Cloning, Characterization, and Activity Analysis of a Flavonol Synthase Gene FtFLS1 and Its Association with Flavonoid Content in Tartary Buckwheat

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ABSTRACT: Evidence from in vitro and in vivo studies indicates that rutin, the main flavonoid in tartary buckwheat (Fagopyrum tataricum), may have high value for medicine and health. This paper reports the finding of a flavonol synthase (FLS) gene, cloned and characterized from F. tataricum and designated FtFLS1, that is involved in rutin biosynthesis. The FtFLS1 gene was expressed in Escherichia coli BL21(DE3), and the recombinant soluble FtFLS1 protein had a relative molecular mass of 40 kDa. The purified recombinant protein showed, with dihydroquercetin as substrate, total and specific activities of 36.55 \( \times \) 10\(^{-3}\) IU and 18.94 \( \times \) 10\(^{-3}\) IU/mg, respectively, whereas the total and specific activities were 10.19 \( \times \) 10\(^{-3}\) IU and 5.28 \( \times \) 10\(^{-3}\) IU/mg, respectively, with dihydrokaempferol. RT-PCR revealed that during F. tataricum florescence there was an organ-specific expression pattern by the FtFLS1 gene, with similar trends in flavonoid content. These observations suggest that FtFLS1 in F. tataricum encodes a functional protein, which might play a key role in rutin biosynthesis.

KEYWORDS: biosynthetic pathways, flavonol synthase, flavonoids, functional expression, tartary buckwheat, Fagopyrum tataricum

INTRODUCTION

Tartary buckwheat (Fagopyrum tataricum Gaertn.) is the main cultivated buckwheat species in Asian countries. It is well-known as a healthy food with a balance of nutrients that include proteins, minerals, lipids, dietary fiber, and flavonoids. The predominant flavonoid in rutin (quercetin 3-O-rutinoside), which is 0.8–1.7% of the dry weight (DW) of the plant and 50–80% of the total flavonoid content.\(^1\) Researchers have reported that rutin is an antioxidant with anti-inflammatory, anticarcinogenic, and vasoprotective properties.\(^2\) There is also evidence that rutin plays a role in physiological functions such as UV-B screening, antioxidant activity, and disease resistance.\(^3\)

Through the phenylpropanoid pathway, flavonoids are synthesized from several individual branches and can be classified into different groups of derivatives. Rutin is synthesized through the flavonol branch and is a flavonol glycoside. In the flavonol class of flavonoids, quercetin, kaempferol, and myricetin differ by the single hydroxyl group on the flavonoid B ring, which can be hydroxylated at the 3\(^{′}\)-position by flavonoid 3′-hydroxylase (F3′H), or at the 5\(^{′}\)- and 7\(^{′}\)-positions by flavonoid 3′,5′-hydroxylase (F3′5′H).\(^4\) Flavonol synthesis is attributed to flavonol synthase (FLS), which competes with F3′H and F3′5′H for substrates and forms two precursor compounds of rutin, quercetin, and kaempferol.\(^5\) The formation of rutin from flavonols is catalyzed by flavonoid glycosyl-transferases (Figure 1).\(^6\)\(^7\)

FLS belongs to the 2-oxoglutarate-dependent dioxygenase (2-ODD) family and controls a key step in flavonol biosynthesis.\(^7\) It is a soluble enzyme that requires ascorbate for stabilization, 2-oxoglutarate as a cosubstrate, and Fe\(^{2+}\) as a cofactor.\(^7\) The first report of FLS activity was from irradiated parsley cells; it was characterized in vitro as a 2-ODD.\(^8\) Later, FLS cDNAs were cloned and identified from other plants. Studies have indicated that FLS is encoded by a multicopy gene in plants, and the different copies are expressed in either an organ- or species-specific pattern.\(^9\)–\(^12\) In addition, FLSs display variable substrate preferences and loose catalytic activities, which may contribute to the different isoforms of FLS.\(^9\)–\(^13\)

