JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

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

Chenglei Li,[†] Yuechen Bai,[†] Shuangjiang Li,[†] Hui Chen,[†] Xueyi Han,[†] Haixia Zhao,[†] Jirong Shao,[†] Sang-un Park,[§] and Qi Wu*[,†]

[†]College of Life Science, Sichuan Agriculture University, Ya'an 625014, Sichuan, People's Republic of China

[§]Department of Crop Science, College of Agriculture and Life Sciences, Chungnam National University, 220 Gung-Dong, Yuseong-Gu, Daejeon, Korea

Supporting Information

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 36.55×10^{-3} IU and 18.94×10^{-3} IU/mg, respectively, whereas the total and specific activities were 10.19×10^{-3} IU and 5.28×10^{-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 is 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 3′- and 5′-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 recombined 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.

Received: December 17, 2011
Revised: May 3, 2012
Accepted: May 7, 2012
Published: May 7, 2012

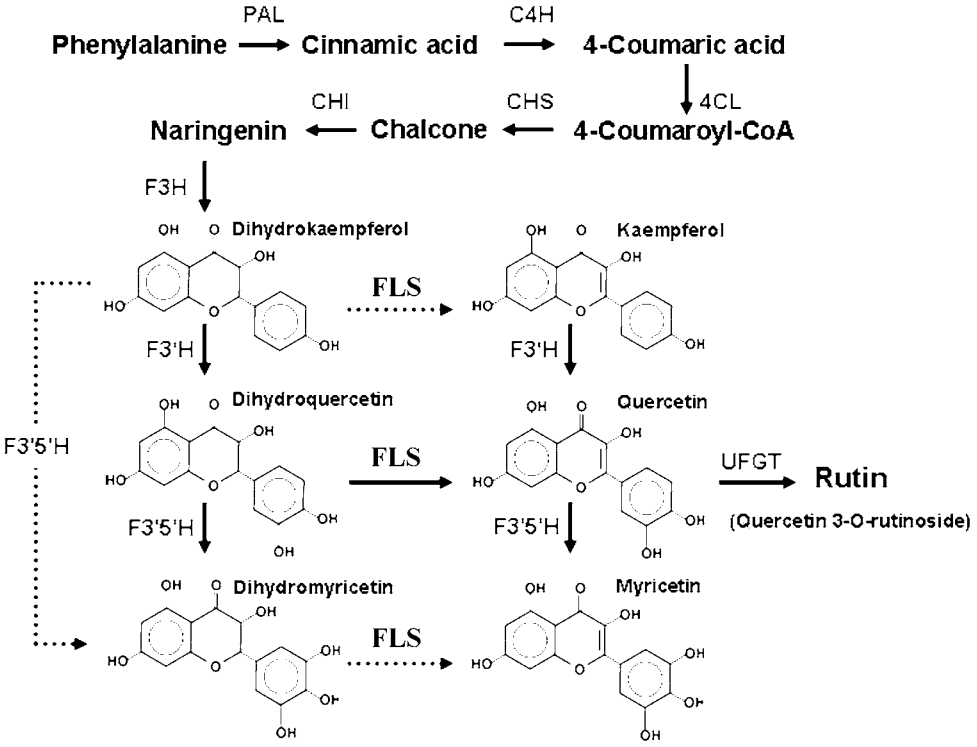

Figure 1. Rutin biosynthesis pathway in *F. tataricum*. Enzyme genes involved in the biosynthesis of rutin are indicated by solid arrows. PAL, Phe ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl-CoA synthase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3β-hydroxylase; 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 flavonoid 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 florescence (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 RNA_{out} kit (Tiandz, China), and cDNA was synthesized with a RevertAid First Strand cDNA Synthesis kit (MBI, USA). The cDNA from flowers was used as a template to amplify the *FtFLS1* gene from *F. tataricum*. A pair of degenerate primers, FLS-f and FLS-r, was designed and synthesized on the basis of the conserved region of known *FLS* genes (Invitrogen, China). To obtain the full-length cDNA sequence of *FtFLS1*, RACE was performed using a Smart Race cDNA Amplification kit (Takara, Japan). Three gene-specific primers were designed to amplify each of the flanking regions of *FtFLS1*: F5W, F5Z, and F5N for the 5′, and F3W, F3Z, and F3N for the 3′. By comparison and alignment, the ORF sequence of *FtFLS1* was obtained with primers FLS-p and FLS-q. The PCR product was subcloned into a pMD@19-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 multialignment was performed with Clustal X, and the secondary structure of the deduced FtFLS1 protein was predicated 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 *Bam*H I and *Hin*dIII 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 assay was performed. 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.[11] 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

