identification and Characters of TCP Family Genes in *Brassica juncea* var. *tumida*

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Abstract

Background Teosinte branched1/Cycloidea/Proliferating cell factor (TCP) proteins are plant-specific transcription factors, which widely involved in leaf development, flowering, shoot branching, and circadian rhythm. So far, TCP proteins function in tumorous stem mustard has not been reported. Here we identified and characterized the entire TCP protein family members in the tumorous stem mustard. **Results** We identified fifty-four TCP genes in *Brassica juncea* var. *tumida*, containing thirty-three Class I subfamily members and twenty-one Class II subfamily members. Fifty-three TCP genes are distributed on 15 chromosomes. Gene structure and conserved motif analysis showed that the same clade genes have similar gene intron/exon structure and conserved motifs. Cis-acting element results showed that the same clade genes also have similar cis-element, however subtle differences also imply the different regulated pathway. More than twice paralogs genes relation to diploid species in some members imply gene duplication events in evolution. The members of BjTCP18s are low expressed in DY strains and un-swelling stage of YA strains. After treatment with GA and SA, it was detected that the expression levels of multiple TCP genes were affected by these two hormones. **Conclusion** In this study, we perform the first genome-wide analysis of the tumorous stem mustard TCP gene family. The results provide valuable information for understanding the classification and functions of TCP genes in tumorous stem mustard.

Background

The Teosinte branched1/Cycloidea/Proliferating cell factor (TCP) family is a group of plant-specific transcription factors (TFs) that involved in embryonic growth, leaf development, branching, flowering, circadian rhythm, hormone signalling, stress responses and so on [1–9]. TCP family proteins contain a highly conserved TCP domain that formed with an N-terminal region enriched in basic amino acids followed by two amphipathic α -helices connected by a disordered loop [10, 11].

Base on their conserved domain, the TCP proteins are divided into two subfamilies: Class I and Class II [7, 9]. Class I TCPs bind the DNA sequence GGNCCCAC and Class II TCPs bind GTGGNCCC [12]. In *Arabidopsis*, TCP2–5, TCP10, TCP13, TCP17 and TCP24 were related to lateral organ organogenesis and controlled leaf development [13–16]. TCP1, TCP12 and TCP18 are similar homology genes in one subfamily, for the reason that Branched1 (TCP18) and Branched2 (TCP12) control branch outgrowth [17–20]. TCP18 interacts with the florigen proteins FLOWERING LOCUS T (FT) and modulates its activity in the axillary buds to repress the floral transition of axillary meristems [21]. TCP21 is involved in circadian clock through interacts with TIMING OF CAB EXPRESSION 1 (TOC1) and CIRCADIAN AND CLOCK ASSOCIATED 1 (CCA1) promoter [22]. The function of TCP proteins in plant growth and development is usually regulated by a series of phytohormone synthesis and metabolism, including brassinosteroids (BRs), jasmonic acid (JA), indole-3-acetic acid (IAA) and so on [7, 16, 23, 24].

In recently years, TCP proteins were demonstrated to be related with defence responses. TCP13, TCP14 and TCP19 were found to be directly targeted by effectors from both *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis* [25]. Kim et al. found TCP8, TCP13, TCP15, TCP20, TCP22 and TCP23 can interact with the *Arabidopsis* immune adaptor SUPPRESSOR OF rps4-RLD1 (SRFR1) that is a negative regulator of effector-triggered immunity [26].

TCP gene family has been identified in many plant species, such as, 24 TCP genes in *Arabidopsis* [9], 28 TCP genes in *Oryza sativa* s, 30 TCP genes in *Lycopersicon esculentum* [27], 33 TCP genes in *Populus euphratica* [28], 27 TCP genes in *Citrullus lanatus* s [29], 66 TCP genes in *Triticum aestivum* [30], 75 TCP genes in *Gossypium barbadense* [31], 39 TCP genes in *Brassica rapa* L. ssp. *Pekinensis* [32] and 39 TCP genes in *Brassica rapa* ssp. *rapa* [33].

Tumorous stem mustard (*Brassica juncea* var. *tumida* Tsen et Lee) is an important crop with great economic benefits in China. Improve the yield of this crop is a key issue for the Chinese pickle industry. The growth of Tumorous stem mustard are divided into four stages: germination, seedling, stem swelling and flowering. Stem swelling is a key character of tumourous stem formation. The balance between stem swelling and flowering were directly related to the quality and yield of tumourous mustards. Tumourous stem mustard is an annual plant, and the stem does not swell except in plants sown between mid-September and mid-October in Chongqing and the other valleys of the Yangtze River, China. Thus, the production period of edible stems is limited. As above reports, TCP proteins were extensively involved in branching, flowering and a series development processes, forming the shape of plant architecture [7, 17, 18, 21, 24, 34–46]. However, no report on tumorous stem mustard TCP family exists, and whether TCP family proteins control the stem swelling and flowering development of tumorous stem mustard are still unknown.

As the whole genome of tumorous stem mustard is sequenced[47], a genome wide analysis of *TCP* genes is performed for the first time in the current study. Fifty-four *BjTCP* genes were identified in the tumorous stem mustard genome, and their phylogenetic relationship, gene structure, protein motifs, chromosome location, and expression profile in different tissue were analysed. The results provide information on the classification and detail of BjTCs and lay the foundation on the stem swelling and flowering regulation mechanism of TCP proteins in tumorous stem mustard.

