Identification and characterization of a rhamnosyltransferase involved in rutin biosynthesis in *Fagopyrum esculentum* (common buckwheat)

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Abstract

Rutin, a 3-rutinosyl quercetin, is a representative flavonoid distributed in many plant species, and is highlighted for its therapeutic potential. In this study, we purified uridine diphosphate-rhamnose: quercetin 3-*O*-glucoside 6″-*O*-rhamnosyltransferase and isolated the corresponding cDNA (*FeF3G6″RhaT*) from seedlings of common buckwheat (*Fagopyrum esculentum*). The recombinant FeF3G6″RhaT enzyme expressed in *Escherichia coli* exhibited 6″-*O*-rhamnosylation activity against flavonol 3-*O*-glucoside and flavonol 3-*O*-galactoside as substrates, but showed only faint activity against flavonoid 7-*O*-glucosides. Tobacco cells expressing *FeF3G6″RhaT* converted the administered quercetin into rutin, suggesting that FeF3G6″RhaT can function as a rhamnosyltransferase *in planta*. Quantitative PCR analysis on several organs of common buckwheat revealed that accumulation of *FeF3G6″RhaT* began during the early developmental stages of rutin-accumulating organs, such as flowers, leaves, and cotyledons. These results suggest that FeF3G6″RhaT is involved in rutin biosynthesis in common buckwheat.

Key words: common buckwheat, flavonoid biosynthesis; rhamnosyltransferase; rutin

Introduction

Flavonoids are typical secondary products produced by plants, in which more than 8,000 derivatives have been reported [1]. Flavonoids have various roles *in planta*, and function as color pigments in flowers and fruits, UV-B protectants, and antimicrobial agents, and are also involved in signaling during plant–animal and plant–microbe interactions [2,3]. Flavonoids usually accumulate in plants in modified form, including those that are glycosylated, acylated, and methylated. Overall, glycosylation is the most common and important modification, since it greatly affects the properties of the compounds, such as their stability, water solubility, and biological activities [4,5].

These glycosylation steps are catalyzed by glycosyltransferases (GTs). Two types of flavonoid GTs have been found in plants; uridine diphosphate (UDP)-sugar dependent glycosyltransferases (UGT), which belongs to the glycosyltransferase family 1 [6], and acylglucose-dependent glucosyltransferases, which belongs to the glycoside hydrolase family 1 [7]; Most of the reported GTs are UGTs. Many GTs involved in flavonoid biosynthesis have been found, most of which are responsible for the formation of a glycosidic bond between the flavonoid molecule and the sugar moiety; in contrast, fewer GTs are responsible for the formation of a sugar-sugar bond in flavonoid glucosides.

Rutin, a 3-rutinosyl quercetin, is a representative flavonoid present in many plant species, including rue (*Ruta graveolens*), buckwheat (*Fagopyrum sp.*), Japanese pagoda tree (*Sophora japonica*), onion (*Allium cepa*), viola (*Viola tricolor*), thyme (*Thymus* sp.), and tobacco (*Nicotiana tabacum*) [8,9]. Rutin protects against UV-irradiation *in planta* owing to its antioxidant capabilities [1,10]. Rutin has also studied for its therapeutic potential, including its antioxidant, anti-diabetic, anti-inflammatory, anti-cancer,

anti-hypotensive, and cardiovascular protective activities [9–11].

The structure of rutin is characterized by the presence of rutinose residue, an α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside that is connected to the 3-hydroxyl group of quercetin, which is produced by glucosylation of quercetin and subsequent rhamnosylation on the glucose moiety of consequent isoquercitrin (Figure 1). At present, some members of rhamnosyltransferases, which catalyze rhamnosylation of the sugar moiety of flavonoid glycosides, have been characterized. These include flavanone 7-*O*-glucoside 6″-*O*-rhamnosyltransferase from *Citrus sinensis* and *C. maxima* [12,13], flavanone 7-*O*-glucoside 2″-*O*-rhamnosyltransferase from *C. maxima* [14], anthocyanidin-3-*O*-glucoside 6″-*O*-rhamnosyltransferase from *Petunia hybrida* [15] and *Lobelia erinus* [16], and flavonoid 3-*O*-glucoside 6″-*O*-rhamnosyltransferase from *Glycine max* [17].

[Figure 1 near here]

Buckwheat (*Fagopyrum* sp.) is a pseudocereal, whose achenes (seeds) are used as food all over the world and are considered a healthy food source [18]. Buckwheat accumulates large amounts of rutin, mainly in its flowers and leaves (c.a. 3–10% of dry weight), and also in immature seeds and cotyledons [19,20]. The biosynthesis of rutin in buckwheat has been well-studied, and the genes encoding enzymes involved in the biosynthesis of rutin have been elucidated, except for the final two glycosylation steps [21,22]. Quercetin 3-*O*-glucosyltransferase has been purified from common buckwheat [23]; however, the corresponding gene has not yet been reported, which may be due to the existence of a wide variety of UGTs in these plants. The final step catalyzed by

rhamnosyltransferase for quercetin 3-*O*-glucoside has not yet been identified [24].

In the present study, we purified quercetin 3-*O*-glucoside 6″-*O*-rhamnosyltransferase (F3G6″RhaT) and isolated the corresponding gene (*FeF3G6″RhaT*) from cotyledons of common buckwheat. We characterized FeF3G6″RhaT activity using a recombinant enzyme expressed in *Escherichia coli* and confirmed its function *in planta* by expressing the *FeF3G6″RhaT* gene in tobacco cells. We also studied the accumulation of *FeF3G6″RhaT* transcripts and flavonoids during different developmental stages in several organs, and used this information to further elaborate the pathway of rutin biosynthesis in common buckwheat.