dx.doi.org/10.1021/jf205192q | J. Agric. Food Chem. 2012, 60, 5161−5168

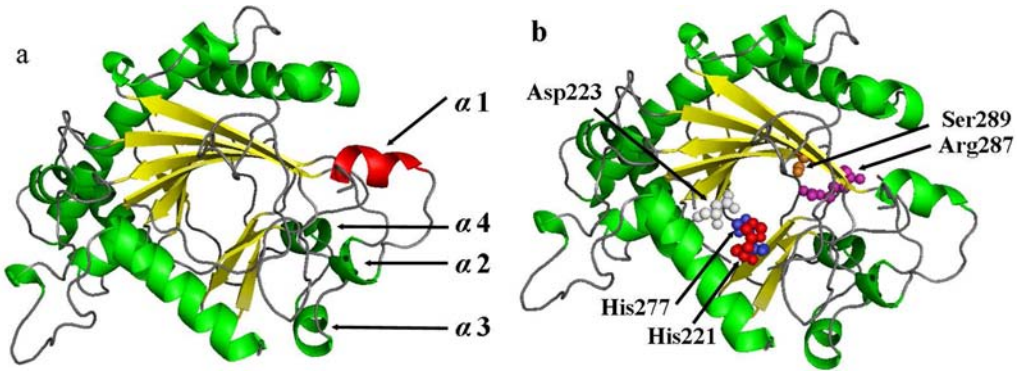

Figure 2. Three-dimensional structures of the deduced FtFLS1 protein: (a) overall structure of FtFLS1, four α-helices (α1−α4) at the N-terminal; (b) iron-binding sites at His221, Asp223, and His277 and 2-oxoglutarate-binding sites at Arg287 and Ser289.

same volume of ethyl acetate twice and separated via silica gel G thin-layer chromatography (TLC; toluene/acetic 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 8000*g* 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 12000*g* 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).[20] 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-squared (R^2) = 0.9968 (Supporting Information, Supplementary Figure 1b).

FLS activity was routinely assayed at 37 °C and pH 5.0.[13] The concentration of purified FtFLS1, without removing $(His)_6$-tags, was measured using Coomassie brilliant blue G250.[21] As previously described,[13] 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.[20] 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.[22]

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.[23] 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 8000*g* 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.[24] The reaction process was as follows: One milliliter of diluted extracts containing flavonoids, 0.3 mL of 5% (w/w) $NaNO_2$, and 4 mL of 70% methanol (v/v) were mixed for 6 min, and then 0.3 mL of 10% $Al(NO_2)_3$ (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 WFJ2000 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

dx.doi.org/10.1021/jf205192q | J. Agric. Food Chem. 2012, 60, 5161−5168

highly similar to previously reported FLSs from other plants, for example, *Fagopyrum esculentum* (AEC33115, 99%), *Camellia nitidissima* (ADZ28516, 77%), *Camellia sinensis* (ACL98052, 76%), *Petunia hybrida* (Q07512, 73%), *Nicotiana tabacum* (ABE28017, 73%), and *Citrus unshiu* (Q9ZWQ9, 72%).

The amino acid sequence of FtFLS1 protein was analyzed using DNAman, SignalP, and TargetP software. The deduced protein contains 335 amino acids, with a molecular mass of 38.9 kDa and an isoelectric point of 6.07, a nonsecretory protein without predicted signal peptides or transmembrane signals. The secondary structure of FtFLS1 consisted of α-helices (31.94%), an extended strand (17.91%), a β-turn (4.78%), and random coils (45.37%; Supporting Information, Supplementary Figure 2). The multiple sequence alignment indicated that the conserved 2-ODD domains A and B were in FtFLS1,[6] and FtFLS1 also contained the conserved iron-binding sites His at 221, Asp at 223, and His at 277 and the conserved 2-oxoglutarate-binding sites Arg at 287 and Ser at 289 (Supporting Information, Supplementary Figure 2).[25]