Results

Identification of members of the TCP family in *Brassica juncea var. tumida*

Fifty-four TCP family genes were identified in *Brassica juncea var. tumida*, based on the similarity with *Arabidopsis thaliana* homology genes, the tumorous stem mustard TCP genes were named with *BjTCP1a-BjTCP27b* (Table 1). The coding amino acids were 171–457, with the molecular weight (MW) of 18.6–50.08 kDa and the isoelectric point (pI) of 5.5–10.18. Except *BjTCP27b* anchored in contig6125, *TCP* genes were located on 15 of 20 chromosomes. There are one TCP gene on chromosomes A10, B01 and B06, two TCP genes on A06, as well as three to seven members on the other chromosomes (Figure 1). Except BjTCPa-c, other BjTCP proteins all are localized in nuclear (Table 1), that is a typical feature of transcription factors.

The phylogenetic tree of *BjTCs* and *AtTCs*

Multiple sequence alignment of TCP proteins showed the conserved region mainly focused on the TCP domain (Figure S1).

To assess the phylogenetic relationships of the TCP family, the predicted TCP protein sequences in *Brassica juncea var. tumida* and *Arabidopsis thaliana* were used to construct a phylogenetic tree. The results indicated that all the TCP proteins were divided into two groups, Class I and Class II TCs (Figure 2a). In the Class II TCs cluster, these proteins were further divided into three subgroups, CYC, TB1 and CIN. The CYC subgroup was mainly cluster with AtTCP1 and AtTCP12, containing four AtTCP1 homology proteins BjTCP1a-d and two AtTCP12 homology proteins BjTCP12a-b. The TB1 subgroup was composed of AtTCP18 and four homology TCP proteins BjTCP18a-d. In CIN cluster, no proteins were found to be homology with AtTCP2–4 and AtTCP10, the other TCP proteins were found two or three homology proteins like AtTCP24 (three homology proteins BjTCP24a-c), AtTCP13 (three homology proteins BjTCP13a-c), AtTCP27 (two homology proteins BjTCP27a-b) and AtTCP5 (three homology proteins BjTCP5a-c). In the Class I TCs cluster, except AtTCP6, AtTCP11, AtTCP16, AtTCP23 cannot found homology proteins in *Brassica juncea var. tumida*, the other TCP proteins all have multiple homology proteins, such as AtTCP15 and AtTCP21 even have six homology proteins, respectively.

The BjTCP proteins of mustard have a typical bHLH motif in all other identified TCP proteins, (Figure 2b). As TCP proteins of *Arabidopsis thaliana*, the main different of Class I and Class II TCP family proteins were the identity of the residue at position 10–15 of the domain. Most Class I BjTCP proteins lose four amino acids between position 9–13 and have a Gly at position 15, whereas Class II BjTCP proteins have an Asp at this position in the TCP domain (Figure 2b).

The gene structures and conserved motifs analysis of BjTCs

The intron-exon gene structure results showed that most *BjTCP* genes only have one exon except *BjTCP18s*, *BjTCP12s*, *BjTCP20b*, *BjTCP13b* containing two and more exons. The genetic structure and evolutionary relationships of all TCP family members of stem mustard are also closely related. Genes within the same subfamily often have similar gene structures. *BjTCP12a* and *BjTCP12b* genes were composed of two exons, and *BjTCP18a-d* were more than three exons (Figure 3A and B). The conserved motifs in these BjTCs also showed the similar characters in the same subgroup, such as three similar motifs in all BjTCP15 homology proteins and five similar motifs in all BjTCP21 homology proteins (Figure 3C).

The gene duplication of BjTCP members

The gene duplication events were further analysed in the tumorous stem mustard TCP gene family. As shown in Table 2, 67 paralogous and/or orthologous pairs were identified. The Ka/Ks ratio is widely applied to measure genetic evolution and selection

pressure. The ratios of forty-eight homologous pairs were greater than 1, in contrast other nineteen homologous pairs were smaller than 1, indicating positive selection and negative selection during evolution, respectively (Table 2). Meanwhile, the divergence times of the TCPs were also calculated. The results indicated that these *BjTCPs* underwent duplication events from ~2.65 to 37.39 million years ago (MYA).

The promoter cis-acting element analysis of *BjTCPs*

The cis-acting element in the promoter of a gene usually regulated the expression and function. Multiple cis-acting elements were found in these TCP gene promoters, such as plant hormone responses element, light responses element, stress responses element, meristem expression, circadian control, low-temperature and wound responses element and so on (Figure 4).

For hormone-related cis-acting elements, the abscisic acid (ABA) responsive elements were identified at least two or more ABRE cis-acting elements in the tumorous stem mustard TCP gene promoters except *BjTCP5s*, *BjTCP18s*, *BjTCP19* and *BjTCP24s*. Auxin responsive elements include AuxRP and TGA elements. AuxRP has a relative small number of components, mainly in the *BjTCP5*, *BjTCP19* and *BjTCP20* homologous gene promoters. The distribution of TGA-element is relatively more extensive. The MeJA responsive related elements CGTCA and TGACG are found on most gene promoters, except for *BjTCP12*, *BjTCP19* and *BjTCP20* homologous genes. A number of other hormones related cis-element, such as ethylene (ET) responsive element ERE, gibberellin (GA) responsive elements GARE, P-box and TATC-box, and salicylic acid (SA) responsive element TCA-element are also present in promoters of some *BjTCP* genes.

