



Article

Genome-Wide Identification and Expression Pattern Analysis of the TCP Gene Family in Radish (*Raphanus sativus* L.)Yi Mei, Zhe Liu, Jiaqiu Zheng, Weiwei Wang, Yanxia Zu, Yongcheng Wu, Lina Zhang, Ruchao Feng and Feng Shen * 

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Abstract: TCP transcription factors are a unique transcription family in higher plants, and play important roles in plant development, responses to environmental stresses and phytohormones. Radish is an important crop and widely cultivated worldwide. However, genome-wide identification and expression analysis of TCP family in radish has not been reported yet. In this study, 32 *RsTCP* genes were identified from the whole genome. Phylogenetic analysis showed that the *RsTCP* family can be divided into two major groups and three subgroups, namely Class I (PCF), Class II (CIN), and Class II (CYC/TB1). Chromosome mapping showed that 32 genes were distributed on all nine chromosomes of radish. Transcriptome data indicated that the transcription of *RsTCP* genes differed between tissues and developmental stages. Multiple phytohormone-related *cis*-acting elements in the promoter region of *RsTCPs* and several post-translational modification sites in *RsTCP* protein sequences were identified. The real-time quantitative PCR (RT-qPCR) analysis indicated that several *RsTCP* genes changed significantly in response to the treatments, including low temperature, drought, salt, gibberellin (GA₃), and abscisic acid. Among them, *RsTCP16* showed significantly higher levels in leaves than in other radish tissues, and the transcription of *RsTCP16* was significantly upregulated under the five treatments, especially during low temperature, salt shock, and GA₃ response, suggesting that *RsTCP16* could be involved in the development of radish leaves and the response to stress. In summary, these results provided a basis for further exploring the molecular mechanism of *RsTCP* genes in radish.

Keywords: radish; TCP gene family; genome-wide analysis; expression profiling



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1. Introduction

Plant transcription factors play key roles in regulating the initiation of transcription of specific genes, which are crucial for development and responses to environmental changes in plants [1,2]. TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1 (TCP) transcription factors are a unique family in higher plants, because they are only detected in plants and are involved in physiological processes, including cell differentiation, seed germination, flowering, and phytohormone response in higher plants [3–6]. TCP is named using the acronyms of the three isolated members, including TEOSINTE BRANCHED 1 (TB1) in *Zea mays*, CYCLOIDEA (CYC) in *Antirrhinum majus*, PROLIFERATING CELL FACTORS 1 and 2 (PCF1 and PCF2) in *Oryza sativa* [7]. With the completion of genome sequencing of plant species, many TCP transcription factors have been identified and analyzed in recent decades. Up to date, a total of 24, 30, 27, 23, 39, 75, 21, and 27 TCP members have been identified in *Arabidopsis thaliana* [8], *Solanum lycopersicum* [9], *Citrullus lanatus* [10], *Phalaenopsis equestris* [11], *Brassica rapa* ssp. *rapa* [12], *Gossypium barbadense* [13], *Oryza sativa* [14], and *Cucumis sativus* [15], respectively.

There is a conserved TCP domain that is important for DNA binding existing in TCP transcription factors. Although the TCP domain has a N-terminal basic helix–loop–helix

(bHLH) structure, there are significant differences between the TCP and bHLH transcription factors [7]. Based on homology analysis of amino acid sequences of the conserved TCP domain, TCP transcription factors can be classified into two classes, namely Class I (PCF or TCP-P) and Class II (TCP-C) [8]. Distinctly different from the Class I, which contains a non-canonical bHLH motif, the Class II has four more amino acids in the conserved domain [7,16,17]. In addition, Class II members can be further subdivided into CIN and CYC/TB1 branches based on the sequence differences in the TCP domain [18,19].

Previous studies revealed that the TCP transcription factors play important roles in plant growth, development, and responses to biotic and abiotic stresses in *A. thaliana* [20]. For example, AtTCP4 regulates jasmonic acid (JA) biosynthesis through interaction with the *JGB* gene, which mediates the pollen germination and gametophyte development [21]. MPK8 can interact with AtTCP14 in the nucleus and phosphorylate AtTCP14 outside the nucleus, promoting seed germination [3]. The TCP transcription factors (Class II) can be used in combination with the FLOWERING LOCUS T (FT)-FD complex, which further acts on the AP1 promoter and actively regulates flowering [4]. TCP transcription factors inhibit the formation of cotyledon epidermal hair by inhibiting the activation of the MYB-bHLH-WD40 (MBW) complex at both transcription and protein levels [5]. In addition, TCP transcription factors are regulated by auxin, gibberellin, brassinolide, and other hormones in *A. thaliana* [6,22,23]. Under adverse environments, TCP transcription factors function in plant resistance and adaptation. For example, *TCP20* acts as an activator of nitrate assimilatory genes and promotes the expression of these genes under the nitrogen starvation condition in *A. thaliana* [24]. In response to cold stress, overexpression of *DgTCP1* improved the cold tolerance of chrysanthemum, while the *DgTCP1* editing line showed decreased survival ratio under cold stress [25]. *OsPCF2* has been proved to promote resistance to salt and drought stress in rice by activating the expression of the *OsNHX1* gene [26]. Overall, these results demonstrate a broad spectrum of TCP functions in plant growth and environmental adaptation.

Radish (*Raphanus sativus* L.) is an important root vegetable crop [27], and the whole genome sequence of radish has been published [28]. Up to date, genome-wide identification of the *TCP* gene family has been performed in several species, but not in radish. In this study, 32 *RsTCP* genes were identified in the radish genome, and the gene structure, conserved motif, phylogenetic and evolutionary relationship were analyzed. The expression pattern of *RsTCP* genes was further detected in different tissues, developmental stages, and stress conditions. Overall, these results preliminarily revealed the sequence information and potential biological functions of *RsTCP* transcription factors in radish, contributing basic information for further exploration of the molecular mechanisms underlying radish growth and responses to environmental stresses.

2. Materials and Methods

2.1. Plant Material and Treatments

The radish variety 'YC-FH1' was used to test the changes in *RsTCP* family expression in response to low temperature, saline and drought shock, as well as to treatments with abscisic acid (ABA) and gibberellin. Radish seeds were disinfected with 5% NaClO solution for 10 min, rinsed with distilled water, and germinated at 25 °C in the dark. Next, the germinated seeds were cultivated in nursery culture pots containing sterilized culture substrate. The photoperiod was 16 h/8 h (light/dark) and the temperature was 25 °C at day time and 16 °C at night. When the fourth or fifth true leaf appeared, healthy seedlings were randomly subjected to treatments with shocks and phytohormones. For low temperature shock, the culture temperature was adjusted to 4 °C [29]. In response to salt shock, 250 mM extra NaCl was added in the culture media [30]. Drought shock was simulated by incorporating 15% PEG6000 in the culture media [31]. For tests of phytohormones, 100 µM ABA solution [32] and 100 µM GA₃ [33] were sprayed on leaves, independently. All treatments were repeated three times, and at least nine seedlings were

used for each treatment. The samples were collected after each treatment and immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation.