For further understanding of the structure−function relationship, the 3D structure of FtFLS1 was built on the basis of the crystal structure of *Arabidopsis thaliana* anthocyanidin synthase (AtANS; PDB ID 1gp6A).[26] The sequence similarity between the FtFLS1 and AtANS homologue was about 43%, and the overall folding patterns of FtFLS1 were similar to the AtANS X-ray structure (Figure 2a). A nine-residue α-helix (α1) was present at the N-terminus of FtFLS1 located at the mouth of the jellyroll β-sheet motif, which may be associated with the structural stability and catalytic activity of FLSs in plants (Figure 2a).[27,28] There were three other α-helices (α2−α4) at 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 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.

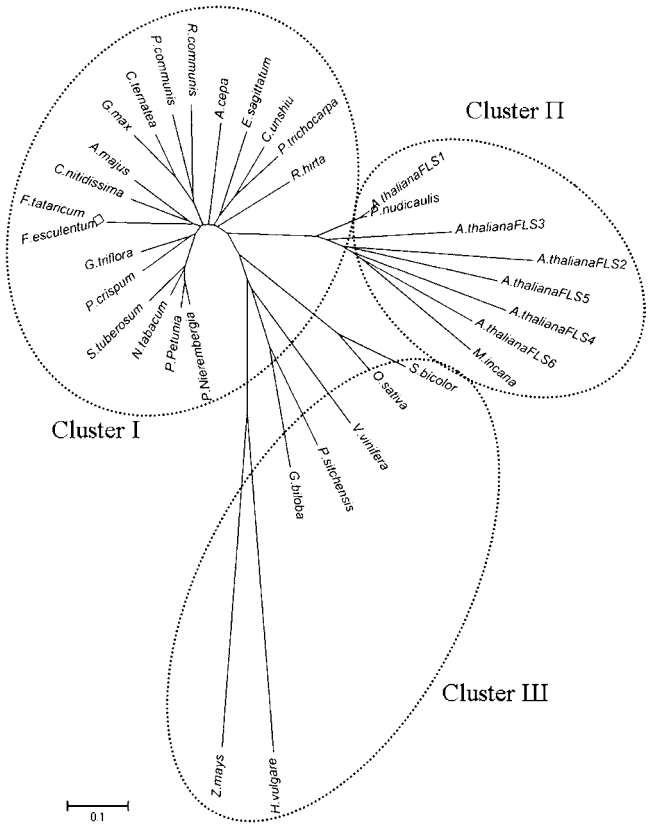

Figure 3. Phylogenetic tree of FtFLS1 and selected sequences. The bars represent evolutionary distance. FtFLS1 is indicated by a square (□). The following sequences, in addition to FtFLS1, were analyzed: *Allium cepa* (AAT68476); *Sorghum bicolor* (XP_002454608); *Zea mays* (NP_001146910); *Hordeum vulgare* (CBC71975); *Oryza sativa* (NP_001048230); *Rudbeckia hirta* (ABN79672); *Fagopyrum esculentum* (AEC33115); *Citrus unshiu* (BAA36554); *Camellia nitidissima* (ADZ28516); *Antirrhinum majus* (ABB53382); *Gentiana triflora* (BAK09226); *Pyrus communis* (ABB70118); *Glycine max* (BAF31231); *Vitis vinifera* (BAE75810); *Petroselinum crispum* (AAP57395); *Ricinus communis* (XP_002513774); *Nicotiana tabacum* (ABE28017); *Picea sitchensis* (EF087398); *Ginkgo biloba* (AY496932); *Epimedium sagittatum* (ABY63659); *Clitoria ternatea* (BAF49296); *Populus trichocarpa* (EEF00079); *Solanum tuberosum* (CAA63092); *Solanum tuberosum* (CAA63092); *Petunioideae Nierembergia* (BAC10995); *Petunia hybrida* (CAA80264); *Parrya nudicaulis* (ADY02654); *Matthiola incana* (AAB58800); *Arabidopsis thaliana*, AtFLS1 (NM_001203337), AtFLS2 (NM_125753), AtFLS3 (BT003134), AtFLS4 (NM_148158), AtFLS5 (AY114035), and AtFLS6 (NM_148083).

