Genome-wide Identification of TCP Transcription Factors and Abiotic Stress Analysis in Tartary Buckwheat (Postprint)

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Abstract

TCP transcription factors are plant-specific and play important roles in plant growth and development. This study conducted genome-wide identification of the TCP family in tartary buckwheat using bioinformatics methods, and analyzed the expression characteristics of tartary buckwheat TCP genes under drought and salt stresses through real-time fluorescence quantitative PCR (qRT-PCR) technology, laying a foundation for functional research and utilization of the tartary buckwheat TCP family. The results showed that: (1) 28 TCP family members were identified in the tartary buckwheat genome, unevenly distributed across 8 chromosomes of tartary buckwheat. (2) Most tartary buckwheat TCP genes contain 1-5 exons. (3) Phylogenetic analysis divided the tartary buckwheat TCP family into 5 subfamilies, with intraspecific TCP proteins mostly clustering on the same branch. (4) Synteny analysis indicated that 5 tartary buckwheat TCP genes originated from whole-genome duplication events. (5) Cis-element analysis revealed that cis-responsive elements in the promoter regions of tartary buckwheat TCP genes mainly comprise two categories: stress-responsive elements and hormone-responsive elements. (6) Transcriptome data analysis showed that all tartary buckwheat TCP genes were expressed in the tested tissues. (7) qPCR results showed that the expression levels of FtTCP3, FtTCP6, FtTCP12, and FtTCP13 genes changed under drought and salt stresses, among which FtTCP3 reached peak expression at 6 h of both drought and salt treatments, indicating that the FtTCP3 gene plays a positive regulatory role in tartary buckwheat's response to drought and salt stresses. This study provides new insights into understanding the evolution and function of the TCP gene family.

Full Text

Preamble

Genome-wide Identification and Abiotic Stress Response Analysis of TCP Transcription Factor Gene Family in Tartary Buckwheat (Fagopyrum tataricum)

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Abstract: TCP transcription factors are a plant-specific group of transcription factors that play crucial roles in plant growth and development. In this study, bioinformatics methods were used to identify the complete TCP gene family in the Tartary buckwheat genome, and real-time quantitative PCR (qRT-PCR) was conducted to investigate the expression characteristics of TCP genes under drought and salt stress. These findings provide a foundation for the functional exploration and utilization of the Tartary buckwheat TCP gene family. The results were as follows: (1) A total of 28 TCP genes were identified in the Tartary buckwheat genome, unevenly distributed across its eight chromosomes. (2) Most Tartary buckwheat TCP genes contained 1–5 exons. (3) Phylogenetic analysis classified the Tartary buckwheat TCP family into five subfamilies, with intraspecific TCP proteins mainly clustering together. (4) Collinearity analysis indicated that five Tartary buckwheat TCP genes originated from genome-wide duplication events. (5) Cis-element analysis revealed that the promoter regions of Tartary buckwheat TCP genes predominantly contained two types of cis-response elements: stress response elements and hormone response elements. (6) Transcriptomic data analysis demonstrated that all Tartary buckwheat TCP genes were expressed in the examined tissues. (7) qRT-PCR results indicated that the expression levels of FtTCP3, FtTCP6, FtTCP12, and FtTCP13 changed under drought and salt stress conditions, with FtTCP3 peaking at 6 hours of both drought and salt treatments, suggesting that it plays a positive regulatory role in Tartary buckwheat's response to these stresses. This study provides new insights into the evolution and function of the TCP gene family.

Keywords: Tartary buckwheat, TCP transcription factors, genome-wide identification, abiotic stress, real-time quantitative PCR

Tartary buckwheat (*Fagopyrum tataricum*), an annual herbaceous plant in the family Polygonaceae and genus *Fagopyrum*, is a dual-purpose crop used for both food and medicine. Research has shown that Tartary buckwheat contains higher levels of protein, fat, vitamins, and trace elements than common grain crops such as corn and wheat. Rich in flavonoid compounds, Tartary buckwheat exhibits anti-hypertensive, free radical scavenging, antioxidant, anti-

aging, and cardiovascular disease prevention functions, giving it exceptional nutritional and medicinal value. Widely cultivated across China, particularly in the mountainous regions of Northeast, North, Northwest, and Southwest China, Tartary buckwheat is characterized by a short growth period, drought and cold tolerance, and strong adaptability to poor soils. Previous studies have demonstrated that drought and salt stress affect seed germination, root morphogenesis, aboveground agronomic traits, and antioxidant enzyme activity in Tartary buckwheat. Therefore, investigating the salt and drought tolerance mechanisms and regulatory strategies of Tartary buckwheat is crucial for achieving sustainable agricultural development and ensuring food security in arid regions.

