Cloning, expression, and in silico analysis of a novel annexin gene \textit{FtANX1} from Tartary buckwheat (\textit{Fagopyrum tataricum} (L.) Gaertn.)

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\textbf{Abstract:} Annexins are generally thought to play an essential role in the regulation of plant growth, development, and stress responses. Here we describe an annexin gene from Tartary buckwheat (\textit{Fagopyrum tataricum} (L.) Gaertn.), designated as \textit{FtANX1}. The full-length coding sequence of \textit{FtANX1} is 942 bp in size, encoding a polypeptide of 313 amino acids with a theoretical molecular mass of 36.0 kDa and pI 5.94. In silico analysis of the sequence revealed that \textit{FtANX1} contains four repeats, with a type-II \(\text{Ca}^{2+}\)-binding site in repeat I. Expression pattern analysis provided evidence that \textit{FANX1} was predominantly expressed in immature achenes, while its level was lowest in leaves. In addition, \textit{FtANX1} was found to be associated with stress responses in Tartary buckwheat, such as drought, cold, UV-B radiation, jasmonic acid, and heavy metals (Cu, Pb, Zn, and Cd). Several stress-related regulatory elements (ABRE, W-box, GATA-box, and \text{CuRE} elements) were found by in silico analysis in the \textit{FtANX1} promoter region. Our results showed that \textit{FtANX1}, a novel annexin gene, may play an important role in the growth and stress tolerance of Tartary buckwheat.

\textbf{Key words:} Annexin, Tartary buckwheat, \textit{FtANX1}, stress responses

\section{1. Introduction}
Tartary buckwheat (\textit{Fagopyrum tataricum} (L.) Gaertn.) is an important human food crop in many areas of the world, especially in southwestern China. In addition to its high-quality proteins, buckwheat is also rich in many rare components that have a healing effect in chronic diseases, such as flavonoids and phytosterols (Zhang et al., 2012). Most importantly, this functional crop can be cultivated and genetically adapted to marginal lands characterized by harsh environmental stress.

\(\text{Ca}^{2+}\)-binding proteins are known to serve as calcium signal transducers in the cell, and are typically involved in regulating stress responses in plants (Mahajan and Tuteja, 2005). Annexin is one of the \(\text{Ca}^{2+}\)-binding proteins responsible for a variety of cellular and physiological responses (Mortimer et al., 2008). Plant annexin was first described in 1989 as a \(\text{Ca}^{2+}\)-dependent phospholipid-binding protein in higher plant cells (Boustead et al., 1989). The general structure of plant annexin is evolutionarily conserved, consisting of a variable N-terminal region and a highly conserved C-terminal core domain of a four-fold repeat (I–IV) of a 70 amino acid sequence. It has been shown that the expression of annexin genes can be found in roots, stems, leaves, pulvinus, flowers, and seeds (De Carvalho-Niebel et al., 2002), and they are tissue- and time-dependently differentially transcribed (Cantero et al., 2006).

Linked to plant growth and development, annexin expression levels also change in response to diverse environmental stress conditions (Mortimer et al., 2008). The transcription level of \textit{Cynanchum komarovii} annexin \textit{CkANN} was found to increase significantly following stress by drought and \(\text{H}_2\text{O}_2\) (Zhang et al., 2011). The maize annexin genes were reported to be induced by heavy metals (Ni, Zn, and Cd) and jasmonic acid (JA) (Zhou et al., 2013). The expression levels of \textit{Arabidopsis} annexin genes \textit{AnxAt4} and \textit{AnxAt1} were also activated by abscisic acid (ABA), drought, and salt stress (Huh et al., 2010). Despite the important antiadversity role of annexin in other plants, its existence and physiological function in Tartary buckwheat remains unknown.

Here we isolated an annexin gene, \textit{FtANX1}, from Tartary buckwheat. We predicted its structure and location by homology modeling analysis and phylogenetic analysis. Further, we explored the \textit{FtANX1} responses to abiotic stresses, such as drought, cold shock, UV-B irradiation, JA, and heavy metal treatments, and we found that \textit{FtANX1} expression levels in Tartary buckwheat seedlings were...
significant effects on the downstream "promoter" region of this gene and identified candidates for the cis-regulatory elements of FtANX1 expression by comparison with databases. To the best of our knowledge, this is the first study that describes an annexin from Tartary buckwheat.