2.2. Identification of *RsTCP* Genes in Radish Genome

The conserved domain of TCP proteins (<http://pfam.xfam.org/family/PF03634>; accessed on 13 March 2020) was used as the probe to search *RsTCP* sequences in the Radish Genome Database (<http://radish-genome.org/>; accessed on 15 May 2020) using the Hidden Markov Model (HMM) with the e-value set to 0.01 [28,34]. Sequences of candidate *RsTCP* genes were further queried against the SMART (<http://smart.embl.de/>; accessed on 15 May 2020) [35,36] and InterProScan (<https://www.ebi.ac.uk/interpro/>; accessed on 15 May 2020) databases to verify the reliability of the results [37]. The sequences of *AtTCP* genes were downloaded from the *A. thaliana* Information Resource (TAIR, <https://www.arabidopsis.org/>; accessed on 15 May 2020), and the sequences of *OsTCP* genes were downloaded from the Rice Genome Annotation Project (RAP) database (<http://rice.plantbiology.msu.edu/index.shtml>; accessed on 25 April 2022).

2.3. Protein Properties and Phylogeny Analysis

The characteristics of *RsTCP* proteins, including the number of amino acids (AA), theoretical molecular weight (MW), instability index (II), and isoelectric point (*pI*), were analyzed through the online ProtParam program (<https://web.expasy.org/protparam/>; accessed on 3 April 2021) [38]. The Online ScanProsite tool (<https://prosite.expasy.org/scanprosite/>; accessed on 2 September 2020) was used to analyze the post-translational modification sites of *RsTCP* proteins [39]. An un-rooted phylogenetic tree of 32 predicted *RsTCP* proteins, 24 reported *AtTCP* proteins, and 26 reported *OsTCP* proteins were generated subsequently using MEGA X software with: 1000 bootstrap replicates (Test of phylogeny), p-distance (Model/Method), and pairwise deletion (Gaps/Missing Data treatment) [36,40]. Evolview (<http://www.evolgenius.info/evolview/>; accessed on 25 April 2022) was used to visualize the un-rooted phylogenetic tree [41].

2.4. Gene Structure, Conserved Motifs, and Cis-Element Analyses

According to the genome sequences and coding sequences (CDS) of the *RsTCP* gene family, the structure of *RsTCPs* were analyzed using TBtools [42]. The conservative motif analysis of *RsTCP* genes was performed using the MEME program. The maximum number of motifs was set to 10 and the optimal motif width was set to 6–50 bp [43]. The online software PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>; accessed on 3 July 2020) was used to investigate the *cis*-elements of the *RsTCP* genes [44].

2.5. Synteny Analysis, Chromosomal Localization, and Prediction of *rsa-miR319* Target Genes

Collinearity analysis was performed according to methods described in the PlantDGD (<http://pdgd.njau.edu.cn:8080/>; accessed on 11 August 2020) [45]. The collinearity block in *RsTCP* genes was identified based on duplication events using MCScanX [46], and then Circos was used to integrate and plot the data [47]. Based on the distribution information of the putative *RsTCP* genes from the radish genome, the location of *RsTCPs* on chromosomes was identified using TBtools software [42]. Finally, psRNATarget (<http://plantgrn.noble.org/psRNATarget/>; accessed on 27 August 2020) was used to identify *rsa-miR319* target genes.

2.6. Transcription Analysis of *RsTCP* Genes

Illumina RNA sequencing data of radish leaf, root tip, cambium, cortex, and xylem tissues at six developmental stages (7, 14, 20, 40, 60, and 90 d) were downloaded from the NODAI Genome [48]. Afterwards, the transcription levels of each *RsTCP* gene were compared by calculating the reads per kilobase of transcript per million mapped reads (RPKM) as described in Mitsui et al. [48] and the heat map was generated using TBtools [42].

2.7. RNA Extraction and RT-qPCR Analysis

Total RNA of all samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instruction, and the isolated RNA was subsequently treated with RNase-Free DNase I (Takara, Dalian, China). Total RNA was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Bioer, Hangzhou, China). Specific primers (Table S1) for *RsTCP* genes were designed using Beacon Designer 7.7 (Premier Biosoft International, Palo Alto, CA, USA), and the SYBR Green RT-qPCR mix (Bioer, Hangzhou, China) was used for RT-qPCR assays on a LightCycler 480 Instrument (Roche, Mannheim, Germany). In addition, the *RsActin* gene was used as the internal control. At least three biological replicates were tested for each treatment, and three technical replicates were run for each reaction. The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta CT}$ method [49]. The data used for expression profile analysis comprised the mean of three biological replicates, and the standard errors of the mean were calculated on the basis of the technical replicated data.

3. Results

3.1. Identification and Classification of *RsTCP* Members in Radish

In order to obtain the candidate TCP members in radish, the TCP domain (PF03634) was used to search against protein sequences of the radish genome. A total of 32 *RsTCP* members were identified from the radish genome and were named from *RsTCP01* to *RsTCP32* based on their positions on the chromosomes. Each *RsTCP* protein contained 109 to 460 amino acids, and their molecular weight ranged from 12.25 to 44.47 kDa. The theoretical *pI* ranged from 5.85 to 9.99, with a mean of 7.71, suggesting that most of *RsTCP*s were weakly alkaline (Table S2). The instability index of each protein ranged from 37.56 to 71.34. Among them, only one *RsTCP* showed an instability index less than 40.00, and thus this protein was predicted as a stable protein. The other 31 *RsTCP*s were structurally unstable. The aliphatic index ranged from 50.93 to 69.82, indicating that most *RsTCP*s contain a large number of aliphatic amino acids. The grand average of the hydropathy (GRAVY) value ranged from -1.043 to -0.204 , indicating that all *RsTCP*s were hydrophilic (Table S2).