Materials and methods

Plant materials

A diploid cultivar of common buckwheat (*Fagopyrum esculentum* Moench cultivar Shinano No. 1) was used in this study. For enzyme preparation, seeds were placed on a paper towel wet with slightly acidic electrolyzed water (Purester, Morinaga Milk Industry, Tokyo, Japan) and germinated in the dark at 25°C. Four days after wetting, cotyledons were collected from etiolated seedlings, frozen with liquid nitrogen, and stored at −80°C until use. For preparation of plant materials, seeds were sown on culture soil and cultured at 22°C under 14 h light/10 h dark conditions.

BY-2 cells of *Nicotiana tabacum* L. cultivar Bright Yellow-2 were maintained using modified Linsmaier and Skoog (LS) medium as described previously [25,26].

Reagents

Substrates used for enzyme reactions were obtained as follows: quercetin, UDP-glucose, UDP-glucuronic acid (Nacalai Tesque, Kyoto, Japan), rutin, kaempferol, phloridzin, (Tokyo Chemical Industries, Tokyo, Japan), quercetin 3-*O*-glucoside, UDP-galactose (Sigma-Aldrich, St-Louis, MO, USA), quercetin 3-*O*-galactoside (Extrasynthèse, Genay, France), daidzin (Fujikko, Kobe, Japan), 4-methylumbelliferone glucoside (Wako Pure Chemical Industries, Osaka, Japan), NAD^+, and NADPH (Oriental Yeast, Tokyo, Japan). Kaempferol 7-*O*-glucoside, quercetin 7-*O*-glucoside, 7-hydroxyflavone glucoside, 3-hydroxyflavone glucoside, kaempferol 3,7-*di*-*O*-glucoside, 1-naphthol glucoside, and 2-naphthol glucoside were obtained from our laboratory's stock [26]. All other chemicals were obtained from Sigma-Aldrich, Nacalai Tesque, Wako Pure Chemical

Industries, and Kanto Chemical (Tokyo, Japan) unless otherwise specified.

PCR primers

PCR primers used in this work are listed in Table S1.

Preparation of UDP-rhamnose

UDP-rhamnose was prepared as previously described [27], with some modifications. cDNA encoding UDP-rhamnose synthase (*Rhm2*, At1g53500) was PCR-amplified using an *Arabidopsis thaliana* leaf cDNA library [28] as a template with the primer pair (AtRhm2-Fw and AtRhm2-Rv, Table S1), and *Ex Taq* (Takara Bio, Kusatsu, Japan) under the following conditions: 94°C for 3 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 2 min; followed by 72°C for 5 min. The amplified fragment was cloned into the pET32a(+) vector (Merck, Darmstadt, Germany) at *Eco* RI and *Xho* I restriction sites and introduced into *E. coli* BL21(DE3) strain (Merck). The recombinant enzyme fused with thioredoxin (Rhm2) was expressed in accordance with the manufacturer's instructions with an addition of 0.4 mM isopropyl thiogalactopyranoside and incubated at 22°C for 16 h. Soluble crude enzymes were extracted from *E. coli* cells using an extraction buffer: 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol and 150 mM KCl. The extracted recombinant enzyme was purified with His-GraviTrap (GE Healthcare, Tokyo, Japan) according to the manufacturer's instructions, and the purified enzyme was then concentrated using Amicon Ultra-15 Ultracel-10k (Merck Millipore, Billerica, MA, USA). The reaction mixture (200 µL) containing an aliquot of Rhm2 enzyme (50 µL), 20 mM UDP-glucose, 5 mM NAD^+, 5 mM NADPH, 50 mM Tris-HCl (pH 8.0), and 5 mM 2-mercaptoethanol was incubated

146 at 30°C for 12 h. Chloroform (200 µL) was added to the reaction mixture, which was
147 then vortexed. After centrifugation, the water phase was recovered and used as
148 UDP-rhamnose (Figure S1).
149
150 ***Analysis of phenolic compounds in buckwheat***
151 Frozen samples of buckwheat ground with a mortar and pestle (100 mg fresh weight)
152 were extracted with methanol (1 mL) overnight at -20°C. The resultant extracts were
153 centrifuged at 17,000 × *g* for 10 min, and the supernatants were filtered through a
154 0.2-µm polytetrafluoroethylene filter (Merck Millipore) and analyzed by HPLC.
155
156 ***HPLC conditions***
157 HPLC-mass spectrometry (MS) was performed using a Waters UPLC ACQUITY SQD
158 system (Waters, Milford, MA, USA) with an electron-spray ionization probe. For
159 separation of phenolic compounds, samples were eluted from an octadecylsilyl (ODS)
160 column (2.1 mm i.d. × 50 mm: ACQUITY UPLC BEH C18 1.7µm Column, Waters) at
161 a flow rate of 0.25 mL min^{-1} at 40°C with 10% solvent B (acetonitrile containing 0.1%
162 formic acid) in solvent A (0.1% formic acid) for 0.5 min, followed by 10 to 50% solvent
163 B in A for 4 min, and finally, 10% solvent B in A for 2 min. Alternatively, samples were
164 eluted from a 100 mm ODS column (2.1 mm i.d. × 100 mm: ACQUITY UPLC BEH
165 C18 1.7µm Column, Waters) with 10% solvent B in A for 0.5 min, followed by 10 to
166 50% solvent B in A for 6 min, and finally, 50% solvent B in A for 0.5 min. For the
167 analysis of tobacco cell extracts, samples were eluted from a 100 mm ODS column with
168 15% solvent B in A for 0.5 min, followed by 15 to 35% solvent B in A for 4 min, and
169 finally, 35% solvent B in A for 0.5 min. For the analysis of methanol extracts from

