

## Research Article

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# Genome-wide analysis of the WRKY gene family and its response to abiotic stress in buckwheat (*Fagopyrum tataricum*)

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**Abstract:** The WRKY gene family is an ancient plant transcription factor (TF) family with a vital role in plant growth and development, especially in response to biotic and abiotic stresses. Although many researchers have studied WRKY TFs in numerous plant species, little is known of them in Tartary buckwheat (*Fagopyrum tataricum*). Based on the recently reported genome sequence of Tartary buckwheat, we identified 78 FtWRKY proteins that could be classified into three major groups. All 77 WRKY genes were distributed unevenly across all eight chromosomes. Exon–intron analysis and motif composition prediction revealed the complexity and diversity of FtWRKYs, indicating that WRKY TFs may be of significance in plant growth regulation and stress response. Two separate pairs of tandem duplication genes were found, but no segmental duplications were identified. Overall, most orthologous gene-pairs between Tartary and common buckwheat evolved under strong purifying selection. qRT-PCR was used to analyze differences in expression among four FtWRKYs (FtWRKY6, 74, 31, and 7) under salt, drought, cold, and heat treatments. The results revealed that all four proteins are related to abiotic stress responses, although they exhibited various expression patterns. In particular,

the relative expression levels of FtWRKY6, 74, and 31 were significantly upregulated under salt stress, while the highest expression of FtWRKY7 was observed from heat treatment. This study provides comprehensive insights into the WRKY gene family in Tartary buckwheat, and can support the screening of additional candidate genes for further functional characterization of WRKYs under various stresses.

**Keywords:** *Fagopyrum tataricum*; WRKY gene family; bioinformatic; genome-wide analysis; abiotic stress

## 1 Introduction

Although most plants grow in specific environments, they experience continual changes in their external conditions, therefore, plants have developed a series of complex mechanisms to withstand stresses [1, 2]. Transcription factors (TFs) are crucial proteins in the response to environmental stimuli by regulating gene expression temporally and spatially [3]. TFs, also called sequence-specific DNA-binding factors, bind to conserved cis-elements in promoter regions, thereby interacting with downstream target genes to influence transcription [4, 5]. The WRKY family is one of the largest and most diverse TF families, and is related to the coordination of many physiological activities in the plant kingdom. Moreover, it has been widely studied for its important role in regulating gene expression under adverse conditions [6–9]. According to the number of domains and features of the zinc-finger motif, the WRKY family can be divided into three distinct groups (I, II, and III) [10]. Groups I and II both exhibit a C<sub>2</sub>H<sub>2</sub> zinc-finger motif, although group I has two WRKY domains whereas group II has only one. Group II can be further classified into five subgroups (IIa–e) based on the amino acid (aa) stretch in the zinc-finger motif [11, 12]. Group III also contains a single WRKY

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domain but a C2HC zinc-finger motif [11, 12]. The domain of WRKY TFs is approximately 60-aa long at the N-terminus and has a typical zinc-finger motif at the C-terminus [13]. It is generally thought that WRKY TFs can specifically interact with the W-box (TTGACT/C) found in the promoter region of many plants [14, 15]. The first WRKY gene, SPF1, was isolated from sweet potato (*Ipomoea batatas*), and was considered to have potential negative impacts on the regulation of sucrose-induced genes [16]. Multiple studies have since been performed in different plants. For example, 72 and 64 WRKYs have been reported in the model herbaceous plants *Arabidopsis thaliana* and *Oryza sativa*, respectively [17], and many other studies (Table S1) have been performed in *Triticum aestivum* [18], *Camellia sinensis* [19], *Populus trichocarpa* [10], and *Glycine max* [20]. Such studies have demonstrated that WRKY proteins not only participate in physiological processes such as seed germination [21] and leaf senescence [22], but are also involved in the response to biotic stresses such as pathogens [23] and pests [24], as well as abiotic stresses such as drought [25], heat [26], cold [27], salinity [28], and heavy metals [8]. For instance, in transgenic tobacco, the BcWRKY46 gene enhanced tolerance to freezing, abscisic acid, salt, and dehydration stresses [29]. Meanwhile, ThWRKY7 improved cadmium tolerance under cadmium stress in combination with ThVHAc1 in woody plants [30]. In *A. thaliana*, AtWRKY25, 33, 46, and 54 have been demonstrated to play vital roles in the response to several types of stress [31, 32]. Moreover, WRKY proteins have vital roles in the biosynthesis of secondary metabolites, such as paclitaxel and benzyloisoquinoline [33, 34].

Tartary buckwheat (*Fagopyrum tatarium*) is an annual eudicot plant belonging to the genus *Fagopyrum*. Tartary buckwheat and common buckwheat (*Fagopyrum esculentum*) are the most commonly cultivated species of this genus [35, 36]. Tartary buckwheat originates in southwest China, and is also known as kuqiao for its bitter seeds. It is grown mainly in farming and ranching areas that overlap with northern China, and exhibits strong abiotic resistance to harsh eco-climatic environments [37 - 39]. As a medicinal and nutrient-rich crop, Tartary buckwheat has higher flavonoid content than common buckwheat and is especially abundant in rutin, accounting for 0.8–1.7% of the dry weight of the plant [40, 41]. Moreover, quercetin, anthocyanins, and other flavonoids in buckwheat have various biological activities, such as antibacterial, antioxidant, and anti-inflammatory effects [41 - 44].

The genome sequence of Tartary buckwheat was recently published [42], enabling the systematic characterization of WRKY genes in this species and the study of their expression. Therefore, we performed

bioinformatics analyses including phylogenetic, gene structure, and motif composition analyses; determined the chromosomal locations of the genes; and calculated the Ka/Ks values of WRKY genes in Tartary buckwheat. Subsequently, the expression patterns of select FtWRKYs under salt, drought, cold, and heat treatments were analyzed. This study helps clarify the functions of WRKY proteins and provides a foundation for further comparative genomic studies in Tartary buckwheat.

## 2 Materials and Methods

### 2.1 Identification of putative WRKY proteins in Tartary buckwheat

To accurately identify WRKY TFs in Tartary buckwheat, we downloaded the whole genome sequence from the MBKbase website (<http://www.mbkbase.org/Pinku1>) [42]. Moreover, the Hidden Markov Model profile of the WRKY domain (PF03106) was downloaded from the Pfam family database (<http://pfam.xfam.org/search>) [45]. All possible WRKY proteins were searched using HMMER 3.0 (<http://hmmer.janelia.org/>) with the default parameters. In addition, we used both HMMER (<http://plants.ensembl.org/hmmer/index.html>) and SMART (<http://smart.embl-heidelberg.de/>) to ascertain the presence of the WRKY domain [46]. Sequences with different domains or redundancies were excluded. AtWRKY data were obtained from the *Arabidopsis* genome TAIR website (<http://www.Arabidopsis.org/index.jsp>) [47].

### 2.2 Phylogenetic analysis and protein properties of FtWRKYs

Multiple alignment of the WRKY domain sequence in the 78 FtWRKY proteins was performed with ClustalW using the default settings [48], and a phylogenetic tree was constructed using the neighbor-joining (NJ) method with MEGA 6.0 software [49] with the following parameters: pairwise deletion, 1000 bootstrap replicates, and Poisson correction. To obtain an accurate classification, two members per group and highly conserved representative AtWRKY proteins were included in the tree building (Table S2). The FtWRKY domains in each group were analyzed, and the sequence logos were produced using WebLogo online software (<http://weblogo.threeplusone.com/>) [8]. The properties of the proteins, including sequence length (aa length), molecular weight (MW), isoelectric point (IP), instability index (II), aliphatic index (AI), and grand

average of hydropathicity (GRAVY) were calculated using the ExPASy website (<http://web.expasy.org/protparam/>), while the subcellular localization of each protein was predicted with Cell-PLoc [50].

### 2.3 Conserved motifs and gene structure analysis of FtWRKY genes

The conserved motifs in the FtWRKY proteins were predicted using MEME Suite (<http://meme-suite.org/tools/meme>), the following parameters were employed in analysis: maximum number of motifs 10; minimum motif width 6; maximum motif width 50. The gene structure of FtWRKY was predicted by comparing the coding sequences and corresponding genomic sequences using the GSDS tool (<http://gsds.cbi.pku.edu.cn>) [51].

### 2.4 Chromosomal location of FtWRKY genes

The physical location of FtWRKYs on chromosomes was retrieved from the annotated genome and chromosome files, and genes were plotted separately onto all eight chromosomes based on the order of their physical position. Finally, an image of their physical location was created with MapInspect software [52].