3.2. Phylogenetic Analysis of *RsTCP* Proteins

To better clarify the phylogenetic relationships among the predicted *RsTCP*s, the 32, 25, and 24 TCP proteins identified from radish, rice, and Arabidopsis were aligned using the ClustalW program, and then an un-rooted phylogenetic tree was constructed using MEGA X (Figure 1). The result showed that these 81 TCP proteins could be clustered into two categories (Class I and Class II) and three groups (PCFs, CINs, and CYCs). The Class I (PCF) subgroup contained 44 members (54.32%), including 17 *RsTCP*s, 14 *OsTCP*s, and 13 *AtTCP*s, respectively. Class II included CIN and CYC/TB1 types. Among them, 13 *RsTCP*s, 8 *OsTCP*s, and 8 *AtTCP*s belonged to the CIN subgroup, while 2 *RsTCP*s, 3 *OsTCP*s, and 3 *AtTCP*s were categorized into the CYC/TB1 type, accounting for 9.88% of the total. In addition, the result also showed that the relationship of TCPs between radish and the dicot Arabidopsis was closer to that of the monocot rice.

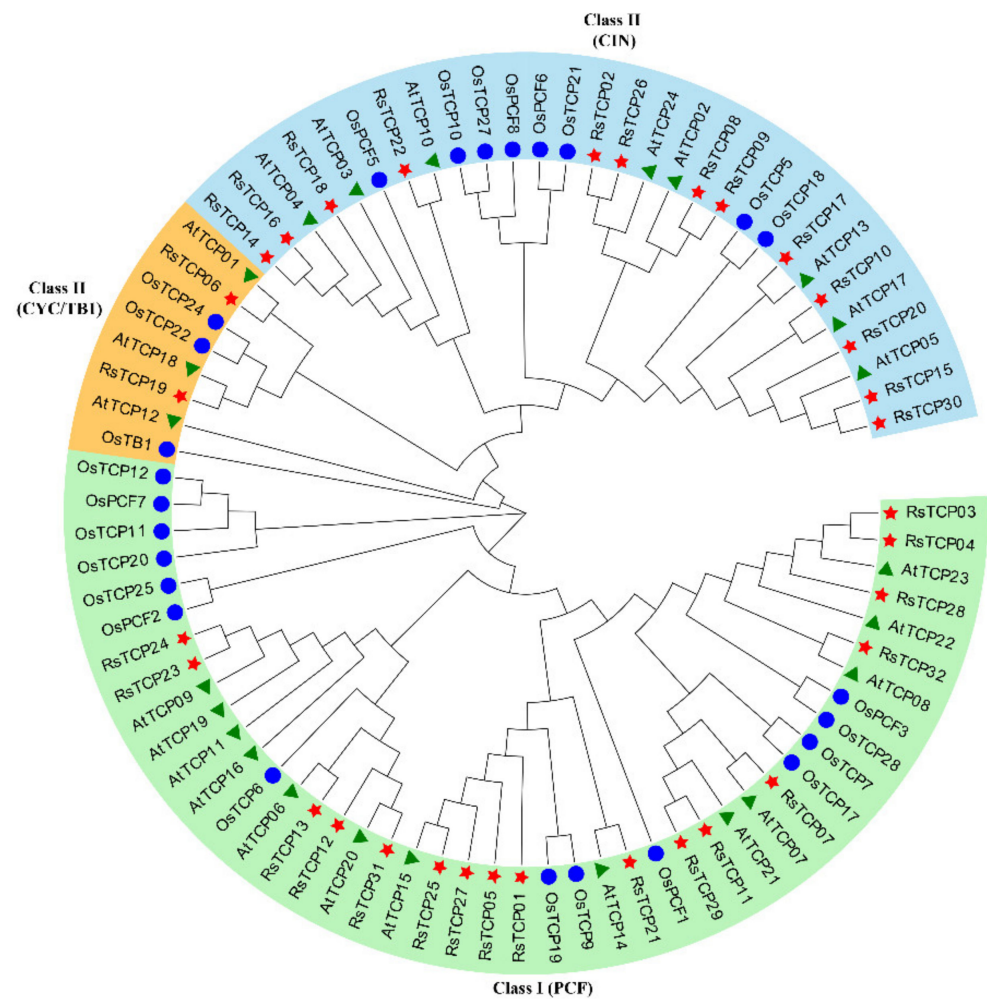


Figure 1. Phylogenetic tree of the predicted TCP proteins from radish, rice, and Arabidopsis.

3.3. Motif and Gene Structure Analyses

In total, 10 conserved motifs were identified from 32 RsTCP proteins using the online software MEME motif search tool [50,51]. Subsequently, each of these 10 identified motifs containing 6–50aa was submitted to the InterProScan and SMART for further identification. Among them, motifs 1 and 2 were the typical characteristic domains of the plant TCP family and were present in all RsTCP members. However, the motif 3 exclusively existed in all Class II RsTCP members but not in Class I, suggesting that motif 3 may play an important role in the structural differences of RsTCP proteins (Figure 2b). Based on the phylogenetic analysis, most RsTCP proteins in the same evolutionary branch had a similar motif distribution. These results indicated that RsTCP proteins from the same evolutionary branch may have similar biological functions.

Exon–intron analysis of *RsTCP* genes indicated that the gene structure of the *RsTCP* gene family was relatively simple, and 28 out of the 32 *RsTCP* genes did not have any introns while *RsTCP32*, *RsTCP06*, *RsTCP20*, and *RsTCP30* contained 1–3 introns (Figure 2c). In addition, except for *RsTCP11*, *RsTCP14*, *RsTCP23*, and *RsTCP24*, the remaining 28 *RsTCP* genes all had untranslated region (UTR) annotations in the radish genome.

