Expression analysis of 19 bZIP genes in Tartary buckwheat and its response to abscisic acid (ABA)

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Abstract

Tartary buckwheat is a kind of plant which can be used as medicine as well as edible. ABA signaling is important for plants to respond to drought. In this study, the germination, root length, stoma, and anthocyanin accumulation of Tartary buckwheat were all significantly affected by ABA. In ABA signaling, the bZIP gene family is a critical member of the ABA signaling pathway in plants, which plays an important role in plants response to drought. Through the analysis of the origin relationship between the bZIP family and related species, found that 19 bZIP genes in Tartary buckwheat were relatively conservative, which laid a foundation for the further study of the bZIP family. qRT-PCR showed that most of the group members were induced by ABA stress, including different times or concentration of ABA. In addition, the expression patterns of FtbZIPs in plant organs were different, indicating that they may have different functions in Tartary buckwheat responding to drought. In a word, this study will be helpful to further analyze and provide clues to improve the genetic breeding of Tartary buckwheat for drought resistance and its economic value.

Introduction

Common buckwheat (*Fagopyrum esculentum*) and Tartary buckwheat (*Fagopyrum tataricum*) are two cultivars of *Fagopyrum* genus of Polygonaceae [1]. The former is widely cultivated in Asia, Europe, Americas, while Tartary buckwheat is most grown in Asia (eg. China, Bhutan, Nepal, and India) with a small quantity of production in the Islek region of Europe [2]. Compared with common buckwheat, Tartary buckwheat has a higher yield and better tolerance towards harsh conditions under which other major crops may fail [2, 3]. Moreover, Tartary buckwheat seeds contains more rutin (0.8–1.7% DW) than common buckwheat seeds (0.01% DW) [4]. The taste of Tartary buckwheat grains is much bitterer than that of common buckwheat due to the much higher concentrations of flavonoids, especially, Tartary buckwheat seeds contained traces of quercitrin and quercetin, which were not found in common buckwheat seeds [2, 4]. By virtue of gluten free nature of protein, balanced amino acid profile and health promoting bioactive flavonoids, Tartary buckwheat is treated as a golden crop of future and is gaining research focus [2] [3].

As sessile organisms, plants face fluctuating environments that are often unsuitable for growth and development, such as drought, high salinity, low temperature, and so on. Among these conditions, drought is considered the most serious environmental factor affecting the geographical distribution of plants, limiting plant productivity in agriculture and threatening food security [5] [6]. Drought causes osmotic pressure and oxidative stress in plants and damages cellular components including membrane lipids, proteins, and nucleic acids. Interestingly, plants have developed complex and diverse mechanisms to cope with such situations. One important mechanism is the accumulation of abscisic acid (ABA), which in turn activates many adaptive responses in plants [6]. Rapidly generates in response to many kinds of abiotic stresses, ABA plays a crucial role in seed dormancy and germination, root growth, leaf senescence, and in adaptive response to environmental stresses [6] [7, 8].

During the past decades, many ABA signaling components have been identified in *Arabidopsis thaliana*, such as ABA receptors, phosphatases, kinases, transcription factors (TFs), and ABA-responsive genes [7]. Upon perception of ABA by specialized receptors, pyrabactin resistance 1 (PYR1)/PYR1-like (PYL)/regulatory components of ABA receptors (RCAR), the signal is transduced via various groups of Ser/Thr kinases, which phosphorylate the basic leucine zipper (bZIP) TFs. Following such post-translational modification of bZIPs, they are activated so that they bind to specific *cis*-acting sequences called abscisic-acid-responsive elements (ABREs) or GC-rich coupling elements (CE), thereby influencing the expression of their target downstream genes [7, 9, 10]. Moreover, ABRE-binding factors (ABFs), a kind of bZIPs, directly bind to the promoters of group A protein phosphatase 2Cs (PP2C) genes, and mediate rapid induction of their expression on exogenous ABA treatments, thus playing a role in the negative feedback regulation of ABA signaling [7].