### 2.5 Calculation of Ka/Ks of orthologous gene-pairs between FtWRKYs and FeWRKYs

The orthologous gene-pairs of WRKY between Tartary and common buckwheat were aligned using ClustalW on the basis of diverse sequence alignment tools. Alignment of the aa sequences and their corresponding original cDNA sequences were used to calculate the synonymous rate (Ks) and nonsynonymous rate (Ka) using the CODEML program in the PAML interface tool of PAL2NAL [5]. Furthermore, the evolutionary constraint (Ka/Ks) was determined. The approximate time (million years ago [Mya]) of the orthologous gene-pairs were estimated using the equation  $T = Ks/2\lambda$ , where the synonymous substitution rate ( $\lambda$ ) was  $1.5 \times 10^{-8}$  [53].

### 2.6 RNA-sequencing data analysis of FtWRKY genes

To investigate the expression patterns of FtWRKYs among different tissues as well as under salt treatment, the

Illumina RNA-sequencing datasets were collected from the NCBI SRA database (<https://www.ncbi.nlm.nih.gov/sra>), including different five tissues, i.e. roots, flowers, stems, and leaves (accession: SRX3974871; SRX3974872; SRX3974873; SRX3974874), and for the salt treatment, plants were disposed with 200 mM NaCl for 0, 24 hours (accession:SRX3210945; SRX3210946). Transcript expression levels were calculated in FPKM units as reads per kilobase of transcript sequence per million mapped reads. FPKM value were transformed by log2 and the heatmap was performed by HemI software [54].

### 2.7 Plant material, growth conditions, and treatments

The seeds of Tartary buckwheat cv. Pinku1 were provided by Dr. Bo Li from the college of agriculture, South China Agricultural University. Plants were grown in pots containing soil and vermiculite mixture (3:1) in an artificial climate chamber, with a program set to 25/22°C (day/night), 16-h photoperiod, and relative humidity of 75%. Stress treatments were initiated in 5-week-old normal seedlings, and the seedlings were disposed with following treatment as described by Zhou *et al.* [38] and Gao *et al.* [40]. For salt and drought treatments, seedlings were irrigated with 15% NaCl and 30% PEG6000, respectively. For cold and heat treatments, seedlings were transferred to 4°C and 40°C in an illuminated incubator, respectively. Whole samples were harvested after 0, 3, and 12 h, while plants at 0 h were used as the control. Samples from three biological replicates were frozen immediately in liquid nitrogen and stored at -80°C until further analysis.

### 2.8 Total RNA extraction and cDNA reverse transcription

Total RNA was isolated from frozen seedlings with an RNAPrep Pure Plant Kit (TIANGEN, Beijing, China) and the concentration of RNA was determined using spectrophotometry. First-strand cDNA was reverse transcribed using a PrimeScript RT Master Mix (Perfect) Real Time Kit (TaKaRa, Dalian, China). All operational procedures were performed according to the manufacturer's instructions. Finally, 1  $\mu$ L cDNA was diluted with 4  $\mu$ L nuclease-free water before quantitative real-time (qRT)-PCR analysis.

## 2.9 qRT-PCR analysis

The expression of FtWRKY6 and 74 were enhanced significantly after treatment with NaCl, and same as FtWRKY7 and 31, they shared a high identity with AtWRKY25, 33, 46, and 54 were identified in plant defense experiments, thus were selected for qRT-PCR analysis. The housekeeping gene histone3 (GenBank ID: HM628903) of Tartary buckwheat was used as an internal control [55]. Specific qRT-PCR primers (Table S3) were designed with Primer Premier 6.0 and synthesized by Sangon (Guangzhou, China). The SYBR Premix Ex Taq Kit (TaKaRa, China) was used for the qRT-PCR reaction, and the reactions were performed using a Roche LightCycler 480 system (Roche, Basel, Switzerland). The qRT-PCR reactions were performed in a total volume of 20  $\mu$ L, including 10  $\mu$ L SYBR Premix Ex Taq, 6  $\mu$ L ddH<sub>2</sub>O, 2  $\mu$ L diluted cDNA, and 1  $\mu$ L each forward and reverse primer. The qPCR program was as follows: initiation with a 3 min denaturation period at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s. Three biological replicates and three technical replicates were included in the qRT-PCR analysis. Finally, gene expression was calculated using the 2<sup>- $\Delta\Delta$ c</sup> method [56], and the means and standard deviation of three biological replicates were calculated. The significance was statistically analyzed by *t* text, and revealed by asterisks (\* *p*<0.05, \*\**p*<0.01).

## 3 Results

### 3.1 Identification and classification of FtWRKYs

In the *F. tatarium* genome sequence, 74 protein sequences were detected that contained the conserved complete WRKY domain based on the HMMER and SMART analyses. Moreover, four additional proteins were identified that were missing the zinc-finger motif. These sequences were included for the subsequent analysis because they have been identified as annotated WRKYs in *Arabidopsis* based on BLAST, and this phenomenon has also been reported in *Capsicum annuum* [57]. Interestingly, one gene (FtPinG0001732900.01) showed two alternative messenger RNA splicing, therefore, 77 genes were detected in total. The 78 identified proteins were named FtWRKY1–77 according to the distribution (from top to bottom) of the corresponding genes on chromosomes (Chr.) 1–8 (Table 1).

The phylogenetic relationship of all FtWRKYs and 14 typical AtWRKYs was constructed based on multiple sequence alignment of their WRKY domains (Fig. 1) The

NJ tree indicated that the FtWRKYs could be divided into three main groups defined in previous research [11]. Figure S1 showed the multiple sequence alignment of the FtWRKY domain among each group. Group II had the most FtWRKYs (56), followed by group I (15). Meanwhile, group III had a different type of zinc-finger compared with groups I and II, and contained only seven members. Group II could be further classified into five subgroups (IIa–e). Subgroup IIa (six members) and IIb (ten members) were in the same branch, while subgroup IIc (12 members) and IIe (11 members) were derived from one clade. Subgroup IId (17 members) was more similar to group I than the other subgroups. Notably, in group I, four FtWRKYs (FtWRKY3, 25, 66, and 59) had only one WRKY domain at the C-terminus, which may have lost or acquired a domain during evolution [58].

Based on the 78 complete aa sequences, we predicted the properties of the FtWRKYs, including the aa length, MW, IP, II, AI, and GRAVY (Table 1). The average length of FtWRKY proteins was approximately 323.12 aa, ranging from 130 (FtWRKY59) to 722 (FtWRKY69) aa, showing a difference of approximately 4.5 times. The MW ranged from 14,210.09 (FtWRKY59) to 78,740.04 (FtWRKY69) kDa, and the IP ranged from 4.85 (FtWRKY66) to 10.04 (FtWRKY33.1). The AI varied from 32.99 (FtWRKY63) to 78.28 (FtWRKY48). Finally, the GRAVY ranged from -1.187 (FtWRKY43) to -0.396 (FtWRKY75), suggesting that all FtWRKY proteins are hydrophilic.

According to the predicted protein stability, almost 93.59% of WRKY proteins were unstable, because their II values were greater than 40, while only five FtWRKYs were stable, among which four members were from subgroup IId. The predicted results of the subcellular localization revealed that all FtWRKYs were localized in the nucleus.

### 3.2 Gene structure and motif composition of FtWRKY proteins

The analysis of the intron–exon structure of full-length cDNA (Fig. 2B) revealed that all FtWRKYs had introns in the translated region, although the number of introns and exons varied from one to five introns and two to six exons, respectively. The majority (52.56%) of the FtWRKY genes contained two introns. Meanwhile, six genes contained five introns and eight genes contained only one intron. Most members of subgroup IIb had two introns, except FtWRKY23 and 47, and five members of group III (71.43%) contained three exons and two introns. Genes with similar structures were always clustered in the same group, which was further confirmed by the results of the FtWRKY classification (Fig. 2A).

Table 1. Description of the WRKY proteins in Tartary buckwheat.