2. Materials and methods

2.1. Plant materials and stress treatments

*Fagopyrum tataricum* (L.) Gaertn. seed "Chuan Qiao 3" was a gift from the Institute of Agricultural Sciences, Liangshan Prefecture, and was grown in a normal flowerpot (27.0 cm H × 21.0 cm W × 29.0 cm D) containing 25 g of perlite at the farm of Sichuan Agricultural University. Eighteen 3-week-old seedlings grown in each container were used to investigate the effects of environmental stress on gene expression. Irrigation duration was at 1-day intervals with 100 mL of solutions throughout the experimental time. For the JA treatment, seedlings were sprayed with 50 µM JA (Sigma) dissolved in DMSO, whereas the control was mock-treated with DMSO. For the cold treatment, seedlings were grown in controlled environment chambers at 4 °C, and the control plants were grown at room temperature (~20 °C). For the UV-B radiation stress, plants were treated under UV light using a UV-B lamp, which has a maximum radiation peak at 302 nm. The intensity of UV-B light on the sample surface was 1.26 µW/cm² and the total energy supply was 0.34 J/cm², whereas the control was exposed to normal daylight. The seedlings were irrigated with 100 mM NaCl for salt treatments. For the drought treatments, the seedlings were irrigated with 30% PEG-6000, and the controls were irrigated with water. The seedlings above the sample surface was 1.26 µW/cm² and the total energy supply was 0.34 J/cm², which were designed based on the conserved sequences from dicotyledon annexins. The 5′ and 3′ end cDNA fragments were amplified using the SMARTer RACE cDNA Amplification Kit (Clontech) following the manufacturer's protocol. The nested gene-specific primers for 3′ RACE were as follows: GPS1 (5′-CGA GGA GTT CTT GGG TAT CAG GAG GG-3′) and GPS2 (5′-AGA TCC TCT CTT TCA CAG CCA AGT TTA G-3′). The 5′ RACE was performed with nested primers GPS3 (5′-TTC AGT TGT GCC TGG TTT CTG TGT CC-3′) and GPS4 (5′-AGT CGC CTT TCT TCA CAG CCA CAT TG-3′). Based on the cDNA sequence of FtANX1, primers were designed (forward: 5′-ATG GCG ACA TTG ATA GCT CC-3′, reverse: 5′-GTA ACC TTC GGC TCC GAG CA-3′) to obtain the full-length FtANX1 DNA. The sequences of the DNA fragments were determined after cloning into the pMD19-T Simple Vector (TaKaRa).

2.2. Cloning of the full-length FtANX1

Total genomic DNA was extracted following the SDS-proteinase method (Jin et al., 2007). Total RNA was isolated using the Column Plant RNA OUT 2.0 kit (TIANDZ) according to the manufacturer's protocol. cDNA synthesis was performed using the PrimeScript RT reagent Kit with the gDNA Eraser (Takara) with oligo-dT primers for RT-PCR. The conserved cDNA fragment of Tartary buckwheat annexin was obtained by degenerate primers as follows: 5′-CTG CGA AAA GCT TGT AAG ggn tgg ggn ac-3′ (forward) and 5′-GAT CTA GTA GAC AGA ATT CTA ggn cct gwt c-3′ (reverse), which were designed based on the conserved sequences from dicotyledon annexins.

2.3. Sequence and phylogenetic analysis

Sequence comparison was conducted using the BLAST program of the NCBI online database (http://www.ncbi.nlm.nih.gov/blast/). Sequence features, such as pl and molecular mass, were calculated using protein analysis tools (http://web.expasy.org/compute_pi/). The localization of protein was predicted on the TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP). Protein structure homology modeling was completed using the Swiss-Model workspace (http://swissmodel.expasy.org). Multiple sequence alignment was performed using ClustalX 2.0 (Thompson et al., 1997). The phylogenetic tree was constructed according to the neighbor-joining method with the Poisson model using MEGA 5.0 (Tamura et al., 2011).

