



# Article Identification, Expression, and Functional Study of Seven NAC Transcription Factor Genes Involved in Stress Response in Tartary Buckwheat (*Fagopyrum tataricum* (L.) Gaertn.)

Juan Huang<sup>+</sup>, Rongrong Ren<sup>+</sup>, Yuping Rong, Bin Tang, Jiao Deng, Qingfu Chen<sup>\*</sup> and Taoxiong Shi<sup>\*</sup>

\* Correspondence: 100043716@gznu.edu.cn (Q.C.); 460181736@gznu.edu.cn (T.S.)

+ These authors contributed equally to this work.

Abstract: Plant NAC transcription factors play vital roles in diverse biological processes, especially in response to stress. However, only a limited number of the FtNAC genes have been cloned and functionally analyzed in Tartary buckwheat (Fagopyrum tataricum (L.) Gaertn.), which is a coarse grain crop that is highly resistant to abiotic stress. Here, we identified seven Tartary buckwheat FtNAC genes, namely FtNAC11, FtNAC12, FtNAC26, FtNAC28, FtNAC65, FtNAC70, and FtNAC78. Multiple-sequence alignments revealed that all of them contained the conserved NAM domain, which consisted of the A, B, C, D, and E subdomains. Seven NAC proteins were classified into six subgroups based on phylogenetic analyses. Cis-acting regulatory elements analyses (CARE) indicated that there were numerous CAREs related to biotic and abiotic responses and hormone responses in the promoters of FtNAC genes. qRT-PCR showed that they were tissue-specifically expressed and could be induced by four out of six treatments, namely PEG, NaCl, cold, MeJA, ABA, and GA, implying that they were abiotic and hormone responsive. Among these, *FtNAC70* was the most strongly induced gene and could be induced by all six treatments. Meanwhile, FtNAC70-overexpressed Arabidopsis showed more resistance to salt and drought. The identification, phylogenetics, expression, and functional study of seven stress-responsive FtNAC genes laid a foundation for further research on the molecular mechanism of abiotic stress regulated by NAC TFs in Tartary buckwheat.

**Keywords:** Tartary buckwheat; NAC transcription factor; phylogenetic tree; qRT-PCR; transgenic *Arabidopsis*; salt stress; drought stress

# 1. Introduction

The plant-specific NAC (petunia NAM and *Arabidopsis thaliana* ATAF1, ATAF2, and CUC2) transcription factors (TFs) play essential roles in diverse biological processes, including plant growth, development, senescence, and responses to biotic and abiotic stress [1,2]. In the plant kingdom, the NAC family is one of the largest TF families; it has been identified at a genome-wide scale in many plant species, and includes 105 genes in *Arabidopsis*, 150 genes in rice (*Oryza sativa* L.), 88 genes in pigeonpea (*Cajanus cajan* (L.) Millsp.), 283 genes in upland cotton (*Gossypium hirsutum* (L.)), 152 genes in maize (*Zea mays* (L.)), 114 genes in *Dimocarpus longan* Lour, and approximately 80 genes in Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) [3–9]. The NAC TFs have a highly conserved consensus sequence known as the NAM domain in the N-terminal region, which has about 150 amino acid residues and consists of five subdomains (A, B, C, D, and E) [4]. Based on the sequence similarity of the NAM domains, NAC TFs are classified into two groups and 18 subgroups in *Arabidopsis* and rice [4].

As is well known, NAC TFs play essential roles in diverse biological processes, including plant growth, development, pattern formation, secondary cell wall formation, senescence, and responses to biotic and abiotic stress [10–13]. Two closely related NAC



Citation: Huang, J.; Ren, R.; Rong, Y.; Tang, B.; Deng, J.; Chen, Q.; Shi, T. Identification, Expression, and Functional Study of Seven NAC Transcription Factor Genes Involved in Stress Response in Tartary Buckwheat (*Fagopyrum tataricum* (L.) Gaertn.). *Agronomy* **2022**, *12*, 849. https://doi.org/10.3390/ agronomy12040849

Academic Editors: Monica Boscaiu and Ana Fita

Received: 23 February 2022 Accepted: 24 March 2022 Published: 30 March 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/).

Research Center of Buckwheat Industry Technology, Guizhou Normal University, Guiyang 550001, China; 201509007@gznu.edu.cn (J.H.); 20010100382@gznu.edu.cn (R.R.); 18010100303@gznu.edu.cn (Y.R.); 19010100309@gznu.edu.cn (B.T.); 201507012@gznu.edu.cn (J.D.)

genes (*CUC1* and *CUC2*) are involved in the control of organ formation, as highlighted by the fact that the *cuc1 cuc2* double mutants in *Arabidopsis* are defective in the separation of cotyledons, sepals, and stamens [12]. The second wall thickenings are completely suppressed in the woody tissues of *nst1-1 nst3-1* double knockout *Arabidopsis*, suggesting *NST1* and *NST2* are key regulators of the secondary wall formation [10]. Three NAC TFs, *ANAC019*, *ANAC055*, and *ANAC072*, have roles in *Arabidopsis* leaf senescence and stress responses, and a gene-regulatory network surrounding them is predicted, including HB, MYB, ABF, CBF, and bZIP TFs and the SA and JA signaling pathways [13]. NAC genes in rice are found to be related to the biotic responses induced by virus-infection microarray analysis [11]. Because of the sequence similarity of NAC proteins in the same subgroup, they often share the same or similar functionality. For instance, SENU5-subgroup genes often regulate leaf senescence [14]. NAC2-subgroup genes often regulate proteotoxic stress in plant survival and maintain protein homeostasis [15]. NAM-subgroup genes often regulate pattern formation in embryos and flowers [16]. Genes in the subgroups of ATAF, NAP, AtNAC3, and OsNAC3 often regulate different kinds of stress [4,17,18].

It is predicted that about 20% to 25% NAC genes respond to at least one abiotic stress, including drought, salt, and cold [19]. These NAC genes are defined as SNACs (stress-responsive NACs) [1]. In plants, the regulation of stress by NAC TFs usually depends on plant hormones; thus, the ABA-dependent and ABA-independent pathways in response to biotic stress and the JA-mediated pathway in response to both abiotic and biotic stress are evolved [1,20]. Three NAC genes in the ATAF subgroup (ATAF1, OsNAC5, and *OsNAC6*) regulate drought, salt, and cold stress via the ABA-dependent pathway [21–23]. Three members of the AtNAC3 subgroup (ANAC019, ANAC055, and ANAC072) and two members of the OsNAC3 subgroup (OsNAC3 and OsNAC4) regulate various abiotic stresses via the ABA-independent pathway [1,13]. SiNAC and TaNAC8 also regulate drought, salt, and cold stresses via the ABA-independent pathway [20]. Furthermore, OsNAC3, OsNAC4, OsNAC5, and OsNAC6 regulate abiotic stresses such as salt and drought via a JA-mediated pathway [1,22]. Notably, some NAC TFs can play more than one role in regulating biological processes. For example, AtNAC4, belonging to the NAM subgroup, is involved in pattern formation in embryos and flowers, pathogen-induced cell death, and salt-stress resistance [24–26].