buckwheat organs, samples were eluted from an ODS column (2.1 mm i.d. × 50 mm: Kinetex 1.7μ C18, Phenomenex, Torrance, CA) with 20% solvent C (methanol containing 0.1% formic acid) in A for 0.5 min, followed by 20 to 30% solvent C in A for 1 min, 30% solvent C in A for 3 min, 30 to 60% solvent C in A for 3 min, and finally, 60% solvent C in A for 2 min. For analysis of UDP-glucose and UDP-rhamnose, a 100 mm ODS column was used for elution with 20 mM triethylamine acetate (pH 7.0) containing 0.5% acetonitrile for 12 min.

Assay of rhamnosyltransferase activity

Rhamnosyltransferase activity was assayed in a reaction mixture (50 μL) composed of the enzyme prepared in the steps below, 200 μM substrate (quercetin 3-*O*-glucoside), 2 μL of UDP-rhamnose solution (approximately 400 μM at final concentration) in buffer A (50 mM Tris-HCl [pH 7.5], 10 mM 2-mercaptoethanol). The reaction was performed at 30°C for 5–30 min. The reaction was stopped by the addition of 10 μL of 1 M HCl, followed by the addition of 100 μL of methanol and an internal standard for HPLC analysis.

Purification of rhamnosyltransferase enzymes from common buckwheat

Frozen etiolated cotyledon (240 g fresh weight) was ground in a mortar in the presence of liquid nitrogen, and then buffer A (500 mL) containing 5% (w/v) polyvinylpolypyrrolidone and 0.1 mM phenylmethylsulfonyl fluoride was added. Enzyme purification was carried out at 4°C. The mixture was sonicated for 30 × 3 sec at an amplitude of 40% (Vibra Cell VCX500, Sonic & Materials, Inc., Newtown, CT, USA). The filtrate was passed through two layers of a non-woven cloth, and the filtrate

194 was centrifuged at 10,000 ×g for 15 min. The supernatant was saved as a crude enzyme
195 fraction. The pellet was extracted again with 250 mL of buffer A, and the resultant
196 supernatant was added to the crude fraction. The enzyme was fractionated by
197 ammonium sulfate precipitation (30–70% saturation), dissolved in buffer A, and then
198 centrifuged at 93,000 ×g for 30 min to remove lipids and the membrane fraction. The
199 supernatant containing the enzyme was added with ammonium sulfate (1 M at final
200 concentration), loaded onto the Phenyl Sepharose CL-4B column (15 mm i.d. × 100
201 mm; GE Healthcare), equilibrated with buffer A containing 1 M ammonium sulfate, and
202 finally, eluted with 40 mL each of buffer A containing 1, 0.5, 0.3, 0.2, 0.1, and 0 M of
203 ammonium sulfate. The fraction with activity was fractionated by ammonium sulfate
204 (70% saturation) and loaded onto the Sephadex G-100 column (20 mm i.d. × 500 mm;
205 GE healthcare) equilibrated with buffer A. Elution was performed with 200 mL of
206 buffer A, and the fraction containing the enzyme was loaded onto the DEAE Sepharose
207 FF column (15 mm i.d. × 100 mm; GE healthcare) equilibrated with buffer A. After
208 washing the column with 60 mL of buffer A, elution was performed with a linear
209 gradient of NaCl (100 mL, 0 to 150 mM) in buffer A. The fraction containing enzymatic
210 activity was pooled, concentrated, and then loaded onto the Reactive Green 19-agarose
211 column (8 mm i.d. × 15 mm; Sigma Aldrich) equilibrated with buffer A containing 10
212 mM NaCl. After washing the column with the same buffer, elution was done with 10
213 mL each of buffer A containing 20, 50, 100, and 1000 mM NaCl. The fraction
214 containing enzymatic activity was concentrated and desalted by Amicon-Ultra 15 and
215 the enzyme was then purified by HPLC (Multi-station LC-8020 Model II, Tosoh, Tokyo,
216 Japan) with a Mono Q™ 5/50 GL column (GE Healthcare). The sample was loaded onto
217 the column equilibrated with buffer A. Elution was performed with buffer A for 5 min,

followed by a linear gradient of 0–150 mM NaCl in buffer A for 45 min at a flow rate of 1 mL min^{-1}.

Sequencing analysis of peptides

Peptide fragments of the purified rhamnosyltransferase digested with lysyl endopeptidase were prepared as previously described [29]. Following this, the peptide fragments were separated using a Waters UPLC Xevo Qtof system (Waters) with a 0–40% linear acetonitrile gradient for 60 min. The data were processed using ProteinLynx Global Server 2.2.5 (Waters). Homology search of the peptide sequences obtained from sequencing was performed against the Genbank database (https://www.ncbi.nlm.nih.gov/) using the BLASTP program.