In addition, a large number of cis-acting elements related to light responsive have been found on these TCP gene promoters, including 3-AF1 binding site, ACE, AE-box, TCT-motif, ATC-motif, Box 4, GATA-motif, G-Box, GT1-motif and I-box. Others include WUN-motif (related to wound), meristem-element (related to meristem), circadian element (related to circadian control), LTR element (related to low-temperature induction), defence and stress responsiveness elements (include MBS, Myb, Myc, STRE, TC-rich, W box and ARE elements). In particular, MYB and MYC-motif elements are identified on almost all TCP gene promoters..

Tissue-specific expression profiles of *BjTCPs*

In *A. thaliana*, TCP proteins were mainly involved in development and defence. Tumorous stem mustard is mainly used for a vegetable crop. The expression patterns of all TCP genes during the different development periods and tissues were analysis base on the previous RNA-seq data[48]. The results showed the expression of *BjTCP1a* and *BjTCP5c* cannot be detected in all samples. Twenty-six *TCP* genes were highly expressed in at least two tissues ($\log_2(\text{FPKM}) \geq 3$, the subgroup half bottom in Figure 5). *BjTCP13b* and *BjTCP5a* were weakly expressed in DA sample. The expression of *BjTCP1b*, *BjTCP1c*, *BjTCP1d*, *BjTCP5c*, *BjTCP7c*, *BjTCP13a*, *BjTCP13c*, *BjTCP18d* and *BjTCP19* were not detected in DY stem tissue. *BjTCP12a*, *BjTCP12b* and *BjTCP18c* have weakly expression in DY, YA1 and/or YA2 (Figure 5).

Expression Analysis of *BjTCP* Genes in response to Exogenous hormones

To predict possible functions of *TCP* genes in environmental adaptation, we investigated the transcriptional profile of *TCP* genes under SA and GA treatment.

For SA treatment, almost all of the detected *BjTCPs* are upregulated after treatment for 2–8 hours expected *BjTCP1*, *BjTCP12* and *BjTCP17*, whereas decreased to be at a low level at 24h. The *BjTCP9*, *BjTCP13*, *BjTCP15*, *BjTCP18*, *BjTCP19*, *BjTCP22* and *BjTCP24* are induced at the early stages and maintained at a relatively high level until 8h. *BjTCP17* are induced slowly and the high expression level appear at 24h after treatment (Figure 6).

Under GA treatment, the levels of *BjTCP12*, *BjTCP17* and *BjTCP20* cannot be detected. In contrast, the expression of other genes were induced at the early stages but then decreased to a low level mainly at 8h (Figure 6).

Discussion

As plant-specific TFs, the TCP TFs play a various function role in plant growth and development processes. In tumorous stem mustard, 54 family members were found. As other plants, the general organization of these TCP family are also conserved and significantly more members in the class I subfamily than those in the class II[28–33, 49–52]. As a tetraploid plant, the number of TCP proteins in tumorous stem mustard is significantly more than twice as abundant as Arabidopsis (24 TCP proteins), which suggest that some genes are doubled in the process of evolution.

In addition, the exon/intron structure, conserved motif distribution patterns and domain of BjTCP homology genes often showed high similarity, such as *BjTCP21a-f*, *BjTCP12a/b* and so on. These similarities cluster homology genes members might play similar function during tumorous stem mustard growth and development.

Interesting, a series of genes such as BjTCP15b, c and BjTCP1b and BjTCP22b are located on the same chromosome A07. But the homology gene of these genes can be found (BjTCP15b and BjTCP15f, BjTCP15e and BjTCP15c, BjTCP1b and BjTCP1c, BjTCP22b and BjTCP22d) in series and showed the same order on the corresponding B03 chromosome (Figure 1 and 2). The allopolyploid tumorous stem mustard (*B.juncea*, AABB) may form by hybridization between the diploid ancestors of *B.rapa* (AA) and *B.nigra* (BB), followed by spontaneous chromosome doubling. These results indicated that the formation of fragments between the four genes of two chromosome A07 and B03 may be obtained from *B.rapa* (AA) and *B.nigra* (BB), respectively. The most similarity of the eight genes were aquered in *B.rapa* (AA) and *B.nigra* (BB) using the blastP program, and four gene were selected out in *B.rapa* (AA) and *B.nigra* (BB), respectively. The evolutionary relationships of the sixteen genes coding proteins showed the four genes located on the same chromosome A07 were cluster with the homology genes of A subgenome ancestor *B.rapa* (AA) in one branch, respectively (Figure S2). And the four genes on chromosome B03 are also corresponding to B subgenome ancestor *B.nigra* (BB), respectively (Figure S2). These results also indicted that the division of BjTCP15b and BjTCP15c may be earlier than the formation of tetraploids.