2.4. Quantitative real-time PCR analysis

The cDNA template for transcript level analysis of FtANX1 expression was prepared as described above. The primers used for qRT-PCR reaction were as follows: 5′-GAT GTC TAT GGT GAT TCC-3′ (forward) and 5′-GAG GTA CTC CTT GGA G-3′ (reverse). The histone H3 was used as an internal control using the following primers: 5′-GAG AGA AAC CAT CAG ACG AC-3′ (forward) and 5′-GAG AGC AGA CAC AGC AGA GC-3′ (reverse) (Timotijevic et al., 2010). The reactions were performed according to the procedure described in the SYBR Premix Ex Taq II (Perfect Real Time) Kit (TaKaRa). At least three replicates of each cDNA sample were performed for qRT-PCR analysis. The data were analyzed using the 2−ΔΔCt method (Livak and Schmittgen, 2001).

2.5. Isolation and computational analysis of promoter regions for regulatory elements

The 5′-upstream sequence of FtANX1 was isolated using the Genome Walking Kit (TaKaRa) with nested specific primers (sp1: 5′-CCA AGC CTC TGA AGC ACC TA-3′, sp2: 5′-ATG AGA TCC TCG CCA TAC TG-3′, and sp3: 5′-GTA ACT TGC AGA TCC GAG TC-3′) and the AP primers...
provided in the kit. The PCR products were cloned into the pMD19-T Simple Vector and sequenced. The transcription start site was searched through the Prediction Server (http://www.fruitfly.org/seq_tools/promoter.html). To identify the possible putative regulatory elements, the whole promoter sequence was analyzed using the PLACE/Signal Scan database (http://www.dna.affrc.go.jp/PLACE/signalscan).

3. Results

3.1. Cloning and sequence analysis of FtANX1

In order to isolate the annexin gene from Tartary buckwheat, RT-PCR was first performed targeting the conserved region of annexin, as described in Section 2. Further analysis of the 5′ and 3′ terminal sequences of the full-length transcript containing this conserved region by RACE revealed a 942-bp coding sequence of the *F. tataricum* annexin gene, called FtANX1. The full-length DNA of FtANX1 was 1650 bp and contained five exons and four introns (GenBank Accession No. KP303383). It was found to share 77% identity with *M. truncatula* annexin (Accession No. XM_003627732).

The full-length 942-bp transcript of the FtANX1 gene encodes 313 amino acid residues with a theoretical molecular mass of 36.06 kDa and pl 5.94. A search against the PROSITE database (http://prosite.expasy.org/scanprosite/) revealed that there were 11 potential phosphorylation sites in the form of cAMP-cGMP-dependent protein kinase, protein kinase C phosphorylation sites, casein kinase II phosphorylation sites, and tyrosine kinase phosphorylation sites. Additionally, an N-myristoylation site was also found. Interestingly, a tripeptide RGD sequence that mediates cell attachment in fibronectin was also present in FtANX1 between residues 297 and 299 (Figure 1).

![Figure 1. Multiple sequence alignment analysis of *F. tataricum* and other annexin domains. The arrangements of five alpha-helices in each of the four domains (I, II, III, and IV) are indicated. His40 key peroxidase residues are highlighted in red; residues highlighted in pink are type II Ca²⁺-binding sequences; conserved tryptophan required for phospholipid binding is shown in purple; residues highlighted in green are the IRI motif, whereas residues highlighted in brown are the RGD motif; asterisk represents the identical amino acid sequences of *F. tataricum* and other annexin proteins.](image-url)
Multiple alignments with other plant annexins revealed that the structure of FtANX1 was evolutionarily conserved (Figure 1). We modeled the 3D structure of FtANX1 using Capsicum annuum annexin (AnnCaP32) with the highest sequence identity (50.16%) as a template (Figure 2). The protein is dominated by four repeats and a type-II Ca\textsuperscript{2+}-binding site (G-X-G-T-[38]-E) in repeat I, in which the W residue (position 27) may associate with phospholipid binding (Mortimer et al., 2008) (Figures 1 and 2). In addition, FtANX1 also contained a highly conserved H residue at amino acid 39 (Figure 1), which may play a role in stabilizing the structure through anchoring the N-terminal region to the C-terminal core (Konupka-Postupolska et al., 2011). Phylogenetic analysis of amino acid sequences of FtANX1 and other plant annexins revealed that annexins can be categorized into four groups of orthologs, and FtANX1 is grouped into Group II together with AnnGh5, Annfaf, and AnnMt1 (Figure 3), suggesting that they may have similar features and perform similar functions.