TCP transcription factors are plant-specific, named after the first letters of three genes: *TB1* from maize, *CYC* from snapdragon, and *PCF* from rice. Based on differences in binding site sequences, they are divided into two classes: Class I (GGNCCCAC), which primarily induces cell division, and Class II (GTGGNCC), which inhibits plant growth and development. TCP proteins possess two conserved domains: a bHLH (basic helix-loop-helix) domain and an R domain. The bHLH domain, encoded by 59 amino acids, is present in all TCP proteins and is highly conserved. Transcription factors with bHLH domains play important roles in plant growth, metabolism, signal transduction, and abiotic stress responses. Only a subset of TCP proteins contain the R domain, which is predicted to form a coiled shape and may mediate protein-protein interactions and other structural functions. Numerous studies have shown that TCP transcription factors play significant roles in plant stress responses by regulating cellular osmotic pressure, altering cell permeability, reducing harmful substances, and modifying hormone sensitivity. For example, overexpressing *PeTCP10* enhances catalase (CAT) activity and antioxidant capacity, improving salt tolerance in transgenic *Arabidopsis* during the vegetative growth stage. Overexpression of the tobacco *bHLH* gene *NtbHLH123* enhances salt tolerance while inducing *NtRbohE* expression and reactive oxygen species production, thereby improving salt tolerance through the NtbHLH123-NtRbohE signaling pathway. Overexpressing *OsTCP19* in *Arabidopsis* reduces water loss and reactive oxygen species accumulation while increasing lipid droplet accumulation, thereby enhancing stress tolerance in both seedlings and mature plants.

To date, the TCP transcription factor family has been identified in many plants, including 24 members in *Arabidopsis*, 19 in plum, 15 in grape, 29 in maize, and 36 in tea. However, identification and expression analysis in Tartary buckwheat have not been reported. With the publication of genomic data, transcription factors in Tartary buckwheat have been gradually characterized. For instance, heterologous expression of *FtMYB12* enhances cold tolerance in transgenic *Arabidopsis*, overexpression of *FtbHLH3* improves drought/oxidative stress tolerance, and overexpression of *FtDREB6* in Tartary buckwheat hairy roots increases SOD and CAT enzyme activities while reducing MDA content, indicating enhanced drought resistance. Nevertheless, the role of Tartary buckwheat TCP transcription factors in abiotic stress remains unclear.

This study employed bioinformatics methods to identify TCP transcription factors in Tartary buckwheat and analyzed their relative expression under drought and salt treatments using qRT-PCR to identify genes associated with abiotic stress. The study aimed to address: (1) analysis of the physicochemical properties, phylogenetic relationships, structural characteristics, and cis-acting elements of the Tartary buckwheat TCP transcription factor family; and (2) expression changes of TCP family members under drought and salt stress to identify stress-responsive TCP genes, laying a foundation for investigating their functions in abiotic stress responses.

1. Materials and Methods

1.1 Plant Materials and Stress Treatments

The Tartary buckwheat cultivar 'Chuanqiao No. 1' was used in this study, cultivated at the experimental base of the College of Agriculture, Yangtze University. Plump, uniformly sized seeds were soaked in water for 24 hours, dehulled, and germinated at 25°C. After germination, seedlings were transferred to germination boxes and grown in an artificial climate chamber at 25°C with a 16 h light/8 h dark photoperiod. One-week-old seedlings were subjected to stress treatments using 15% PEG6000 and 200 $mol \cdot L^{-1}$ NaCl solutions, with water treatment serving as the control. Samples of all leaves were collected at 0, 3, 6, and 9 h for both treatments and the control, immediately frozen in liquid nitrogen, and stored at -80°C. Three biological replicates were established for each treatment.