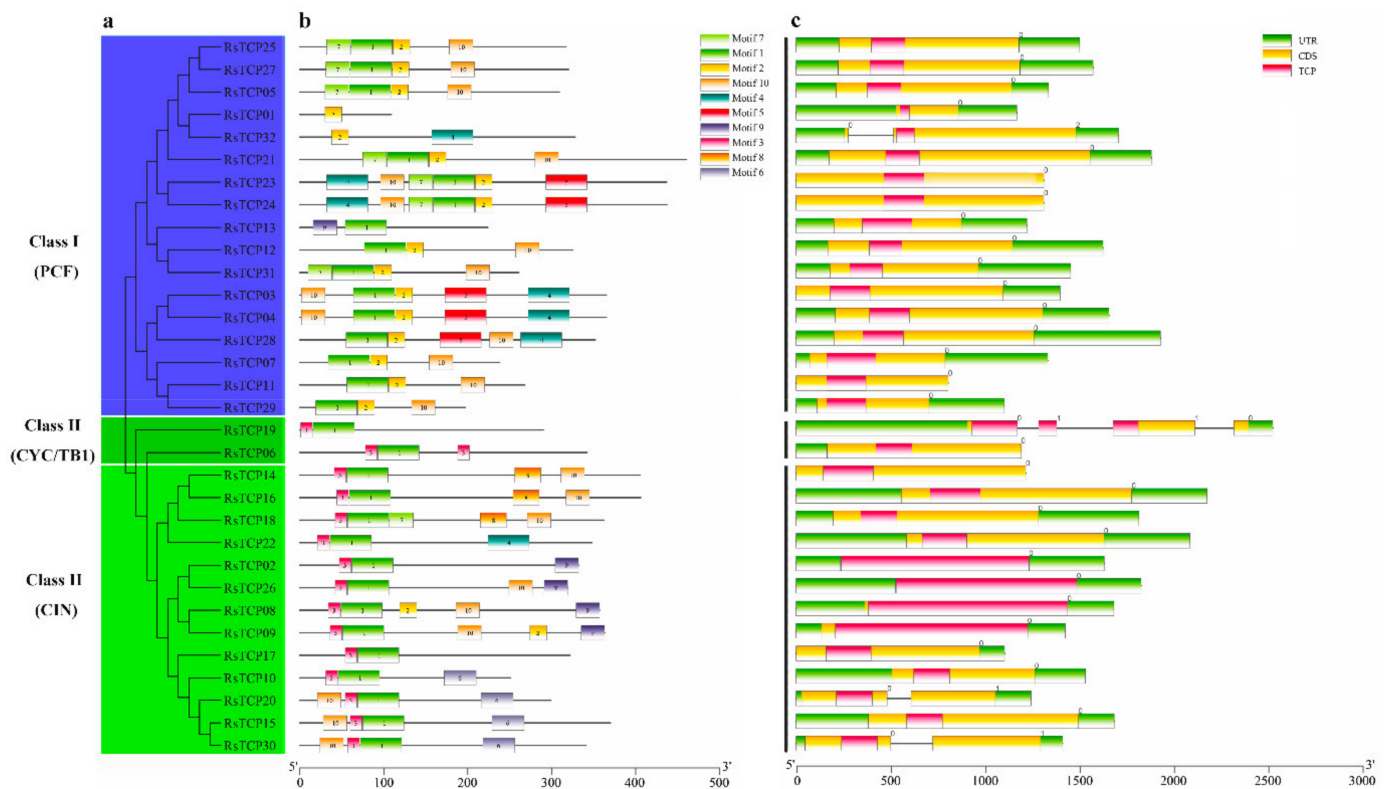


Figure 2. Distribution of conserved motif and gene structure of *RsTCP* genes. (a) Phylogenetic tree of 32 *RsTCP* proteins; (b) distribution of the conserved motif for *RsTCP* proteins; (c) exon–intron structure of *TCP* genes in radish.

3.4. Cis-Element Analysis of *RsTCP* Genes

It is commonly accepted that the *cis*-elements play a critical role in regulation of gene transcription. In this study, the promoter sequences of all 32 *RsTCP* genes (within 2 kb upstream of the start codon) were analyzed using PlantCARE, and a total of 692 *cis*-elements were detected. In addition to some basic elements such as TATA-box and CAAT-box, nine important *cis*-elements potentially related to plant development and responses to various stresses were identified (Figure 3). The light responsive elements were observed in the promoter regions of all *RsTCP* genes. The drought responsive elements were detected in *RsTCP25*, *RsTCP05*, *RsTCP21*, *RsTCP31*, *RsTCP03*, *RsTCP11*, *RsTCP29*, *RsTCP19*, *RsTCP06*, *RsTCP14*, and *RsTCP16*. In addition, ABA, GA₃ salicylic acid (SA), auxin (AUX), and low-temperature responsive elements presented in promoter regions of some *RsTCP* genes. These findings suggested that *RsTCP* genes might respond to changes in light, temperature, water supply, and be regulated by ABA, GA₃, SA, and AUX.

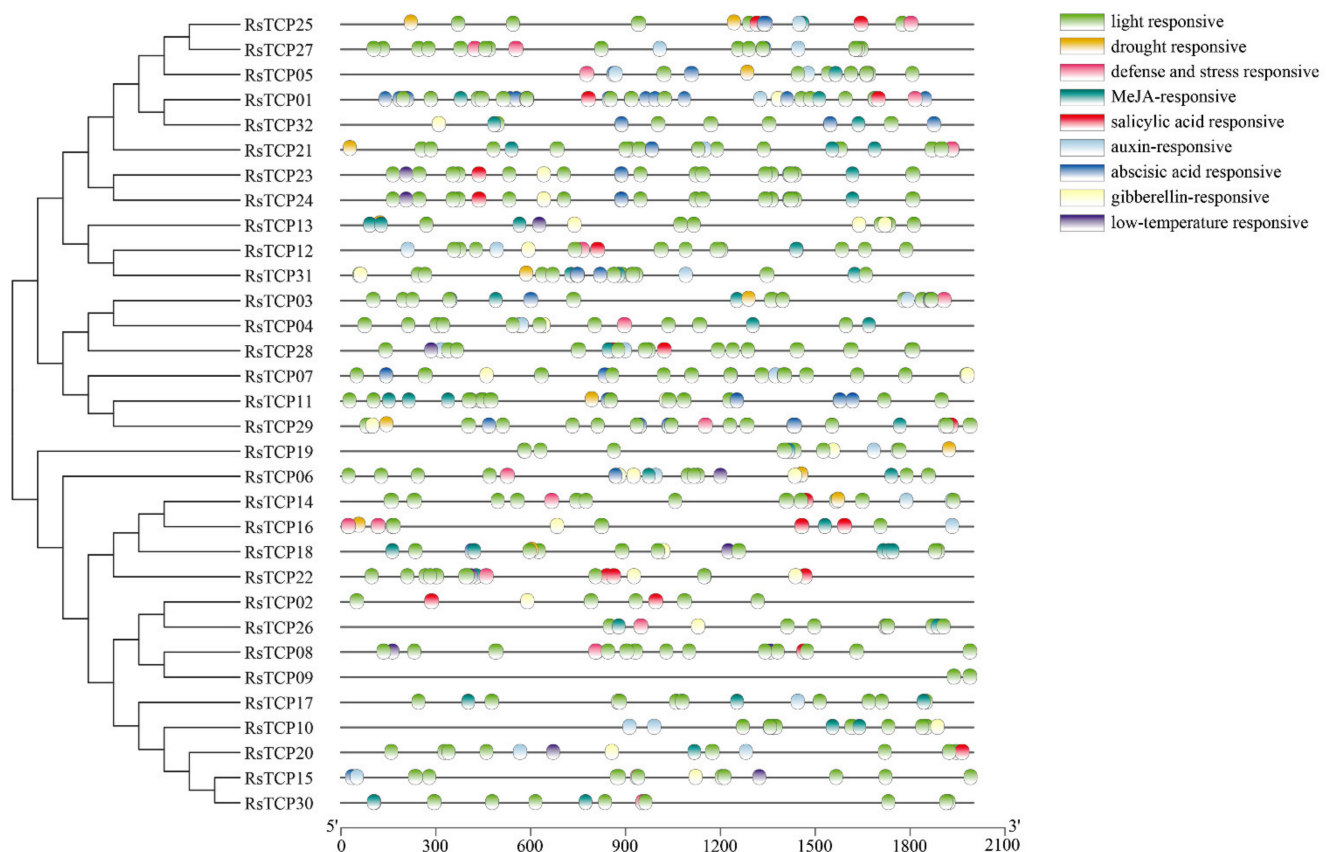


Figure 3. Cis-acting elements on promoters of *RsTCP* genes.