Functional Expression of FtFLS1 in *E. coli*. After IPTG-induced expression of FtFLS1, SDS-PAGE showed that the recombinant FtFLS1 was successfully expressed as a major protein product in the total cellular proteins of the bacterial culture (Supporting Information, Supplementary Figure 3), that is, ∼10% of the total protein. Its molecular mass was ∼40 kDa, which was in good agreement with both the deduced FtFLS1 protein estimate and the FLSs in other papers.[28] The standard samples in TLC were effectively separated, and the R_f values for standard dihydroquercetin, quercetin, dihydrokaempferol, and kaempferol were 0.33, 0.45, 0.54, and 0.62, respectively. The recombinant protein could catalyze the formation of quercetin and kaempferol from dihydroquercetin and dihydrokaempferol, whereas the negative control group could not (Figure 4). The

results demonstrated that *FtFLS1* encodes a functional protein with FLS activity.

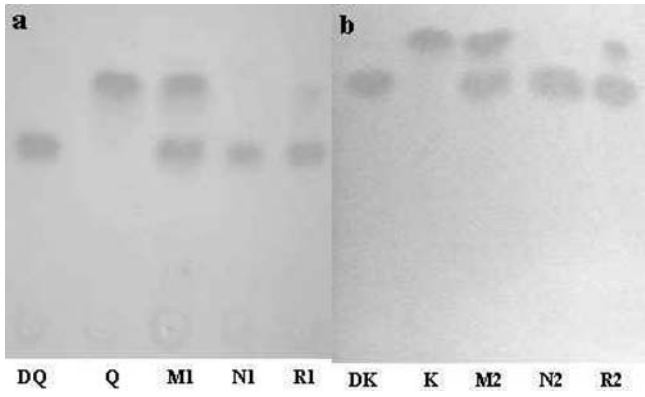

Figure 4. Functional examination of recombinant FtFLS1 in *E. coli*: (a) TLC of reaction products using dihydroquercetin as substrate (DQ, dihydroquercetin standard; Q, standard quercetin; M1, mixture of dihydroquercetin−quercetin; N1, only dihydroquercetin was detected in the negative control; R1, the substrate (dihydroquercetin) and product (quercetin) were detected in the experimental group); (b) TLC of reaction products using dihydrokaempferol as substrate (DK, dihydrokaempferol standard; K, standard kaempferol; M2, mixture of dihydrokaempferol−kaempferol; N2, only dihydrokaempferol was detected in the negative control; R2, the substrate (dihydrokaempferol) and product (kaempferol) were detected in the experimental group).

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

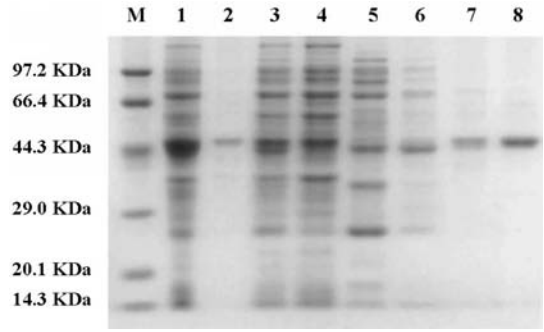

Figure 5. SDS-PAGE analysis of recombinant FtFLS1 in the purification process. Lanes: 1, sample of soluble fraction (5 mL); 2, sample of insoluble fraction; 3, sample collected after His binding process (5 mL); 4−7, samples collected after gradient rinsing with 40 mM (10 mL), 50 mM (1 mL), 100 mM (1 mL), and 150 mM (1 mL) imidazole buffers; 8, sample of purified FtFLS1 (1 mL).

total protein of the soluble fraction was 2.56 g/L; total activity and specific activity, using dihydroquercetin as substrate, were 66.03×10^{-3} IU and 5.16×10^{-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 36.55×10^{-3} IU and 18.94×10^{-3} IU/mg, respectively, and the total activity and specific activity for dihydrokaempferol were 10.19×10^{-3} IU and 5.28×10^{-3} IU/mg, respectively. Taken together, these results demonstrate that FtFLS1 corresponds to a bona fide flavonol synthase.[29]

Analysis of *FtFLS1* and Flavonoids during Florescence 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