Cloning and sequencing of the FeF3G6″RhaT gene

Degenerate primers (GDQ-Rv1 and SFF-Fw1, Table S1) were designed from the sequences of peptide fragments that showed similarity to reported UGTs. Partial fragments of the *FeF3G6″RhaT* gene were amplified by PCR using a cDNA library that was constructed using common buckwheat cotyledon [29] as a template. The PCR was performed three times with three distinct primer pairs: SFF-Fw1 and GDQ-Rv1, vector (pDONR222) sequence (M13-FL) and GDQ-Rv1, SFF-Fw1 and vector sequence (M13-Rv), using iProof™ High Fidelity DNA polymerase (Bio-Rad, Hercules, CA, USA) under the following conditions: 98°C for 30 sec; 30 cycles of 98°C for 10 sec, 50°C for 20 sec, and 72°C for 1 min; followed by 72°C for 5 min. The resulting fragments were cloned into the pCR4Blunt-TOPO vector (ThermoFisher Scientific, Yokohama, Japan) and sequenced using a DNA sequencer (3130xl Genetic Analyzer,

242 ThermoFisher Scientific). The DNA fragments corresponding to the coding region were
243 amplified from the cDNA library with FeRhaT-F1 and FeRhaT-R1 using *Ex Taq*
244 (Takara Bio, Kusatsu, Japan) under the following conditions: 94°C for 3 min; 30 cycles
245 of 94°C for 30 sec, 68°C for 30 sec, and 72°C for 105 sec; followed by 72°C for 5 min.
246 The amplified fragment was cloned into a dT-protruding pBlueScript SK^- vector and
247 sequenced.
248
249 ***RNA-sequencing analysis***
250 Total RNA was extracted from etiolated cotyledons (4 days after wetting for
251 germination), matured leaves, and flowers of common buckwheat as described
252 previously [29]. RNA quality was evaluated using a BioAnalyzer 2100 (Agilent
253 Technologies, Santa Clara, CA, USA). The cDNA libraries were constructed using the
254 TruSeq Prep Kit v2 (Illumina, San Diego, CA, USA), and the resulting cDNA libraries
255 were sequenced by next-generation sequencing GAIIx (Illumina) with 100-bp
256 paired-end reads. Total reads obtained from GAIIx and public dbEST
257 (https://www.ncbi.nlm.nih.gov/dbEST/) of *Fagopyrum_esculentum* were
258 hybrid-assembled using CLC Genomics Workbench version 4.8 (CLC Bio) with the
259 following parameters: minimum contig length, 300; performed without scaffolding to
260 obtain assembled contigs after adaptor sequences and low-quality reads were removed.
261 These contig sequences were used to construct an expressed sequence tags (EST)
262 database using a BLASTx program [30]-based homology search against the NCBI-nr
263 protein database (http://www.ncbi.nlm.nih.gov) using a cutoff E-value $<10^{-5}$ as queries.
264
265 ***Heterologous expression of FeF3G6″RhaT in E. coli***

The coding regions of *FeF3G6″RhaT* were subcloned into the pET28a(+) vector (Merck, Darmstadt, Germany) at *Nde* I and *Xho* I restriction sites. The resulting plasmids (pET-FeRhaT) were introduced into *E. coli* Rosetta™ 2(DE3) (Merck). The recombinant *FeF3G6″RhaT* was expressed and extracted according to the manufacturer's instructions. The recombinant proteins were purified using a His-GraviTrap and concentrated using Amicon Ultra-15.

Characterization of the recombinant FeF3G6″RhaT

The enzymatic assay for recombinant FeF3G6″RhaT was performed as follows: a reaction mixture (50 µL) was prepared with 200 µM flavonoid substrate, 2.5 µL of UDP-rhamnose solution (approximately, 500 µM at final concentration), and 1–500 ng of the purified enzymes in the reaction buffer [50 mM Tris-HCl (pH 8.5), containing 0.01% BSA and 5 mM 2-mercaptoethanol]. The reaction was initiated by adding substrate and incubated at 30°C for 5–30 min. The reaction was terminated by adding 10 µL of 1M HCl. To check the effect of pH on enzymatic activity, 100 mM Bistris propane buffer (pH 6.5 to 10.0) was used as a buffer and quercetin 3-*O*-glucoside was used as a substrate. To investigate the optimal temperature, the reaction was performed at 20 to 60°C. Substrate specificity of the recombinant FeF3G6″RhaT was confirmed using the flavonoid glycosides and related phenolic compounds (100 µM) as substrates.

Quantitative RT-PCR analysis

Total RNA was extracted from several organs of common buckwheat [cotyledons (2–3 days and 2 weeks after wetting for germination), young root from seedling, hypocotyl (2–3 days after sowing), stem, leaves (leaf bud; primary leaf after development;

matured leaf), flowers (floral bud in early stage; flowering stage), and immature seed (achene)] using the phenol-SDS method, as described previously [29]. Root samples were prepared from seedlings only, because isolation of RNA from the matured root was not successful. Total RNA (0.5 µg each) was used for first-strand cDNA synthesis in a 10 µL reaction using a PrimeScript RT Reagent Kit (Perfect Real Time) (Takara Bio) in accordance with the manufacturer's instructions. Each of the reaction mixture was diluted by adding 30 µL of water and then used as a template for PCR. Quantitative PCR was performed in a Thermal Cycler Dice Real Time System TP800 (Takara Bio) using SYBR *Premix Ex Taq II* (Takara Bio) and 2 µL of first-strand cDNA. The primer sets used were as follows: FeRhaT-548F and FeRhaT-653R for *FeF3G6″RhaT*, FeCHS-221F and FeCHS-323R for chalcone synthase (CHS) from *F. esculentum* (*FeCHS*, Genbank Accession No. HM149787), and FeCHI-215F and FeCHI-343R for chalcone isomerase (CHI) from *F. esculentum* (*FeCHI*, HM149788). For normalization, glyceraldehyde-3-phosphate dehydrogenase (*Fe-gapdh*, AB919116) was used as a housekeeping gene with the primer set FeGAPDH-543F and FeGAPDH-689R. Transcript levels were calculated using Real-Time System software (Takara Bio) using the ddCt method based on the second derivative maximum and three biological replicates. Statistical analyses were performed with Tukey's test using EZR software [31].