3.5. Chromosomal Localization and Gene Distribution Analysis

All these 32 *RsTCP* genes identified in this study were mapped to the nine chromosomes (from R1 to R9) in radish (Figure 4). The results showed that R2 and R6 contained the most *RsTCP* genes with seven *RsTCP*s (21.86%) on each chromosome, followed by R9 with six *RsTCP*s (18.75%). R5, R4, and R1 contained four, three, and two *RsTCP*s (12.5%, 9.38%, and 6.25%), respectively. In addition, each of R3, R7, and R8 contained only one *RsTCP* gene.

Genome duplication events have been proven to promote the expansion of plant gene families, including whole-genome duplication (WGD)/segmental duplication (SD), transposed duplication (TRD), dispersed duplication (DD), proximal duplication (PD), and tandem duplication (TD) [52]. In this study, MCScanX analysis revealed that a total of 15 pairs of *RsTCP* genes were collinear, and 18 (56.25%) *RsTCP* genes were duplicated and retained in the WGD/SD (Figure 4). These results suggested that the WGD/SD event might play an important role in the extension of the *RsTCP* gene family.

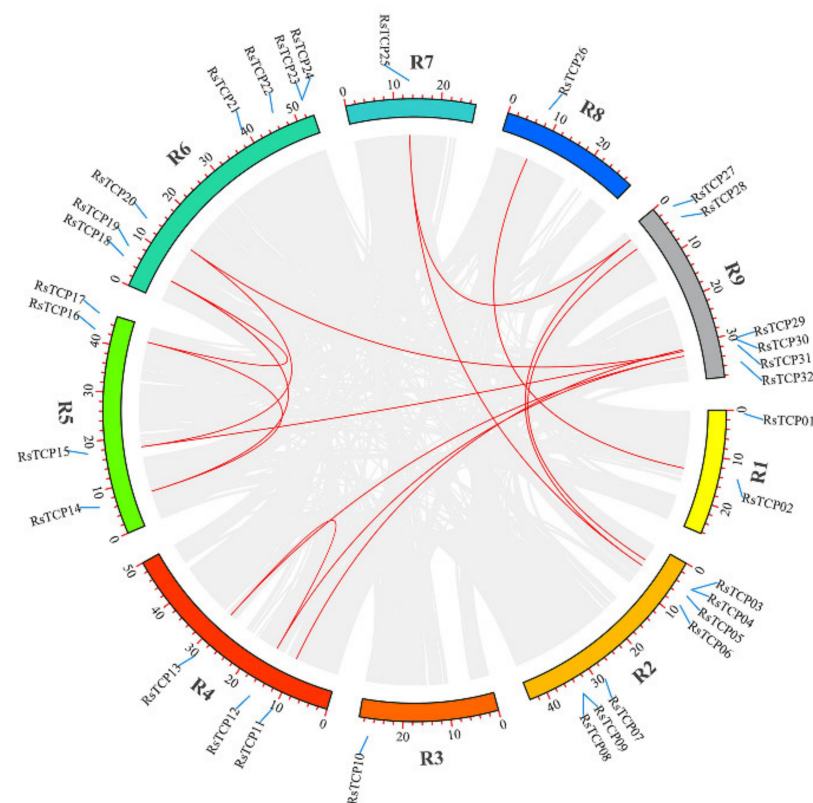


Figure 4. The chromosomal location and synteny analysis of the *R*sTCP family. Red lines indicate the collinear relation.

3.6. Evolution Analysis of the *R*sTCP Genes and Target Site Analysis of *rsa*-miR319

In order to explore the evolutionary history and the origin of TCP genes, syntenic regions were compared between radish and *A. thaliana* (Figure 5). The result demonstrated that 34 pairs of collinear genes were obtained, including 26 *R*sTCP genes and 18 *At*TCP genes. Among them, six pairs of orthologous genes (one to one) were identified, including *R*sTCP06 and *At*TCP01, *R*sTCP09 and *At*TCP02, *R*sTCP23 and *At*TCP09, *R*sTCP22 and *At*TCP10, *R*sTCP21 and *At*TCP14, as well as *R*sTCP32 and *At*TCP08, suggesting that these genes might have the same common ancestors between *A. thaliana* and radish. In addition, one *R*sTCP gene may correspond to multiple *A. thaliana* TCP genes, for example, *R*sTCP11 and *At*TCP21/07, *R*sTCP18 and *At*TCP03/04. Similarly, multiple radish genes may correspond to one *A. thaliana* gene, such as *At*TCP03 and *R*sTCP14/16/18, *At*TCP15 and *R*sTCP05/25/27 (Figure 5, Table S3). These synchronous events revealed that some TCP genes evolved before the divergence of the *A. thaliana* and radish lineages.

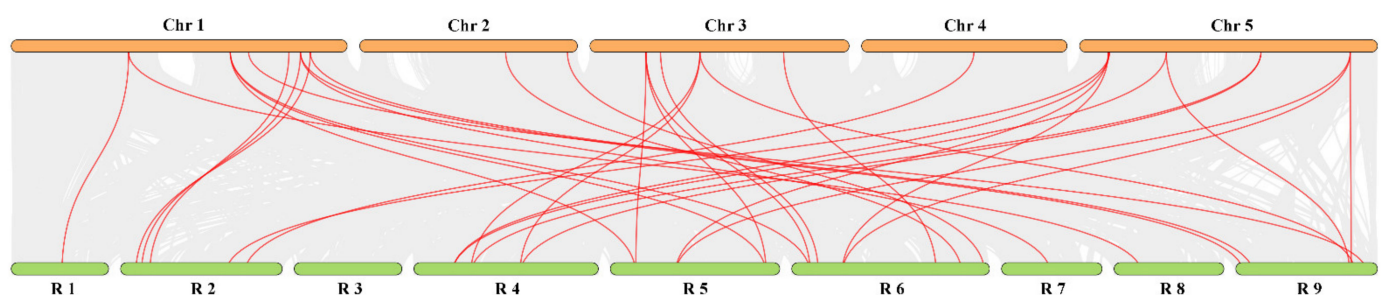


Figure 5. Synteny analysis of TCP genes between radish and Arabidopsis. Colored lines connecting two chromosomal regions indicate syntenic regions between radish (R1–9) and Arabidopsis (Chr1–5) chromosomes.