In Fagopyrum spp., the complete open reading frame (ORF) of the FLS gene has not previously been cloned, nor has its biological function been identified, although rutin biosynthesis and related genes have been studied in F. tataricum.\(^14\)–\(^16\) Due to the pivotal role of FLS in rutin biosynthesis, our study focused on the cloning and molecular characterization of a functional FLS gene from tartary buckwheat, designated FtFLS1. We performed rapid amplification of cDNA ends (RACE) technology to clone FtFLS1. The plasmid pET-30b(+)-FtFLS1 was constructed to express the FtFLS1 protein, and immobilized metal ion affinity chromatography was used to purify the recombinated protein. In addition, the organ-specific association between FtFLS1 expression and flavonoid content in tartary buckwheat was investigated by comparing total flavonoid content with the results of semiquantitative reverse transcription (RT)-PCR.
Figure 1. Rutin biosynthesis pathway in *F. tataricum*. Enzyme genes involved in the biosynthesis of rutin are indicated by solid arrows. PAL, Phe ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl-CoA synthase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3′-hydroxylase; F3′H, flavonoid-3′-hydroxylase; F3′5′H, flavonoid-3′,5′-hydroxylase; FLS, flavonol synthase; UFGT, flavonoid glycosyltransferases.

### MATERIALS AND METHODS

**Plant Materials.** *F. tataricum* seeds were grown under field conditions on a farm at Sichuan Agricultural University, Ya’an, Sichuan, China. Ya’an city is located in southwestern China (29°59’ N, 102°59’ E), ~800 m above sea level. The average annual rainfall is 1000–1800 mm, and the annual mean temperature is ~16.2 °C. To study the total flavonol content and relative amount of *FtFLS1* mRNA, healthy *F. tataricum* flower buds, flowers, immature seeds, stems, and leaves were collected at the three different developmental stages of fluorescence (initial, full, and postbloom) during ~30 days over June–July 2010. Initial bloom occurred from June 21 to June 30, full bloom from July 1 to July 10, and postbloom from July 11 to July 20. All samples were collected on the fifth day of each blooming stage, frozen in liquid nitrogen, and stored at ~80 °C for RNA and flavonoid extraction.

**Extraction of Total RNA and Cloning of *FtFLS1*.** Total RNA was extracted from the various plant organs using an RNAout kit (Tiangen, China), and cDNA was synthesized with a RevertAid First Strand cDNA Synthesis kit (MBI, USA). The cDNA from *FtFLS1* was obtained with a 5′- and 3′-primers FLS-p and FLS-q. The PCR product was subcloned into a pMD19-T simple vector (designated pMD19-*FtFLS1*), transformed into *E. coli* DH5α, and then sequenced (Takara, Japan). The primer sequences are presented in Supplementary Table 1 of the Supporting Information.

**Molecular Characterization of *FtFLS1*.** The amino acid sequence of the *FtFLS1* protein was deduced from the cDNA sequence and then analyzed using the DNAMan and BLAST programs in NCBI. SignalP and TargetP were used to analyze the signal peptide and subcellular location of *FtFLS1*. The multidomain was performed with Clustal X, and the secondary structure of the predicted *FtFLS1* protein was predicted by using the self-optimized prediction method (SOPM).17 The three-dimensional (3D) structure of *FtFLS1* was built using the SWISS-MODEL program and illustrated with the PyMOL Viewer.18 The phylogenetic tree was drawn using the MEGA (5.0) program with the neighbor-joining method.19

**Functional Expression of *FtFLS1* in *Escherichia coli*.** Primers pFLS-f and pFLS-r (Supporting Information, Supplementary Table 1) were designed to amplify the ORF sequence of *FtFLS1*. Using the pMD19-*FtFLS1* plasmid as a template, PCR was performed under the following conditions: 4 min at 95 °C for 1 cycle, then 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C for 30 cycles. The PCR products were purified and digested with BamHI and HindIII and then ligated to the pET-30b(+) vector (Novagen, Germany), here called pET-30b(+)-*FtFLS1*. (His)6-tags were fused to the N- and C-termini of the recombinant protein to facilitate protein purification. The resultant plasmid was transformed into *E. coli* BL21(DE3) cells (Novagen, Germany). A single colony was then inoculated and cultured at 37 °C in Luria–Bertani medium containing kanamycin (50 μg/mL) with shaking (200 rpm) until the optical density at 600 nm (OD600) reached 0.5. To express *FtFLS1* as a soluble protein, the final expression conditions were induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM at 25 °C for 10 h.