There are 24 TCP genes in Arabidopsis, whereas some corresponding homologous TCP genes cannot find in tumorous stem mustard, such as TCP2–4, 6, 11 and 16, which may due to occurred losing event during the evolution process. Tumorous stem mustard is a tetraploid plant that belongs to the cruciferous near-source species of Arabidopsis. In theory, each of the Arabidopsis TCP genes has two orthologous genes in the stem mustard. However, some TCP genes can find out more than two paralogous genes, such as BjTCP1 (four homology genes), BjTCP18 (four homology genes), BjTCP21 (six homology genes), BjTCP15 (six homology genes) and so on. These genes may be caused by multiple gene doubling events, the function of these paralogous genes have similarity and gradual differentiation in the process of evolution. First of all, from the characteristics of the cis-elements of these genes, most of the paralogous genes have similar cis-acting elements, but there are also a few differences, such as the four paralogs of BjTCP18 are no ABA and auxin cis-acting elements, but BjTCP18b is the only member of the four genes with circadian regulatory elements, suggesting that the BjTCP18b gene may be involved in circadian rhythm (figure 4). Correspondingly, the expression patterns of these paralogous genes are also different (Figure 5).

Previous studies demonstrated that *B.juncea* genome underwent genome duplication events about 0.039–0.055 million years ago [47]. In this study, all the duplication pair gene was occurred before 2.65 MYA, suggesting that the gene duplication events occurred before the *B.juncea* forming. This results also proved the guess above that the *BjTCP* genes clustered together on the chromosome A07 and B03 were formed before the formation of tetraploids.

The Arabidopsis *BRANCHED1(BRC1)*, the rice *TB1* and maize *TB1* gene were demonstrated for its role as a negative regulator in the growth of axillary buds and branching[17, 36, 53, 54]. *BRC1* is ortholog with *TB1* and rice *TB1* that may play a similar roles and molecular mechanism in development of the primary shoot architecture and negatively regulating lateral branching[17–20, 36, 53, 55]. Moreover, OsTB1 can be regulated by IPA1 to suppress tillering in rice and TB1 can interact with FT1 to regulat inflorescence architecture in bread wheat[36, 54, 56]. For tumorous stem mustard, a close phylogenetic relationship with Arabidopsis, BjTCP18s may also play the similar function in branching. But four BjTCP18s homology genes in tumorous stem mustard, which may form by gene duplication. Functional differentiation may occur between the four TCP18 genes. From the perspective of their expression patterns, the four genes are not consistent during tissue development. In this study, the expression profiles of tumorous stem mustard seedlings and tumour stems were used, and the expression levels of these four genes were low in these tissues. The expression levels of the four genes gradually decreased with the swelling of the tumorous stems (Figure S3). The flowering stage of the tumorous stem mustard is mainly characterized by the swelling of the tumorous stem. At this time, there will be a bolting and flowering phenomenon similar to *Arabidopsis*. Since BRC1 has the function of inhibiting branching and flowering, the gradual down-

regulation of its mRNA levels may reflect the gradual decrease ability in inhibition of branching and flowering. The results also indicated that the tumorous mustard is about to enter the period of reproductive growth.

According to reports, there are 16 varieties of mustard species identified, which the mainly difference of these vegetables tissues are used for food and shape, including root, stem, leaf and branch[57]. BRC1 gene controls plant branching and flowering, and four identified BjBRC1 may imply the further function differentiation of branch development and flowering during the development of mustard. Among these *BjTCP* genes, multiple BjTCP15s and BjTCP21s in DY and/or the early stage of seedling and tumour stem per-swelling stage were higher expression than swelling stage. DY is a no swelling mutant line tissues sample, and YA1 and YA2 are also not begin to swelling. This result may indicate that these genes are involved in the process of stem swelling in tumorous stem mustard.

Increasing evidence verified that TCP proteins are involved in responses to plant hormone[37, 58, 59]. In this study, most of tumorous stem mustard TCP genes appear to be regulated by SA and GA (Figure 6). In *Arabidopsis thaliana*, several TCPs interact with the SA biosynthetic enzyme ISOCHORISMATE SYNTHASE 1 gene and enhanced the gene's expression through binding to the TCP-binding motif in its promoter region [60]. In our results, there are many SA-related cis-elements in the promoter regions of BjTCPs (Figure 4), and the expression levels of several BjTCP genes significantly increased after the SA treatment (Figure 6), indicating that BjTCPs may be involved in the signal transduction of SA. In addition, most *BjTCP* genes have polytype gibberellin (GA) responsive elements GARE, P-box and TATC-box in their promoters (Figure 4), which may lead to more complex regulation of their expression and more diverse expression patterns (Figure 6).

This study was the first to identify 54 *BjTCP* gene family members in tumorous stem mustard and to investigate their roles in stem development. Our results provide a foundation to further determine the molecular mechanisms of TCP genes in the development of tumorous stem.

Conclusions

We performed a genome-wide analysis and identify 54 TCP genes in *Brassica juncea var. tumida*. These genes are divided into two subfamilies, 33 Class I and 21 Class II. Chromosomal mapping showed that 53 *BjTCP* genes were heterogeneously distributed on 15 chromosomes. Structural analyses of *BjTCP* genes showed that 44 genes had no introns, most of the *BjTCP* genes in the same cluster had similar patterns of exon length, intron number, and conserved motifs. We identified several genes that are highly expressed in development of tumorous stem mustard and branching relation genes were low expression in swelling stage of vegetative growth.

Materials And Methods

Plant materials growth conditions and treatments.