The target sites of *rsa-miR319* in the *RsTCP* genes were predicted by psRNATarget online software, and it was found that eight putative genes contained *rsa-miR319* recognition site, namely *RsTCP02*, *RsTCP08*, *RsTCP09*, *RsTCP14*, *RsTCP16*, *RsTCP18*, *RsTCP22*, and *RsTCP26* (Figure 6), while the other *RsTCP* genes did not contain a putative *rsa-miR319* recognition site. This result suggested that these eight putative genes may play more critical roles in the growth and development of radish than other *RsTCP* members.

<i>rsa-miR319</i>	UUGGACUGAAGGGAGCUCCCU
<i>RsTCP02</i>	AACCUGACUUCCCUAGGGGGAU
<i>RsTCP08</i>	AACCUGACUUCCCAGGGGGAC
<i>RsTCP09</i>	AACCUGACUUCCCAGGGGGAC
<i>RsTCP14</i>	GACCUGACUUCCCUCGGGGAG
<i>RsTCP16</i>	GACCUGACUUCCC AUGGGGAG
<i>RsTCP18</i>	GACCUGACUUCCC CUGGGGAG
<i>RsTCP22</i>	GACCUGACUUCCC AUGGGGCA
<i>RsTCP26</i>	AACCUGACUUCCCAGGGGGAU

Figure 6. Alignment of putative target sites for *rsa-miR319*. Mismatches are represented in yellow.

3.7. Characterization of Deduced *RsTCP* Proteins

Multiple post-translational modification sites were identified in the *RsTCP* protein using ScanProsite. Except *RsTCP03*, several putative phosphorylation sites were identified in other *RsTCP* proteins in the form of cAMP-cGMP kinase (cAMP-cGMP), protein kinase C (PKC), and casein kinase II (CK II). Additionally, N-Glycosylation (N-Glyc) and N-Myristoylation (N-Myr) were also found in deduced *RsTCP* protein sequences (Table 1). Interestingly, *RsTCP* proteins in the same evolutionary branch showed similar composition and number of post-translational modification sites. For example, both *RsTCP23* and *RsTCP24* in Class I contained nine PKC, three cAMP-cGMP, five CK II, five N-Myr, and one N-Glyc modification site. Both *RsTCP08* and *RsTCP09* had four PKC, seven CK II, and four N-Glyc modification sites. Similar results were also found in Class II members. These modification sites should be important for the regulation of protein activity and cell signal transduction.

Table 1. Predicted *RsTCP* protein sequence features.

Name	PKC	CK II	cAMP-cGMP	N-Myr	N-Glyc
<i>RsTCP01</i>	3	1	0	0	3
<i>RsTCP02</i>	11	8	0	20	6
<i>RsTCP03</i>	0	0	0	0	0
<i>RsTCP04</i>	5	3	1	14	5
<i>RsTCP05</i>	3	5	1	12	2
<i>RsTCP06</i>	6	7	0	3	1
<i>RsTCP07</i>	2	1	0	7	0
<i>RsTCP08</i>	4	7	0	10	4

Table 1. Cont.

Name	PKC	CK II	cAMP-cGMP	N-Myr	N-Glyc
RsTCP09	4	7	0	11	4
RsTCP10	6	3	0	2	4
RsTCP11	3	4	0	6	2
RsTCP12	5	5	1	16	2
RsTCP13	3	4	1	6	2
RsTCP14	6	4	0	3	1
RsTCP15	5	4	0	3	5
RsTCP16	6	6	1	7	4
RsTCP17	8	4	0	6	4
RsTCP18	5	7	0	2	0
RsTCP19	4	7	3	3	4
RsTCP20	5	3	0	3	3
RsTCP21	3	7	1	14	3
RsTCP22	4	5	0	5	4
RsTCP23	9	5	3	5	1
RsTCP24	9	5	3	5	1
RsTCP25	4	6	1	15	3
RsTCP26	7	9	0	7	2
RsTCP27	5	6	1	17	5
RsTCP28	5	7	1	23	3
RsTCP29	2	2	0	4	1
RsTCP30	4	2	0	4	5
RsTCP31	4	7	1	12	1
RsTCP32	8	9	0	16	4

The post-translational modifications were predicted using ScanProsite (<http://ca.expasy.org/tools/scanprosite/>; accessed on 2 September 2020).

3.8. Transcription Profiling of RsTCP Genes in Radish

From the published database, RPKM of *RsTCP* genes in leaf, root tip, cambium, cortical, and xylem at different developmental stages were obtained and further analyzed (Figure 7, Table S4). Overall, the transcript levels of Class I members were significantly higher than those of Class II members in different growth periods and tissues. In the Class I, the expression of *RsTCP31* was lower in all samples, while the other 16 members showed higher levels in some samples, especially *RsTCP03*, *RsTCP04*, *RsTCP07*, *RsTCP11*, and *RsTCP28*. The expression profiles of these *RsTCP* genes varied greatly among developmental stages, suggesting their potential roles in radish development. In contrast, significantly higher expression levels of *RsTCP02*, *RsTCP08*, *RsTCP09*, *RsTCP14*, *RsTCP16*, *RsTCP18*, *RsTCP22*, and *RsTCP26* were observed in leaves at different stages, suggesting that these eight members may play crucial roles in the development of radish leaves. However, the expression profiles of other Class II members were extremely low in all samples, especially the two CYC/TB1 members in Class II, which were barely detected in all tissues at all stages. In addition, the expression of *RsTCP03*, *RsTCP04*, and *RsTCP28* showed significant upregulation in cortical at 40- and 60-day-old stages and root tip at 60- and 90-day-old stages, demonstrating that these three *RsTCP* genes might be specifically involved in regulating specific stages of radish development. Overall, these results suggested that transcription of *RsTCP* genes was spatial- and temporal-specific, indicating that they may play different functions in the growth and development process.