To investigate whether the recombinant *FtFLS1* protein had a potential function in flavonol biosynthesis, a rough and qualitative FLS activity was measured. Cells of the negative control group (containing the plasmid pET-30b(+)), and the experimental group (pET-30b(+)-*FtFLS1*) were harvested after a 10 h induction and assayed for FLS activity. Both dihydroquercetin and dihydrokaempferol are commercially available; they are the substrates for flavonol biosynthesis.20 Thus, a 50 μL crude extract sample was used in a 500 μL reaction mixture in a rough FLS assay.20 The mixture was extracted with the

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same volume of ethyl acetate twice and separated via silica gel thin-layer chromatography (TLC; toluene/acetie ether/formic acid, 5:2:0.5). Dihydroquercetin, quercetin, dihydrokaempferol, kaempferol, dihydrokaempferol–kaempferol mixture, and a mixture of dihydroquercetin and quercetin were used as the internal standards (Chromadex, USA). The reaction products were identified either by using standard samples or according to their retention factor ($R_f$) values.

**Purification of FtFLS1.** After 10 h of induction, the cultures were harvested by centrifugation at 8000g for 10 min at 4 °C and suspended in 5 mL of His- Bind buffer (0.5 M NaCl, 40 mM imidazole, and 20 mM sodium phosphate, pH 7.4). The crude extract obtained after sonication was centrifuged at 12000g for 15 min. The soluble protein fraction was subsequently purified using a Hitrap FF column (GE Healthcare, USA). After rinsing sequentially with 40 mM (10 mL), 50 mM (1 mL), 100 mM (1 mL), and 150 mM (1 mL) imidazole buffer (0.5 M NaCl and 20 mM sodium phosphate, pH 7.4), the recombinant FtFLS1 protein was eluted with 200 mM imidazole elution buffer (1 mL of 0.5 M NaCl and 20 mM sodium phosphate, pH 7.4). All samples were analyzed by 12.5% SDS-PAGE.

**Activity Assay of FtFLS1.** A full spectral scan (wavelengths 200–1000 nm) was performed using a UV–vis spectrophotometer (Shimadzu, Japan). The best measurement wavelength and the −1000 nm) was performed using a UV enzyme assays. The calibration curve had the regression equation ascorbate, ferrous sulfate, succinate, 2-oxoglutaric acid). The optimum pH 7.4). All samples were analyzed by 12.5% SDS-PAGE.

A full spectral scan (wavelengths 200–1000 nm) was performed using a UV–vis spectrophotometer (Shimadzu, Japan). The best measurement wavelength and the calibration curve were determined by scanning different concentrations of products (0.5, 1, 2, 3, 5, 7, 9, 12, and 15 μg/mL quercetin or kaempferol) dissolved in the reaction buffer (sodium acetate, sodium ascorbate, ferrous sulfate, succinate, 2-oxoglutaric acid). The optimum wavelength of 365.5 nm was used for measuring quercetin in the enzyme assays. The calibration curve had the regression equation $A = 0.03C$ ($A$ = absorbance; $C$ = concentration, μg/mL), with an $R$-square ($R^2$) = 0.9980 (Supporting Information, Supplementary Figure 1a). The optimum wavelength of 367 nm was used for measuring kaempferol in the enzyme assays. The calibration curve was calculated with the regression equation $A = 0.0274C$, where $A$ is the absorbance and $C$ is the concentration (μg/mL), and $R^2$ = 0.9968 (Supporting Information, Supplementary Figure 1b).

FLS activity was routinely assayed at 37 °C and pH 5.0. The concentration of purified FtFLS1, without removing (His) 6-tags, was measured using Coomassie brilliant blue G250. As previously described, it is assumed that parameters used for FLS proteins were within the linearity range; the dihydroflavonol substrates and the other substrates were at saturating levels to reach a maximum activity. Therefore, each 500 μL of reaction buffer contained 100 μM dihydroquercetin or dihydrokaempferol as substrates, 111 mM sodium acetate, 83 μM 2-oxoglutaric acid, 42 μM ferrous sulfate, 2.5 mM sodium ascorbate, and 12.5 μg of FtFLS1 enzyme. The reaction mixture was incubated in open vials under gentle shaking for 20 min before the absorbance of the product was measured at 365.5 nm for quercetin and at 367 nm for kaempferol. A single unit (IU) was defined as the amount of FLS that catalyzed the production of 1 μmol of product from the substrates per minute at 37 °C (μmol/min). Specific activity was defined as IU/mg.