Because of their beneficial health effects, flavonoids metabolic pathways and the medicine use of Tartary buckwheat and its products were intensively studied [2, 3, 11]. There is still a lack of basic information on ABA signaling pathway in Tartary buckwheat, thus hindering the further development of Tartary buckwheat as a sustainable crop. In this study, the effect of ABA on Tartary buckwheat growth and stoma movement were investigated, and tissue-specific expression and ABA-induced expression of 19 bZIP A group genes of Tartary buckwheat (abbreviated as FtbZIP) were analyzed. These results may provide a clue for further uncovering how Tartary buckwheat response to abiotic stress.

Results

Inhibitive effect of ABA on seed germination and root elongation of Tartary buckwheat

There were 50 seeds of Tartary buckwheat by different ABA treatment to observe germination rates (Fig. 1A). The seed germination and the root elongation of Tartary buckwheat were inhibited by ABA significantly. In which, the germination rates of seeds and the root elongation of Tartary buckwheat by 0.9 and 1.5 μM ABA were markedly reduced compared with no ABA, and the tendency lasted until 120 h (fig.1D). The germination rate in the normal environment usually reaches 99%, while it only reaches 80% percent, 78% percent, and 62% percent respectively with ABA treatment of 0.3, 0.9 and 1.5 μM at 120 h (fig.1A). The hypocotyl of Tartary buckwheat by ABA treatment was obviously shorter than that of no ABA treatment. The average length of 30 seedling roots of Tartary buckwheat grown in 0, 0.3, 0.9, and 1.5 μM ABA environment was 7.46, 5.56, 3.18, and 2.68cm respectively (fig.1D). The lower part of the hypocotyl displays visible gradient red by ABA treatment, especially at 0.9 μM ABA, suggesting that ABA may cause anthocyanin accumulation in hypocotyl (fig.1C).

The stomatal aperture of Tartary buckwheat in response to ABA

The leaves of Tartary buckwheat were completely immersed in the stomatal opening solution to ensure that all pores are fully opened. The length and width of 90 stomas were measured in each group, and the length/width ratios were sorted by the following ranges: 1 $\times$ 2, 2 $\times$ 5, 5 $\times$ 8, 8 $\times$ 14, 14 $\times$ 17 and $\geq$ 17. The length/width ratios of most stomas in control group were mostly between 1-2. Following the increasing of

ABA concentration, the numbers of the stomata with larger ranges of length/width ratio were added up, for instance 30 μ M ABA with largest in the ranges of 5-8 and 8-14, while 50 μ M ABA with the majority in the range of 17. When the concentration of ABA reached 70 μ M, the stomata had been completely closed (fig.2 B).

Homology analysis about the genes from group A of FtbZIP family with eight other species

The phylogenetic tree was constructed with group A FtbZIPs and ABF from eight other species (Supplementary fig.1). Group A of FtbZIP family were divided into three subgroups according their positional relationship on the phylogenetic tree (fig.3 A). Among these genes, FtbZIP3, FtbZIP85, FtbZIP75, FtbZIP40, FtbZIP45 had a relatively close evolutionary relationship, FtbZIP5, FtbZIP12, FtbZIP83, FtbZIP28 genes were close on the evolutionary tree, so were FtbZIP2 and FtbZIP39. FtbZIP2 and FtbZIP39 were closed to GBF4 from other species on the subtree (fig.3 B). The FtbZIP78 that might have high homology to the ABI5 compared with other species was focused (fig. 3 C and D). The predicted structures of the FtbZIP78 and StABI5 had a high similarity.

Tissue-specific expression of FtbZIP A group in Tartary buckwheat

The expressions of 19 FtbZIP genes was examined in five organs including roots, stems, leaves, flowers, and fruits by qRT-PCR (fig.4 A). Not all genes are expressed in all tissues, some genes are expressed lowly, even in some tissues that they are almost undetectable. For example, the expression of FtbZIP68 in fruit was very low, FtbZIP45 expressions in flower and fruit were low, and FtbZIP1 expression in root, stem, and leaves was very few. However, some genes were highly expressed in a particular organ. There are four genes (bZIP2, bZIP45, bZIP69, bZIP67) show the highest expression level in the Tartary buckwheat roots, the expression of bZIP67 is particularly high. The highest expression level of eleven genes (bZIP3, bZIP5, bZIP12, bZIP21, bZIP28, bZIP40, bZIP45, bZIP68, bZIP69, bZIP75, bZIP83, bZIP85) were found in stems. And there were nine genes (bZIP1, bZIP49, bZIP69, bZIP75, bZIP78, bZIP39, bZIP74, bZIP64, bZIP75) expressed highly in flowers, six genes (bZIP1, bZIP5, bZIP39, bZIP74, bZIP49, bZIP64) expressed highly in fruit. The expression level of FtbZIP5 was the highest in flowers.