Harsh environments, such as drought and high salinity, make the growth and development of staple food crops difficult. Furthermore, it is necessary to breed and cultivate coarse grain crops that are highly resistant to abiotic stress. Tartary buckwheat is one such crop that has a high tolerance to aluminum, salt, and drought [27–29]. It is widely planted in high-altitude mountainous areas in Asian countries where the staple food crops are difficult to grow, such as the southwest China karst mountain area [30]. In recent years, the mechanism of its high-tolerance ability has gathered attention and some TFs have been reported to be involved in abiotic stress in Tartary buckwheat. Members of the WRKY, bZIP, MYB, bHLH, and NAC TF families are identified in response to salt, drought, oxidative, and cold stress [31–38]. Based on the Tartary buckwheat genome data, approximately 80 FtNAC genes are identified, among which 11 genes are cloned and functionally analyzed, including FtNAC2-9, FtNAC15, FtNAC16, and FtNAC17. All of the above genes have a role in response to salt or drought stress [8,9,27,35–38]. In spite of the limited number of the cloned NAC TFs, dozens of FtNAC genes remain unknown. In this study, we cloned and identified seven FtNAC genes that were stress-related in Tartary buckwheat. Multiple-sequence alignments, phylogenetic analyses, cis-acting regulatory element analyses, expression patterns in different tissues, and under different abiotic stresses and hormone treatments were performed to obtain a better understanding of their functions. In addition, FtNAC70, which was most strongly expressed in response to stress, was transformed into Arabidopsis to further clarify its function in drought and salt stress. This work will provide valuable information about the molecular mechanism of abiotic stress regulated by NAC TFs in Tartary buckwheat.

# 2. Materials and Methods

#### 2.1. Plant Growth and Stress Treatment of Tartary Buckwheat

A Tartary buckwheat variety "Guimiku NO. 11", which is principally cultivated in Guizhou Province, China, was used. It was planted in an experimental field with regular field management. For tissue-specific expression analysis, the roots, stems, leaves, and flowers were harvested at the flowering stage, while the mature seeds were harvested at the mature stage. Mature seeds were divided into two kinds: one with green shells and the other with yellow shells. Then, the shells were detached on ice. All samples were frozen in liquid nitrogen and stored at -80 °C.

Stress treatment was performed in accordance with a previous method, with some modifications [28]. First, the surfaces of Tartary buckwheat seeds were sterilized with 10% H<sub>2</sub>O<sub>2</sub> solution and rinsed with ddH<sub>2</sub>O three times. To accelerate germination, they were placed on wetted filter papers in the center of petri dishes for two days. Then, the uniformly sprouted seeds were transformed to rolls of paper soaked with ddH<sub>2</sub>O for about seven days when seedlings grew two true leaves. The growth conditions were set as 16/8 h light/dark cycles at 25 °C. The consistent seedlings were subsequently treated with 20% polyethylene glycol (PEG), 150 mM NaCl, 1 mM methyl jasmonate (MeJA), and 1 mM gibberellin (GA), and cold (4 °C), and sampled at 0 h, 3 h, 6 h, 12 h, and 24 h [28,38]. Three independent biological replicates were performed; each included at least five seedlings.

# 2.2. RNA Isolation, Identification, and Cloning of FtNACs

RNA isolation was performed with a plant RNA purification kit (RNAprep Pure Plant Plus Kit, DP441, TianGen Biotech Co., Ltd., Beijing, China). RNA integrity was detected by agarose gel electrophoresis. RNA concentration and quality was detected by microspectrophotometer. Then, first-strand cDNA was synthesized using the PrimeScript<sup>™</sup> II 1st Strand cDNA Synthesis Kit (6210A, Takara Biomedical Technology Co., Ltd., Beijing, China). Seven pairs of gene-specific primers were designed by oligo 7 primer analysis software [39]. Primer sequences are listed in Table S1. Gene cloning was carried out using the T-Vector pMD<sup>™</sup> 20 kit (3270, Takara Biomedical Technology Co., Ltd., Beijing, China). The products were transformed into DH5a competent cells. After the spreading of the plates, the selection of monoclones, and PCR identification, the positive monoclones were sequenced by Sangon Biotech Co., Ltd., Shanghai, China.

#### 2.3. Bioinformatics Analyses of FtNACs

Seven candidate FtNAC genes were selected from the transcriptome data [40] and the full DNA, coding sequences (CDSs), and 1500 bp promoter sequences were obtained from the Tartary buckwheat genome data [27] using local python scripts. All seven FtNAC sequences were uploaded to GenBank using the BankIt website (https://www.ncbi.nlm.nih. gov/WebSub/ accessed on 11 December 2021). Protein molecular weight and theoretical pI were predicted using an online website (https://web.expasy.org/protparam/ accessed on 11 December 2021). Protein subcellular location was predicted by WoLF PSORT (https://wolfpsort.hgc.jp/ accessed on 11 December 2021). Gene structure was visualized using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/ accessed on 11 December 2021). CAREs in the promoters of FtNAC genes were predicted using plantCARE [41] and visualized with TBtools [42].

Multiple amino acid sequence alignments of the NAM domain of seven FtNAC proteins were performed using DNAMAN software (version 6.0, Lynnon Corp., Quebec, QC, Canada). The sequence logos of five subdomains, namely A, B, C, D, and E, among the NAM domain were visualized by WebLogo (http://weblogo.berkeley.edu/logo.cgi accessed on 11 December 2021) [43]. Eighteen representative NAC TFs from each subgroup were selected based on previous reported publications that classified the plant NAC proteins to 18 subdomains [4]. Among these, nine representative NAC TFs were retrieved from the TAIR database (https://www.arabidopsis.org/ accessed on 11 December 2021). Their accession numbers are AT4G29230.1 (ONAC003 subgroup), AT3G55210.1 (ANAC063 subgroup), AT2G17040.1(ONAC022 subgroup), AT1G69490.1 (NAP subgroup), AT3G15500.1 (AtNAC3 subgroup), AT5G63790.1 (ATAF1 subgroup), AT1G32510.1 (ANAC011 subgroup), AT1G01010.1 (ANAC001 subgroup), and AT5G24590.2 (TIP subgroup). Nine remaining representative NAC TFs were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/ accessed on 11 December 2021). Their accession numbers are BAG87473.1 (ONAC001 subgroup), BAG96778.1 (OsNAC3 subgroup), AAF09254.1 (NAC2 subgroup), BAG95726.1 (OsNAC8 subgroup), BAA89801.1 (OsNAC7 subgroup), AAF21437.1 (NAC1 subgroup), CAA63101.1 (NAM subgroup), BAA78417.1 (TERN subgroup), and CAA99760.1 (SENU5 subgroup). Finally, the phylogenetic tree was constructed in MEGA X software, using the neighbor-joining method with 5000 replicates taken to represent the evolutionary history and pairwise deletion option [44].

## 2.4. Quantitative Reverse Transcription PCR (qRT-PCR) Analyses of FtNACs

Gene-specific primers for qRT-PCR were designed using the Primer3Plus website (https://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi, accessed on 11 December 2021; Table S1). Universal primers of FtActin genes (FtActUniv) and AtActin (AtAcUniv) genes were used as the housekeeping gene for Tartary buckwheat and *Arabidopsis*, respectively (Table S1) [28,45]. For each tissue, 1 ug total RNA was used for first-strand cDNA synthesis. qRT-PCR was performed using the TB Green<sup>®</sup> Premix Ex Taq<sup>TM</sup> II kit (RR820A, Takara Biomedical Technology Co., Ltd., Beijing, China) on an ABI 7500 Fast Real-Time PCR system (Thermofisher, Waltham, MA, USA) in accordance with the manufacturer's instructions, with three technical replicates. Gene expression data were calculated using the  $2^{-\Delta\Delta CT}$  method. The statistical analysis was performed using SPSS Statistics software (Version 20, IBM, New York, NY, USA), with the Student's *t* test being used to evaluate the statistical significance. The results were visualized using Office Excel software (version 2019, Microsoft, Redmond, DC, USA).