**Analysis of FtFLS1 Expression and Flavonoid Content during Florescence in Different Organs.** Semiquantitative RT-PCR was performed to analyze the expression pattern of FtFLS1 in different organs of F. tataricum during florescence. To remove trace DNA from samples, total RNA extractions from different organs were treated with RNAse free DNase I. The products were used as template, with specific primers for semiquantitative RT-PCR (Supporting Information, Supplementary Table 1). For FtFLS1, primers FLS-sf and FLS-sr were synthesized to amplify the fragment. The housekeeping gene H3 (Histone 3, Genbank ID HM628903) was used as the internal standard, using specific primers H3-sf and H3-sr. RNA of FtFLS1 was quantified relative to the level of the housekeeping gene H3. FtFLS1 expression in all organs was detected by RT-PCR under 24-cycle amplification conditions. Electrophoresis images were scanned with a Bio-Rad Gel imaging system (Universal Hood II, Bio-Rad, USA).

All samples of F. tataricum (500 mg each) were homogenized in 5 mL of 70% methanol containing 10% phosphoric acid (final, 0.1%) with a hand homogenizer. Flavonoids were extracted using an ultrasonic method. After cooling, the test tubes were centrifuged at 8000g for 10 min. The obtained supernatants were stored at −20 °C. F. tataricum extracts were analyzed with a spectrophotometer according to a previous method with a little modification. The reaction process was as follows: One milliliter of diluted extracts containing flavonoids, 0.3 mL of 5% (w/w) NaNO2, and 4 mL of 70% methanol (v/v) were mixed for 6 min, and then 0.3 mL of 10% Al(NO3)3 (w/w) was added and mixed. Six minutes later, 4 mL of 1% NaOH (w/w) was added. The solution was diluted to 10 mL with 70% (v/v) methanol for measurement. After 15 min, the absorbance of the solution was measured at 500 nm with a UNICO WJF2000 spectrophotometer (Unico, China).

**Statistical Analyses.** The intensity of specific bands was quantified in triplicate using Quantity One software (Bio-Rad, USA). The expression levels of FtFLS1 were presented as the ratio of the intensity of FtFLS1 to H3 (i.e., OD$_{FtFLS1}$/OD$_{H3}$). The total flavonoid contents were expressed as the percentage of rutin (mg) in 500 mg of fresh weight (FW) of plant material and compared to a rutin standard curve made under the same conditions. Quantitative analysis of the total flavonoids in F. tataricum was performed in triplicate. The relative flavonoid content differences and the expression levels of FtFLS1 were compared using a post hoc test with Dunnett’s multiple comparison. Statistical analyses were performed with SPSS software (SPSS 13.0).

**RESULTS**

**Molecular Characterization of FtFLS1.** Using homology cloning and RACE technology, FLS cDNA was isolated from flowers of F. tataricum, which contained a 1008 bp ORF (Genbank ID JF274262, designated FtFLS1). The results of protein–protein BLAST (BLASTp) in NCBI revealed that the length of the amino acid sequence of FtFLS1 was similar to 2-ODDs in other plants. The predicted amino acid sequence is
The topology of the phylogenetic tree revealed that FLSs from one eudicotyledon FLS (FeFLS) was 334/335, and these proteins exhibited di-the most highly related. The identity between the FtFLS1 and their amino acid sequences in only one residue in position 56.

Cluster III might have become species-speci-uncertain pattern and had lower homology among its members evolutionary relationship among FLSs in cluster III showed an (Supporting Information, Supplementary Figure 2).25 oxoglutarate-binding sites Arg at 287 and Ser at 289 221, Asp at 223, and His at 277 and the conserved 2-FtFLS1 also contained the conserved iron-binding sites His at (AtANS; PDB ID 1gp6A).26 The sequence similarity between the jellyroll β-ray structure (Figure 2a). A nine-residue overall folding patterns of FtFLS1 were similar to the AtANS X- motif and might help form the enclosed active site the N-terminus, which were arranged around the back face of the jellyroll motif and might help form the enclosed active site (Figure 2b). Importantly, the conserved iron- and 2-oxoglutarate-binding sites were defined by the location of the jellyroll core (Figure 2b).