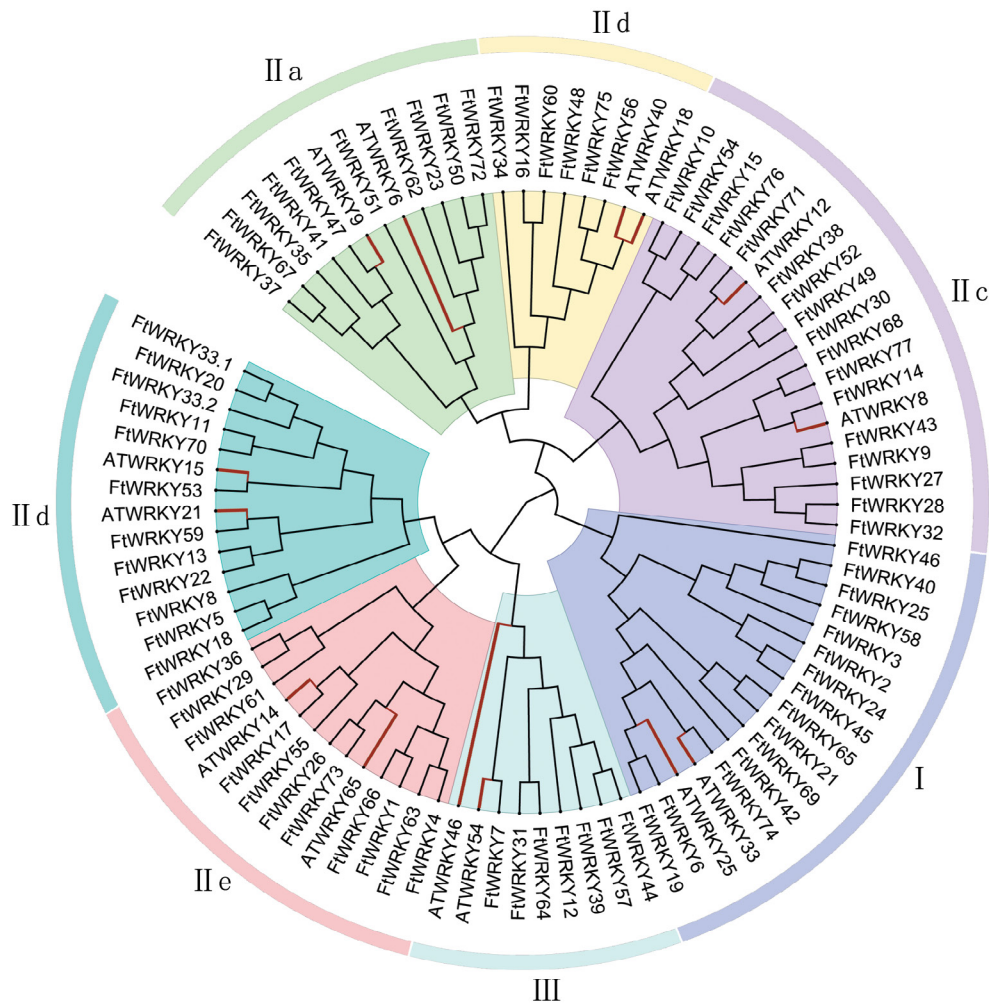
Protein name	Gene ID	Accession number	Group	Chr.	Protein length(aa)	Molecular weight(Da)	Theoretical IP	Aliphatic index	Grand average of instability hydrophaticity index	subcellular localization	
FWRKY1	FtPmg0000171800.01	MK161300	Ile	Chr.1	379	41987.38	5.28	65.78	-0.686	54.04	Nucleus
FWRKY2	FtPmg0000299100.01	MK161305	I	Chr.1	582	62963	6.45	66.36	-0.649	51.36	Nucleus
FWRKY3	FtPmg00001428200.01	MK161291	I	Chr.1	153	17133.75	5.96	46.41	-1.166	51.73	Nucleus
FWRKY4	FtPmg00002493300.01	MK161272	Ile	Chr.1	272	29546.32	5.7	56.32	-0.7	55.51	Nucleus
FWRKY5	FtPmg00003193100.01	MK161301	Ild	Chr.1	144	15924.29	9.53	43.33	-0.655	53.4	Nucleus
FWRKY6	FtPmg00001577500.01	MK161279	I	Chr.1	523	57475.12	6.58	55.18	-0.852	58.96	Nucleus
FWRKY7	FtPmg00004829100.01	MK161280	III	Chr.1	335	37272.07	6.82	51.85	-0.848	73.87	Nucleus
FWRKY8	FtPmg00001878200.01	MK161333	Ild	Chr.1	334	36276.22	9.82	67.72	-0.519	45.02	Nucleus
FWRKY9	FtPmg00005486000.01	MK161313	Ilc	Chr.1	172	19952.73	9.3	62.21	-0.843	58.86	Nucleus
FWRKY10	FtPmg00006017000.01	MK161273	Ilc	Chr.1	293	32346.18	5.36	67.2	-0.619	59.74	Nucleus
FWRKY11	FtPmg00001921200.01	MK161289	Ild	Chr.1	263	29160.81	9.62	62.28	-0.807	59.99	Nucleus
FWRKY12	FtPmg00003025100.01	MK161335	III	Chr.1	360	40227.07	6.07	66.92	-0.517	52.83	Nucleus
FWRKY13	FtPmg00004319300.01	MK161332	Ild	Chr.1	175	20144.18	9.88	49.54	-0.894	57.16	Nucleus
FWRKY14	FtPmg00008726200.01	MK161345	Ilc	Chr.2	274	30477.75	9.04	58.32	-0.897	48.22	Nucleus
FWRKY15	FtPmg00005008400.01	MK161327	Ilc	Chr.2	178	20159.06	6.21	44.33	-0.938	42.88	Nucleus
FWRKY16	FtPmg00005548400.01	MK161299	Ila	Chr.2	321	35837.17	6.96	67.17	-0.711	54.25	Nucleus
FWRKY17	FtPmg00006962300.01	MK161326	Ile	Chr.2	248	28409.05	6.72	64.84	-0.85	53.55	Nucleus
FWRKY18	FtPmg00007313500.01	MK161312	Ild	Chr.2	294	33406.33	9.54	68.3	-0.709	41.77	Nucleus
FWRKY19	FtPmg00004914600.01	MK161339	I	Chr.2	639	69367.94	5.91	55.85	-0.737	52.69	Nucleus
FWRKY20	FtPmg00003001300.01	MK161309	Ild	Chr.2	327	35664.56	9.88	62.1	-0.599	53.04	Nucleus
FWRKY21	FtPmg00003701200.01	MK161287	I	Chr.2	476	52191.55	9.23	58.17	-0.871	48.96	Nucleus
FWRKY22	FtPmg00003722700.01	MK161306	Ild	Chr.2	330	36055.55	9.62	63.24	-0.641	56.15	Nucleus
FWRKY23	FtPmg00003761600.01	MK161334	Ilb	Chr.2	537	57589.63	6.41	62.42	-0.683	53.67	Nucleus
FWRKY24	FtPmg00006884300.01	MK161274	I	Chr.2	480	52906.17	5.72	63.56	-0.617	46.41	Nucleus
FWRKY25	FtPmg00003815100.01	MK161283	I	Chr.2	319	35182.41	8.84	67.43	-0.784	61.55	Nucleus
FWRKY26	FtPmg00004814300.01	MK161318	Ile	Chr.3	289	32264.84	6.66	55.95	-0.922	41.54	Nucleus
FWRKY27	FtPmg00006083600.01	MK161319	Ilc	Chr.3	304	33816.57	8.63	61.18	-0.687	50.98	Nucleus
FWRKY28	FtPmg00007458900.01	MK161275	Ilc	Chr.3	291	31784.28	6.44	67.01	-0.629	53.33	Nucleus
FWRKY29	FtPmg00005089800.01	MK161338	Ile	Chr.3	228	26122.18	8.22	49.91	-0.86	50.3	Nucleus
FWRKY30	FtPmg00005089400.01	MK161298	Ilc	Chr.3	195	21966.61	9.05	64.9	-0.808	38.5	Nucleus
FWRKY31	FtPmg00006388000.01	MK161325	III	Chr.3	278	31217.17	5.97	47.41	-0.879	55.09	Nucleus

continued  
Table 1.