#### 2.5. Arabidopsis Transformation and Stress Treatment of FtNAC70

The full-length CDS of *FtNAC70* was linked to the pBI121 vector using the In-Fusion<sup>®</sup> HD Cloning Kit (639648, Takara Biomedical Technology Co., Ltd., Beijing, China) in accordance with the manufacturer's instructions. *Bam*H I and *Sac* I were used as the double restriction site. The primers for vector construction (pBI121-*FtNAC70*, Table S1) were designed using the following website: https://www.takarabio.com/learning-centers/cloning/primer-design-and-other-tools, accessed on 12 February 2022. After transformation, plate spreading, and sequencing of positive monoclones, the plasmid-positive monoclones were extracted and transformed into *Agrobacterium tumefaciens* (GV3101).

The wild-type (WT) Col-0 *Arabidopsis* was planted under long-day conditions (16/8 h light/dark cycle) at 25 °C and the *Agrobacterium*-mediated transformation was performed using the floral dip method [46]. The transgenic seeds were selected on the 1/2 MS medium with kanamycin resistance and the positive seedlings were transferred into nutritional soil for normal growth. At the flower stage, the positive overexpression (OE) lines were identified by PCR and qRT-PCR. PCR was performed using the vector-specific 35S as the forward primer and a gene-specific reverse primer (Table S1). Seeds at the third transgenic generation were used for stress-treatment experiments.

Salt and drought stress treatments of WT and OE *Arabidopsis* were performed as reported previously with slight modifications [47]. Briefly, WT and three OE lines were planted on 1/2 MS medium in a vertical orientation for five days. Then, the consistent seedlings were transferred to 1/2 MS medium with 150 mM NaCl (or 25% PEG) for salt (or drought) treatment in a vertical orientation. Six days later, the seedlings were photographed and the root length was measured. For drought treatment, no obvious difference appeared in the seedlings' root length. So, we further transferred the seedlings into nutritional soil for normal growth. A month later, while the WT under drought stress were at the reproductive stage, they were photographed. For each line or WT, three independent stress-treatment experiments were taken for biological repeats.

# 3. Results

### 3.1. Isolation and Analyses of Seven NAC Genes from Tartary Buckwheat

Previously, genome-wide identification of NAC TFs was performed based on the released genomic data of Tartary buckwheat [8,9]. Among these identified genes, we selected seven NAC genes that were highly expressed in our transcriptome data of the seed development of Tartary buckwheat [40] and named them as *FtNAC11*, *FtNAC12*, *FtNAC26*, *FtNAC28*, *FtNAC65*, *FtNAC70*, and *FtNAC78* in accordance with Liu's research [8]. Subsequentially, gene-specific primers were designed to isolate and verify the CDSs of seven NAC genes from Tartary buckwheat cDNA (Table S1). The verified CDSs were uploaded to GenBank under the accession numbers of MW355481–MW355487. As shown in Table 1, the CDS length of seven FtNACs ranged from 729 to 1260 bp. The DNA length of seven FtNACs ranged from 922 to 4908 bp. The deduced protein length of seven NAC ranged from 242 to 419 aa. The molecular weight of seven NAC proteins ranged from 2.01 to 47.14 kDa. The theoretical pI of seven NAC proteins ranged from 5.39 to 8.97. The predicted cellular locations of seven NAC proteins were all in the nucleus. Furthermore, they were located on the chromosomes Ft1, Ft2, Ft7, and Ft8, respectively.

Table 1. Overview of seven FtNACs identified in Tartary bu
--

Gene Name	Genome Data Accession	CDS Length (bp)	DNA Length (bp)	Protein Length (Amino Acids)	Protein Molecular Weight (kDa)	Protein Theoretical pI	Protein Predicted Location	GenBank Accession	Chromosome
FtNAC11	FtPinG0006563600	840	1023	279	31.82	6.91	Nucleus	MW355481	Ft1
FtNAC12	FtPinG0002560000	888	1144	295	33.25	8.86	Nucleus	MW355482	Ft1
FtNAC26	FtPinG0000059500	1242	4032	413	46.30	5.53	Nucleus	MW355483	Ft2
FtNAC28	FtPinG0002920400	942	1439	313	35.52	8.82	Nucleus	MW355484	Ft2
FtNAC65	FtPinG0005688900	729	922	242	28.01	8.97	Nucleus	MW355485	Ft7
FtNAC70	FtPinG0002339300	1020	1535	339	38.15	5.39	Nucleus	MW355486	Ft8
FtNAC78	FtPinG0005906600	1260	4908	419	47.14	6.43	Nucleus	MW355487	Ft8

Gene structure analyses revealed that NAC family genes were conservative in structure (Figure 1). Among seven FtNACs, five (*FtNA11*, *FtNA12*, *FtNA28*, *FtNA65*, and *FtNA70*) possessed three exons and two introns. For the two introns in each gene, the first was a phase 1 intron and the second was a phase 0 intron. *FtNAC26* possessed four exons and three introns. *FtNAC78* possessed six exons and five introns.



Exon — Intron 0 1 2: intron phase

Figure 1. Gene structure of seven FtNACs.

## 3.2. Amino Acids and Phylogenetic Analyses of Seven FtNAC Proteins

It is well known that NAC TFs own a conserved NAM domain in the N termini, and it comprises five conserved subdomains, namely subdomain A, B, C, D, and E [4]. We therefore performed multiple-sequence alignments to see whether the NAM domain existed in seven FtNACs. Similar to other species, FtNACs possessed a conserved NAM domain that could be divided into five subdomains (A, B, C, D, and E) (Figure 2A). The sequence logos of these subdomains were predicted (Figure 2B). Furthermore, they were similar to that in rice and *Arabidopsis* [4]. The phylogenetic analysis of NAC families in

rice and *Arabidopsis* led to the classification of NAC TFs into two groups, in which group I contained 14 subgroups (TERN, ONAC022, SENU5, NAP, AtNAC3, ATAF, OsNAC3, NAC2, ANAC011, TIP, OsNAC8, OsNAC7, NAC1, and NAM) and group II contained four subgroups (ANAC001, ONAC003, ONAC001, and ANAC063) [4]. Eighteen representative NAC TFs in each subgroup were retrieved from Genbank or the TAIR database. They were used to make a phylogenetic tree along with seven FtNACs. As shown in Figure 2C, all 25 NAC proteins were clustered into six groups. Among these, FtNAC11 and FtNAC28 were closest to AT5G63790.1 in the ATAF subgroup. FtNAC12 was closest to AT1G69490.1 in the NAP subgroup. FtNAC65 was closest to CAA99760.1 in the SENU5 subgroup. FtNAC26 was closest to AAF09254.1 in the NAC2 subgroup. FtNAC70 was closest to CAA63101.1 in the NAM subgroup. *FtNAC78* was closest to AT4G29230.1 in the ONAC003 subgroup. Closer relationships indicate that they may have similar functions during the regulation of plant growth, development, and stress response.