A phylogenetic tree was drawn to investigate the evolutionary relationship between FtFLS1 and other FLSs involved in plant flavonol biosynthesis (Figure 3). The topology of the phylogenetic tree was an asymmetric clover shape, comprising clusters I, II, and III. It became clear that the phylogenetic tree was an asymmetric clover shape, comprising clusters I, II, and III. It became clear that the phylogenetic tree was generally in good agreement with the traditional taxonomic classification. Cluster I consisted of 19 FLSs, all belonging to the eudicotyledons, and some of them with demonstrated activity. Cluster II contained eight dicotyledon FLSs, including 6 FLSs from A. thaliana (AtFLS1 to –AtFLS6), Parrya nudicaulis FLS, and Matthiola incana FLS; functional activity was attributed only to AtFLS1 and AtFLS3.10 The results suggested that most FLSs in the eudicotyledons shared a high homology at the amino acid level, and the duplications leading to the amplification of FLS genes in some species were ancient events. Cluster III consisted of four monocotyledon FLSs (Sorghum bicolor, Oryza sativa, Hordeum vulgare, and Zea mays), one eudicotyledon FLS (Vitis vinifera), and two gymnosperm FLSs (Ginkgo biloba, and Picea sitchensis). Interestingly, the evolutionary relationship among FLSs in cluster III showed an uncertain pattern and had lower homology among its members than did clusters I and II. It may be suggested that the FLSs in cluster III might have become species-specific during evolution. The topology of the phylogenetic tree revealed that FLSs from F. tartaricum and F. esculentum, both belonging to cluster I, are the most highly related. The identity between the FtFLS1 and FeFLS was 334/335, and these proteins exhibited differences in their amino acid sequences in only one residue in position 56.
results demonstrated that *FtFLS1* encodes a functional protein with FLS activity.

### Purification of FtFLS1
To purify the recombinant protein, FtFLS1 was successfully expressed, and most of the protein was present in the soluble fraction (Figure 5, lanes 1 and 2). The total protein of the soluble fraction was 2.56 g/L; total activity and specific activity, using dihydroquercetin as substrate, were $66.03 \times 10^{-3}$ IU and $5.16 \times 10^{-3}$ IU/mg, respectively. The soluble fraction was loaded into a HiTrap FF column, and the gradient was rinsed with His-Bind and imidazole buffers. The recombinant FtFLS1 protein was then eluted with 200 mM imidazole buffers (Figure 5, lanes 3–8). The results suggested that the protein-binding process was efficient, and most miscellaneous protein had been removed in the 50 and 100 mM imidazole buffers (Figure 5, lanes 5 and 6). Compared with the 150 mM imidazole buffers (Figure 5, lane 7), the recombinant protein of FtFLS1 was eluted with 200 mM imidazole buffers more effectively and yielded one major band (Figure 5, lane 8). The yield of the purification was 38.6 g/L of culture, the purity of recombinant FtFLS1 was >95%, and total protein was 1.93 g/L. The total activity and specific activity for dihydroquercetin were $36.55 \times 10^{-3}$ IU and $18.94 \times 10^{-3}$ IU/mg, respectively, and the total activity and specific activity for dihydrokaempferol were $10.19 \times 10^{-3}$ IU and $5.28 \times 10^{-3}$ IU/mg, respectively. Taken together, these results demonstrate that FtFLS1 corresponds to a bona fide flavonol synthase.

### Analysis of FtFLS1 and Flavonoids during Florecence in Different Organs
The expression pattern of FtFLS1 in different organs of *F. tataricum* was investigated. From the results shown in Figure 6, *FtFLS1* was found to exhibit distinct organ-specific expression patterns ($P < 0.05$), which is consistent with *FcFLS* in *F. esculentum* and the recent study in *F. tataricum*. Although *FtFLS1* was expressed in every organ of *F. tataricum*, the overall expression levels were the highest in leaves and flowers, showed a moderate level in stems, and were found to lesser extents in flower buds and immature seeds ($P < 0.05$). *FtFLS1* was most highly expressed in leaves at initial bloom, with a little decrease at full bloom ($P > 0.05$), then sharply reduced at postbloom ($P < 0.05$). The expression level of *FtFLS1* in reproductive organs peaked at full bloom and then decreased postbloom ($P < 0.05$); *FtFLS1* was stably expressed in stems during florecence, with little change ($P > 0.05$).