Construction of vectors for overexpression and transformation of tobacco cells

Coding region of *FeF3G6″RhaT* was amplified using pET-FeRhaT as a template with FeRhaT-F2 and FeRhaT-R1 as primers. The iProof™ High Fidelity DNA polymerase was used for the PCR under the following conditions: 98°C for 30 sec; 35 cycles of

98°C for 10 sec, 62°C for 20 sec, and 72°C for 30 sec; followed by 72°C for 5 min. The fragment was cloned into pENTR/D-TOPO (ThermoFisher Scientific) and subcloned into the binary vector pGWB402 [32] by LR-reaction using LR clonase 2 (ThermoFisher Scientific) to produce pGWB402-FeRhaT. It was then introduced into *Agrobacterium tumefaciens* LBA4404 (Takara Bio). The tobacco BY-2 cells were transformed by co-cultivating with *A. tumefaciens* harboring pGWB402-FeRhaT for 48 h. Transformants were then selected on 1% agar plates of modified LS medium supplemented with kanamycin (100 mg L^{-1}) and cefotaxime (200 mg L^{-1}, Tokyo Chemical Industry).

The transformants obtained were sub-cultured several times, transferred to the modified LS medium supplemented with the same antibiotics, and then sub-cultured at weekly intervals. The 4-day tobacco cell culture (3 mL) was mixed with 3 µL of quercetin solution (50 mM in DMSO) and incubated for 24 h. Cells were recovered from the culture by filtration and then extracted with methanol.

Phylogenetic analysis

A phylogenetic tree was created using the neighbor-joining method with 1000 bootstrap replicates. The tree was constructed with MEGA7 software [33] using related UGT sequences (Table S2) aligned with the Muscle Program.

Results

Purification of rhamnosyltransferase from common buckwheat cotyledons

Rutin is found in most common buckwheat tissues, especially the flowers, leaves, and cotyledons [24]. We used etiolated cotyledons for enzyme purification because their extracts exhibited strong rhamnosyltransferase activity against quercetin 3-*O*-glucoside (Figure S2). Furthermore, they can be prepared easily and are expected to reduce contamination by photosynthetic enzymes.

We purified the 6″-*O*-rhamnosyltransferase enzyme from the etiolated cotyledons of common buckwheat via eight purification steps while monitoring rhamnosylation activity with quercetin 3-*O*-glucoside and UDP-rhamnose (Table 1, Figure 2). As UDP-rhamnose was not available commercially, we synthesized it from UDP-glucose through an enzyme reaction using recombinant *Arabidopsis* rhamnose synthase expressed in *E. coli*. We used a UDP-rhamnose solution containing approximately 10 mM each of UDP-rhamnose and UDP-glucose as sugar-donor (Figure S1). Among the affinity resins tested, i.e. dye-ligand agarose resins (Sigma-Aldrich) and UDP-glucuronic acid agarose, only a Reactive Green-19 agarose was effective for the adsorption of rhamnosyltransferase activity. After the final step using Mono Q chromatography, we detected a major peak (fraction 25–26) and a minor peak (fraction 30) of rhamnosyltransferase activity between the NaCl gradient of 50–100 mM (Figure S3). Analysis of these fractions with SDS-PAGE revealed two to three major protein bands, one of which was present at approximately 50 kDa and was closely associated with the enzymatic activity, suggesting that they could be the rhamnosyltransferase proteins. We used the major peak of rhamnosyltransferase activity (fraction 25–26) as a

purified enzyme. Successive purification steps resulted in 107-fold purification and 0.9% recovery (Table 1).

[Figure 2 near here]

[Table 1 near here]

Peptide sequence determination and isolation of the corresponding gene encoding rhamnosyltransferase

The purified enzyme, with a molecular mass of ca. 50 kDa, was separated on SDS-PAGE, blotted onto a polyvinylidene difluoride (PVDF) membrane, treated with lysyl endopeptidase, and analyzed by HPLC-MS/MS. The data obtained were analyzed by Protein Lynx software and used to perform a BLASTP search on the Genbank protein database. Three peptide sequences obtained (GDQFLNSK, LPEGFLERVK, and ISFFSAPGNIPRIK) showed significant similarity to the internal sequences of the reported glycosyltransferases.