**Figure 2.** Amino acids and phylogenetic analyses of seven FtNAC proteins. (**A**) Multiple amino acid sequence alignments of the NAM domains in seven FtNAC proteins. Five conserved subdomains (A, B, C, D, and E) were marked by black boxes. (**B**) Sequence logos of five subdomains in NAM domain. (**C**) Phylogenetic tree of seven FtNACs (green dots) and 18 representative NAC sequences.

## 3.3. CARE Analyses of Seven FtNAC Promoters

Plant NAC TFs are involved in various biological processes and their promoters contain numerous CAREs that are responsive to biotic and abiotic processes [3,5]. We therefore analyzed the CAREs in the promoters of seven FtNAC genes. As a result, 7, 10, 5, 8, 7, 5, and 6 CAREs related to biotic and abiotic responses or hormone responses were identified in the promoter of *FtNAC11*, *FtNAC12*, *FtNAC26*, *FtNAC28*, *FtNAC65*, *FtNAC70*, and *FtNAC78*, respectively (Figure 3). Among these CAREs, ABRE appeared as the most abundant CARE (17 ABREs in six FtNAC promoters), followed by ARE (seven AREs in four FtNAC promoters), and CGTCA-motif (five CGTCA-motifs in three FtNAC promoters). ABRE, ARE, and CGTCA-motif are cis-acting elements involved in the responsiveness



of abscisic acid (ABA), anaerobic induction, and MeJA-responsiveness, respectively [41], which indicated that the FtNACs might be induced by these factors.

**Figure 3.** CARE Analyses of Seven FtNAC Promoters. ABRE, CARE involved in the ABA responsiveness; MBS, MYB-binding site involved in drought-inducibility; O<sub>2</sub>-site, CARE involved in zein metabolism regulation; ARE, CARE essential for the anaerobic induction; TGA-box, part of an auxinresponsive element; CGTCA-motif, CARE involved in MeJA-responsiveness; TGA-element, auxinresponsive element; AuxRR-core, CARE involved in auxin responsiveness; TC-rich repeats, CARE involved in defense and stress responsiveness; P-box, GA-responsive element; TCA-element, CARE involved in salicylic acid responsiveness; LTR, CARE involved in low-temperature responsiveness.

## 3.4. Tissue-Specific Analyses of Seven FtNAC Genes

Genes expressed in different tissues often perform different functions, so we examined the expression patterns of seven FtNAC genes in different tissues of Tartary buckwheat by qRT-PCR, including root, stem, flower, seed with green shell (filling was finished and the shell was green), and seed with yellow shell (filling was finished and the shell was yellow). The results showed that seven FtNAC genes exhibited different expression patterns (Figure 4). *FtNAC11* was more highly expressed in flower but hardly expressed in leaf. *FtNAC12* showed a high expression level in root and leaf rather than the other tissues. *FtNAC26* was expressed in every tissue: the highest expression was in stem but the lowest expression was in seed with yellow shell. *FtNAC28* was also expressed in every tissue: the highest expression was in flower but the lowest expression was in seed with green shell. *FtNAC65* was predominantly expressed in root, stem, leaf, and flower rather than seed. On the contrary, *FtNAC70* was expressed thousands of times more highly in seeds than the other four tissues. *FtNAC78* was preferentially expressed in seeds and stem and hardly expressed in root, leaf, and flower.

### 3.5. Expression Analyses of Seven FtNAC Genes under Abiotic Stress and Phytohormones Treatment

As many abiotic and hormone-response CAREs in the promoters of FtNACs have been identified (Figure 3), we performed qRT-PCR analyses to investigate the expression patterns of seven FtNAC genes in response to three abiotic stresses (PEG, NaCl, and cold) and three phytohormones (MeJA, ABA, and GA) (Figure 5). For PEG mimic drought stress, *FtNAC11*, *FtNAC12*, *FtNAC28*, and *FtNAC70* could be induced by drought stress, whereas *FtNAC26*, *FtNAC65*, and *FtNAC78* could be inhibited by drought stress. For NaCl mimic salt stress, *FtNAC12*, *FtNAC26*, *FtNAC26*, *FtNAC28*, and *FtNAC78* were inhibited by salt stress. For MeJA treatment, six FtNAC genes were induced by it except for *FtNAC65*. For ABA treatment, *FtNAC12* and *FtNAC26* were induced by ABA; *FtNAC78* was inhibited by ABA; whereas *FtNAC11*, *FtNAC28*, *FtNAC65*, and *FtNAC70* showed no response to ABA. For GA treatment, *FtNAC11*, *FtNAC26*, *FtNAC65*, and *FtNAC70* were induced by GA; *FtNAC12*.

and *FtNAC28* were inhibited by GA; whereas *FtNAC78* showed no response to GA. For cold treatment, *FtNAC11*, *FtNAC12*, *FtNAC26*, *FtNAC28*, and *FtNAC78* were induced by cold, whereas *FtNAC65* and *FtNAC70* showed no response to cold. Among seven FtNAC genes, we found that the expression level of *FtNAC70* was 1.3, 3.0, 7.2, 2.9, 13.8, and 4.8 times higher after the PEG, NaC1, MeJA, ABA, GA, and cold treatments, respectively, which suggested that *FtNAC70* was important in the response of Tartary buckwheat to various stresses. Overall, the results showed that seven FtNAC genes showed different expression patterns under different abiotic stresses or hormone treatments. Furthermore, each FtNAC genes were involved in abiotic stress in Tartary buckwheat, as has been reported previously [8,35–38].



**Figure 4.** Tissue-specific analyses of seven FtNAC genes, namely *FtNAC11* (**A**), *FtNAC12* (**B**), *FtNAC26* (**C**), *FtNAC28* (**D**), *FtNAC65* (**E**), *FtNAC70* (**F**), and *FtNAC78* (**G**). The columns indicate the means of the relative mRNA expression levels. The error bars indicate the standard deviations of the replicates.



**Figure 5.** Expression analyses of seven FtNAC genes under PEG, NaCl, MeJA, ABA, GA, and cold treatment, namely *FtNAC11* (**A**), *FtNAC12* (**B**), *FtNAC26* (**C**), *FtNAC28* (**D**), *FtNAC65* (**E**), *FtNAC70* (**F**), and *FtNAC78* (**G**). The columns indicate the means of the relative mRNA expression levels. The error bars indicate the standard deviations of the replicates. The lowercase letters show the statistical significance evaluated by Student's *t* test. For one treatment, different lowercase letters indicate significant difference, whereas the same lowercase letters indicate no significant difference between two treatment times.

# 3.6. The Effects of FtNAC70 on Salt and Drought

We subsequently chose *FtNAC70* for further functional analysis, in order to investigate its function in the stress response, especially in drought and salt stresses. The pBI121-*FtNAC70* vector was constructed and transformed into *Arabidopsis*. As a result, we obtained three *FtNAC70* OE lines, whose expression level of *FtNAC70* were hundreds of times higher than that of WT *Arabidopsis* (Figure 6A).