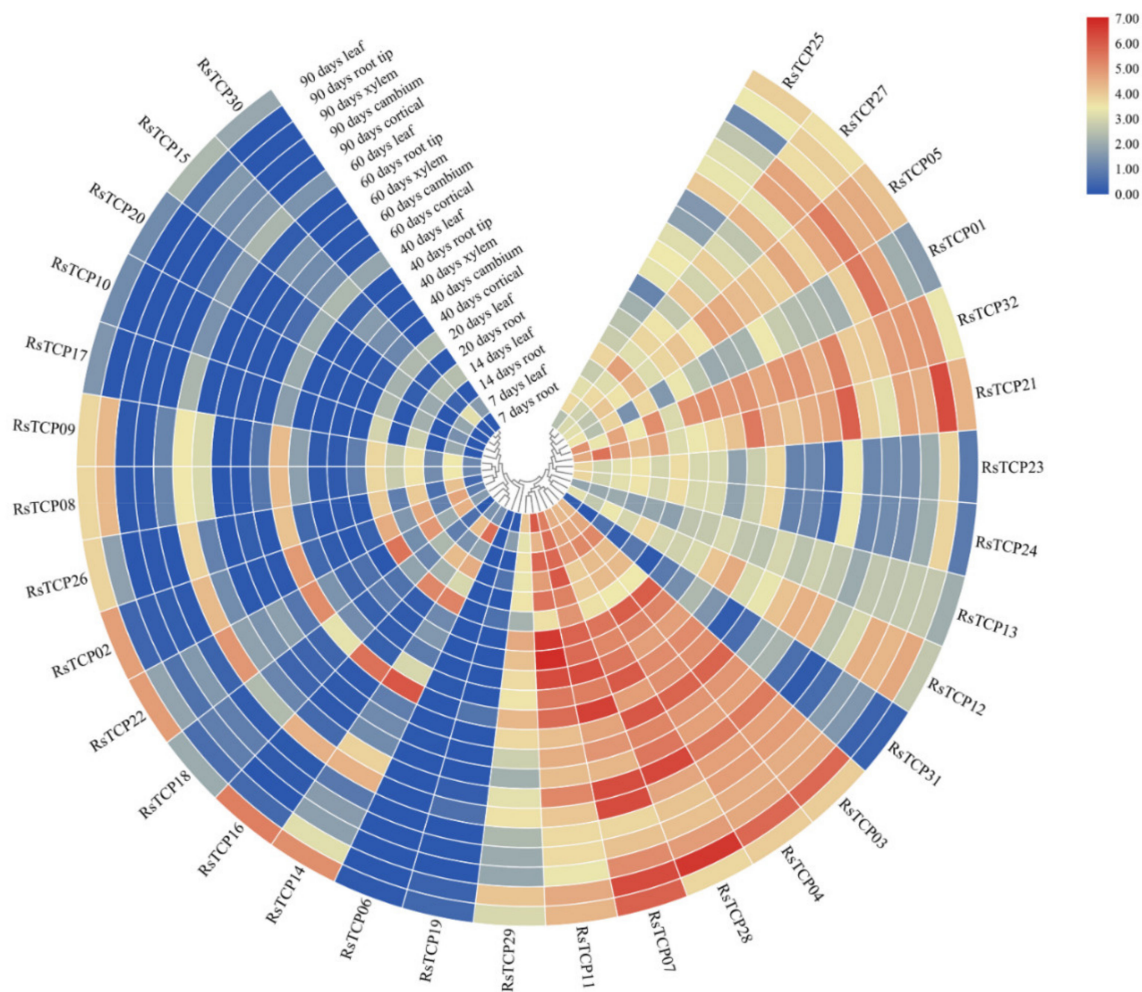


Figure 7. Expression profile of *RsTCP* genes in different stages and tissues.

3.9. Transcription of *RsTCP* Genes in Response to Environmental Shocks and Phytohormones

Based on the results of transcriptome data, nine *RsTCP* genes were selected for further investigation of the expression patterns of *RsTCP* genes after 4 °C, 250 mM NaCl, 15% *w/v* PEG6000, 100 µM ABA, and 100 µM GA₃ treatments. In response to low temperature, except for *RsTCP11* and *RsTCP27*, which showed slight downregulation, and *RsTCP21* showed slight upregulation, the remaining six *RsTCP* genes were significantly upregulated compared with the control (Figure 8a). After NaCl treatment for 24 h, only *RsTCP11*, *RsTCP16*, and *RsTCP28* showed significant upregulation, and *RsTCP21* and *RsTCP22* showed significant downregulation, the expression levels of the remaining four genes only showed slight changes (Figure 8b). Under drought shock, *RsTCP32* exhibited a unique and significant downregulation, whereas the expression of the remaining eight *RsTCP* genes all showed upregulation to varying degrees (Figure 8c). Moreover, a slight increase in the transcript abundance of *RsTCP03* was observed, while the expression level of the others showed significant upregulation under 100 µM ABA treatment for 24 h (Figure 8d). In addition, *RsTCP* genes were all highly sensitive to GA₃ exposure, and all *RsTCP* genes were significantly upregulated after 24 h treatment of 100 µM GA₃ solution (Figure 8e). The expression level of *RsTCP16* showed significant upregulation under the five treatments, especially under 44 °C, NaCl and GA₃. In summary, the transcript levels of *RsTCP* genes were differentially regulated under various treatments, indicating that the *RsTCP* genes might play an important role in response to abiotic stresses and phytohormones in radish.