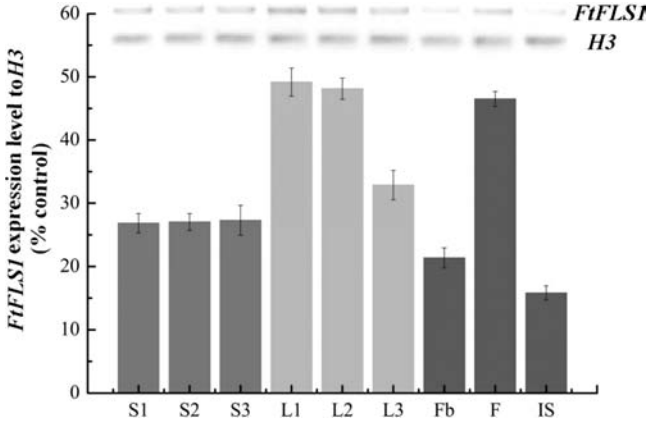

Figure 6. Expression analysis of *FtFLS1* in different organs during florescence. S1−S3, expression level of *FtFLS1* in stems; L1−L3, expression level of *FtFLS1* in leaves; Fb, expression level of *FtFLS1* in flower buds; F, expression level of *FtFLS1* in flowers; Is, expression level of *FtFLS1* in immature seeds. There was no significant difference ($P > 0.05$) among S1, S2, and S3 in stems or between L1 and L2 in leaves. There was a significant difference ($P < 0.05$) among L1 and L3, L2 and L3, and three stages in reproductive organs.

organ-specific expression patterns ($P < 0.05$), which is consistent with *FeFLS* in *F. esculentum*[15] and the recent study in *F. tataricum*.[14] 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 florescence, with little change ($P > 0.05$).

Results of the total flavonoids in *F. tataricum* are presented in Table 1. During florescence, organ differences in total flavonoid content were apparent. The average total flavonoid content during florescence 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 inflorescence. In stems, the total flavonoid contents at initial, full, and postbloom were 0.16 ± 0.05, 0.17 ± 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

dx.doi.org/10.1021/jf205192q | J. Agric. Food Chem. 2012, 60, 5161−5168

Table 1. Flavonoid Content (Milligrams per 500 mg Fresh Weight, Percent) in Different Organs of *F. tataricum*[a]

organ	initial bloom	full bloom	postbloom
stems	$0.16 \pm 0.05_{c}$	$0.17 \pm 0.02_{c}$	$0.23 \pm 0.09_{c}$
leaves	$1.61 \pm 0.12^{ab}_{b}$	$1.71 \pm 0.17^{a}_{b}$	$1.12 \pm 0.11^{b}_{b}$
reproductive organs	$2.11 \pm 0.21^{c}_{a}$ (flower buds)	$3.52 \pm 0.12^{b}_{a}$ (flowers)	$2.50 \pm 0.12^{a}_{a}$ (immature seeds)

[a]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.[1] 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.[6,7,11]

In the 2-ODD family, four structural genes are involved in flavonoid biosynthesis, namely FLS, flavanone 3β-hydroxylase (F3H), flavone synthase (FNS), and anthocyanidin synthase (ANS).[7] By comparing the nucleotide and amino acid sequences of FtFLS1 to other FLSs, we found that it has the conserved motifs and active sites typical of FLSs (Supporting Information, Supplementary Figure 2), which corresponds with other studies.[20,27,28] In addition, the residue His at 75, which is located in the conserved domain A, had a marginal effect on the enzyme activity of *P. hybrida* FHT (a F3H enzyme).[30] The residue Pro at 207, which is located in the conserved domain B, did not affect the activity to a significant extent, and the replacement of the Gly residue by Ala reduced the FLS activity in *C. unshiu*.[13]

In the current study, the deduced secondary structure indicated that FtFLS1 is rich in random coils and α-helices (Supporting Information, Supplementary Figure 2). Importantly, the absence of the first α-helix in AtFLS1 could cause loss of activity,[9] and the mutation or deletion of this region in AtFLS2 to AtFLS6 proteins would cause the inactivation of expression products.[27] Accordingly, these loose but neatly arranged α-helices in FtFLS1 may provide the conformational flexibility for the polypeptide backbone. Together, all of the molecular features of FtFLS1 suggest that it could catalyze the formation of flavonol from dihydroflavonol in *F. tataricum*.