Protein name	Gene ID	Accession number	Group	Chr.	Protein length(aa)	Molecular weight(Da)	Theoretical IP	Aliphatic index	Grand average of instability index	Grand average of hydrophobicity	subcellular localization
FWRKY32	FtPinG0000403800.01	MK161322	Ilc	Chr.3	286	31863.57	6.66	60.28	-0.771	55.06	Nucleus
FWRKY33.1	FtPinG0001732900.01	MK161323	Ild	Chr.3	155	17303.72	10.04	53.48	-0.87	47.98	Nucleus
FWRKY33.2	FtPinG0001732900.01	MK161323	Ild	Chr.3	363	40614.9	9.85	65.84	-0.804	55.88	Nucleus
FWRKY34	FtPinG0004464200.01	MK161285	Ila	Chr.3	304	34327.33	6.16	69.9	-0.763	55	Nucleus
FWRKY35	FtPinG0004460400.01	MK161337	Ilb	Chr.3	538	57612.46	8.9	57.47	-0.706	44.3	Nucleus
FWRKY36	FtPinG0002374400.01	MK161331	Ile	Chr.3	148	16780.86	5.13	61.08	-0.81	56.55	Nucleus
FWRKY37	FtPinG0006311800.01	MK161284	Ilb	Chr.3	359	37811.64	9.35	57.66	-0.522	41.67	Nucleus
FWRKY38	FtPinG0008419400.01	MK161311	Ilc	Chr.4	189	21067.67	9.23	61.27	-0.807	38.41	Nucleus
FWRKY39	FtPinG0007629700.01	MK161271	Ill	Chr.4	342	38260.19	4.97	54.53	-0.753	55.17	Nucleus
FWRKY40	FtPinG0005111700.01	MK161316	I	Chr.4	494	53873.54	7.09	54.88	-0.791	62.48	Nucleus
FWRKY41	FtPinG0005105500.01	MK161310	Ilb	Chr.4	578	63106.91	5.16	60.45	-0.744	48.21	Nucleus
FWRKY42	FtPinG0006617300.01	MK161320	I	Chr.4	490	53787.68	7	55.12	-0.81	65.75	Nucleus
FWRKY43	FtPinG000497200.01	MK161304	Ilc	Chr.5	171	19859.8	8.97	42.75	-1.187	44.96	Nucleus
FWRKY44	FtPinG0002524500.01	MK161295	Ill	Chr.5	321	36241.7	5.24	71.31	-0.585	49.24	Nucleus
FWRKY45	FtPinG0004671500.01	MK161329	I	Chr.5	548	60094.97	5.92	67.08	-0.635	47.41	Nucleus
FWRKY46	FtPinG0004682800.01	MK161290	I	Chr.5	459	49709.24	6.58	55.05	-0.763	65.13	Nucleus
FWRKY47	FtPinG0001532600.01	MK161293	Ilb	Chr.5	368	39517.67	9.69	49.92	-0.649	55.13	Nucleus
FWRKY48	FtPinG0004237400.01	MK161342	Ila	Chr.5	163	18194.55	6.22	78.28	-0.528	45.55	Nucleus
FWRKY49	FtPinG0007732600.01	MK161315	Ilc	Chr.5	176	20396.98	9.41	56.36	-1.035	50.62	Nucleus
FWRKY50	FtPinG0006202300.01	MK161286	Ilb	Chr.5	246	26678.17	8.25	61.63	-0.408	50.03	Nucleus
FWRKY51	FtPinG0006201300.01	MK161282	Ilb	Chr.5	575	62401.23	6.66	61.63	-0.688	43.59	Nucleus
FWRKY52	FtPinG0002688700.01	MK161277	Ilc	Chr.6	230	26476.19	6.12	55.04	-1.112	39.34	Nucleus
FWRKY53	FtPinG0002710400.01	MK161307	Ild	Chr.6	319	34893.69	9.58	64.73	-0.559	44.81	Nucleus
FWRKY54	FtPinG0007768300.01	MK161281	Ilc	Chr.6	198	22167.05	9.27	60.05	-0.868	38.85	Nucleus
FWRKY55	FtPinG0006322300.01	MK161346	Ile	Chr.6	301	34764.78	6.22	72.19	-0.664	71.23	Nucleus
FWRKY56	FtPinG0008708000.01	MK161347	Ila	Chr.6	379	41222.95	8.89	67.18	-0.642	42.6	Nucleus
FWRKY57	FtPinG0000354800.01	MK161296	Ill	Chr.6	312	35162.28	5.63	63.4	-0.707	55.94	Nucleus
FWRKY58	FtPinG0003093800.01	MK161341	I	Chr.7	203	22759.07	9.65	61.82	-0.779	42.43	Nucleus
FWRKY59	FtPinG0002810600.01	MK161297	Ild	Chr.7	130	14210.09	9.77	54.69	-0.655	73.08	Nucleus

continued  
Table 1.

Protein name	Gene ID	Accession number	Group	Chr.	Protein length(aa)	Molecular weight(Da)	Theoretical IP	Aliphatic index	Grand average of instability index	Grand average of hydrophobicity	subcellular localization
FWRKY60	FtPinG0002864000.01	MK161328	Ila	Chr.7	322	33750.82	6.62	63.91	-0.811	44.82	Nucleus
FWRKY61	FtPinG0009522600.01	MK161294	Ila	Chr.7	180	20755.01	9.13	52.44	-1.158	37.64	Nucleus
FWRKY62	FtPinG0004132200.01	MK161317	Ilb	Chr.7	508	55108.2	6.29	63.82	-0.79	46.43	Nucleus
FWRKY63	FtPinG0004155300.01	MK161321	Ile	Chr.7	184	20475.32	5.26	32.99	-1.034	45.65	Nucleus
FWRKY64	FtPinG0001212400.01	MK161308	III	Chr.7	305	34022.56	6.04	54.72	-0.794	57.2	Nucleus
FWRKY65	FtPinG0007552800.01	MK161278	I	Chr.7	170	18754.78	5.97	65.29	-0.81	43.92	Nucleus
FWRKY66	FtPinG0004764100.01	MK161330	Ile	Chr.7	292	30632.72	4.85	59.25	-0.514	53.56	Nucleus
FWRKY67	FtPinG0002334500.01	MK161292	Ilb	Chr.8	458	50521.77	5.91	54.56	-0.834	50	Nucleus
FWRKY68	FtPinG0002325700.01	MK161303	Ilc	Chr.8	273	31425.68	5.77	53.22	-1.021	58.13	Nucleus
FWRKY69	FtPinG0002177700.01	MK161336	I	Chr.8	722	78740.04	5.59	61.02	-0.75	47.25	Nucleus
FWRKY70	FtPinG0002142900.01	MK161302	Ild	Chr.8	326	35492.1	9.69	66.99	-0.624	53.59	Nucleus
FWRKY71	FtPinG0009255800.01	MK161314	Ilc	Chr.8	247	27933.96	7.67	50.49	-0.887	63.85	Nucleus
FWRKY72	FtPinG0005364100.01	MK161288	Ilb	Chr.8	548	59507.15	5.84	64.53	-0.657	49.24	Nucleus
FWRKY73	FtPinG0009798800.01	MK161276	Ile	Chr.8	302	32652.77	5.84	49.21	-0.766	61.06	Nucleus
FWRKY74	FtPinG0009186100.01	MK161340	I	Chr.8	472	52360.55	6.18	51.44	-0.833	64.76	Nucleus
FWRKY75	FtPinG0007226700.01	MK161343	Ila	Chr.8	147	16177.46	9.23	75.65	-0.396	44.21	Nucleus
FWRKY76	FtPinG0007227700.01	MK161344	Ilc	Chr.8	139	16039.78	7.67	47.63	-0.917	42.13	Nucleus
FWRKY77	FtPinG0001770900.01	MK161324	Ilc	Chr.8	270	29952.42	7.23	55.26	-0.781	56.55	Nucleus