Control Drought Control Drought Control Drought Control Drought

**Figure 6.** The effects of *FtNAC70* on salt and drought. (**A**) Expression level of *FtNAC70* in WT and three transgenic *Arabidopsis* lines (*FtNAC70*-OE2, *FtNAC70*-OE3, and *FtNAC70*-OE4). (**B**) Phenotype of the effect of *FtNAC70* on salt stress. (**C**) Root length statistics of *FtNAC70* on salt stress. (**D**) Phenotype of the effect of *FtNAC70* on drought stress. The error bars indicate the standard deviations of the replicates. Asterisks show the statistical significance evaluated by Student's *t* test.

The effect of *FtNAC70* on salt stress was investigated by treating the seedlings with 200 mM NaCl (Figure 6B,C). The result indicated that the root length of WT and three *FtNAC70*-OE lines had no significant difference under control conditions (1/2 MS medium). Salt stress inhibited the growth rate of seedlings, and the root lengths of WT and three *FtNAC70*-OE lines under salt stress were shorter than that under control conditions. However, the root length of three *FtNAC70*-OE lines were longer than that of WT, among which *FtNAC70*-OE2 and *FtNAC70*-OE3 reached the level of significance (p < 0.05). These results suggested that the root inhibition level of the *FtNAC70*-OE lines were less than that of WT after salt stress, which meant that *FtNAC70* could rescue the root inhibition caused by salt stress in *Arabidopsis*.

The effect of *FtNAC70* on drought stress was also investigated by treating the seedlings with 25% PEG (Figure 6D). No obvious difference appeared in the phenotype and root length of the seedlings after six days' drought treatment. However, the difference occurred one month later after transplanting the seedlings to soil (Figure 6D). At this time, WT *Arabidopsis* under control (normal growth) conditions were in the reproductive stage with numerous siliques, while WT under drought stress just begun bolting and flowering, indicating their growth was inhibited by drought stress. Three *FtNAC70*-OE lines under control conditions. Although the growth of *FtNAC70*-OE lines under drought stress were also retarded compared to *FtNAC70*-OE lines under control conditions, they were in the reproductive stage with siliques, which were faster than WT *Arabidopsis* under drought stress. This suggested that *FtNAC70* could rescue the growth retardation caused by drought stress in *Arabidopsis*.

#### 4. Discussion

It is well known that the plant NAC TF family plays essential roles in diverse biological processes, including plant growth, development, senescence, and responses to biotic and abiotic stress [1,2]. To date, the NAC members have been identified at a genome-wide scale in many species, including Tartary buckwheat [3–5,8,9]. It was reported that there were approximately 80 NAC TFs in Tartary buckwheat [8,9]. Except for the genome-wide identification, only a limited number (11) of the FtNAC genes were cloned and functionally analyzed in Tartary buckwheat. Among these, nine FtNAC genes, namely *FtNAC2-9* and *FtNAC17*, were related to stress [36,38]. One gene (*FtNAC16*) was related to pod cracking and salinity tolerance [35]. Furthermore, one gene (*FtNAC15*) was related to seed development and drought response [37]. Dozens of FtNAC genes need to be cloned and their functions need to be clarified in Tartary buckwheat. In this study, we reported seven FtNAC genes and named them according to previous research (Table 1) [8]. This would not only accelerate the research of NAC gene functional analyses in Tartary buckwheat, but also lay a foundation for future research.

Multiple-sequence alignments of seven FtNAC proteins indicated that they possessed the plant NAC TF-family-specific conserved NAM domain, which consists of the A, B, C, D, and E subdomains [4]. Based on the sequence logo, five subdomains in seven Ft-NACs in Tartary buckwheat were highly similar to the reported subdomains in other plant species (Figure 2A). Subdomain A (LPPGFRFHPTDxELV-xYL), C (EWYFFxxRDxKYPNG-R-NRAxGSGYWKATGxD), and D (GxKKxLVFY-GKxPKGxKTNWIMHEYRL) had higher levels of conservation than subdomain B (IxExDxYK-xPWxLP) and D (xxDWVLCRIxxK–) (Figure 2B), which conformed with the previous reports [4,38]. The subdomains of each FtNAC protein were also highly conserved to its evolutionarily closest NAC protein. The proteins that were closer to each other in the evolutionary tree often had similar biological functions. The seven FtNAC proteins were clustered to six subgroups, among which five subgroups belonged to the group I subclass whereas one subgroup belonged to the group II subclass [4] (Figure 2C). Every subgroup has its specific functions, which were determined on the basis of studies conducted on *Arabidopsis*, tomato (*Solanum lycopersicum* (L.)), and rice. Among seven FtNAC proteins, FtNAC11 and FtNAC28 belonged to the ATAF1

subgroup. Arabidopsis ATAF1, the representative gene in this subgroup, was involved in the detoxification and abiotic response [17,23]. FtNAC12 belonged to the NAP subgroup. Genes in this subgroup had a role in nitrate response and senescence in *Arabidopsis* [18,48]. FtNAC65 belonged to the SENU5 subgroup. SENU5, the representative gene in this subgroup, was identified as a senescence marker gene in tomato [14]. FtNAC26 belonged to the NAC2 subgroup. Double mutants disrupting NAC78 and NAC53 in the NAC2 subgroup of Arabidopsis showed that they were involved in plants surviving proteotoxic stress and protein homeostasis maintenance [15]. All representative genes of the above subgroups could be taken as stress-response genes, and FtNAC11, FtNAC28, FtNAC12, FtNAC65, and FtNAC26 might have similar functions to their homologous genes. It was determined in the study of their tissue-specific expression that FtNAC11, FtNAC28, FtNAC12, FtNAC65, and FtNAC26 were expressed in different tissues (Figure 4), implying that they could function in different tissues to respond to various stresses. FtNAC70 belonged to the NAM subgroup. Members of this subgroup were required for pattern formation in embryos and flowers and were also involved in pathogen-induced cell death and salt-stress resistance [24–26]. FtNAC78 belonged to the ONAC003 subgroup, which belonged to the group II subclass, whereas the other six proteins belonged to the group I subclass on the phylogenetic tree [4,49]. Meanwhile, on the basis of the multiple-sequence alignments of the NAM domain, it was determined that *FtNAC78* was most divergent to the other six proteins. And *FtNAC78* it was highly expressed in seeds and stems, as determined by its tissue-specific expression. These findings suggested that *FtNAC78* might have a distinct role in regulating the development process of Tartary buckwheat.