In *A. thaliana*, the recombinant AtFLS1 was more effective at converting dihydrokaempferol to kaempferol than dihydroquercetin to quercetin[27] and had a specific activity similarto that of FtFLS1 in this study.[20] Wellmann et al.[13] studied a variety mutations of *C. unshiu* 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 cloned form *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.[11] 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.[10] 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.[27] 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^{2+}, 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,[31] and its expression influenced flavonoid levels and the root gravitropic response in seedlings under nonstressed conditions.[27] *FLS1* could be activated by flavonol-specific transcription factors (TFs) MYB11, MYB12, and MYB111 of *A. thaliana*,[32] and these TFs caused different spatial accumulation of specific flavonol derivatives in leaves, stems, inflorescences, siliques, and roots.[33] It is reported that the anthocyanin (C1/PL1 + R/B) and 3-deoxy flavonoid (P1) TFs mediate the expression of *FLS1* in *Z. mays*, an expression that is also induced by UV-B.[11] In *C. unshiu*, *CtFLS* transcript levels were higher in leaves during the early developmental stage than at maturity and increased in the peel during fruit maturation.[29] In a recent case described by Gupta et al.,[14] the flavonoid synthesis genes in *F. tataricum* (*FLS*, *PAL*, *CHS*, and *CHI*) were more highly expressed than in *F. esculentum*. In our study, the expression pattern of *FtFLS1* was similar to that of *FtCHS* in *F. tataricum*.[23] 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.[14,15,34] Fabjan et al.[1] 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.[34] analyzed the seeds of 11 cultured buckwheat

species and found that both the rutin and total favonoid contents were signifcantly 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.,[35] 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* tt4 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.[37] 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.[38] 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 in *A. thaliana*.[40] 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 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.

■ ASSOCIATED CONTENT

Supporting Information

Additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: wuqiwq@yahoo.cn.

Funding

This work was supported by the Department of Science and Technology of Sichuan Province, China (2006Z08-012).

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.

■ REFERENCES

(1) Fabjan, N.; Rode, J.; Košir, I. J.; Wang, Z.; Zhang, Z.; Kreft, I. Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) as a source of dietary rutin and quercitrin. *J. Agric. Food Chem.* **2003**, *51* (22), 6452−6455.

(2) Conesa, C. M.; Ortega, V. V.; Gascón, M. J. Y.; Baños, M. A.; Jordana, M. C.; Benavente-García, O.; Castillo, J. Treatment of metastatic melanoma B16F10 by the flavonoids tangeretin, rutin, and diosmin. *J. Agric. Food Chem.* **2005**, *53* (17), 6791−6797.

(3) Harborne, J. B.; Williams, C. A. Advances in flavonoid research since 1992. *Phytochemistry* **2000**, *55* (6), 481−504.

(4) Hagmann, M. L.; Heller, W.; Grisebach, H. Induction and characterization of a microsomal flavonoid 3′-hydroxylase from parsley cell cultures. *Eur. J. Biochem.* **1983**, *134* (3), 547−554.

(5) Kaltenbach, M.; Schröder, G.; Schmelzer, E.; Lutz, V.; Schröder, J. Flavonoid hydroxylase from *Catharanthus roseus*: cDNA, heterologous expression, enzyme properties and cell-type specific expression in plants. *Plant J.* **1999**, *19* (2), 183−193.

(6) Holton, T. A.; Brugliera, F.; Tanaka, Y. Cloning and expression of flavonol synthase from *Petunia hybrida*. *Plant J.* **1993**, *4* (6), 1003−1010.

(7) Turnbull, J. J.; Nakajima, J.; Welford, R. W.; Yamazaki, M.; Saito, K.; Schofield, C. J. Mechanistic studies on three 2-oxoglutarate-dependent oxygenases of flavonoid biosynthesis: anthocyanidin synthase, flavonol synthase, and flavanone 3β-hydroxylase. *J. Biol. Chem.* **2004**, *279*, 1206−1216.

(8) Britsch, L.; Heller, W.; Grisebach, H. Conversion of flavanone to flavone, dihydroflavonol and flavonol with an enzyme system from cell culture of parsley. *Z. Naturforsch.* **1981**, *36* (9/10), 742−750.