We performed RNA sequencing using total RNA extracted from common buckwheat to construct EST, and then searched for sequences similar to the EST corresponding to the purified enzyme using tBLASTn program with the peptide sequences as queries. However, we were unable to detect the homologous sequences using this method. Therefore, we obtained cDNA encoding 6″-*O*-rhamnosyltransferase by PCR using degenerated primers constructed from the peptide sequences and the cDNA library constructed using mRNA extracted from cotyledons of common buckwheat [29] as templates. The deduced amino acid sequence of the obtained *FeF3G6″RhaT* cDNA contained the three partial peptide sequences stated above (Figure

S4), suggesting that the obtained cDNA corresponded to the purified enzyme. After cloning *FeF3G6″RhaT*, the EST was searched using the BLASTn program, and a corresponding contig was found with a few substitutions. This sequence variety occurs in allogamous plants, such as common buckwheat, which frequently recombine by cross-fertilization; we also detected these sequence varieties with other genes isolated from this plant [29].

FeF3G6″RhaT (Accession No. LC312144) is composed of an open reading frame of 1398 bp encoding a polypeptide of 465 amino acids and a calculated molecular mass of 51.7 kDa. The amino acid sequence of FeF3G6″RhaT showed 51–57% identity with flavonoid glycoside 6″-*O*-rhamnosyltransferases from citrus, petunia, lobelia, and soybean, 51% identity with a flavonoid glycoside 6″-*O*-glucosyltransferase from soybean, and 26–28% identity with other reported rhamnosyltransferases, such as flavonoid glucoside 2″-*O*-rhamnosyltransferase from citrus and soyasaponin III rhamnosyltransferase from soybean. FeF3G6″RhaT possessed no signal sequences in its *N*-terminus. FeF3G6″RhaT was termed UGT79A8 by the UGT nomenclature committee.

Properties of recombinant FeF3G6″RhaT

The coding region of *FeF3G6″RhaT* cDNA was subcloned into the pET28a(+) vector and introduced into *E. coli* Rosetta 2(DE3), in which the recombinant protein was expressed and purified using histidine tag. SDS-PAGE analysis revealed a single protein band of approximately 52 kDa, corresponding to recombinant FeF3G6″RhaT (Figure S5A), which was used for an enzyme reaction with UDP-rhamnose and quercetin 3-*O*-glucoside as the sugar donor and acceptor, respectively. After the reaction, a new

405 peak exhibiting a $[M-H]^-$ ion at a mass-to-charge ratio (*m/z*) of 609 was observed. This
406 *m/z* value increased by 146 (corresponding to the molecular weight of rhamnose
407 moiety) from an *m/z* of 463 (corresponding to the molecular weight of quercetin
408 3-*O*-glucoside) (Figure 3). In addition, the retention time of the product corresponded to
409 that of rutin. These results clearly indicate that rutin was produced from quercetin
410 3-*O*-glucoside, suggesting that FeF3G6″RhaT is involved in the biosynthesis of rutin.
411
412 ***[Figure 3 near here]***
413
414 Recombinant FeF3G6″RhaT exhibited 70–80% of the maximum activity (at pH
415 9.5) observed within the pH range 7.5–10, when the reaction was performed at pH 6.5–
416 10 (Figure S5B). The optimum temperature for the recombinant FeF3G6″RhaT reaction
417 was 50°C (Figure S5C).
418 Substrate preference of recombinant FeF3G6″RhaT was examined using several
419 related compounds (Figure S6) as sugar acceptors (Table 2). In addition to quercetin
420 3-*O*-glucoside, the enzyme exhibited rhamnosyltransferase activity against kaempferol
421 3-*O*-glucoside. The enzyme also reacted with quercetin 3-*O*-galactoside at a similar
422 level to its reaction with quercetin 3-*O*-glucoside, but presented lower activity with
423 3-hydroxyflavone glucoside. Conversely, the enzyme exhibited low activity with
424 quercetin 7-*O*-glucoside, kaempferol 7-*O*-glucoside, and 7-hydroxyflavone glucoside,
425 no activity with kaempferol 3,7-di-*O*-glucoside and flavonoid aglycones, such as
426 quercetin and kaempferol. The enzyme did not utilize UDP-glucose, UDP-galactose, or
427 UDP-glucuronic acid as sugar donors. These results indicate that FeF3G6″RhaT is a
428 rhamnosyltransferase specific for flavonol 3-*O*-glycoside. Interestingly, the enzyme also

429 significantly utilized phloridzin (dihydrochalcone glucoside) and naphthol glucosides
430 (simpler structure than flavonoids) as sugar acceptors at a significant level.
431
432 ***[Table 2 near here]***
433
434 ***Accumulation of flavonoids in the organs of common buckwheat***
435 Common buckwheat accumulates several flavonoids, including rutin, quercitrin
436 (quercetin 3-*O*-rhamnoside) and *C*-glucosylflavones [18,24]. To confirm the
437 accumulation of these flavonoids in each organ of common buckwheat, they were
438 extracted with methanol and flavonoid contents were analyzed using HPLC-MS (Figure
439 S7 and Table 3; Figure S8). The accumulated flavonoids varied in each organ. Rutin
440 (peak g) was detected in most of the organs tested, mainly in flowers, leaves, and
441 cotyledons. Rutin levels were increased in flowers during flower formation and
442 blooming, in leaves during leaf maturation, and in cotyledons during seed germination,
443 and were decreased in achene (seed) formation after flowering. *C*-glucosylflavones,
444 namely orientin (peak a), isoorientin (peak b), vitexin (peak c), and isovitexin (peak d)
445 accumulated mainly in the cotyledon, with some accumulation in immature seeds.
446 Quercitrin (peak h) was one of the major flavonoids in the flower and immature seeds.
447 We also detected quercetin 3-*O*-galactoside (peak e) in the stem and immature seeds, a
448 compound predicted to be quercetin 3-*O*-robinobioside (peak f) exhibiting the $[M-H]^-$
449 ion at *m/z* 609 in the cotyledon and hypocotyl, and quercetin (peak i) in the flower as
450 minor components.
451
452 ***[Table 3 near here]***