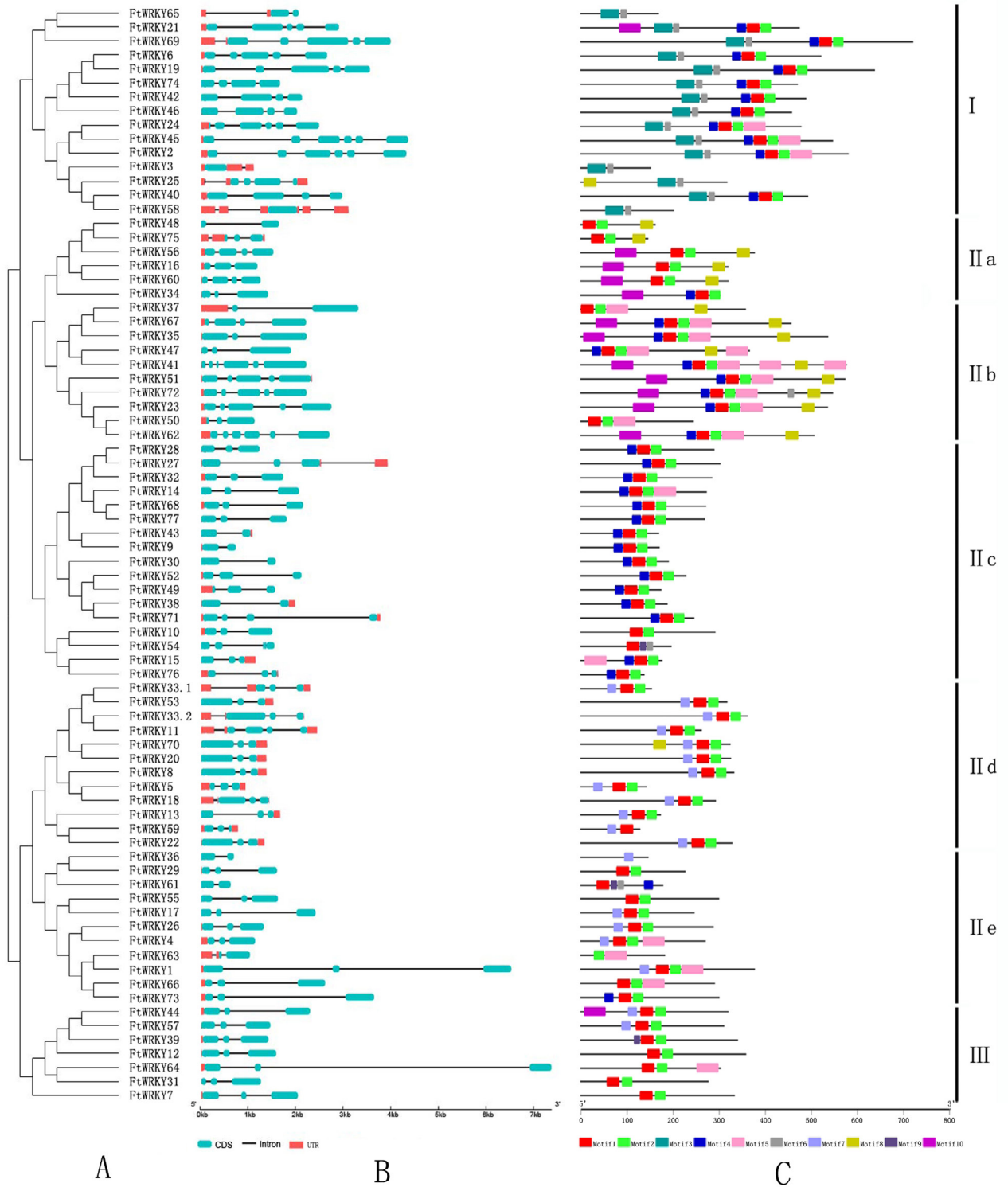


**Figure 1.** Relationship of the 78 FtWRKYs and 14 representative AtWRKYs illustrated with an unrooted phylogenetic tree. The domain sequences of all WRKYs were aligned with ClustalW and the phylogenetic tree was constructed with MEGA 6.0 software using the neighbor joining method. The branches of the AtWRKYs are in bold and highlighted red. The seven groups are highlighted with various colors (see color figure online).

Ten motifs were identified in Tartary buckwheat using MEME software. Table S4 presents detailed information of these motifs and Figure 2C presents the motif compositions of each FtWRKY. The length of the 10 motifs ranged from 15 (motif 9) to 49 (motif 5) bp. Motifs 3 and 6 partly represented the distribution of the conserved domain at the C-terminus and were shared by all 15 members of group I. Meanwhile, motifs 1 and 2 partly represented the distribution of the conserved domain at the N-terminus and were found in the majority of FtWRKYs. The other six motifs appeared around the WRKY domain, but were distributed uniquely, and their function remains unclear. In group I, 11 of 15 FtWRKYs contained motifs 1, 2, and 4, while FtWRKY3, 25, 58, and 65 contained only one WRKY

domain at the C-terminus. These three motifs were also found in 15 of the 17 proteins of subgroup Iic, because FtWRKY54 lacked the zinc-finger-like motif and FtWRKY10 did not contain motif 4. Similarly, in subgroup Iid, motifs 1, 2, and 7 were found in 11 of the 12 members, except the one member without a zinc finger (FtWRKY59). This indicated that the motifs were selectively distributed among the groups. Motif 8 was found in all members of subgroup Iia and nine of the ten members of subgroup Iib. Moreover, all members in subgroup Iib contained motif 5. Motif 10 was mostly found in subgroups Iia and Iib, while motif 9 was only identified in FtWRKY39, 54, and 61. Overall, the variety and complexity of these motifs suggests that these proteins likely have additional functions [59].





**Figure 2.** Phylogenetic relationship, gene structure, and conserved motif analysis of FtWRKYs. (A) Unrooted phylogenetic tree constructed with MEGA 6.0 software based on the 78 WRKY domain sequences with 1000 bootstrap replicates, where the colors represent different groups. (B) Gene structure of the 78 FtWRKYs predicted with GSDS, where *blue boxes* indicate exons, *red boxes* indicate untranslated regions, and *dark lines* indicate introns. The scale at the bottom can be used to estimate the lengths of the exons, introns, and untranslated regions. (C) Analysis of the conserved motif composition performed using MEME. The ten identified motifs are indicated with different colored boxes (see color figure online).

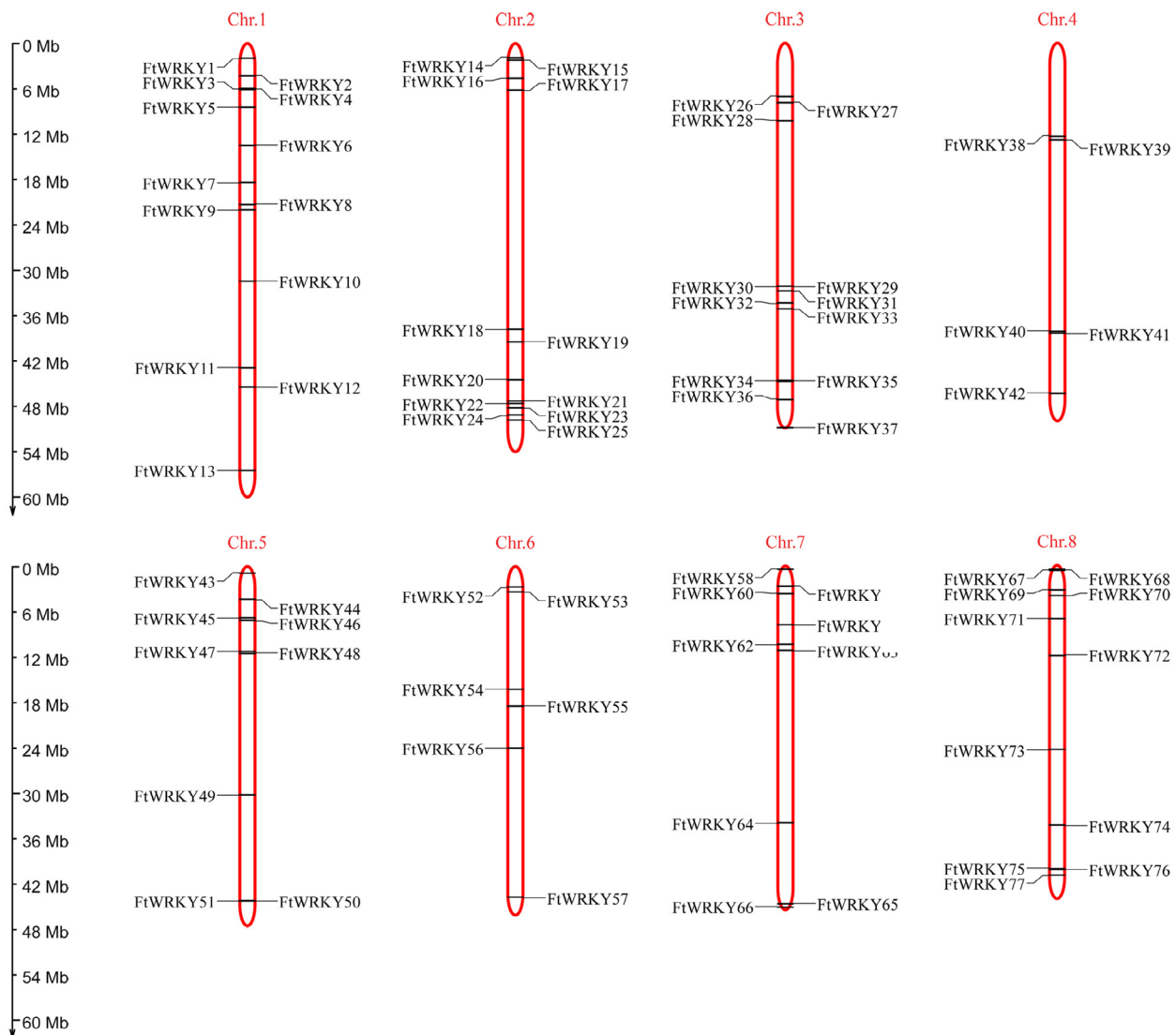
### 3.3 Chromosomal locations of FtWRKY genes