3.2. Tissue- and developmental stage-specific expression of FtANX1

The expression pattern of FtANX1 was investigated in roots, stems, leaves, flowers, mature achenes, and immature achenes. As shown in Figure 4, FtANX1 turned out to be expressed in all organs, with the highest expression in immature achenes, being relatively less abundant in roots, stems, flowers, and mature achenes. The least expression was in the leaves, indicating tissue- and developmental stage-specific expression patterns of FtANX1.

3.3. Differential expression of FtANX1 in seedlings under various stresses

Since phytohormone, cold, drought, NaCl, and UV-B could influence the transcript level of stress-related genes in plants, we investigated whether FtANX1 is responsive to these stresses in F. tataricum. The seedlings were treated with JA (50 µM), cold (4 °C), drought (30% PEG-6000), NaCl (100 mM), and UV-B radiation (1.26 µW/cm\textsuperscript{2}) and were collected at 0, 12, and 24 h after treatment. Under JA and UV-B stress, we observed an 8.7- and 9.7-fold increase in FtANX1 transcript levels at 24 h, respectively, compared to the control group (Figure 5A). However, the FtANX1 mRNA level was elevated at 12 h and decreased at 24 h when treated with NaCl. Cold and drought stress also caused an increase in FtANX1 transcript levels, and the highest increase in annexin expression was observed at 24 h after exposure to cold and drought stress (16.3- and 16.5-fold, respectively) when compared to the control group (Figure 5A).

Heavy metals are well recognized as playing a dual role in higher plants during growth and development (Cia et al., 2011). Here we examined the effects of seven heavy metals on the transcription of FtANX1 in Tartary buckwheat. As shown in Figure 5B, exposure to copper or lead resulted in a 3.6- and 1-fold increase at 7 days, respectively, whereas a slight decrease was observed at 14 days, but this was still higher than in the controls (2.6- and 0.4-fold, respectively). Zinc exposure resulted in a 3-fold increase in FtANX1 transcript level at 7 days, whereas a significant decrease was observed at 14 days after treatment. For iron or cadmium, FtANX1 mRNA level was downregulated at 7 days, whereas the FtANX1 expression reached the control level for iron treatment at 14 days, and cadmium treatment yielded a 1.2-fold increase at 14 days. However, there was a dramatic decrease (7 days and 14 days) in the FtANX1 transcript levels in response to nickel and manganese treatment. In general, the highest upregulation

![Figure 2. A diagram of the predicted 3D structure of FtANX1. The key to the color-coding for the structural profile is as follows: N-terminal domain (red), repeat I (pink), repeat II (blue), repeat III (green), repeat IV (yellow), and C-terminal domain (brown). The dark green sphere represents the calcium ion bound to module I. The H39 residue is indicated by its location in the side chains (shown as sticks). A and B show the front view and convex membrane-binding surface of the FtANX1 protein, respectively.](image-url)
was observed at 7 days after exposure to copper, and the most drastic decrease in the expression pattern of \( \text{FtANX1} \) was seen at 14 days after manganese exposure (Figure 5B).

### 3.4. Stress-related cis-elements in promoter of \( \text{FtANX1} \)

Since transcriptional regulatory elements within promoter regions govern the regulation of gene expression, studies on the promoter will lead to a better understanding of the specific regulation of gene expression and stress tolerance mechanisms. As \( \text{FtANX1} \) was differentially regulated by various stresses, we cloned and analyzed a 1563-bp promoter fragment upstream of the translational start site of \( \text{FtANX1} \). The putative transcription start site and TATA box were found to be 31 and 54 bp upstream of the translation initiation codon, respectively (Figure 6).