1.2 Identification of Tartary Buckwheat FtTCP Gene Family Members

Protein sequences were downloaded from the Tartary buckwheat genome database Pinku1 (http://mbkbase.org/Pinku1/), and *Arabidopsis* protein sequences were obtained from TAIR (http://www.Arabidopsis.org/). Using *Arabidopsis* TCP protein sequences as references, the Tartary buckwheat genome data were compared and screened using the BLAST module in TBtools software. Duplicate sequences and those lacking TCP conserved domains were removed to obtain candidate TCP protein sequences. All candidate proteins were submitted to NCBI CDD (https://www.ncbi.nlm.nih.gov/cdd) and PFAM (http://pfam.xfam.org/) databases. Proteins containing TCP conserved domains were confirmed as Tartary buckwheat TCP family members.

1.3 Phylogenetic Analysis of Tartary Buckwheat FtTCP Gene Family

Common buckwheat protein sequences were downloaded from the BGDB database (http://buckwheat.kazusa.or.jp/), while rice and sugar beet protein sequences were obtained from Ensembl Plants (http://plants.ensembl.org/index.html). TBtools software was used to perform homology comparisons between Tartary buckwheat TCP proteins and those from sugar beet, rice, *Arabidopsis*, and common buckwheat to obtain TCP homologous proteins. Multiple sequence

alignment of TCP proteins from the five species was performed using Cluster-X 2.0 software, and a phylogenetic tree was constructed using MEGA 6.0 software with the Neighbor-Joining (NJ) method. Bootstrap validation parameters were set to 1,000 replicates, with other parameters set to default values.

1.4 Analysis of Gene Structure, Physicochemical Properties, and Promoter Sequences of Tartary Buckwheat FtTCP Genes

The MEME website (https://meme-suite.org/) was used to predict TCP protein motifs online. NCBI CDD (https://www.ncbi.nlm.nih.gov/) was used to predict Tartary buckwheat TCP protein domains. The Protparam online tool (https://web.expasy.org/protparam) was used to predict the physicochemical properties of Tartary buckwheat TCP proteins. WOLF PSORT (https://wolfpsort.hgc.jp/) was used to predict subcellular localization. TBtools software was used to extract promoter sequences of TCP genes from the Tartary buckwheat genome. The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze cis-acting elements.

1.5 Chromosomal Localization and Collinearity Analysis of Tartary Buckwheat FtTCP Genes

The complete genome file and GFF3 annotation file of Tartary buckwheat were downloaded from the Pinku1 database. TBtools software was used for chromosomal localization of Tartary buckwheat TCP genes. MCScanX software was used to analyze gene duplication events between Tartary buckwheat and four other species: common buckwheat, rice, sugar beet, and *Arabidopsis*.

1.6 Transcriptomic Data Analysis

Transcriptomic data for different Tartary buckwheat tissues were obtained from NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126576). RNA-seq data for TCP genes in root, stem, leaf, flower, and post-anthesis days 13, 19, and 25 were extracted and normalized. TBtools software was used to generate tissue expression profiles of Tartary buckwheat TCP genes.

1.7 Real-Time Fluorescent Quantitative PCR Detection

One hundred milligrams of Tartary buckwheat leaves were ground into fine powder in liquid nitrogen using a mortar. Total RNA was extracted using an RNA kit, and its quality was verified using 1% agarose gel electrophoresis. Concentration was measured using an ultra-micro nucleic acid analyzer, and RNA was reverse-transcribed into cDNA using a reverse transcription kit. The cDNA was diluted to 100 ng $\cdot$ L^{-1} and stored at -4°C. Primer 5.0 software was used to design qRT-PCR specific primers (Table 1), with *FtH3* (HM628903) as the reference gene. The PCR reaction mixture contained 10 L of 2× mix, 0.8 L each of forward and reverse primers, 1 L of cDNA, and 7.4 L of ddH_2O.

The amplification program consisted of 95°C for 1 min; 40 cycles of 95°C for 10 s, 60°C for 30 s; followed by 95°C for 10 s, 60°C for 30 s, and 95°C for 30 s. Four biological replicates were established for each sample, and relative gene expression levels were calculated using the 2^(-ΔΔCt) method.