In this study, tumorous stem mustard cultivar Yong An Xiao Ye (YA) was used for gene expression pattern analysis. The seeds were sowed into 2:1 vermiculite: turfy soil, cultured under constant light at 22 °C with a 16/8 h light/dark regime in culture room. Three-week-old seedlings were used for exogenous hormone treatments. For SA and GA3 treatment, the seedlings were sprayed with 100 µM SA and 100 µM GA, respectively. The second true leaf on each plant was sampled at 0 (control), 2, 4, 6, 8 h and 24h after spraying. All treatments were repeated three times and each treatment contained at least 20 seedlings. All materials were frozen at –70 °C until RNA isolation.

Identification of TCP protein in tumorous stem mustard

The genome sequences of *Brassica juncea var. tumida*, *Brassica nigra* and *Brassica rapa* were downloaded from the *Brassica database* (BRAD, <http://brassicadb.org/brad/index.php>)[47, 61]. The TCP domain in the Pfam database with accession number PF03634 were download[62], and searched for the domain in the *Brassica juncea var. tumida* databases using HMMER 3.0 with an E-value<1e–6[63]. To confirm the results obtained using the HMMER algorithm, the protein domain were further verify in Pfam and Smart databases[62, 64, 65]. The TCP family protein sequence of *Arabidopsis thaliana* were download the Arabidopsis information resource website (<https://www.arabidopsis.org>).

Sequence and phylogenetic analyses

Multiple alignments of TCP protein sequences from *Brassica juncea* var. *tumida* and *Arabidopsis thaliana* were performed using the ClustalW programme[66]. Phylogenetic tree were constructed with the MEGA 7.0 software using the neighbour joining method and a bootstrap test replicated 1000 times[67]. The gene structure diagram was drawn using the online software of the GSDS2.0 server (<http://gsds.cbi.pku.edu.cn/>)[68]. The physical location data of *BjTCP* genes were retrieved from the *Brassica juncea* var. *tumida* genomes. The mapping of these *TCP* genes was subsequently performed using MapInspect software. Default parameters were used for the Multiple Em for Motif Elicitation (MEME, <http://meme-suite.org/>) programme for the identification of conserved protein motifs and a maximum number of 12 motifs. Subcellular localizations of *BjTCPs* were predicted using ProComp9.0. The identified protein motifs were further annotated using Weblogo (<http://weblogo.berkeley.edu/>). The 2000 bp of the 5' sequence as the promoter domain of each *TCP* genes were used to analyse the cis-acting elements using the online software PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [69].

Chromosomal location and gene duplication

The physical location data of *BjTCP* genes were retrieved from the *Brassica juncea* var. *tumida* genomes. The mapping of these *TCP* genes was subsequently performed using MapInspect software. Gene duplication was defined according to the criteria described in previous studies: the aligned region of two sequences covers over 80% of the longer sequence, and the similarity of the aligned region is over 80%. In addition, the KaKs_Calculator software was employed to calculate Ka (nonsynonymous substitution rate) and Ks (synonymous substitution rate). The divergence time was calculated with the formula $T = Ks/2r$, the r was taken to be 1.5×10^{-8} synonymous substitutions per site per year for dicotyledonous plants.

The expression profile of TCP genes

The RNA-seq data were we previously reported and can downloaded from NCBI SRA database (<http://www.ncbi.nlm.nih.gov/sra/>) with the accession number were SRX108496(DY), SRX108498(YA1), SRX108499(YA2), SRX108500(YA3), SRX108501(YA4) and SRX108502(YAr), respectively[48]. Then the clean reads that were filtered from the raw reads were mapped onto *B. juncea* genome using Tophat2 [70, 71]. Gene expression levels of individual genes were quantified using RPKM values (fragments per kilobase of exon per million fragments mapped) by the Cufflinks 2.2.1 [70].

RNA extraction and real-time quantitative PCR analysis

The total RNA was extracted from different plant materials using RNA plant plus reagent (Tiangen Biotech Co., Ltd., Beijing, China). The total RNA samples were treated with DNase I (Takara, Qingdao, China) to remove contaminating genomic DNA. First-strand cDNA was synthesized from the total RNA using a Hiscript II 1st strand cDNA synthesis Kit (Vazyme, Nanjing, China). Real-time qPCR (qRT-PCR) was performed using TB Green™ *Premix Ex Taq*™ II (Tli RNaseH Plus) (Takara, Qingdao, China). *BjActin* was used as the internal reference gene for qRT-PCR, and the gene-specific primers are listed in Supplementary Table S1. The relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Declarations

Authors' contributions

QS conceived and designed the experiments, carried out the main bioinformatics analysed, and drafted the manuscript. JH and XHH performed the experiments. PAC helped to improve the manuscript. DPG collected the public dataset and assisted with data analysis. HZJ and XHH participated in its design and helped to revisions the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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Tables