All 77 FtWRKY genes were separately mapped onto the eight chromosomes of Tartary buckwheat (Chr.1–8). Most FtWRKY genes were observed at the top and bottom arms of the chromosomes (Fig. 3). Chr.1 contained the most FtWRKY genes, with 13 out of 77 genes (16.88%), whereas Chr.4 contained the fewest, with five genes (6.49%). Chr.2 and Chr.3 each contained 12 genes (15.58%), while Chr.5 and Chr.7 each contained nine genes (11.69%). In addition, Chr.6 and Chr.8 contained six (7.79%) and 11 genes (14.29%), respectively. Each chromosome contained more than five classes of FtWRKYs. The FtWRKYs of subgroup IIc were the most widely distributed, and found on seven chromosomes (except Chr.7), while each of the other

groups were located on six chromosomes. Interestingly, the FtWRKYs of subgroups IIId and IIe were located on the same chromosomes, except Chr.4 and Chr.5. The results revealed a non-uniform distribution of WRKY genes among the chromosomes of Tartary buckwheat.

### 3.4 Duplication and evolution of FtWRKY

Gene duplication plays a vital role in the enlargement of gene families [60]. Chromosome regions shorter than 100 kb containing more than two genes with a similarity greater than 40% are considered to be caused by a tandem duplication event [61]. We found two pairs of tandem duplicates, FtWRKY29 and 30 and FtWRKY50 and 51,



**Figure 3.** Location and distribution of the FtWRKY genes in all eight chromosomes mapped based on their genomic position.

**Table 2.** Ka/Ks ratios and estimated divergence times of orthologous WRKY proteins in Tartary buckwheat and common buckwheat.

Protein names (Tartary buckwheat)	Protein IDs (buckwheat)	Ks	Ka	Ka/Ks	Time <sup>a</sup> (MYA)
FtWRKY2	Fes_sc0008092.1.g000001.a.a.1	0.0933	0.0123	0.1322	3.110
FtWRKY57	Fes_sc0004855.1.g000007.a.a.1	0.35	0.1955	0.5587	11.667
FtWRKY43	Fes_sc0004942.1.g000002.a.a.1	0.4657	0.051	0.1096	15.523
FtWRKY64	Fes_sc0000377.1.g000010.a.a.1	0.501	0.1124	0.2243	16.700
FtWRKY3	Fes_sc0011045.1.g000001.a.a.1	0.5287	0.235	0.4444	17.623
FtWRKY47	Fes_sc0001199.1.g000019.a.a.1	0.3288	0.1271	0.3866	10.960
FtWRKY6	Fes_sc0000044.1.g000018.a.a.1	0.1673	0.0252	0.1505	5.577
FtWRKY33.2	Fes_sc0000926.1.g000002.a.a.1	0.087	0.0158	0.1814	2.900
FtWRKY77	Fes_sc0013031.1.g000003.a.a.1	0.2838	0.0438	0.1542	9.460
FtWRKY8	Fes_sc0014831.1.g000001.a.a.1	0.1268	0.0355	0.2803	4.227
FtWRKY70	Fes_sc0000037.1.g000002.a.a.1	0.2145	0.0301	0.1404	7.150
FtWRKY69	Fes_sc0000011.1.g000128.a.a.1	0.0818	0.0284	0.347	2.727
FtWRKY67	Fes_sc0009561.1.g000007.a.a.1	0.17	0.058	0.3411	5.667
FtWRKY4	Fes_sc0008506.1.g000001.a.a.1	0.1878	0.0351	0.1872	6.260
FtWRKY44	Fes_sc0005050.1.g000005.a.a.1	0.1876	0.0523	0.2788	6.253
FtWRKY52	Fes_sc0006770.1.g000001.a.a.1	0.5152	0.1731	0.336	17.173
FtWRKY53	Fes_sc0069080.1.g000001.a.a.1	0.1253	0.0159	0.1268	4.177
FtWRKY60	Fes_sc0000003.1.g000030.a.a.1	0.2816	0.1011	0.3588	9.387
FtWRKY20	Fes_sc0029985.1.g000001.a.a.1	0.1534	0.0253	0.1646	5.113
FtWRKY12	Fes_sc0011190.1.g000002.a.a.1	0.2406	0.1457	0.6054	8.020
FtWRKY5	Fes_sc0003720.1.g000003.a.a.1	0.2283	0.0331	0.1449	7.610
FtWRKY21	Fes_sc0007586.1.g000004.a.a.1	0.1271	0.05	0.3939	4.237
FtWRKY22	Fes_sc0011986.1.g000003.a.a.1	0.1273	0.0175	0.1378	4.243
FtWRKY25	Fes_sc0000035.1.g000037.a.a.1	0.1216	0.0589	0.484	4.053
FtWRKY62	Fes_sc0000187.1.g000019.a.a.1	0.1237	0.0178	0.1438	4.123
FtWRKY63	Fes_sc0000026.1.g000039.a.a.1	0.0489	0.0072	0.1473	1.630
FtWRKY48	Fes_sc0000472.1.g000012.a.a.1	0.2265	0.0915	0.4037	7.550
FtWRKY13	Fes_sc0029335.1.g000001.a.a.1	0.2211	0.0304	0.1376	7.370
FtWRKY35	Fes_sc0008226.1.g000001.a.a.1	0.194	0.049	0.2528	6.467
FtWRKY34	Fes_sc0001280.1.g000011.a.a.1	0.1692	0.1863	1.1014	5.640
FtWRKY45	Fes_sc0001079.1.g000012.a.a.1	0.1131	0.0374	0.3307	3.770
FtWRKY46	Fes_sc0002930.1.g000006.a.a.1	0.1755	0.0355	0.2023	5.850
FtWRKY66	Fes_sc0005571.1.g000002.a.a.1	0.0956	0.0258	0.2703	3.187
FtWRKY26	Fes_sc0012248.1.g000002.a.a.1	0.2519	0.089	0.3535	8.397
FtWRKY7	Fes_sc0015209.1.g000003.a.a.1	0.1533	0.0635	0.4143	5.110
FtWRKY19	Fes_sc0004112.1.g000005.a.a.1	0.1388	0.0408	0.2941	4.627
FtWRKY15	Fes_sc0019573.1.g000002.a.a.1	0.2846	0.0344	0.1209	9.487
FtWRKY30	Fes_sc0002003.1.g000003.a.a.1	0.3094	0.1056	0.3413	10.313
FtWRKY29	Fes_sc0039378.1.g000001.a.a.1	0.4246	0.1549	0.3649	14.153
FtWRKY41	Fes_sc0000009.1.g000030.a.a.1	0.1215	0.0138	0.114	4.050
FtWRKY40	Fes_sc0000009.1.g000064.a.a.1	0.1256	0.0211	0.1676	4.187
FtWRKY72	Fes_sc0002754.1.g000009.a.a.1	0.3706	0.0839	0.2265	12.353
FtWRKY9	Fes_sc0004559.1.g000004.a.a.1	0.2475	0.0377	0.1523	8.250
FtWRKY16	Fes_sc0001592.1.g000015.a.a.1	0.1999	0.0477	0.2386	6.663
FtWRKY10	Fes_sc0007337.1.g000004.a.a.1	0.1715	0.0807	0.4707	5.717