2. Results

2.1 Identification and Analysis of Tartary Buckwheat FtTCP Family Genes

Based on conserved domain analysis, 28 TCP family members were identified from the Tartary buckwheat genome and named FtTCP1 through FtTCP28 according to their protein molecular weights. The physicochemical properties of the Tartary buckwheat TCP family varied considerably, with amino acid lengths ranging from 84 to 475 aa, molecular weights (MW) from 9.50 to 51.04 kD, and isoelectric points (pI) between 4.93 and 9.71. Acidic and basic proteins were evenly distributed. The instability index ranged from 34.88 to 71.12, and the aliphatic index from 53.29 to 97.50. Subcellular localization prediction revealed that, except for FtTCP1 and FtTCP2, which were localized in the cytoplasm, the remaining 26 proteins were localized in the nucleus (Table 2).

2.2 Phylogenetic Analysis of Tartary Buckwheat FtTCP Gene Family

To comprehensively analyze the functions of the Tartary buckwheat TCP family, we first examined their evolutionary relationships. A phylogenetic tree was constructed using 121 TCP protein sequences from five species: Tartary buckwheat, rice, *Arabidopsis*, sugar beet, and common buckwheat (Figure 1 [Figure 1: see original paper]). The results showed that the Tartary buckwheat TCP gene family was divided into five subfamilies (A1–A5), containing 20, 39, 21, 17, and 24 members, respectively. Tartary buckwheat TCP genes were most abundant in subfamily A2 (9 members) and least abundant in subfamily A3 (2 members). Common buckwheat and Tartary buckwheat belong to the same genus and showed the closest genetic relationship, with 20 pairs of TCP proteins clustering together at the terminal branches of the phylogenetic tree. Tartary buckwheat showed a more distant evolutionary relationship with rice, consistent with the evolutionary divergence between monocots and dicots. Within each subfamily, intraspecific TCP proteins tended to cluster together.

2.3 Gene Structure and Protein Conserved Motif Analysis of Tartary Buckwheat FtTCP Genes

A phylogenetic tree was constructed using 28 Tartary buckwheat TCP protein sequences (Figure 2 [Figure 2: see original paper]A), dividing them into four groups: Group I contained 5 members, Group II contained 6, Group III contained 12, and Group IV contained 5. Adjacent branches showed similar gene structures. Conserved domain analysis revealed that the 28 Tartary buckwheat TCP proteins shared 10 motifs (Figure 2 [Figure 2: see original

paper]B). FtTCP8, FtTCP9, FtTCP6, FtTCP12, FtTCP11, FtTCP4, and FtTCP1 lacked motif1, while motif4 was located near the N-terminus. Most Tartary buckwheat TCP proteins contained 2–3 conserved motifs, with FtTCP1 containing only one, FtTCP7 containing four, and FtTCP26 and FtTCP28 containing six. All Tartary buckwheat TCP genes contained a single typical TCP domain (Figure 2 [Figure 2: see original paper]C). Gene structure analysis (Figure 2 [Figure 2: see original paper]D) showed that 11 Tartary buckwheat TCP genes contained introns, with Group III having the most introns, suggesting intron insertion events during evolution of this subfamily. Intron length varied considerably, contributing to substantial differences in gene length, with FtTCP28 being the longest gene, its introns accounting for approximately 50% of total gene length.

2.4 Analysis of Cis-Acting Elements in Tartary Buckwheat FtTCP Gene Promoters

Cis-acting element analysis (Figure 3 [Figure 3: see original paper]) revealed that Tartary buckwheat TCP gene promoter regions primarily contained stress response elements and hormone response elements. Hormone response elements were particularly diverse and widely distributed, suggesting that Tartary buckwheat TCP genes play important roles in hormonal regulation. Among 39 response elements identified, light-responsive elements were most abundant (24 elements, ~61.53%), indicating that Tartary buckwheat TCP gene transcription may be regulated by photoperiod. Abscisic acid (ABA) response elements (ABRE) were present in 26 FtTCP genes, with the highest response level in FtTCP12. Light-responsive elements G-BOX and BOX4 existed in 24 TCP genes, anaerobic response element ARE in 5 genes, while Gap-box, CAG-motif, and GTGGC-motif were specific to FtTCP19, FtTCP2, and FtTCP20, respectively. The auxin response element TGA-box was found only in FtTCP6. Abiotic stress response elements such as MBS, LAR, Gc-motif, and ARE were distributed in most TCP genes, though ARE was absent in FtTCP6, FtTCP13, and FtTCP17. These findings suggest that Tartary buckwheat TCP genes play important roles in photoperiod regulation, hormone signaling, and stress responses.