Results of the total flavonoids in *F. tataricum* are presented in Table 1. During florecence, organ differences in total flavonoid content were apparent. The average total flavonoid content during florecence was lowest in stems, with moderate level in leaves and higher amounts in reproductive organs ($P < 0.05$). The total flavonoid content was also significantly different depending on the developmental stage of florecence. In stems, the total flavonoid contents at initial, full, and postbloom were $0.16 \pm 0.05$, $0.17 \pm 0.02$, and $0.23 \pm 0.09$, respectively, a slightly rising trend ($P > 0.05$). The total flavonoid in leaves reached a peak at full bloom ($1.7 \pm 0.17$%), with lower levels at...
Table 1. Flavonoid Content (Milligrams per 500 mg Fresh Weight, Percent) in Different Organs of F. tataricum

<table>
<thead>
<tr>
<th>organ</th>
<th>initial bloom</th>
<th>full bloom</th>
<th>postbloom</th>
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<tbody>
<tr>
<td>stems</td>
<td>0.16 ± 0.05e</td>
<td>0.17 ± 0.02</td>
<td>0.23 ± 0.09</td>
</tr>
<tr>
<td>leaves</td>
<td>1.61 ± 0.12f</td>
<td>1.71 ± 0.17g</td>
<td>1.12 ± 0.11i</td>
</tr>
<tr>
<td>reproductive organs</td>
<td>2.11 ± 0.21j (flower buds)</td>
<td>3.52 ± 0.12n (flowers)</td>
<td>2.50 ± 0.12f (immature seeds)</td>
</tr>
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</table>

“Results are expressed as the mean ± SD (n = 3). There is no significant differences (P > 0.05) among three different stages in stems. Letters a–c indicate significant differences (P < 0.05). Superscripts indicate comparison between columns, and subscripts indicate comparison between rows.

initial bloom (1.61 ± 0.12%) and postbloom (1.13 ± 0.12%; P < 0.05 for both in comparison to full bloom). The total flavonoid content was most abundant in the reproductive organs, with a maximum in flowers (3.52 ± 0.12%), followed by immature seeds (2.50 ± 0.12%) and flower buds (2.11 ± 0.21%; P < 0.05 for both in comparison to flowers). The results indicated that flavonoids in F. tataricum accumulated much more highly in the reproductive organs, ~2-fold more than leaves and 15-fold more than stems. In addition, the flavonoid content in stems did not change significantly any time during inflorescence (P > 0.05). However, during the change from flower buds to immature seeds the flavonoid content in flowers at full bloom was ~1.5-fold that at initial and postbloom (P < 0.05).

**DISCUSSION**

In this study, we successfully isolated and characterized FtFLS1 cDNA from F. tataricum, demonstrated that FtFLS1 encodes a functional protein, and also showed that its expression pattern is organ-specific during florescence.

Previous studies reported that rutin is the most abundant flavonoid in F. tataricum seeds and leaves. However, although F. tataricum is both a medicinal and food crop, there has been little research using this plant to study the structural genes involved in rutin metabolism. Thus, we sought to clone the full-length cDNA of the FLS gene in F. tataricum (FtFLS1), which is putatively responsible for the conversion of dihydroflavonol (dihydroquercetin and dihydrokaempferol) to flavonol (quercetin and kaempferol), the precursor compounds of rutin. In A. thaliana, the recombinant AtFLS1 was more effective at converting dihydroykaempferol to kaempferol than dihydroquercetin to quercetin and had a specific activity similar to that of FtFLS1 in this study. Wellmann et al. studied a variety mutations of C. ussii FLS in vitro, and results revealed that FLS had a higher affinity to dihydrokaempferol (K_m = 45 μM) than to dihydroquercetin (K_m = 272 μM). A functional FLS (ZmFLS1) was clone from Z. mays recently, and it had a different K_m for converting dihydrokaempferol (K_m = 151 μM) and dihydroquercetin (K_m = 58 μM) to the corresponding products. In our study, the activity of FtFLS1 was demonstrated by TLC and spectrophotometric assays. Examination of the specific activities of dihydroquercetin and dihydrokaempferol revealed different catalytic efficiencies, which may be explained by the variable degrees of substrate preferences and loose catalytic activities of various 2-ODDs. Although we did not examine the K_m values of FtFLS1 for each substrate, our result might raise the possibility that dihydroquercetin, which has a higher specific activity than dihydrokaempferol, is the predominant substrate of FtFLS1. This might suggest rutin is mainly synthesized from dihydrokaempferol to dihydroquercetin, then quercetin, and finally rutin in F. tataricum. Hence, the study of K_m values of FLSs for different substrates, such as Fe^3+, 2-oxoglutaric acid, and other flavonoids, will be an important avenue for future research.