453

454 ***FeF3G6"RhaT expression in the organs of common buckwheat***

455 To examine the contribution of *FeF3G6"RhaT* to rutin biosynthesis in common
456 buckwheat, we determined the expression level of *FeF3G6"RhaT*. Total RNA was
457 isolated from several organs of common buckwheat and then subjected to quantitative
458 RT-PCR analysis (Figure 4A). *FeF3G6"RhaT* transcripts accumulated to significantly
459 high levels in the early developing stages of flowers, leaves, and cotyledons. The
460 transcript levels then decreased as the organs matured. These results suggest that rutin
461 biosynthesis begins during the early stages of development and that rutin accumulates in
462 the same organs of common buckwheat. To investigate further, we analyzed the
463 accumulation of chalcone synthase (*FeCHS*) and chalcone isomerase (*FeCHI*)
464 transcripts, key enzymes in flavonoid biosynthesis (Figure 4B and C). *FeCHI* transcripts
465 accumulated significantly during the early developmental stages of rutin-accumulating
466 organs compared with the mature stages. This pattern was similar to that observed for
467 *FeF3G6"RhaT*. The accumulation of *FeCHS* transcripts showed a tendency similar to
468 that observed for *FeF3G6"RhaT* and *FeCHI*, although no significant differences
469 between developmental stages were observed. *FeCHS* and *FeCHI* were also expressed
470 in immature seeds, stems, and young roots, in which the expression of *FeF3G6"RhaT*
471 was low.

472

473 ***[Figure 4 near here]***

474

475 ***Heterologous expression of FeF3G6"RhaT in tobacco cells***

476 *FeF3G6"RhaT* was over-expressed in tobacco cells to investigate whether

FeF3G6″RhaT enzyme promotes rutin production *in planta* through its rhamnosyltransferase activity. Tobacco plants can produce rutin; however, tobacco BY-2 cells do not produce rutin [26]. A construct designed to express *FeF3G6"RhaT* under control of the cauliflower mosaic virus 35S promoter was transformed into tobacco BY-2 cells. The accumulation of *FeF3G6"RhaT* transcript was studied in 12 lines of transformed cells (RhaT-ex) by RT-PCR; two lines that accumulated significant levels of *FeF3G6"RhaT* transcripts were selected and cultured in liquid media. The resulting RhaT-ex cells, as well as wild-type BY-2 cells, were treated with quercetin (Figure 5). Wild-type BY-2 cells mainly converted incorporated quercetin into 3-*O*-glucoside and malonylglucoside in a similar way as reported by us previously [26], whereas RhaT-ex cells produced rutin with decreased accumulation of 3-*O*-glucoside and malonylglucoside. These results indicate that *FeF3G6"RhaT* functions as a rhamnosyltransferase *in planta*.

[Figure 5 near here]

Phylogenetic analysis of FeF3G6"RhaT

A phylogenetic analysis of FeF3G6″RhaT (UGT79A8) and some UGTs related to the formation of rhamnosides and disaccharides (Table S2) was performed (Figure 6). FeF3G6″RhaT clustered into the clade composed of flavonoid glycoside 6″-*O*-rhamnosyltransferases and a flavonoid glycoside 6″-*O*-glucosyltrnasferase from soybean (GmF3G6″GT), which belong to UGT79A, while flavonoid glycoside 2″-*O*-rhamnosyltrnasferase from citrus (Cm1,2RhaT) clustered into the other clade composed of 2″-*O*-rhamnosyltrnasferase, 6″-*O*-glucuronosyltransferase, and

6′-*O*-glycosyltranasferases for diverse compounds such as sesaminol (lignan) glucoside (SiSG6′GT), crocetin (carotenoid) glucoside (GjUGT9) and flavonoid glucoside (CaUGT3), which belong to UGT94. Rhamnosyltransferases that form saponin glycosides (GmSGT3), or those that catalyze the rhamnosylation of flavonoid skeletons (AtA3RhaT and AtF7RhaT) are out of these clades.

Discussion

Rutin has been the focus of much attention, since it is a compound that promotes good health [11]. Buckwheat is a good source of rutin [18,20]. The enzymes involved in rutin biosynthesis have been well-studied in buckwheat in terms of the formation of quercetin [24]. However, the rhamnosyltransferase that catalyzes this final step in the pathway remains poorly understood. Thus, in this study, we characterized the enzyme involved in rutin biosynthesis.

We purified an enzyme with rhamnosyltransferase activity from common buckwheat cotyledons. After fractionation via the Mono Q anion exchange chromatography, we detected a protein band of approximately 50 kDa on the SDS-PAGE gel, which retained most of the remaining activity. A relatively low purification efficiency (approximately 110-fold) was observed, which was apparently caused by inactivation of the enzyme during dye-ligand affinity chromatography purification, even though it effectively removed several contaminated proteins. We also detected a minor peak of proteins with rhamnosyltransferase activity after separation with Mono Q (Fraction 30, Figure S3). These varieties of rhamnosyltransferases are likely to have resulted from frequent allelic recombination, during which partial substitutions of amino acid residues led to a small change in their affinity for the column; this also occurred for *C*-glucosyltransferases in common buckwheat [29]. However, we cannot rule out the possibility that this minor enzyme is another type of rhamnosyltransferase working for rutin biosynthesis at different conditions. Further studies are required to clarify the role of this minor enzyme.