Protein names (Tartary buckwheat)	Protein IDs (buckwheat)	Ks	Ka	Ka/Ks	Time <sup>a</sup> (MYA)
FtWRKY27	Fes_sc0003240.1.g000006.aua.1	0.1237	0.0468	0.3786	4.123
FtWRKY51	Fes_sc0045576.1.g000001.aua.1	0.2371	0.0421	0.1775	7.903
FtWRKY50	Fes_sc0014867.1.g000001.aua.1	0.155	0.1329	0.8579	5.167
FtWRKY42	Fes_sc0000437.1.g000022.aua.1	0.1495	0.0318	0.2124	4.983
FtWRKY24	Fes_sc0001536.1.g000007.aua.1	0.1069	0.028	0.2618	3.563
FtWRKY17	Fes_sc0003509.1.g000001.aua.1	0.2287	0.0523	0.2285	7.623
FtWRKY75	Fes_sc0008827.1.g000003.aua.1	0.2043	0.0441	0.2159	6.810
FtWRKY18	Fes_sc0001529.1.g000004.aua.1	0.4757	0.1315	0.2764	15.857
FtWRKY28	Fes_sc0038083.1.g000001.aua.1	0.3283	0.0723	0.2201	10.943
FtWRKY65	Fes_sc0001852.1.g000019.aua.1	0.1255	0.0274	0.2182	4.183
FtWRKY39	Fes_sc0001496.1.g000021.aua.1	0.1397	0.0392	0.2805	4.657
FtWRKY49	Fes_sc0008656.1.g000006.aua.1	0.2406	0.0151	0.0626	8.020
FtWRKY38	Fes_sc0000408.1.g000020.aua.1	0.2339	0.0355	0.1517	7.797
FtWRKY56	Fes_sc0000224.1.g000018.aua.1	0.2222	0.0278	0.125	7.407
FtWRKY14	Fes_sc0012847.1.g000001.aua.1	0.2519	0.0525	0.2083	8.397
FtWRKY74	Fes_sc0004443.1.g000014.aua.1	0.2923	0.0539	0.1845	9.743
FtWRKY61	Fes_sc0000022.1.g000019.aua.1	0.1677	0.1266	0.7551	5.590
FtWRKY73	Fes_sc0000894.1.g000012.aua.1	0.0982	0.0115	0.1166	3.273

which were located on Chr.3 and Chr.5, respectively. Both pairs of tandem duplicates were found in group II.

We calculated the Ka/Ks ratios of the orthologous pairs between Tartary buckwheat and common buckwheat (Table 2) and found 63 orthologous gene-pairs of WRKY TFs. The Ka/Ks values ranged from 0.0626 to 1.1014 (average: 0.2833). The overwhelming majority of gene pairs (62 pairs) had Ka/Ks ratios < 1, indicative of strong purifying selection acting on these genes, while only one pair had a Ka/Ks ratio > 1 (FtWRKY34 and Fes\_sc0001280.1.g000011.aua.1). Moreover, none of the genes had Ka/Ks ratios of ~1, indicating that neutral selection did not occur. The divergence time of Tartary and common buckwheat was predicted to have occurred between 1.630 and 17.623 Mya.

### 3.5 Expression patterns of FtWRKY in different tissue and under salt treatment

The heatmap of 78 FtWRKYs was constructed by log<sub>2</sub> transformed FPKM values (Fig.4). FtWRKY37, 50, 54 and 55 were barely expressed in any of the selected tissues. Other 74 of 78 (94.9%) FtWRKYs were expressed at least in one tissue. Some genes, like FtWRKY8, 70 and 74, were highly expressed in all the tissues, suggesting these FtWRKYs may play an important role in the plant development of

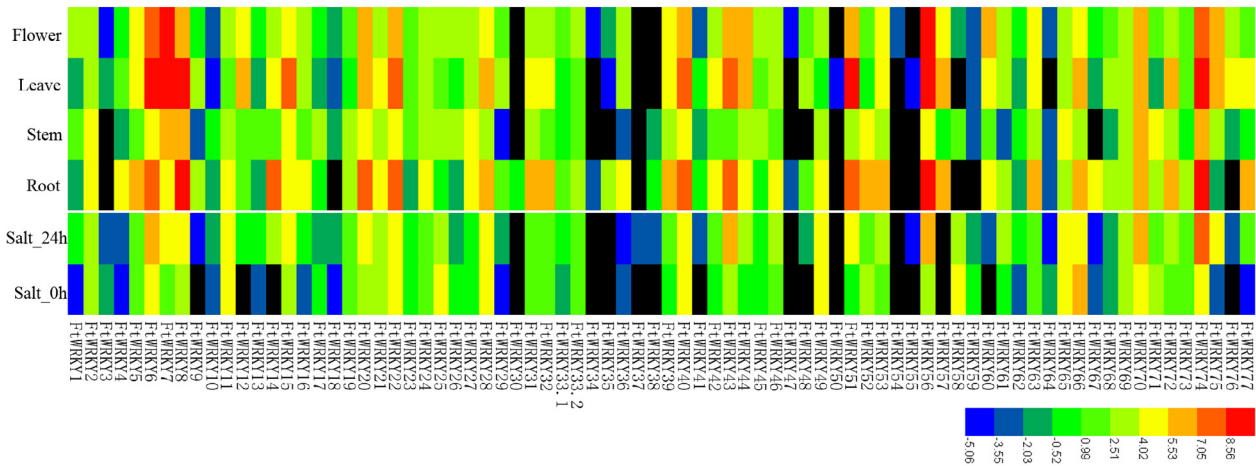
Tartary buckwheat. Additionally, some genes showed high expression simultaneously in flower, leaf and root, such as FtWRKY6, 43 and 51, while most of the genes showed lower expression levels in stem. Interestingly, a small number of genes presented tissue-specific expression profiling, for example, FtWRKY14 was only particularly expressed in root, and it may play key role in this tissue.

A total of 71 FtWRKYs (91.03%) were detected as being expressed under salt stress, indicating that most FtWRKYs genes were related to dealing with environment change. In total, 50 genes were upregulated, among these FtWRKY74 and 6 were most significantly followed by FtWRKY43 and 56.

### 3.6 Expression analysis of selected FtWRKYs under abiotic stress

TFs typically contain various types of DNA-binding domains and can improve expression at the transcription level under different abiotic stresses. Because the members of the same subgroups exhibit functional similarities [13], we selected four FtWRKYs (6, 7, 31, and 74) for an abiotic stress expression study in seedlings (Fig. 5), which were clustered in same groups with AtWRKY25, 33, 46, and 54.

Under salt stress, all FtWRKY genes were upregulated during the first 3 h and then decreased, while FtWRKY6, 74, and 31 were significantly up-regulated. The highest



**Figure 4.** Expression profile of FtWRKY genes among different tissues (i.e. root, stem, leaf and flower) and under salt stress. Levels of transcript accumulation from low to high are shown by using the color from blue to red. (see color figure online).

expression (13-fold increase) was detected after 3 h in FtWRKY31, followed by FtWRKY74. Three FtWRKY genes (FtWRKY74, 31, and 7) were significantly induced by drought conditions, and maintained a highly increased expression of at least 2.67-fold until 24 h. Meanwhile, FtWRKY6 showed an almost 5-fold increase within 3 h, but decreased a few hours later. Under cold treatment, the relative expression of FtWRKY74 increased by more than 4.5-fold within 3 h, while the expression of FtWRKY6 and 7 was significantly upregulated at the 12 h. Meanwhile, FtWRKY31 fluctuated slightly with an upregulation of less than 1.3-fold after 3 h and downregulation below that of the control after 12 h. All genes exhibited similar expression patterns under heat treatment, with no clear changes within 3 h and significantly upregulation of at least 2.95-fold within 12 h. FtWRKY74 showed the highest expression (almost 10-fold increase), whereas FtWRKY7 showed only a small change.

All selected FtWRKY genes responded to the abiotic stress treatments. FtWRKY6 and 74 showed rapid and significant upregulation with all treatments within 3 h. Under heat treatment, all genes were induced after 3 h. Except for FtWRKY31 under cold treatment within 12 h and FtWRKY6 under heat treatment within 3 h, the expression levels of all genes under the various stresses showed greater fold-changes than the control. Within 3 h, the average fold-change with salt treatment was 9.24-fold, which was the most significant change, but only 1.09-fold with heat treatment. Meanwhile, the average fold-changes were 3.24 and 2.34 under drought and cold conditions, respectively. By contrast, after 12 h, the heat condition showed the highest average fold change (5.26-fold), while

the salt treatment induced a fold-change of only 2.66. In addition, the drought and cold treatments caused fold-changes of 3.41 and 2.85 within 12 h, respectively.

## 4 Discussion

The WRKY family is one of the largest TF families in higher plants, and its members play an essential role in many physiological processes. In this study, a total of 78 FtWRKYs were identified, and all proteins presented clear differences, suggesting a high degree of complexity among FtWRKYs. The 78 FtWRKYs could be divided into three main groups, which was consistent with previous studies [6, 8]. Comparing with other species (Table S1), the number in Tartary buckwheat (78 FtWRKYs) was closest to that of *Arabidopsis* (72 AtWRKYs) [17]. Therefore, they likely underwent similar evolutionary patterns. Interestingly, Group II was always the largest group among the species (e.g., 70.5% in Tartary buckwheat and 69.1% in soybean), suggesting that group II may have undergone significant expansion during the course of evolution and that group II mainly accounts for the diversity of the WRKY family among species.