Sequence analysis showed many cis-acting elements are involved in abiotic and biotic stress responses in the \( \text{FtANX1} \) promoter region at different locations (Figure 6). Regulatory element ACGT is reported to be involved in upregulation of the \( \text{erd1} \) gene in \textit{Arabidopsis thaliana} by dehydration stress (Simpson et al., 2003), and six copies of this element were
present on the FtANX1 promoter. A typical ABA-responsive element, ABRE, was found at position 253 bp, which is reported to be linked to dehydration susceptibility (Jami et al., 2009). Motifs (GATA-box, T-box, and I-box) involved in light responsiveness were also present in the FtANX1 promoter region. Regulatory element GTAC (CuRE) was reported be involved in copper signaling, also found in the promoter of the FtANX1 gene. In addition, many other cis-acting elements for the regulation of genes under abiotic stresses, such as salicylic acid signaling (W-box), cold (LTRE element), and water (MYB, MYB core), were observed during the analysis of the FtANX1 promoter region (Figure 6).

4. Discussion
In this paper, a novel annexin gene (designated as FtANX1) was isolated from Tartary buckwheat, which was homologous to other higher plant annexins. Sequence analysis revealed that FtANX1 had four annexin repeats and contained a typical Ca\textsuperscript{2+}-binding site in repeat I. FtANX1 also had a conserved H residue (H39 in FtANX1 numbering in Figure 1) in repeat I, which may play a role in stabilizing the structure of the first domain (Konupka-Postupolska et al., 2011). Phylogenetic analysis indicated that FtANX1 was classified into Group II with AnnMt1, Annfaf, and AnnGh5 (Figure 3). These results demonstrated that FtANX1 belongs to the plant annexin family. Functionally, AnnMt1 has been suggested to participate in Nod factor signaling as a nonspecific ion channel (Kodavali et al., 2013). Research in strawberry indicates that Annfaf may be involved in the ripening progress of strawberry fruit (Chen et al., 2016). AnnGh5 has been described to be preferentially expressed in fibers and is mainly accumulated in rapidly elongating fibers (Li et al., 2013). As FtANX1 is grouped into Group II together with AnnMt1, Annfaf, and AnnGh5, we suspect that FtANX1 might have similar functions and be involved in cell signaling and ripening processes. In addition, many potential posttranslational modification sites were found, which might engage in specific protein–protein interactions (Konopka-Postupolska et al., 2011).

During the florescence of Tartary buckwheat, the expression pattern of FtANX1 in 6 different tissues was investigated. The transcripts of FtANX1 were detected in all the tested organs; however, they were more abundant in roots, stems, and achenes, and especially in immature achenes (Figure 4). This expression pattern was similar to those of AnnSl1.1 and AnnSl7 in tomato. AnnSl7 was
expressed in all organs examined and was most abundant in the pericarp of mature green fruits, whereas AnnSl1.1 was expressed to a lesser extent in young stems and leaves (Lu et al., 2012). Annexins may play a significant role in roots, such as in root hair elongation growth (Mortimer et al., 2008). The function of annexins in young stems is suggested to be engaged in long-distance signal transduction via sensing Ca^{2+} (Giavalisco et al., 2006). The exact functions of FtANX1 in leaves and flowers remain unknown. However, since manufacturing of nutrients and sexual reproduction occur in these organs during this stage, the expression of FtANX1 may be related to these processes. Annexins have been found in forming or regulating Ca^{2+} channels in membranes (Mortimer et al., 2008). Interestingly, our data show that the FtANX1 transcript has a dramatic change during achenes’ development from immature to mature. We hypothesize that FtANX1 might be closely associated with achenes ripening. The results bring new insight to understanding the functions of FtANX1 in Tartary buckwheat.
Regulation of gene expression is the starting point for higher plants to cope with a changing environment. The stimuli-responsive expression of annexin genes has been reported in Arabidopsis (Weber et al., 2006), rice (Jami et al., 2012), and tomato (Lu et al., 2012). Our analysis also showed that the transcript of *FtANX1* was regulated by several stresses. JA widely exists in plants, and its exogenous application could stimulate gene expression during plant defense. It has been reported that *ZmAnx6.1* and *ZmAnx7* were induced by JA, which implies that...
these annexins play a putative role in pathogen stress tolerance (Zhou et al., 2013). In our study, FtANX1 also showed an induced expression pattern under exogenous JA treatment, suggesting that this annexin was engaged in JA-dependent stress responses in Tartary buckwheat (Figure 5A). It is known that the generation of reactive oxygen species is one of the earliest responses of plant cells to various abiotic stresses (such as cold, salt, and drought), and annexins were reported to be involved in balancing reactive oxygen species with their peroxidase activity (Kush and Sabapathy, 2001). Moreover, annexins could control and repair the damaged cell wall and/or membrane and result in stress tolerance (Mahajan and Tuteja, 2005). It is reasonable that abiotic stress (cold, salt, and drought) could result in the accumulation of FtANX1 transcript compared to the controls (Figure 5A). There is some evidence that UV-B radiation increases the production of rutin in buckwheat (Kreft et al., 2002; Suzuki et al., 2005), which could result in UV-B screening, antioxidant activity, and disease resistance (Harborne and Williams, 2000). Consistent with these results, we observed that FtANX1 was responsive to UV-B radiation.