Based on the CARE analyses of seven FtNAC promoters, many CAREs related to drought, anaerobic, and low-temperature response were identified, such as MBS, ARE, TC-rich repeats, and LTR [41]. Meanwhile, CAREs related to hormone response, namely ABA, auxin, MeJA, GA, and SA, were also identified, such as ABRE, TGA-box, CGTCAmotifs, TGA-elements, AuxRR-core, P-box, and TCA-elements (Figure 3) [41]. It has been reported that the drought, high-salinity, and cold response in rice and Tartary buckwheat is divided into two pathways: the ABA-dependent pathway and the ABA-independent pathway [1,20,38,50]. In the qRT-PCR results, all seven FtNAC genes were responsive to drought and high-salinity stress (Figure 5). Among these, FtNAC11, FtNAC12, FtNAC28, and FtNAC70 could be induced, whereas FtNAC26, FtNAC65, and FtNAC78 could be inhibited by drought stress. FtNAC26, FtNAC12, FtNAC28, and FtNAC70 could be induced, whereas FtNAC11, FtNAC65, and FtNAC78 could be inhibited by high-salinity stress. Furthermore, five FtNAC genes (FtANC11, FtNAC12, FtNAC26, FtNAC28, and FtNAC78) were induced by cold (Figure 5). In the promoters of seven FtNAC genes, an ABA-responsive element, ABRE, was found in six FtNAC promoters, but not in FtNAC26 (Figure 3). Interestingly, the expression patterns of *FtNAC11*, *FtNAC28*, *FtNAC65*, and *FtNAC70* were not altered after ABA treatment. This suggested that at least two genes, FtNAC12 and *FtNAC78*, might respond to drought, high-salinity, and cold stress via the ABA-dependent pathway. For the remaining five genes, more evidence should be provided to determine whether they regulate drought, high-salinity, and cold stress in an ABA-dependent manner. Evidence that NAC TFs of Tartary buckwheat could regulate stress responses via both ABA-dependent and ABA-independent pathways was also provided previously [38]. JA is an important hormone that mediates both abiotic and biotic stress signaling [1]. In the qRT-PCR results, all seven FtNAC genes were responsive to MeJA, which suggested that they might be both abiotic and biotic regulators. Interestingly, a MeJA-responsive element, the CGTCA-motif, was found in the promoters of *FtNAC12*, *FtNAC26*, and *FtNAC28*. This implied that FtNAC11, FtNAC65, FtNAC70, and FtNAC78 were regulated by JA in an indirect manner.

Notably, *FtNAC70* was the most strongly induced gene in response to the abiotic stresses (PEG, NaCl, and cold) and hormone treatment (ABA, MeJA, and GA) among seven FtNAC genes, though it was highly expressed in seeds. This suggested that it might not only be a stress-response regulator but also have a distinct role in seed development in Tartary

buckwheat. The FtNAC70 protein was classified to the NAM subfamily. Some identified NAM subfamily genes were also reported to be both development related and stress responsive, such as Arabidopsis AtNAC4 [16,25,26,51–53]. Under normal conditions, AtNAC4 was predominantly expressed in seeds, especially seeds at the middle and late seed developmental stage [54], and was involved in seed germination and petal development [16,51]. Under high salinity, *AtNAC4* was a regulator of *AtSZF1* in auxin-dependent salt-stress responses [26]. Meanwhile, it was also a nitrate regulator, a dehydration stress memory gene, and a regulator involved in pathogen-induced cell death [25,52,53]. After transforming FtNAC70 into Arabidopsis, we found that FtNAC70-overexpressed Arabidopsis showed more resistance to salt and drought (Figure 6). The root inhibition of Arabidopsis seedlings caused by salt stress could be rescued by *FtNAC70*. Meanwhile, the growth retardation of Arabidopsis caused by drought stress could be rescued by FtNAC70. This is consistent with the NAM subfamily genes in *Arabidopsis* and confirmed that *FtNAC70* might be a positive regulator of salt and drought in Tartary buckwheat. In the future, we could screen the suitable Tartary buckwheat varieties that are tolerant to drought and salt based on the sequence polymorphism of FtNAC70, which would promote the molecular breeding of Tartary buckwheat. Besides, *FtNAC70* should be further studied to decipher its role in the developmental process of Tartary buckwheat.

## 5. Conclusions

This study identified and preliminarily analyzed seven NAC TFs in Tartary buckwheat. All of them contained the conserved NAM domain and were clustered to six subgroups based on the phylogenetic analyses. In addition, all seven FtNACs were tissue-specifically expressed and abiotic and hormone responsive, and in their promoter regions, CAREs related to biotic and abiotic response and hormone response were also possessed. At last, the function of *FtNAC70* was further studied and it was found that *FtNAC70*-overexpressed *Arabidopsis* showed more resistance to salt and drought. The results of this study would not only enrich the sequence resources of NAC TFs, but also lay a foundation for future research related to functional analyses of NAC genes in Tartary buckwheat. It would be useful for screening suitable Tartary buckwheat varieties that are tolerant to drought and salt through molecular breeding.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12040849/s1, Table S1: Primers used in this study.

**Author Contributions:** Conceptualization, T.S. and Q.C.; methodology, R.R. and Y.R.; software, J.H. and R.R.; validation, J.H., T.S. and Q.C.; data curation, B.T.; writing, J.H.; writing—review and editing, J.H. and T.S.; supervision, J.D.; project administration, J.H. and Q.C.; funding acquisition, J.H. and Q.C. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the National Natural Science Foundation of China (31760419, 32060508, 31860408), the Science and Technology Foundation of Guizhou Province (QianKeHeJiChu-ZK [2021] General 109), and The Earmarked Fund for China Agriculture Research System (CARS-07-A5).

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

#### References

- Nakashima, K.; Takasaki, H.; Mizoi, J.; Shinozaki, K.; Yamaguchi-Shinozaki, K. NAC transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta* 2012, 1819, 97–103. [CrossRef]
- Diao, P.; Chen, C.; Zhang, Y.; Meng, Q.; Lv, W.; Ma, N. The role of NAC transcription factor in plant cold response. *Plant Signal. Behav.* 2020, 15, 1785668. [CrossRef] [PubMed]
- Munir, N.; Yukun, C.; Xiaohui, C.; Nawaz, M.A.; Iftikhar, J.; Rizwan, H.M.; Xu, S.; Yuling, L.; Xuhan, X.; Zhongxiong, L. Genome-wide identification and comprehensive analyses of NAC transcription factor gene family and expression patterns during somatic embryogenesis in *Dimocarpus longan* Lour. *Plant Physiol. Biochem.* 2020, 157, 169–184. [CrossRef] [PubMed]
- 4. Ooka, H.; Satoh, K.; Doi, K.; Nagata, T.; Kikuchi, S. Comprehensive Analysis of NAC Family Genes in *Oryza sativa* and *Arabidopsis thaliana*. *DNA Res.* **2004**, *10*, 239–247. [CrossRef]