The expression of some genes in plant organs is significantly correlated, that they may play a role in a collaborative approach. For example, As shown in the heat map, there was a high correlation between bZIP74, bZIP1, and bZIP64, which were highly expressed in fruits. The correlation between bZIP2 and bZIP67, which are highly expressed in the root. The correlation coefficients of bZIP3, bZIP45, and bZIP12 were very high, and they were all relatively expressed in stems much higher than other tissues. bZIP39, bZIP64, and bZIP74 were high expression levels in stems and flowers. However, five pairs FtbZIP genes (FtbZIP1 and FtbZIP68; FtbZIP2 and FtbZIP83; FtbZIP12 and FtbZIP74; FtbZIP39 and FtbZIP45; FtbZIP67 and FtbZIP83) are significantly negative correlated (fig.4B).

Expression variance of 19 FtbZIPs under ABA treatment

Most of FtbZIP group A expression at different concentrations ABA treatment for 3.5 h were changed (fig.5 A), however, four genes (bZIP5, bZIP21, bZIP78, bZIP83) whose expression at different concentrations of ABA treatment almost were not changed (fig.S2). Nevertheless, they were changed at different timescales of 50 μ M ABA treatment (fig. S3A). Only FtbZIP39 showed no significant change at current time with concentration gradients of ABA (fig.5A) (fig. S3 A). The expression levels of four genes Ftbzip5, Ftbzip21, Ftbzip78 and Ftbzip83 increased significantly ($P<0.001$) among different timeframe and different ABA concentrations as well. Ftbzip69 and Ftbzip75 are down-regulated at significantly different levels at above 30 μ M ABA ($P<0.001$) (fig.5A) (fig. S3 A).

Discussion

ABA is an important plant stress hormone that accumulates at osmotic stresses such as drought and high salinity [12]. In *Arabidopsis thaliana*, ABI5 is mainly expressed in vegetative tissues and seed, and is necessary for the regulation of certain ABA genes in these organs. FtABI5 appears to be regulated by ABA, most other known ABI genes as well as possibly be regulated by ABA and itself [13]. In addition, ABI5 enhanced sensitivity to ABA during germination, seedling establishment, and subsequent vegetative growth. ABA highly regulates ABI5 protein accumulation, phosphorylation, stability, and activity during germination and early seedling growth [14]. It shows that AtABI5 plays a very important role in the growth and development of *Arabidopsis thaliana* and its tolerance to stress. The evolutionary tree shows that the FtbZIP78 gene has a close evolutionary relationship with the AtABI5 gene of eight other species (*B. vulgaris*, *S. lycopersicum*, *S. tuberosum*, *V. vinifera*, *P. trichocarpa*, *A. thaliana*, *T. cacao*, *G. max*). We speculate that FtbZIP78 may also have similar or the same function with AtABI5. FtbZIP78 is expressed in major vegetative organs, such as flowers and fruits (Fig. 4A), the same tissue expression pattern as AtABI5. It also shows that FtbZIP78 was significantly up-regulated under ABA induction (Fig. 5A, S3 A). We speculated that FtbZIP78 plays an important role in the growth and development of Tartary buckwheat and improves its resistance to drought stress.