2.5 Chromosomal Localization and Collinearity Analysis of Tartary Buckwheat FtTCP Genes

The 28 Tartary buckwheat TCP genes were mapped to eight chromosomes (Figure 4 [Figure 4: see original paper]). Chromosome 8 contained the most genes (7 genes, 25% of the total), indicating its important role in TCP gene family evolution. Chromosomes 1, 5, and 7 contained the fewest genes, with only two each. To analyze evolutionary relationships within the species, collinearity analysis was performed across the Tartary buckwheat genome (Figure 5 [Figure 5: see original paper]), revealing four segmental duplication pairs involving five TCP genes (19% of the family). These collinear pairs were distributed only

on chromosomes Ft1, Ft3, Ft5, and Ft8, with two members on Ft3 and one each on the others. To further investigate the evolutionary history of Tartary buckwheat TCP genes, interspecific collinearity was analyzed among Tartary buckwheat, sugar beet, *Arabidopsis*, and rice (Figure 6 [Figure 6: see original paper]). Seven collinear pairs were identified between Tartary buckwheat and sugar beet, eight between Tartary buckwheat and *Arabidopsis*, and seven between rice and *Arabidopsis*. Notably, Tartary buckwheat chromosomes Ft2, Ft4, Ft6, and Ft7 showed no collinearity with other species.

2.6 Expression Pattern Analysis of Tartary Buckwheat FtTCP Family Genes

To investigate the expression patterns of Tartary buckwheat TCP genes across different tissues, an expression profile heatmap was generated based on FPKM values from transcriptomic data (Figure 7 [Figure 7: see original paper]). FtTCP18 showed highest expression in stems, FtTCP22 was specifically expressed in roots and stems, FtTCP2 and FtTCP4 were specifically expressed in flowers, and FtTCP14, FtTCP15, FtTCP12, FtTCP9, FtTCP7, and FtTCP11 showed high expression at 13 days post-anthesis. FtTCP3, FtTCP6, and FtTCP13 exhibited highest expression in leaves. FtTCP8 showed increasing expression in flowers over time, peaking at 25 days post-anthesis, while FtTCP1 showed highest expression in flowers that gradually decreased over time. These results indicate that Tartary buckwheat TCP genes participate in the development of roots, stems, leaves, and flowers.

Based on transcriptomic data, four tissue-specific Tartary buckwheat TCP genes (FtTCP3, FtTCP6, FtTCP12, and FtTCP13) were selected for drought and salt stress analysis. qRT-PCR analysis (Figure 8 [Figure 8: see original paper]) revealed that under drought stress, FtTCP3 expression was significantly upregulated, showing a trend of initial increase followed by decrease, peaking at 6 h. In contrast, FtTCP6, FtTCP12, and FtTCP13 expression levels decreased, with FtTCP6 and FtTCP12 showing significant reduction after 3 h of treatment, while FtTCP13 remained slightly below control levels throughout the treatment period. Under salt stress, FtTCP3 showed a similar expression pattern as under drought stress. FtTCP13 expression increased slightly before decreasing, with no significant overall change, while FtTCP6 and FtTCP12 expression levels decreased significantly.

Discussion

This study identified 28 TCP family members in Tartary buckwheat, revealing considerable variation in molecular weight and isoelectric points, indicating structural complexity and suggesting functional diversity. Subcellular localization prediction showed that 26 Tartary buckwheat TCP proteins were localized in the nucleus, consistent with findings in tea plants. Phylogenetic analysis divided TCP proteins from Tartary buckwheat, common buckwheat, *Arabidopsis*, rice, and sugar beet into five subfamilies (A1–A5), with Tartary buckwheat TCP