- Satheesh, V.; Jagannadham, P.T.; Chidambaranathan, P.; Jain, P.K.; Srinivasan, R. NAC transcription factor genes: Genome-wide identification, phylogenetic, motif and cis-regulatory element analysis in pigeonpea (*Cajanus cajan* (L.) Millsp.). *Mol. Biol. Rep.* 2014, 41, 7763–7773. [CrossRef] [PubMed]
- 6. Sun, H.; Hu, M.; Li, J.; Chen, L.; Li, M.; Zhang, S.; Zhang, X.; Yang, X. Comprehensive analysis of NAC transcription factors uncovers their roles during fiber development and stress response in cotton. *BMC Plant Biol.* **2018**, *18*, 150. [CrossRef]
- 7. Shiriga, K.; Sharma, R.; Kumar, K.; Yadav, S.K.; Hossain, F.; Thirunavukkarasu, N. Genome-wide identification and expression pattern of drought-responsive members of the NAC family in maize. *Meta Gene* **2014**, *2*, 407–417. [CrossRef] [PubMed]
- 8. Liu, M.; Ma, Z.; Sun, W.; Huang, L.; Wu, Q.; Tang, Z.; Bu, T.; Li, C.; Chen, H. Genome-wide analysis of the NAC transcription factor family in Tartary buckwheat (*Fagopyrum tataricum*). *BMC Genom.* **2019**, *20*, 113. [CrossRef]
- 9. Rong, Y.; Zhang, W.; Deng, J.; Shi, T.; Liang, C.; Wang, Y.; Zhang, X.; Li, H.; Meng, Z.; Huang, J. Bioinformatics analysis of NAC gene family in Tartary Buckwheat. *J. Hunan Agric. Univ. (Nat. Sci. China)* **2019**, *45*, 273–280.
- Mitsuda, N.; Iwase, A.; Yamamoto, H.; Yoshida, M.; Seki, M.; Shinozaki, K.; Ohme-Takagi, M. NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of *Arabidopsis*. *Plant Cell* 2007, *19*, 270–280. [CrossRef] [PubMed]
- Nuruzzaman, M.; Sharoni, A.M.; Satoh, K.; Karim, M.; Harikrishna, J.; Shimizu, T.; Sasaya, T.; Oomura, T.; Haque, M.; Kikuchi, S. NAC transcription factor family genes are differentially expressed in rice during infections with Rice dwarf virus, Rice blackstreaked dwarf virus, Rice grassy stunt virus, Rice ragged stunt virus, and Rice transitory yellowing virus. *Front. Plant Sci.* 2015, *6*, 676. [CrossRef] [PubMed]
- 12. Aida, M.; Ishida, T.; Fukaki, H.; Fujisawa, H.; Tasaka, M. Genes involved in organ separation in *Arabidopsis*: An analysis of the cup-shaped cotyledon mutant. *Plant Cell* **1997**, *9*, 841–857. [CrossRef] [PubMed]
- Hickman, R.; Hill, C.; Penfold, C.A.; Breeze, E.; Bowden, L.; Moore, J.D.; Zhang, P.; Jackson, A.; Cooke, E.; Bewicke-Copley, F.; et al. A local regulatory network around three NAC transcription factors in stress responses and senescence in *Arabidopsis* leaves. *Plant J.* 2013, 75, 26–39. [CrossRef]
- 14. John, I.; Hackett, R.; Cooper, W.; Drake, R.; Farrell, A.; Grierson, D. Cloning and characterization of tomato leaf senescence-related cDNAs. *Plant Mol. Biol.* **1997**, *33*, 641–651. [CrossRef] [PubMed]
- 15. Gladman, N.P.; Marshall, R.S.; Lee, K.H.; Vierstra, R.D. The Proteasome Stress Regulon Is Controlled by a Pair of NAC Transcription Factors in *Arabidopsis*. *Plant Cell* **2016**, *28*, 1279–1296. [CrossRef] [PubMed]
- 16. Baker, C.C.; Sieber, P.; Wellmer, F.; Meyerowitz, E.M. The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in *Arabidopsis. Curr. Biol.* **2005**, *15*, 303–315. [CrossRef] [PubMed]
- D'Alessandro, S.; Ksas, B.; Havaux, M. Decoding beta-Cyclocitral-Mediated Retrograde Signaling Reveals the Role of a Detoxification Response in Plant Tolerance to Photooxidative Stress. *Plant Cell* 2018, 30, 2495–2511. [CrossRef]
- Gonzalez-Bayon, R.; Shen, Y.; Groszmann, M.; Zhu, A.; Wang, A.; Allu, A.D.; Dennis, E.S.; Peacock, W.J.; Greaves, I.K. Senescence and Defense Pathways Contribute to Heterosis. *Plant Physiol.* 2019, 180, 240–252. [CrossRef]
- 19. Puranik, S.; Sahu, P.P.; Srivastava, P.S.; Prasad, M. NAC proteins: Regulation and role in stress tolerance. *Trends Plant Sci.* 2012, 17, 369–381. [CrossRef] [PubMed]
- Nuruzzaman, M.; Sharoni, A.M.; Kikuchi, S. Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. *Front. Microbiol.* 2013, 4, 248. [CrossRef] [PubMed]
- Takasaki, H.; Maruyama, K.; Kidokoro, S.; Ito, Y.; Fujita, Y.; Shinozaki, K.; Yamaguchi-Shinozaki, K.; Nakashima, K. The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. *Mol. Genet. Genom.* 2010, 284, 173–183. [CrossRef] [PubMed]
- 22. Ohnishi, T.; Sugahara, S.; Yamada, T.; Kikuchi, K.; Yoshiba, Y.; Hirano, H.Y.; Tsutsumi, N. OsNAC6, a member of the NAC gene family, is induced by various stresses in rice. *Genes Genet. Syst.* 2005, *80*, 135–139. [CrossRef] [PubMed]
- Wu, Y.; Deng, Z.; Lai, J.; Zhang, Y.; Yang, C.; Yin, B.; Zhao, Q.; Zhang, L.; Li, Y.; Yang, C.; et al. Dual function of *Arabidopsis* ATAF1 in abiotic and biotic stress responses. *Cell Res.* 2009, *19*, 1279–1290. [CrossRef] [PubMed]
- 24. Souer, E.; van Houwelingen, A.; Kloos, D.; Mol, J.; Koes, R. The no apical meristem gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. *Cell* **1996**, *85*, 159–170. [CrossRef]
- 25. Lee, M.H.; Jeon, H.S.; Kim, H.G.; Park, O.K. An *Arabidopsis* NAC transcription factor NAC4 promotes pathogen-induced cell death under negative regulation by microRNA164. *New Phytol.* **2017**, *214*, 343–360. [CrossRef] [PubMed]
- Garrido-Vargas, F.; Godoy, T.; Tejos, R.; O'Brien, J.A. Overexpression of the Auxin Receptor AFB3 in *Arabidopsis* Results in Salt Stress Resistance and the Modulation of NAC4 and SZF1. *Int. J. Mol. Sci.* 2020, 21, 9528. [CrossRef] [PubMed]
- 27. Zhang, L.; Li, X.; Ma, B.; Gao, Q.; Du, H.; Han, Y.; Li, Y.; Cao, Y.; Qi, M.; Zhu, Y.; et al. The Tartary Buckwheat Genome Provides Insights into Rutin Biosynthesis and Abiotic Stress Tolerance. *Mol. Plant* **2017**, *10*, 1224–1237. [CrossRef]
- 28. Huang, J.; Chen, Q.; Rong, Y.; Tang, B.; Zhu, L.; Ren, R.; Shi, T.; Chen, Q. Transcriptome analysis revealed gene regulatory network involved in PEG-induced drought stress in Tartary buckwheat (*Fagopyrum tararicum*). *PeerJ* **2021**, *9*, e11136. [CrossRef]
- Wu, Q.; Bai, X.; Zhao, W.; Xiang, D.; Wan, Y.; Yan, J.; Zou, L.; Zhao, G. De Novo Assembly and Analysis of Tartary Buckwheat (*Fagopyrum tataricum* Garetn.) Transcriptome Discloses Key Regulators Involved in Salt-Stress Response. *Genes* 2017, *8*, 255. [CrossRef]
- Wang, Y.; Campbell, C. Tartary buckwheat breeding (*Fagopyrum tataricum* L. Gaertn.) through hybridization with its Rice-Tartary type. *Euphytica* 2007, 156, 399–405. [CrossRef]