Despite the high conservation of the WRKYGQK sequence in the WRKY family, there were still some interesting cases. The WRKYGKK sequence present in FtWRKY15 and 76 was similar to those identified in many species, such as tomato, apple, and peach [62 - 64], this kind may have lost their ability to combine with the W-box [28]. Meanwhile, the variant type of WRKYDQK was only observed in FtWRKY50, and has also been reported

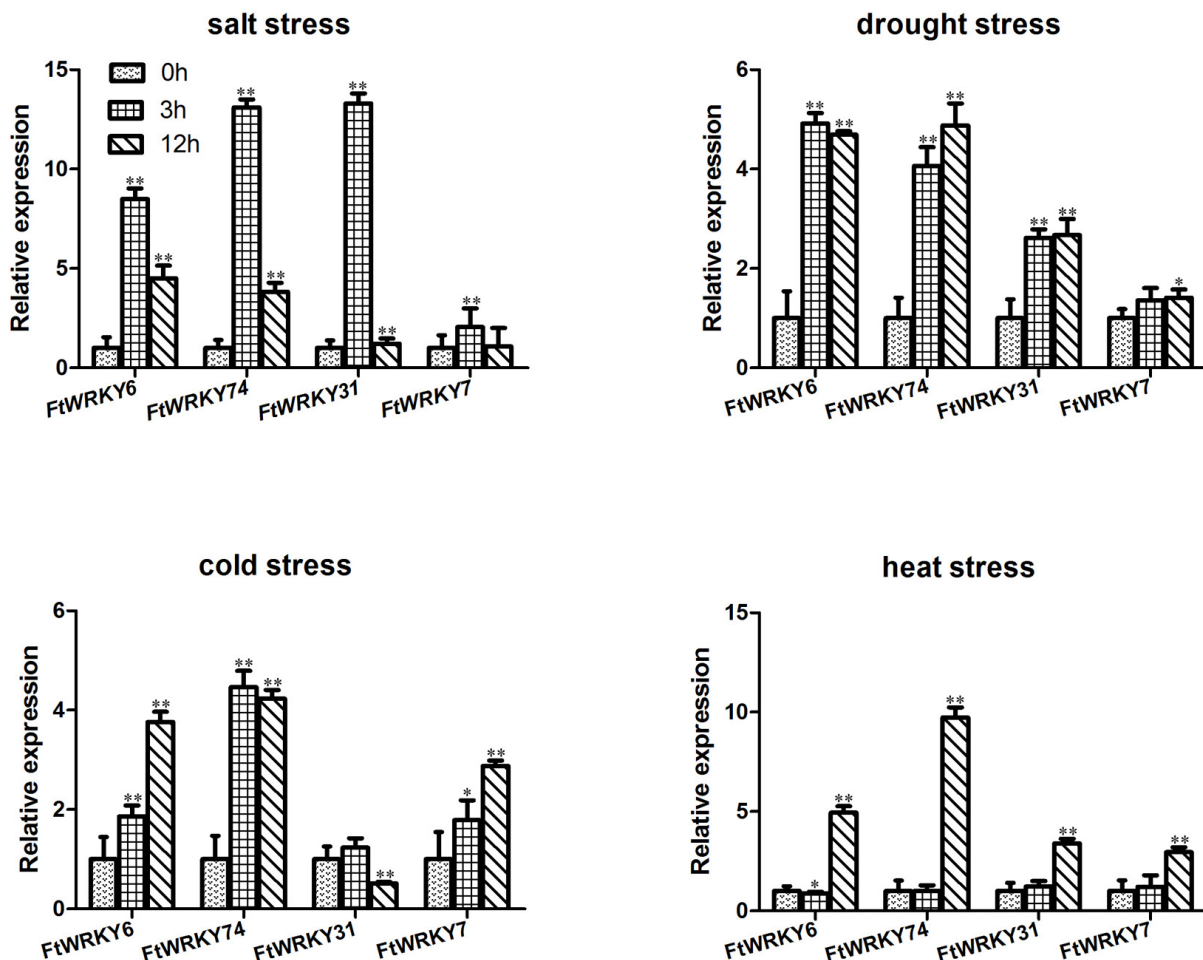
in carrot [65]. Similar to other plants, the properties of FtWRKY proteins showed differences among groups and individuals [11].

From the phylogenetic tree, we identified at least one FtWRKY and AtWRKY in each subgroup, illustrating that the differentiation time of the WRKY family was earlier than the divergence time of Tartary buckwheat and *A. thaliana*. The FtWRKY members of the specific subgroups likely shared closely related motif compositions and functional similarities, which was supported by the subsequent gene structure analysis. Meanwhile, genes containing six exons were clustered in groups I and II, indicating that groups I and II constituted the ancestral genes.

Gene duplication plays a vital role in the enlargement of gene families [60]. Although a whole-genome duplication event occurred in Tartary buckwheat [42],

only two pairs of tandem duplicates were found in the FtWRKY family, FtWRKY29 and 30 (90% similarity) and FtWRKY 50 and 51 (44% similarity). Both pairs of tandem duplicates were found in group II. By contrast, most duplication events in *Arabidopsis* and rice are found in group III. Surprisingly, no segmental duplications were identified in the FtWRKYs, therefore, duplication likely had a limited contribution to the expansion of the WRKY family in Tartary buckwheat. At the same time, a total of 63 orthologous gene-pairs between Tartary buckwheat and common buckwheat were identified, and the Ka/Ks ratios of 62 to 63 pairs (98.41%) were  $< 1$ , indicative of strong purifying selection acting on these genes.

Like the expression pattern of WRKY in other species, different genes showed incongruous expression patterns, suggesting they have various function and



**Figure 5.** Relative expression patterns of four selected FtWRKY genes under four abiotic stresses. Results are presented as the means  $\pm$  standard deviation. Samples were collected at 0, 3, and 12 h, and 0 h was used as the control. Asterisks indicate the gene significantly upregulated or downregulated under abiotic stresses using *t* test (\*  $p < 0.05$ , \*\*  $p < 0.01$ ). (A) Relative expression under drought treatment (30% PEG6000). (B) Relative expression under salinity treatment (15% NaCl). (C) Relative expression under cold treatment (4°C, growth chamber). (D) Relative expression under heat treatment (40°C, growth chamber).

diversity regulatory mechanisms [12, 66]. According to the RNA-seq data of Tartary buckwheat, some genes were not expressed in any tissues, the reason may be some of them are pseudogenes, or they were expressed in other tissues we failed to collect. FtWRKY74 and 6 showed the highest expression level after salt treatment, which revealed these genes can improve the tolerance under osmotic stress, therefore were selected as the candidate genes to develop qRT-PCR analysis.

Substantial evidence has shown that WRKY TFs can improve the stress tolerance of plants by modulating their molecular and physiological metabolism [8, 20]. In *Arabidopsis*, at least 26 AtWRKY genes have been demonstrated to participate in abiotic stress responses [67]. For example, overexpression of AtWRKY25 and 33 was reported under heat and salt treatment [32]. The expression pattern results suggested that the four assessed FtWRKYs were involved in the regulation of abiotic stress responses. Under heat treatment, all four genes were upregulated within 12 h, indicating that the WRKY TFs participated in a complex cross-regulation network under such stresses. FtWRKY6 and 74 simultaneously responded to salt and drought treatments, suggesting that they can co-regulate more than one adverse condition, possibly based on synergistic or antagonistic mechanisms. Overall, the results indicate that the selected four FtWRKY genes play an important role in the establishment of salt, drought, cold, and heat tolerance.

Furthermore, it is indicative of the selection method of responsive genes in our study is efficient. RNA-sequencing analysis can help us to find the genes with higher expression under stress, while sequence alignment suggest the genes clustered with the reported stress resistance genes have similar functions.

## 5 Conclusions

We identified 78 FtWRKY proteins (77 genes) from the *F. tataricum* genome sequence, which could be divided into three groups. Although the proteins were diverse, the members in same groups and subgroups exhibited similar properties, such as gene structure and motif composition. The results suggested that duplication events contributed little to the expansion and evolution of FtWRKYs, and most FtWRKYs experienced purifying selection during the course of evolution. Finally, the expression analysis indicated that all four studied FtWRKYs (6, 74, 31, and 7) were regulated by abiotic stresses. In particular, FtWRKY6 and 74 were highly responsive to both salt and drought treatment. Based on results above, these four selected

genes show potential for transgenic applications in Tartary buckwheat. This study is the solid foundation for expression analyses of additional FtWRKYs and related mechanistic studies, and can prove the fundamental theory to clone specific functional genes.

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