proteins distributed across all subfamilies. Group A2 contained nine Tartary buckwheat TCP proteins. FtTCP26, FtTCP4, FtTCP14, and FtTCP15 were located on independent branches of the phylogenetic tree, suggesting these genes may have evolved separately in Tartary buckwheat. Generally, closer clustering relationships indicate similar structures and functions. FtTCP11 clustered with AtTCP2, while FtTCP1 and FtTCP2 clustered with AtTCP19, suggesting that FtTCP1, FtTCP2, and FtTCP11 may respond to abiotic stress in Tartary buckwheat. FtTCP6 and FtTCP12, located at the terminal branch of the intraspecific phylogenetic tree, share similar conserved motifs and structures and showed similar responses to drought and salt stress (Figure 8), further supporting that genes within the same subfamily often have similar biological functions.

Promoter analysis helps elucidate gene expression regulation and response mechanisms. By analyzing cis-acting elements in promoter regions, important elements related to plant abiotic stress can be identified to understand the complex metabolic regulatory networks formed by various interacting elements. The promoter regions of Tartary buckwheat TCP genes contain numerous light-responsive, hormone-responsive, and abiotic stress-responsive elements, consistent with analyses of TCP family genes in switchgrass and petunia. MYB transcription factors are widely involved in plant cell morphogenesis, growth and development, and stress responses. bZIP transcription factors play crucial roles in resisting biotic and abiotic stresses. ARF is a transcription factor that binds to auxin-responsive gene promoters, participating in plant growth, development, and stress responses. The abundance of MYB, bZIP, and ARF elements in Tartary buckwheat TCP family promoters suggests these transcription factors function in abiotic stress responses. Additionally, 26 Tartary buckwheat TCP gene promoters contained numerous ABRE response elements. ABRE is a cis-acting element involved in abscisic acid (ABA) responses, and the presence of multiple ABRE elements indicates that Tartary buckwheat TCP transcription factors may directly regulate downstream genes in ABA signaling pathways. Studies have shown that ABRE elements are widely distributed in *Ntbhlh15*, *NtbHLH135.1*, and *NtbHLH40.1*, all of which respond to low-temperature stress, suggesting ABRE plays an important role in cold stress responses. Exogenous ABA application can upregulate *OsTCP19* transcription, thereby regulating drought tolerance in rice. Moso bamboo TCP10 can positively regulate drought tolerance through ABA signaling pathways while negatively regulating lateral root growth through methyl jasmonate (Me-JA)-mediated signaling pathways. Therefore, we hypothesize that Tartary buckwheat TCP genes may participate in hormone signal transduction pathways such as ABA to function in abiotic stress responses, though the specific regulatory mechanisms require further investigation.

Tartary buckwheat, primarily cultivated in Southwest China, exhibits strong adaptability to harsh environments. Transcriptomic data showed that most Tartary buckwheat TCP genes were expressed in roots, stems, leaves, and flowers, indicating their involvement in the development of these tissues. Based on tissue-specific transcriptomic data, four Tartary buckwheat TCP genes

(FtTCP3, FtTCP6, FtTCP12, and FtTCP13) were selected for drought and salt stress treatments. Differential expression analysis based on qRT-PCR data revealed that all four genes showed altered expression under drought and salt stress. FtTCP6, FtTCP12, and FtTCP13 expression decreased significantly under drought stress, while FtTCP6 and FtTCP12 showed significant decreases under salt stress. FtTCP3 expression increased initially and then decreased under both salt and drought stress, peaking at 6 h of treatment. These results suggest that FtTCP3, FtTCP6, FtTCP12, and FtTCP13 respond to drought and salt stress to varying degrees, indicating that the Tartary buckwheat TCP gene family functions in stress responses.

In conclusion, this study identified 28 TCP gene family members in Tartary buckwheat, belonging to five subfamilies. Gene expression analysis revealed distinct tissue-specific expression patterns. qRT-PCR detection showed that FtTCP3, FtTCP6, FtTCP12, and FtTCP13 responded to drought and salt stress to varying degrees, suggesting that Tartary buckwheat TCP genes may participate in regulating responses to drought, salt, and other adverse conditions. This study provides new insights into the evolution and function of the TCP gene family and offers important guidance for research on stress resistance and growth and development in Tartary buckwheat.

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Note: Figure translations are in progress. See original paper for figures.