The expression of individual flavonoid genes is regulated by various mechanisms. FLS1 of A. thaliana could be induced by white light in plant seedlings, and its expression influenced flavonoid levels and the root gravitropic response in seedlings under nonstressed conditions. FLS1 could be activated by flavonol-specific transcription factors (TFs) MYB11, MYB12, and MYB111 of A. thaliana, and these TFs caused different spatial accumulation of specific flavonol derivatives in leaves, stems, inflorescences, siliques, and roots. It is reported that the anthocyanin (C1/PL1 + R/B) and 3-deoxy flavonoid (P1) TFs mediate the expression of FLS in Z. mays, an expression that is also induced by UV-B. In C. ussii, CHFS transcript levels were higher in leaves during the early developmental stage than at maturity and increased in the peel during fruit maturation. A recent case described by Gupta et al. showed the flavonoid synthesis genes in F. tataricum (FLS, PAL, CHS, and CHI) were more highly than in F. esculentum. In our study, the expression pattern of FtFLS1 was similar to that of FcCHS in F. tataricum. Because FLS has a central function at the branch point of flavonol derivative metabolism, our results suggest that the expression of FLS genes among buckwheat species is disparate and has distinct organ-specific patterns.

Many studies have investigated the differences in flavonoid content among cultivated buckwheat species. Fabjan et al. reported that rutin content was as high as 3% (DW) in F. tataricum herb and in seeds ranged from 0.8 to 1.7% (DW), which was much higher that in F. esculentum seeds (0.01%, DW). Jiang et al. analyzed the seeds of 11 cultivated buckwheat...
species and found that both the rutin and total flavonoid contents were significantly different among the species, ranging from 0.02 to 0.04% in F. esculentum, from 0.10 to 0.35% in F. homotropicum, and from 1.67 to 2.04% in F. tataricum. In a case described by Kim et al., investigating six phenolic compounds, rutin accounted for 90% of the total phenolic content in tartary buckwheat sprouts, but only 20% of that in common buckwheat sprouts. Rutin contents in the seeds (nongerminated/germinated) and edible parts of tartary buckwheat were 49- and 5-fold higher, respectively, than those of common buckwheat. In the present study, the reproductive organs of F. tataricum contained the highest amounts of flavonoid. Thus, F. tataricum, as an excellent dietary source of flavonoid, might have higher commercial value than F. esculentum.

Recent studies have indicated that the accumulation of flavonoids might contribute to the transport of flavonoids.\(^{36,37}\) In a study involving A. thaliana \(tt\) mutants, researchers demonstrated that dihydrokaempferol and dihydroquercetin could travel long distances to distal organs (at the root tip, midroot, or cotyledons) and convert to quercetin and kaempferol.\(^{38}\) Some enzymes that are involved in the flavonoid biosynthesis exist as enzyme complexes and appear to be associated with flavonoid transport.\(^{36}\) CHS and CHI were located in the nucleus in several cell types in Arabidopsis and might be involved in the deposition of flavonoid.\(^{39}\) AN9, a P. hybrida glutathione S-transferase, was involved in anthocyanin binding and transport,\(^{39}\) and TT19 was involved in both anthocyanin and proanthocyanidins transport.\(^{39}\) In the present study, the accumulation of total flavonoids in leaves and flowers reached a peak at full bloom (\(P < 0.05\)), which corresponded with the expression of \(FtFLS1\) in F. tataricum. However, our results also showed a weak relationship between the flavonoid content and \(FtFLS1\) expression in flower buds and immature seeds. This may be attributed to the dynamic physiological and morphological processes of reproductive organs when transitioning from flower buds to immature seeds and supports the hypothesis of transport.\(^{15,37}\)

In summary, we successfully isolated and characterized a FLS cDNA from F. tataricum. This is the first report of the cloning of the complete ORF of a FLS gene in tartary buckwheat and the first identification of its biological function in a buckwheat species. Our study demonstrates that \(FtFLS1\) encodes a functional protein, and its expression pattern is organ-specific during florescence. Our studies on the cloning, characterization, and expression of \(FtFLS1\) will provide information for further investigation into the roles that FLS plays in the flavonoid metabolism of F. tataricum. This work also points to potential methods for enhancing the flavonoid content of F. tataricum by metabolic engineering.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank Anhu Wang at the Xichang College, Sichuan, China, for the gift of F. tataricum seeds. We also thank Medjaden Bioscience Limited for the critical reading of the manuscript.

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