It was showed that the correlation of FtbZIP5, FtbZIP12, FtbZIP28, and FtbZIP83 was relatively high, and they were significantly up-regulated by ABA treatment (Fig. 5). It was reported that indicated that, Tartary buckwheat transcription factor FtbZIP83 improves the drought/salt tolerance of *Arabidopsis* via an ABA-mediated pathway [15]. Tartary Buckwheat transcription factor FtbZIP5, regulated by FtSnRK2.6, can improve salt/drought resistance in transgenic *Arabidopsis* [16]. It shows that both FtbZIP5 and FtbZIP83 can induce Tartary buckwheat responses to ABA through extremely complex ABA signaling, thus helping plants to improve salt tolerance and drought tolerance. It was showed that the expression levels of these four genes in roots and leaves are higher than those in flowers and fruits. Therefore, we speculated that FtbZIP12 and FtbZIP28 may have the same function on resisting adversity stress. (Fig. 4)

Clustering analysis showed that FtbZIP2 and FtbZIP39 are highly homologous, and the homology is up to 100% (Fig. 3). In Fig. 5A, the expression of FtbZIP2 and FtbZIP39 are both not affected by ABA treatment. They are negative correlated (Fig. 5B, S2). We found that AtGBF4, FtbZIP2, and FtbZIP39 are highly homologous in many species [17]. GBF was a bZIP transcription factor. Previous studies administrated

that 97% of AtGBF4 proteins are located in the nucleus and it participated in the monochromatic light response in *Arabidopsis thaliana* [18]. we speculation that FtbZIP2 and FtbZIP39 might also be nuclear localization genes. They may be involved in optical signaling pathways.

The expression of FtbZIP1 is unique, which expressed low in roots, almost no expression level in stems and leaves, but it has very high expression level in reproductive organs such as fruits and flowers. We speculated that bZIP1 might be related to the yield and quality of Tartary buckwheat fruit.

The study of FtbZIPs genes by ABA treatment will be helpful to clarify the role of FtbZIP in plant [19], It also provides important insights for studying Tartary buckwheat to deal with abiotic stress [20, 21], to screen more high-quality Tartary buckwheat varieties [22].

Materials And Methods

Preparation of plant samples

The Tartary buckwheat used in this study was XIQIAO 1. In the appropriate development stage of Tartary buckwheat, we obtained roots, stems, and leaves from 7-day-old seeding, flowers and fruits were got from green fruit stage seedlings.

All organ samples were taken from three plants with the same growth condition. To facilitate use in further experiments, the collected plant samples were stored in -80°C refrigerator after rapid treatment with liquid nitrogen. For non-sterile cultures, all plants were grown in sterilized soil, a mixture of vermiculite and soil (1:3, v/v) on the balcony of School of Life Science, Sichuan University [23].

About inhibitive effect of ABA on seed and root growth of Tartary buckwheat

To remove the peel easily, seeds were soaked in water for 20 minutes in advance. Disinfect the seeds using 75% ethanol for 30 s. Replace the ethanol with 5% sodium hypochlorite and disinfect for 15 min, washed with sterilized and distilled water. After that, seeds were planted on Murashige and Skoog (MS) medium supplemented with concentration gradient as 0, 0.3, 0.9 and 1.5 μ M ABA [5].

The MS medium PH is 6.0, containing 3% (w/v) sucrose and 0.65% (w/v) agar. We recorded data on seed germination every day.

To measure the root length, seeds were placed on MS culture dish for 48 h, then transplanted germinating seeds on the MS supplemented with 0, 0.3, 0.9, and 1.5 μ M ABA, 3% sugar, and 0.55% agar. After 20 days, the root length was measured and the data were processed (30 Tatary buckwheat seeds were taken as the sample size), to calculate the average length to collect images.

About effect of ABA on leaf stomatal aperture of Tatary buckwheat

The plants were left in the dark for 12 hours to ensure that all stomata are closed. The top leaves of the 20-day-old plants were removed. Then they were immersed in the stomatal opening solution in a climate incubator for 3 h. The stomatal opening solution was made of 10mM MES (pH value 6.15) 10ml, 50mM KCl 5mL, 10 μ M CaCl₂ 10 μ M, and 85 ml deionized water. ABA were added with gradient concentration as 0, 10, 15, 20, 30, 50, and 70 μ M, respectively. After 3 hours, the subepidermal stmata of the leaves were photographed with a microscope (DMI6000B, Leica, Germany). Three fields were randomly selected from each sample, and 30 stomata were randomly selected from each field to measure its length and width with Image J, and the number of cells in long and short sides were counted.