Table 1. The TCP protein family members in *Brassica juncea* var. *tumida*

ID	pfam domain(start- end)		name	chr	star	end	sence+/antisense-	Subcellular localization	homolog	PI	MW(kD)	protein(aa)
BjuA007230	84	242	BjTCP1a	A02	10613454	10614491	-	Nuclear	AtTCP1	5.5	39.28	346
BjuA027377	84	232	BjTCP1b	A07	29105069	29106097	-	Nuclear	AtTCP1	6.68	38.96	343
BjuB030534	84	233	BjTCP1c	B03	29932727	29933758	-	Nuclear	AtTCP1	5.98	39.1	344
BjuB043984	85	235	BjTCP1d	B03	27732982	27734025	-	Nuclear	AtTCP1	5.96	39.57	348
BjuA041558	100	193	BjTCP12a	A02	11715044	11716146	-	Nuclear	AtTCP12	8.78	37.88	322
BjuB010789	103	231	BjTCP12b	B05	55023718	55024870	-	Nuclear	AtTCP12	8.45	39.61	352
BjuA011472	59	220	BjTCP13a	A03	18170796	18171755	+	None	AtTCP13	6.73	35.69	320
BjuA021096	86	243	BjTCP13b	A05	31265695	31266872	-	None	AtTCP13	6.63	35.72	321
BjuB026804	58	214	BjTCP13c	B07	14926766	14927692	-	None	AtTCP13	7.94	34.47	309
BjuA007311	58	201	BjTCP15a	A02	12233735	12234697	+	Nuclear	AtTCP15	6.91	33.84	321
BjuA016487	55	195	BjTCP15b	A07	30212223	30213185	+	Nuclear	AtTCP15	6.91	34.06	321
BjuA027170	56	197	BjTCP15c	A07	27540133	27541098	-	Nuclear	AtTCP15	7.43	33.96	322
BjuB000709	50	186	BjTCP15d	B05	51941921	51942820	-	Nuclear	AtTCP15	8.05	32.02	300
BjuB003932	57	194	BjTCP15e	B03	29008395	29009351	+	Nuclear	AtTCP15	7.43	33.74	319
BjuB030482	55	196	BjTCP15f	B03	32626814	32627776	+	Nuclear	AtTCP15	7.15	33.49	321
BjuA022523	1	178	BjTCP14a	A06	12060176	12061564	-	Nuclear	AtTCP14	5.98	36.45	344
BjuB019669	103	292	BjTCP14b	B08	54134488	54135858	-	Nuclear	AtTCP14	6.48	49.33	457
BjuA012606	154	319	BjTCP18a	A03	22282211	22283992	+	Nuclear	AtTCP18	8.56	48.45	425
BjuB007175	150	311	BjTCP18b	B07	5163145	5165165	+	Nuclear	AtTCP18	7.32	40.12	350
BjuB007177	150	311	BjTCP18c	B07	5138717	5140787	+	Nuclear	AtTCP18	6.66	50.08	437
BjuB025473	159	292	BjTCP18d	B01	38022785	38024462	-	Nuclear	AtTCP18	8.4	46.6	404
BjuA007026	52	174	BjTCP19	A02	9082363	9083205	+	Nuclear	AtTCP19	5.5	30.18	281
BjuA024339	67	304	BjTCP20a	A06	25596111	25597025	+	Nuclear	AtTCP20	7.97	32.22	305
BjuA031722	53	239	BjTCP20b	A09	3333673	3334532	-	Nuclear	AtTCP20	5.17	25.25	241
BjuB037176	65	309	BjTCP20c	B02	56079378	56080310	-	Nuclear	AtTCP20	7.3	32.61	311
BjuA009108	31	206	BjTCP21a	A03	1689417	1690118	+	Nuclear	AtTCP21	10.18	24.26	234
BjuA041017	32	207	BjTCP21b	A02	1466529	1467236	+	Nuclear	AtTCP21	9.29	24.54	236
BjuA047338	31	208	BjTCP21c	A10	17491637	17492344	+	Nuclear	AtTCP21	7.99	24.73	236
BjuB012430	31	209	BjTCP21d	B05	17001527	17002243	-	Nuclear	AtTCP21	9.57	25.05	239
BjuB040955	33	209	BjTCP21e	B08	2452006	2452713	+	Nuclear	AtTCP21	10.18	24.46	236
BjuB048495	28	209	BjTCP21f	B02	50550592	50551305	-	Nuclear	AtTCP21	7.99	24.94	238
BjuA007449	57	190	BjTCP22a	A02	13365484	13366605	+	Nuclear	AtTCP22,AtTCP23	8.63	39.06	374
BjuA043373	40	202	BjTCP22b	A07	31378278	31379348	+	Nuclear	AtTCP22,AtTCP23	6.87	37.34	357
BjuB010697	56	186	BjTCP22c	B05	59790473	59791573	-	Nuclear	AtTCP22,AtTCP23	8.63	38.41	367
BjuB044035	45	168	BjTCP22d	B03	38473563	38474609	+	Nuclear	AtTCP22,AtTCP23	6.31	36.59	349
BjuA034777	46	138	BjTCP24a	A09	31311472	31312425	+	Nuclear	AtTCP24	7.8	35.29	318
BjuB029526	47	139	BjTCP24b	B04	35033858	35034823	-	Nuclear	AtTCP24	6.90	35.7	322
BjuB032913	66	306	BjTCP24c	B03	5929399	5930376	+	Nuclear	AtTCP24	7.16	36.57	326
BjuA009092	32	164	BjTCP17a	A03	1624898	1625620	+	Nuclear	AtTCP17	8.53	19.12	171
BjuO008355	37	254	BjTCP17b	Contig6125	66534	67304	+	Nuclear	AtTCP17	6.7	28.54	257
BjuB027204	34	231	BjTCP5a	B04	184613	186371	-	Nuclear	AtTCP5	9.33	30.31	275
BjuB037760	63	364	BjTCP5b	B02	59342015	59343112	+	Nuclear	AtTCP5	6.21	40.73	365
BjuB039698	62	262	BjTCP5c	B07	12846872	12847951	-	Nuclear	AtTCP5	6.95	34.36	304
BjuA023481	42	223	BjTCP7a	A06	20165661	20166410	-	Nuclear	AtTCP7	9.69	27	250
BjuA032507	14	189	BjTCP7b	A09	5290320	5290967	+	Nuclear	AtTCP7	9.38	23.12	216