- Lv, B.; Wu, Q.; Wang, A.; Li, Q.; Dong, Q.; Yang, J.; Zhao, H.; Wang, X.; Chen, H.; Li, C. A WRKY transcription factor, FtWRKY46, from Tartary buckwheat improves salt tolerance in transgenic *Arabidopsis thaliana*. *Plant Physiol. Biochem.* 2020, 147, 43–53. [CrossRef] [PubMed]
- Li, Q.; Zhao, H.; Wang, X.; Kang, J.; Lv, B.; Dong, Q.; Li, C.; Chen, H.; Wu, Q. Tartary Buckwheat Transcription Factor FtbZIP5, Regulated by FtSnRK2.6, Can Improve Salt/Drought Resistance in Transgenic *Arabidopsis*. *Int. J. Mol. Sci.* 2020, 21, 1123. [CrossRef]
- Yao, P.F.; Li, C.L.; Zhao, X.R.; Li, M.F.; Zhao, H.X.; Guo, J.Y.; Cai, Y.; Chen, H.; Wu, Q. Overexpression of a Tartary Buckwheat Gene, FtbHLH3, Enhances Drought/Oxidative Stress Tolerance in Transgenic *Arabidopsis*. Front. Plant Sci. 2017, 8, 625. [CrossRef] [PubMed]
- 34. Yao, P.; Sun, Z.; Li, C.; Zhao, X.; Li, M.; Deng, R.; Huang, Y.; Zhao, H.; Chen, H.; Wu, Q. Overexpression of *Fagopyrum tataricum* FtbHLH2 enhances tolerance to cold stress in transgenic *Arabidopsis*. *Plant Physiol. Biochem.* **2018**, 125, 85–94. [CrossRef]
- Wang, J.; Ma, Z.; Tang, B.; Yu, H.; Tang, Z.; Bu, T.; Wu, Q.; Chen, H. Tartary Buckwheat (*Fagopyrum tataricum*) NAC Transcription Factors FtNAC16 Negatively Regulates of Pod Cracking and Salinity Tolerant in Arabidopsis. *Int. J. Mol. Sci.* 2021, 22, 3197. [CrossRef] [PubMed]
- Rong, Y.; Tang, B.; Li, P.; Zhang, J.; Chen, Q.; Zhu, L.; Deng, J.; Huang, J. Identification and Expression of NAC Transcription Factor FtNAC17 in Tartary Buckwheat. *Biotechnol. Bull.* 2021, 37, 174.
- 37. Huang, J.; Rong, Y.; Meng, Z.; Tang, B.; Zhang, J.; Xia, Z.; Chen, Q. Cloning and Expression of FtNAC15 Transcription Factor in *Fagopyrum tataricum. Acta Agric. Univ. Jiangxiensis* **2019**, *41*, 1183–1191.
- Deng, R.Y.; Zhao, H.X.; Xiao, Y.H.; Huang, Y.J.; Yao, P.F.; Lei, Y.L.; Li, C.L.; Chen, H.; Wu, Q. Cloning, Characterization, and Expression Analysis of Eight Stress-Related NAC Genes in Tartary Buckwheat. Crop Sci. 2019, 59, 266–279. [CrossRef]
- 39. Rychlik, W. OLIGO 7 primer analysis software. *Methods Mol. Biol.* 2007, 402, 35–60. [CrossRef]
- 40. Huang, J.; Deng, J.; Shi, T.; Chen, Q.; Liang, C.; Meng, Z.; Zhu, L.; Wang, Y.; Zhao, F.; Yu, S.; et al. Global transcriptome analysis and identification of genes involved in nutrients accumulation during seed development of rice tartary buckwheat (*Fagopyrum tararicum*). *Sci. Rep.* **2017**, *7*, 11792. [CrossRef] [PubMed]
- 41. Lescot, M.; Dehais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van de Peer, Y.; Rouze, P.; Rombauts, S. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327. [CrossRef] [PubMed]
- 42. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* 2020, *13*, 1194–1202. [CrossRef] [PubMed]
- 43. Crooks, G.E.; Hon, G.; Chandonia, J.M.; Brenner, S.E. WebLogo: A sequence logo generator. *Genome Res.* 2004, 14, 1188–1190. [CrossRef] [PubMed]
- 44. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
- 45. Brauc, S.; De Vooght, E.; Claeys, M.; Geuns, J.M.; Hofte, M.; Angenon, G. Overexpression of arginase in *Arabidopsis thaliana* influences defence responses against Botrytis cinerea. *Plant Biol.* **2012**, *14* (Suppl. 1), 39–45. [CrossRef]
- Clough, S.J.; Bent, A.F. Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 1998, 16, 735–743. [CrossRef] [PubMed]
- 47. Verslues, P.E.; Agarwal, M.; Katiyar-Agarwal, S.; Zhu, J.; Zhu, J.K. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J.* **2006**, *45*, 523–539. [CrossRef] [PubMed]
- Alvarez, J.M.; Moyano, T.C.; Zhang, T.; Gras, D.E.; Herrera, F.J.; Araus, V.; O'Brien, J.A.; Carrillo, L.; Medina, J.; Vicente-Carbajosa, J.; et al. Local Changes in Chromatin Accessibility and Transcriptional Networks Underlying the Nitrate Response in *Arabidopsis* Roots. *Mol. Plant* 2019, *12*, 1545–1560. [CrossRef]
- 49. Kan, C.; Zhang, Y.; Wang, H.L.; Shen, Y.; Xia, X.; Guo, H.; Li, Z. Transcription Factor NAC075 Delays Leaf Senescence by Deterring Reactive Oxygen Species Accumulation in *Arabidopsis*. *Front. Plant Sci.* **2021**, *12*, 634040. [CrossRef]
- Meng, X.; Li, G.; Yu, J.; Cai, J.; Dong, T.; Sun, J.; Xu, T.; Li, Z.; Pan, S.; Ma, D. Isolation, Expression Analysis, and Function Evaluation of 12 Novel Stress-Responsive Genes of NAC Transcription Factors in Sweetpotato. *Crop Sci.* 2018, 58, 1328–1341. [CrossRef]
- Dekkers, B.J.; Pearce, S.; van Bolderen-Veldkamp, R.P.; Marshall, A.; Widera, P.; Gilbert, J.; Drost, H.G.; Bassel, G.W.; Muller, K.; King, J.R.; et al. Transcriptional dynamics of two seed compartments with opposing roles in *Arabidopsis* seed germination. *Plant Physiol.* 2013, 163, 205–215. [CrossRef] [PubMed]
- 52. Vidal, E.A.; Alvarez, J.M.; Gutierrez, R.A. Nitrate regulation of AFB3 and NAC4 gene expression in *Arabidopsis* roots depends on NRT1.1 nitrate transport function. *Plant Signal. Behav.* **2014**, *9*, e28501. [CrossRef]
- 53. Ding, Y.; Liu, N.; Virlouvet, L.; Riethoven, J.J.; Fromm, M.; Avramova, Z. Four distinct types of dehydration stress memory genes in *Arabidopsis thaliana*. *BMC Plant Biol.* **2013**, *13*, 229. [CrossRef] [PubMed]
- 54. Winter, D.; Vinegar, B.; Nahal, H.; Ammar, R.; Wilson, G.V.; Provart, N.J. An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLoS ONE* **2007**, *2*, e718. [CrossRef] [PubMed]