Homology analysis by constructing phylogenetic trees and predicting protein structures

A phylogenetic tree of Tartary buckwheat has been constructed and *Arabidopsis thaliana* to identify the phylogenetic relationship between the two species.

The genes of the FtbZIP family were classified into 11 groups after constructing a phylogenetic tree with *A. thaliana* [24]. In group A, most genes of *Athaliana* were ABA-responsive element (ABRE) binding factors. According to the phylogenetic relationship of two species, we assumed that the genes in group A of *F. tataricum* were also related to ABA response. The genes sequences of group A FtbZIP family were obtained from the TBGP database (<http://www.mbkbase.org/Pinku1/>) [22].

To study the probable function of these genes during the growth of *F. tataricum*, we constructed the phylogenetic tree of these genes and other genes described as ABRE binding factors from eight other species closely related to *F. tataricum*. Eight species are monocotyledons, which had a relatively close evolutionary relationship with *F. tataricum* (*B. vulgaris*, *S. lycopersicum*, *S. tuberosum*, *V. vinifera*, *P. trichocarpa*, *A. thaliana*, *T.cacao*, *G. max*.) [22]. Then we got the gene NCBI-IDs of those genes of the eight species by searching the Kegg database (<https://www.kegg.jp/>) [25], of which KO number was K14432 [26]. Next, we retrieved and consulted the information of those genes in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) [27], and finally recorded them in supplementary materials (supplementary data 1). We also downloaded the protein sequences of those genes from the NCBI database.

As for the paralogous analysis, at first, the genes in group A of *F. tataricum* were aligned by using MUSCLE [28] with default parameters. Then the phylogenetic tree was constructed by using RAXML [29] with custom parameters (-x 12345 -p 12345 -# 1000 -m PROTGAMMAJTTF -f a -k -s input -n run_id -T 4). We selected JTTF model by running a script provided by the RAXML author and set the bootstrap value as 1000. As for the ortholog analysis of the genes in group A of *F. tataricum* with eight other plant species, we used the same pipeline as described above. Finally, the protein structures were predicted by using phyre2 [30] with ab initio modeling methods.

Analysis of FtbZIP in group A of different tissues expression by qRT-PCR

Expression analysis of FtbZIP in group A in different tissues by qRT-PCR. The expression of FtbZIP genes in five organs (fruit, flower, leaf, stem, root) was analyzed by qRT-PCR. we performed three biological replicates. We used the Primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/>) to obtain qRT-PCR primers of FtbZIP genes (Table S1) [31]. In qRT-PCR experiments, the internal reference gene was Fth3 gene. The cDNA was synthesized by The Prime Script RT Reagent Kit (Takara) using 1 g RNA. The cDNA was synthesized with SYBR Premix Ex Taq II (TAKARA). Finally, we analyzed the results and obtained acquired mRNA expression information by the $2^{-(\Delta\Delta Ct)}$ [32]. qRT-PCR was performed using the SYBR Premix Ex Taq[®] kit (Takara) and an Applied Biosystems 7500 real-time PCR system. Fth3 was used as the internal reference primer for QRT-PCR, and the primer sequences were shown in Table 1.

Test of quatiy variance of FtbZIP with different ABA treatments

The expressions of FtbZIP in A group in Tartary buckwheat treated by concentrations ABA were analyzed. The top leaves of 20-day-old Tartary buckwheat were treated by 0, 15, 30, 50 and 70 μ M ABA for 3h. After drying on the surface with absorbent paper, RNA was extracted. The gene expression was analyzed by qrt-PCR method. When analyzing the expression of FtbZIP in A group in Tartary buckwheat treated by ABA at different times, 7-day-old Tartary buckwheat seedlings were treated by 50 μ M ABA for 0, 0.5, 2, 4, 8, 16, 24 h respectively, and then RNA was extracted for expression analysis using qrt-pcr method.