BjuA045586	1	151	BjTCP7c	A02	34582679	34583206	-	Nuclear	AtTCP7	7.92	18.6	176
BjuB039713	14	190	BjTCP7d	B07	12703979	12704632	-	Nuclear	AtTCP7	9.82	23.3	218
BjuB044801	42	235	BjTCP7e	B02	60858548	60859336	+	Nuclear	AtTCP7	9.51	28.37	263
BjuA033567	53	218	BjTCP8a	A09	15197090	15198274	+	Nuclear	AtTCP8	6.09	41.43	395
BjuB028163	54	232	BjTCP8b	B04	9742480	9743682	-	Nuclear	AtTCP8	6	42.26	401
BjuA010736	71	197	BjTCP9a	A03	13517323	13518273	-	Nuclear	AtTCP9	9.86	33.84	317
BjuA018039	62	178	BjTCP9b	A05	3386569	3387543	-	Nuclear	AtTCP9	9.41	33.43	325
BjuB016827	72	201	BjTCP9c	B08	26586920	26587915	-	Nuclear	AtTCP9	9.67	35.15	332
BjuB020003	67	184	BjTCP9d	B06	3171113	3172111	-	Nuclear	AtTCP9	9.58	35.15	333

Table 2 *Ks*, *Ka* calculation and divergent time of the duplicated *BjTCP* genes