(9) Pelletier, M. K.; Burbulis, I. E.; Winkel-Shirley, B. Disruption of specific flavonoid genes enhances the accumulation of flavonoid enzymes and end-products in *Arabidopsis* seedlings. *Plant Mol. Biol.* **1999**, *40* (1), 45−54.

(10) Preuß, A.; Stracke, R.; Weisshaar, B.; Hillebrecht, A.; Matern, U.; Martens, S. *Arabidopsis thaliana* expresses a second functional flavonol synthase. *FEBS Lett.* **2009**, *583* (12), 1981−1986.

(11) Ferreyra, M. L. F.; Rius, S.; Emiliani, J.; Pourcel, L.; Feller, A.; Morohashi, K.; Casati, P.; Grotewold, E. Cloning and characterization of a UV-B-inducible *maize* flavonol synthase. *Plant J.* **2010**, *62* (1), 77−91.

(12) Kim, B. G.; Joe, E. J.; Ahn, J. H. Molecular characterization of flavonol synthase from poplar and its application to the synthesis of 3-*O*-methylkaempferol. *Biotechnol. lett.* **2010**, *32* (4), 579−584.

(13) Wellmann, F.; Lukačin, R.; Moriguchi, T.; Britsch, L.; Schiltz, E.; Matern, U. Functional expression and mutational analysis of flavonol synthase from *Citrus unshiu*. *Eur. J. Biochem.* **2002**, *269* (16), 4134−4142.

(14) Gupta, N.; Sharma, S. K.; Rana, J. C.; Chauhan, R. S. Expression of flavonoid biosynthesis genes vis-à-vis rutin content variation in different growth stages of *Fagopyrum* species. *J. Plant Physiol.* **2011**, *168* (17), 2117−2123.

(15) Li, X.; Park, N. I.; Xu, H.; Woo, S. H.; Park, C. H.; Park, S. U. Differential expression of flavonoid biosynthesis genes and accumulation of phenolic compounds in common buckwheat (*Fagopyrum esculentum*). *J. Agric. Food Chem.* **2010**, *58* (23), 12176−12181.

(16) Park, N. I.; Li, X.; Suzuki, T.; Kim, S. J.; Woo, S. H.; Park, C. H.; Park, S. U. Differential expression of anthocyanin biosynthetic genes and anthocyanin accumulation in tartary buckwheat cultivars 'Hokkai T8' and 'Hokkai T10'. *J. Agric. Food Chem.* **2011**, *59* (6), 2356−2361.