Statistical analysis

Anova of the above data was conducted by SPSS 24 statistical program. We also used the SPSS 24 to analyze the Pearson's correlation coefficient, both for the expression quantity of FtbZIP genes and the DAP. Then we used Origin Pro 2018B (OriginLab Corporation, Northampton, Massachusetts, USA) to draw the diagrams. The Pearson method was used to to analyze the quantity correlation of the genes. In addition, the heatmap was drawn with matplotlib packages [33].

Declarations

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Author contributions:

Shuya Xiao performed the experiment and wrote the manuscript;

Yaodong Liu provided the analysis of bioinformatics

AnhuWang provided the seed of Tatary buckwheat

Yu Liu contributed significantly to data analysis;

Jianmei Wang contributed to the conception of the study;

Others assisted with the experiment

All authors consent to publish this article

The research does not raise ethical questions

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Figures

Figure 1

The effect of ABA on seed germination and root elongation of Tartary Buckwheat. (A) and (B) The germination of Tartary buckwheat seeds by ABA treatment. Tartary Buckwheat seeds were distilled and planted MS supplemented with 0, 0.3, 0.6, 0.9, and 1.5 μM ABA. The germinated seeds of each group were counted every 12 hours. The values are the average of three individual biological replications. Error bars represent $\pm$ SD ($n=50$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, t-test). (C) and (D) Phenotype and root length of Tartary buckwheat. The germinated seeds were cultured vertically 20 days on MS with different concentrations of ABA and the root length was measured. Error bars represent $\pm$ SD ($n=20$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, t-test).

A

B

Figure 2

The stomatal aperture of Tartary buckwheat in response to ABA. (A) The representative stoma which was treated by 0, 10, 15, 30, 50, 70 μM ABA. Leaves of 3-week-old plants were precultured with stomatal opening solution for 3 h, and then incubated with indicated concentration of ABA for 3.5 h. Bars represent 1mm. (B) The length and width of 90 stomas were measured using Image J in each group, and then length/width ratio was calculated.

Figure 3

Paralogous analysis of FtbZIP group A and Ortholog analysis of the genes in the group. (A) Phylogenetic tree of 19 genes of FtbZIPs group A in Tatar buckwheat. (B) The subtree of the genes symbolled by G-box binding factor 4 (GBF) from nine plant species (*B. vulgaris*, *S. lycopersicum*, *S. tuberosum*, *V. vinifera*, *P. trichocarpa*, *A. thaliana*, *T. cacao*, *G. max*). (C) The subtree which revealed the phylogenetic relationship of the genes symbolled by the protein ABSCISIC ACID-INSENSITIVE 5 (ABI 5) from nine plant species. (D)

The structure of AtABI5, SIABI5, StABI5, and FtbZIP78 predicted by Phyre2 with ab initio modelling methods.

Figure 4

Tissue specific expression of group A of bZIPs in Tartary buckwheat (A) The expression of bZIPs genes in group A of Tartary buckwheat in different tissues. Expression patterns of the genes in roots, stems, leaves, flowers, and fruits were examined by qRT-PCR. Error bars were obtained from three replications.

Lowercase letters indicate significant differences ($\alpha = 0.05$, LSD). (B) The correlation of group A of bZIPs in Tatar buckwheat. A positive number indicates a positive correlation; a negative number indicates a negative correlation. The red numbers indicate a significant correlation at the 0.05 level.

A

B

Figure 5

Transcriptional expression of group A of bZIPs in Tatar buckwheat in response to different ABA concentration. (A) Expression levels of group A in bZIPs family were assayed by qRT-PCR . Total RNA was

obtained from 20-d-old seedlings leaf which were treated by 0, 15, 30, 50, 70 μ M ABA for 3.5h. Data represent means $\pm$ SD (n = 3) from three independent replicates. Asterisks indicate significant differences (*P < 0.05; **P < 0.01, ***P < 0.001) according to Student's t-test compared with the corresponding no ABA treatment plants values. (B) A positive number indicates a positive correlation; a negative number indicates a negative correlation. The red numbers indicate a significant correlation at the 0.05 level.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryData1.pdf](#)
- [SupplementaryTable1.xlsx](#)
- [supplementaryfigureR.pdf](#)