Duplicated Gene Pairs	Ka	Ks	Ka/Ks	P-Value(Fisher)	Divergence-Time[Mya]
BjTCP20a&BjTCP9a	0.999293	1.00215	0.997151	1	33.41
BjTCP20a&BjTCP9d	1.01737	0.946396	1.075	0.179341	31.55
BjTCP20a&BjTCP22d	0.978041	1.06173	0.921176	0.0709694	35.39
BjTCP20a&BjTCP8a	1.01664	0.954319	1.06531	0.207383	31.81
BjTCP20a&BjTCP15d	1.0129	0.962903	1.05192	0.30836	32.10
BjTCP20a&BjTCP13a	1.01885	0.942488	1.08102	0.122359	31.42
BjTCP20c&BjTCP21a	0.989309	1.0313	0.959288	0.482644	34.38
BjTCP20c&BjTCP21c	1.01341	0.960297	1.05531	0.326765	32.01
BjTCP20c&BjTCP9b	1.03638	0.894697	1.15836	0.0455288	29.82
BjTCP20c&BjTCP1b	0.999191	1.00276	0.996436	1	33.43
BjTCP20c&BjTCP1d	1.02992	0.898138	1.14672	0.0072974	29.94
BjTCP20c&BjTCP1a	1.01515	0.949568	1.06907	0.181948	31.65
BjTCP20c&BjTCP5c	1.05632	0.7937	1.33088	9.74E-06	26.46
BjTCP20c&BjTCP5a	1.04315	0.84631	1.23258	0.000574845	28.21
BjTCP20c&BjTCP24a	1.02067	0.937008	1.08929	0.109641	31.23
BjTCP20c&BjTCP24b	1.03281	0.892867	1.15673	0.00840374	29.76
BjTCP21a&BjTCP21c	0.194528	0.145215	1.33958	0.150138	4.84
BjTCP21a&BjTCP9b	0.98743	1.03637	0.952782	0.394321	34.55
BjTCP21a&BjTCP1b	1.08642	0.715504	1.5184	6.06E-08	23.85
BjTCP21a&BjTCP1d	1.01932	0.929203	1.09698	0.176264	30.97
BjTCP21a&BjTCP1a	1.09159	0.714456	1.52787	3.78E-08	23.82
BjTCP21a&BjTCP5c	1.02937	0.908039	1.13362	0.10476	30.27
BjTCP21a&BjTCP5a	1.0257	0.919267	1.11578	0.126757	30.64
BjTCP21a&BjTCP24a	1.07085	0.797343	1.34302	9.27E-05	26.58
BjTCP21a&BjTCP24b	1.09267	0.738083	1.48042	2.30E-08	24.60
BjTCP21c&BjTCP9b	1.0336	0.914091	1.13074	0.0274717	30.47
BjTCP21c&BjTCP1b	1.08258	0.754275	1.43526	2.56E-07	25.14
BjTCP21c&BjTCP1d	1.02796	0.912875	1.12607	0.0551837	30.43
BjTCP21c&BjTCP1a	1.09313	0.73226	1.49282	3.21E-08	24.41
BjTCP21c&BjTCP5c	1.03523	0.897532	1.15342	0.0394119	29.92
BjTCP21c&BjTCP5a	0.993188	1.01984	0.973868	0.695805	33.99
BjTCP21c&BjTCP24a	1.08745	0.769013	1.41408	3.00E-06	25.63
BjTCP21c&BjTCP24b	1.11075	0.706962	1.57116	6.11E-11	23.57
BjTCP9a&BjTCP9c	1.00947	0.974612	1.03577	0.449742	32.49
BjTCP9c&BjTCP9d	0.335531	0.219283	1.53013	0.00527418	7.31
BjTCP9c&BjTCP22d	1.01022	0.971431	1.03993	0.450774	32.38
BjTCP9c&BjTCP8a	1.02033	0.945662	1.07896	0.135508	31.52
BjTCP9c&BjTCP15d	1.01052	0.970874	1.04083	0.436843	32.36
BjTCP9c&BjTCP13a	1.01175	0.966716	1.04659	0.369085	32.22
BjTCP9b&BjTCP1b	0.971449	1.08511	0.895253	0.0226791	36.17
BjTCP9b&BjTCP1d	0.959468	1.12182	0.85528	0.000601298	37.39
BjTCP9b&BjTCP1a	0.988291	1.03402	0.955776	0.34941	34.47
BjTCP9b&BjTCP5c	0.994713	1.0156	0.979437	0.746027	33.85
BjTCP9b&BjTCP5a	1.00184	0.994804	1.00707	0.913911	33.16
BjTCP9b&BjTCP24a	0.979059	1.05884	0.924653	0.164264	35.29
BjTCP9b&BjTCP24b	1.02257	0.942692	1.08473	0.132606	31.42
BjTCP1b&BjTCP1d	0.177095	0.186257	0.950813	0.73976	6.21
BjTCP1b&BjTCP1a	0.150689	0.154738	0.973836	0.900433	5.16
BjTCP1b&BjTCP5c	1.01566	0.944069	1.07584	0.380558	31.47
BjTCP1b&BjTCP5a	1.06615	0.787473	1.35388	0.00101769	26.25
BjTCP1b&BjTCP24a	0.994005	1.0184	0.976049	0.780298	33.95
BjTCP1b&BjTCP24b	0.984745	1.05014	0.937726	0.460408	35.00
BjTCP1d&BjTCP1a	0.145628	0.109236	1.33314	0.300704	3.64
BjTCP1d&BjTCP5c	1.03058	0.889865	1.15813	0.0776737	29.66
BjTCP1d&BjTCP5a	1.10372	0.690437	1.59858	9.46E-08	23.01
BjTCP1d&BjTCP24a	1.00635	0.979309	1.02762	0.775895	32.64
BjTCP1d&BjTCP24b	1.00846	0.97284	1.03662	0.647405	32.43
BjTCP1a&BjTCP5c	1.01142	0.959512	1.0541	0.488766	31.98
BjTCP1a&BjTCP5a	1.05563	0.823698	1.28158	0.00486352	27.46
BjTCP1a&BjTCP24a	1.02273	0.929274	1.10057	0.255428	30.98
BjTCP1a&BjTCP24b	1.00908	0.972336	1.03779	0.646827	32.41
BjTCP5c&BjTCP5a	0.150359	0.287462	0.523056	0.00041802	9.58
BjTCP5c&BjTCP24a	0.86782	0.888744	0.976457	0.774868	29.62

BjTCP5c&BjTCP24b	0.940256	1.01511	0.926263	0.402502	33.84
BjTCP5a&BjTCP24a	0.803529	0.715009	1.1238	0.250546	23.83
BjTCP5a&BjTCP24b	0.892262	1.00693	0.886122	0.193768	33.56
BjTCP24a&BjTCP24b	0.108959	0.0796321	1.36828	0.286969	2.65

*Mya, million years ago

Figures

Figure 1

The gene locations of BjTCP genes family. The chromosome name is at the top of each bar. The scale of the chromosome is in millions of bases (Mb).

Figure 2

Evolutionary relationships of taxa. A. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 78 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 636 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. B. A conserved motif in the Class I subfamily of the BjTCP gene family. C. A conserved motif in the Class II subfamily of the BjTCP gene family. The consensus sequences were displayed using Weblogo (<http://weblogo.berkeley.edu>) .

Figure 3

Genomic structure and motif composition of BjTCPs. A. The phylogenetic tree of BjTCP proteins. B. Genomic structure of BjTCPs family members in tumorous stem mustard. Exons and introns are represented with blank boxes and blank lines. C. The conserved motifs in tumorous stem mustard TCP proteins were identified using MEME. Each motif is represented with a specific color and the characters sequence were showed below.

Figure 4

Cis-acting elements on promoters of BjTCP genes. The colour bar showed the number of cis-acting elements.

Figure 5

Expression patterns of TCP genes in different tissues and development stages of *Brassica juncea* var. *tumida*. DY, Dayeje stems were collected 22 weeks after seeding (daye3bianzhong); YA1-4, The stems of Yong'an were collected 18, 20, 22, and 25 weeks after seeding; YAr, The mix roots samples of 18 and 22 weeks after seeding. The expression levels are represented by the color bar (log2-transformed).

Figure 6

Expression levels of BjTCPs under SA and GA treatment by qRT-PCR. The number represented the treatment times (hours). The colour scales represented relative expression data.

Supplementary Files

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