(17) Geourjon, C.; Deleage, G. SOPM: a self-optimized method for protein secondary structure prediction. *Protein Eng.* **1994**, *7* (2), 157−164.
(18) Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics* **2006**, *22* (2), 195−201.
(19) Kumar, S.; Tamura, K.; Nei, M. MEGA: molecular evolutionary genetics analysis software for microcomputers. *Comput. Appl. Biosci.* **1994**, *10* (2), 189−191.
(20) Chua, C. S.; Biermann, D.; Goo, K. S.; Sim, T. S. Elucidation of active site residues of *Arabidopsis thaliana* flavonol synthase provides a molecular platform for engineering flavonols. *Phytochemistry* **2008**, *69* (1), 66−75.
(21) Sedmak, J. J.; Grossberg, S. E. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* **1977**, *79* (1−2), 544−552.
(22) Prescott, A. G.; John, P. Dioxygenases: molecular structure and role in plant metabolism. *Annu. Rev. Plant Biol.* **1996**, *47* (1), 245−271.
(23) Wu, Q.; Li, C.; Chen, H.; Shao, J.; Zhao, H.; Gou, L. Molecular structure characterization of *Fagopyrum tataricum* chalcone synthase and its tissue-specific expression during florescence. *Chin. J. Biochem. Mol. Biol.* **2010**, *26* (12), 1151−1160.
(24) Liu, B.; Zhu, Y. Extraction of flavonoids from flavonoid-rich parts in tartary buckwheat and identification of the main flavonoids. *J. Food Eng.* **2007**, *78* (2), 584−587.
(25) Kim, B. G.; Kim, J. H.; Kim, J.; Lee, C.; Ahn, J. Accumulation of flavonols in response to ultraviolet-B irradiation in soybean is related to induction of flavanone 3-β-hydroxylase and flavonol synthase. *Mol. Cells* **2008**, *25* (2), 247−252.
(26) Wilmouth, R. C.; Turnbull, J. J.; Welford, R. W. D.; Clifton, I. J.; Prescott, A. G.; Schofield, C. J. Structure and mechanism of anthocyanidin synthase from *Arabidopsis thaliana*. *Structure* **2002**, *10* (1), 93−103.
(27) Owens, D. K.; Alerding, A. B.; Crosby, K. C.; Bandara, A. B.; Westwood, J. H.; Winkel, B. S. J. Functional analysis of a predicted flavonol synthase gene family in *Arabidopsis*. *Plant Physiol.* **2008**, *147* (3), 1046−1061.
(28) Lin, G. Z.; Lian, Y. J.; Ryu, J. H.; Sung, M. K.; Park, J. S.; Park, H. J.; Park, B. K.; Shin, J. S.; Lee, M. S.; Cheon, C. I. Expression and purification of His-tagged flavonol synthase of *Camellia sinensis* from *Escherichia coli*. *Protein Express. Purif.* **2007**, *55* (2), 287−292.
(29) Moriguchi, T.; Kita, M.; Ogawa, K.; Tomono, Y.; Endo, T.; Omura, M. Flavonol synthase gene expression during *Citrus* fruit development. *Physiol. Plant.* **2002**, *114* (2), 251−258.
(30) Lukačin, R.; Britsch, L. Identification of strictly conserved histidine and arginine residues as part of the active site in *Petunia hybrida* flavanone 3β-hydroxylase. *Eur. J. Biochem.* **1997**, *249* (3), 748−757.
(31) Pelletier, M. K.; Murrell, J. R.; Shirley, B. W. Characterization of flavonol synthase and leucoanthocyanidin dioxygenase genes in *Arabidopsis* (further evidence for differential regulation of "early" and "late" genes). *Plant Physiol.* **1997**, *113* (4), 1437−1445.
(32) Stracke, R.; Ishihara, H.; Huep, G.; Barsch, A.; Mehrtens, F.; Niehaus, K.; Weisshaar, B. Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. *Plant J.* **2007**, *50* (4), 660−677.
(33) Stracke, R.; Jahns, O.; Keck, M.; Tohge, T.; Niehaus, K.; Fernie, A. R.; Weisshaar, B. Analysis of production of flavonol glycosides-dependent flavonol glycoside accumulation in *Arabidopsis thaliana* plants reveals MYB11-, MYB12- and MYB111-independent flavonol glycoside accumulation. *New Phytol.* **2010**, *188* (4), 985−1000.
(34) Jiang, P.; Burczynski, F.; Campbell, C.; Pierce, G.; Austria, J. A.; Briggs, C. J. Rutin and flavonoid contents in three buckwheat species *Fagopyrum esculentum*, *F. tataricum*, and *F. homotropicum* and their protective effects against lipid peroxidation. *Food Res. Int.* **2007**, *40* (3), 356−364.
(35) Kim, S. J.; Zaidul, I. S. M.; Suzuki, T.; Mukasa, Y.; Hashimoto, N.; Takigawa, S.; Noda, T.; Matsuura-Endo, C.; Yamauchi, H. Comparison of phenolic compositions between common and tartary buckwheat (*Fagopyrum*) sprouts. *Food Chem.* **2008**, *110* (4), 814−820.
(36) Zhao, J.; Dixon, R. A. The 'ins' and 'outs' of flavonoid transport. *Trends Plant Sci.* **2010**, *15* (2), 72−80.
(37) Buer, C. S.; Muday, G. K.; Djordjevic, M. A. Implications of long-distance flavonoid movement in *Arabidopsis thaliana*. *Plant Signal. Behav.* **2008**, *3* (6), 415−417.
(38) Saslowsky, D. E.; Warek, U.; Winkel, B. S. J. Nuclear localization of flavonoid enzymes in *Arabidopsis*. *J. Biol. Chem.* **2005**, *280* (25), 23735−23740.
(39) Alfenito, M. R.; Souer, E.; Goodman, C. D.; Buell, R.; Mol, J.; Koes, R.; Walbot, V. Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione *S*-transferases. *Plant Cell* **1998**, *10* (7), 1135−1150.
(40) Kitamura, S.; Shikazono, N.; Tanaka, A. Transparent testa 19 is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *Plant J.* **2004**, *37* (1), 104−114.