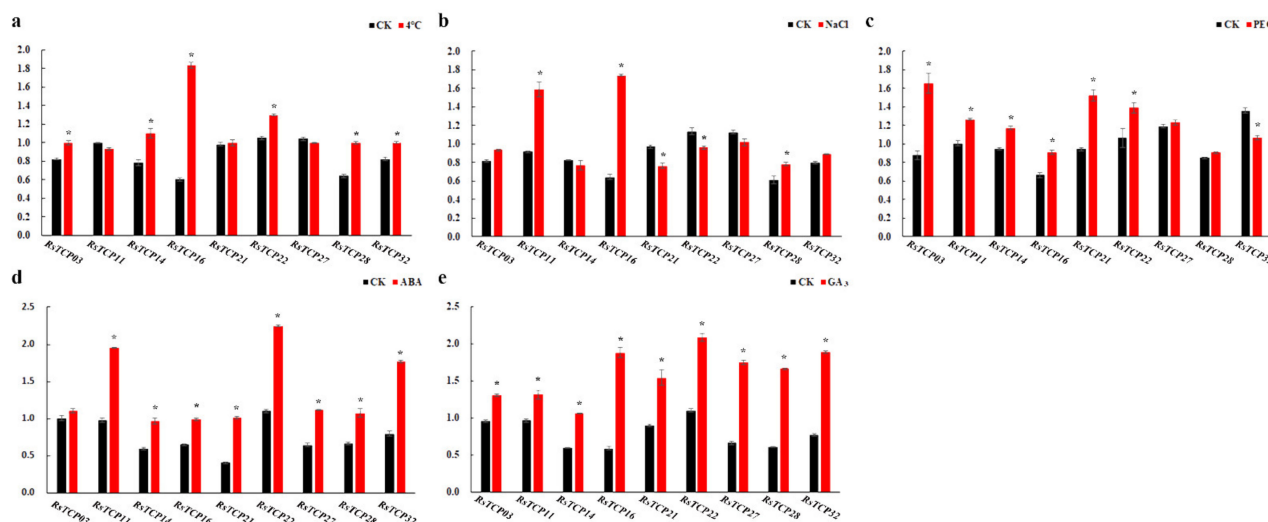


Figure 8. The expression of *RsTCP* genes after 24 h treatment in response to environmental shocks and phytohormones. (a) Low temperature treatment (44 °C); (b) NaCl (250 mM); (c) PEG6000 (15% w/v); (d) ABA (100 μM); (e) GA₃ (100 μM). The *RsActin* gene was used as the internal control, and three biological and technical replicates were conducted. The relative expression levels of each gene were calculated using the $2^{-\Delta\Delta CT}$ method. Each bar shows the mean $\pm$ SE of the triplicate assay, and * indicates significant difference at $p < 0.05$ according to Duncan's multiple range test.

4. Discussion

Based on whole-genome sequences, the *TCP* gene family has been identified in many species [8–15]. Although previous studies have shown that some *TCP* genes in radish could not only participate in anthocyanin biosynthesis, but also respond to Pb, Cr, and heat stress, the members of the *TCP* gene family in radish have not been identified yet, and the structure, evolution, and expression patterns of these members have not been analyzed [53–56]. In this study, 32 *TCP* genes were identified in the radish genome according to the conserved domain of *TCP* proteins.

TCP genes are generally conserved in different plant species. In this study, the phylogenetic tree showed that a total of 32 *RsTCP* genes were divided into two major groups and three subgroups (Figure 1), consistent with the previous studies in other species [15,57,58]. As shown in Figure 1, the phylogenetic analysis showed 13 *AtTCP*s in Class I and 11 *AtTCP*s in Class II, which was consistent with Navaud et al. [8], demonstrating the reliability of the phylogenetic tree. Moreover, the expansion of the gene family was mainly caused by gene replication [59], and duplicated genes could obtain new functions to improve plant adaptability to the environment in the process of plant evolution [60]. In this study, a total of 34 pairs of collinear genes were obtained based on evolution analysis, including 26 *RsTCP* genes and 18 *AtTCP* genes (Figure 5, Table S3), while a total of 32 *RsTCP* genes were identified in this study, exactly 8 more than the 24 *AtTCP* genes identified in a previous study [8], indicating that *TCP* family members may have been replicated or expanded in the radish genome. In addition, similar to other plants, genes in the evolutionary branch shared similar gene structure and motif distribution [61,62], and a duplicated gene pair tended to be clustered into one branch. For examples, the pairs of *RsTCP03-RsTCP04* and *AtTCP15-RsTCP05/25/27* were clustered into Class I (PCF), the pairs of *RsTCP02-RsTCP26* and *AtTCP03-RsTCP14/16/18* were clustered into Class II (CIN) (Figures 1, 4 and 5). Overall, these results provided a reliable and informative basis of the *TCP* gene family in radish, which may be useful for further investigation of the regulatory functions of *TCP* genes in radish.

TCP transcription factors could affect plant growth, development and multiple physiological processes [63], including gametophyte development, seed germination, floral organ and leaf development [3,4,21,64,65]. Moreover, it is well known that microRNA

(miRNA) control key biological processes in plants [66–70]. Among them, miR319 has been extensively studied for its roles in regulating leaf shape development due to its close relationship with *TCP* genes. For example, a previous study showed that *BrTCP4*, as an miR319 target gene, could regulate the round shape of leafy heads of Chinese cabbage [71]. Recent research revealed that *AtTCP2* could play multiple roles through miR319, including regulating leaf morphogenesis and leaf senescence in *A. thaliana* [72]. In this study, eight putative *rsa-miR319*-targeted genes were identified (Figure 6). Interestingly, the phylogenetic tree showed that all these eight putative *rsa-miR319*-target genes belonged to the Class II (CIN) subfamily, which was similar to the result in cucumber [15]. Moreover, the expression profile analysis showed that these eight genes had relatively high expression levels in leaves at various developmental stages (Figure 7), indicating that these eight genes might play a key role in leaf development in radish. In addition, sequence characterization and phylogenetic analysis suggest that *RsTCP16* is a homologue to *AtTCP4* in *A. thaliana* (Figures 1 and 5, Table S3), which not only regulates leaf growth but also inhibits cell proliferation [73,74]. Therefore, we speculated that the *RsTCP16* gene might similarly regulate leaf development in radish.

Increasing evidence indicated that *TCP* genes could not only participate in the biosynthesis of phytohormones, but also play a key role in the regulation of phytohormone-mediated development and growth [26,75]. In this study, multiple phytohormone-related *cis*-acting elements were identified in the promoter regions of *RsTCP* genes (Figure 3), consistent with the results in cucumber [15]. Post-translational modification is also crucial for plant growth and stress tolerance [76]. In this study, multiple post-translational modification sites were predicted in *RsTCPs* (Table 1), indicating that the *RsTCP* gene family may participate in a variety of biological processes. The results of RT-qPCR suggested that only some of these nine *RsTCP* genes were sensitive to low temperature shock, salt shock and drought shock, and almost all *RsTCP* genes were significantly upregulated in response to ABA and GA₃ treatments. In addition, the transcription of *RsTCP16* was significantly upregulated under the five treatments, suggesting that *RsTCP16* could play an important role in response to abiotic stresses and phytohormones (Figure 8). In conclusion, our results suggested that *RsTCP16* might play key roles in the growth and development of radish and lay the foundation for further investigation on the mechanism underlying the abiotic stress and phytohormones response in radish.

5. Conclusions

In summary, we undertook the first identification of a total of 32 *RsTCP* genes from the radish genome, which localized on nine radish chromosomes. Meanwhile, several *cis*-elements involved in plant development and abiotic stress response were detected in the promoter regions. Moreover, post-translational modification sites were found in the *RsTCP* proteins and evolution analysis suggested that some *TCP* genes were evolved before the divergence of the radish and *A. thaliana* lineages. In addition, the results of this study also showed that *RsTCP* genes exhibited tissue-specific expression patterns and revealed that *RsTCP16* might play a key role in response to abiotic stresses and phytohormones.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae8070656/s1>, Table S1: Primer sequences for RT-qPCR of *RsActin* and *RsTCP* genes; Table S2: The information of the *TCP* gene family in radish; Table S3: Synteny blocks of *TCP* genes between radish and Arabidopsis genomes; Table S4: Tissue-specific expression of *RsTCP* genes.

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