Plants possess a range of cellular mechanisms that may be involved in the detoxification and tolerance of heavy metals (Sharma and Chakraverty, 2013). Annexin genes’ expression or abundance had been previously demonstrated to change under metal stress, e.g., zinc affected the expression levels of ZmAnx1 and ZmAnx5 (Zhou et al., 2013), copper affected AtANN3 and AtANN4 (Weber et al., 2006), and cadmium affected pea root annexin abundance (Repetto et al., 2003). Our findings showed that the expression of FtANX1 was upregulated after treatment with cadmium, zinc, and copper compared to the controls (Figure 5B). However, after exposure to nickel, the transcript of FtANX1 was decreased. ZmAnx7 and ZmAnx8 expression was also reduced in response to nickel (Zhou et al., 2013). These data indicate that FtANX1 is associated with the response to heavy metal stress in Tartary buckwheat, suggesting that Tartary buckwheat annexin may have specific signaling functions in these stress transduction pathways.

As the transcript of FtANX1 was linked to abiotic and biotic stresses, there might be regulatory elements that can confer developmental stage- and tissue-specific gene expression in the upstream regions of the FtANX1 gene. Therefore, we analyzed the corresponding promoter region of FtANX1 to further understand its functions. Putative cis-elements were observed on the FtANX1 promoter (Figure 6). For example, the ACGT motifs in the Arabidopsis erd1 promoter involved in upregulation by dehydration stress (Simpson et al., 2003) were also found in the FtANX1 promoter region. The light-stress motifs present on the FtANX1 promoter were also in accordance with the upregulation of FtANX1 by UV-B radiation. It was previously reported that the CuRE motif plays an important role in copper and oxygen response (Quinn et al., 2000), and our motif analysis showed the existence of a regulatory element CuRE in the promoter region of the FtANX1 gene (Figure 6). Since the expression levels of FtANX1 were significantly induced at 7 days and 14 days after exposure to copper (Figure 5B), we speculate that the CuRE motif in the FtANX1 promoter region may participate in the regulation of copper response, and point mutation or deletion mutagenesis of the CuRE element could be performed to explore its role in copper-responsive signal transduction in Tartary buckwheat.

In conclusion, we identified an annexin gene (FtANX1) in Tartary buckwheat. The general motif analysis of the gene and promoter region, which were in line with our experimental data, suggested that FtANX1 may play diverse roles in stress tolerance. However, more annexin genes may exist in buckwheat, which could be identified by optimizing primer or PCR experiments. Further in-depth investigations using knocked-out or heterologously expressed plants are required to elucidate the functions of FtANX1 and the significance of the promoter in regulation.

Acknowledgment
The authors thank Dr Faliang Li, Institute of Agricultural Sciences, Liangshan Prefecture, P.R. China, who generously contributed the Tartary buckwheat seeds.

References