The recombinant FeF3G6″RhaT enzyme showed significant activity against

quercetin 3-*O*-glucoside and produced rutin (quercetin 3-*O*-rutinoside). It also showed significant activity with other flavonol 3-*O*-glucosides and 3-*O*-galactosides, but only faint activity with flavonoid 7-*O*-glucosides, which was less than one-thousandth of that observed for quercetin 3-*O*-glucoside. These results suggest that FeF3G6″RhaT is the flavonoid 3-*O*-glycoside (glucoside/galactoside) 6″-*O*-rhamnosyltransferase. Several flavonoid 3-*O*-(6″-*O*-rhamnosyl) glycosides, such as rutin, quercetin 3-*O*-robinobioside [34], and kaempferol 3-*O*-rutinoside [35] have been found in buckwheat plants, and we detected rutin and a compound predicted to be quercetin 3-*O*-robinobioside in the common buckwheat cultivar Shinano No.1 used in this study (Figure S7); these compounds are likely to be produced by FeF3G6″RhaT. The enzymatic property of FeF3G6″RhaT is similar to that of 6″-*O*-rhamnosyltransferase from *Glycine max* (GmF3G6″RT) [17], which showed 52% identity with FeF3G6″RhaT. In contrast, 6″-*O*-rhamnosyltransferases from *Citrus* (Cs1,6RhaT and Cm1,6RhaT), which shared 57% identity with FeF3G6″RhaT, reacted with flavonoid 7-*O*-glucosides as major substrates and reacted with flavonol 3-*O*-glucosides as minor substrates [12]. Further studies are required to elucidate the differences in substrate recognition among these enzymes. In terms of their sugar-donor specificity, Rojas Rodas et al. (2016) reported that 6″-*O*-rhamnosyltransferase and 6″-*O*-glucosyltransferase in soybean shared 82% identity and suggested that a few amino acids would determine UDP-sugar specificity [36]. FeF3G6″RhaT also conserves these amino acid residues specific for rhamnosyltransferase (Pro_{25}, Val_{141}, Lys_{219}); which is consistent with this suggestion.

In the present study, we found that the genes *FeF3G6"RhaT*, *FeCHS,* and *FeCHI*, were highly expressed in the flower, leaf, and cotyledon during the early stages of development, and rutin accumulation was increased in the same organs during

554 development. Enzymes involved in flavonoid biosynthesis form membrane-embedded
555 cytoplasmic complexes, which channel the substrate from phenylalanine to flavonoid
556 glucosides [37,38]. Thus, our results indicate that rutin synthesis is initiated at an early
557 stage of development and that subsequent rutin accumulation persists during the mature
558 stages in common buckwheat organs, such as flowers, leaves, and cotyledons (Figure 4
559 and Table 3). These results are consistent with the findings of some previous reports.
560 For example, the expression of flavone synthase 1 was associated with the accumulation
561 of flavonoids in tartary buckwheat [39] and genes associated with the synthesis of
562 flavonoids are highly expressed in the inflorescences at the full flowering stage of
563 buckwheat species [40]. Additionally, some of the flavonoid aglycons, such as
564 naringenin and dihydrokaempferol, which are intermediates in rutin biosynthesis, could
565 be transported over long distances in Arabidopsis [41]. Li et al. (2010) proposed that
566 flavonoids in buckwheat might be transported into their accumulating organ (flowers
567 and leaves) after being synthesized in stems and roots, because the accumulation of
568 transcripts of these genes was higher in stems and roots compared with the flowers and
569 leaves [42]. This inconsistency might be due to differences in organ stages between the
570 studies, i.e., we also tested the early developmental stages, and these biosynthetic genes
571 are expressed higher in the early stages compared with the later stages. In fact, the
572 patterns of *FeCHS* and *FeCHI* accumulation in stems, matured leaves, and blooming
573 flowers were similar between the two studies.
574 Rutin is considered to protect buckwheat leaves against from UV light [10,42]. Our
575 results showed that *FeF3G6"RhaT* mRNA accumulated in cotyledons regardless of light
576 exposure, as reported for quercetin 3-*O*-glucosyltransferase activity involved in the rutin
577 biosynthesis [23]. However, we cannot rule out the possibility that rutin also has other

roles in the development of buckwheat, e.g., including tolerance to environmental stress.

During the preparation of this manuscript, the draft genome sequence of common buckwheat has been published (http://buckwheat.kazusa.or.jp/cgi-bin/blast.cgi). A blast search on the database using the *FeF3G6″RhaT* sequence showed that *FeF3G6″RhaT* has not been assembled as a full-length in the database; however, there are corresponding gene fragments, suggesting that *FeF3G6″RhaT* has a long intron (Figure S9). A homologous gene fragment showing high homology is found in the buckwheat genome; this could be an allele of *FeF3G6″RhaT*. Two other homologues are also found, whose encoding proteins show 56–57% amino acid identity with *FeF3G6″RhaT*. The enzyme of minor peak detected after separation with Mono Q chromatography may correspond to one of these homologues. It must wait for further works to clarify whether these proteins would be involved in the rutin biosynthesis.

Conclusion

In this study, we isolated and identified the rhamnosyltransferase FeF3G6″RhaT, which catalyzes the last step of rutin biosynthesis in common buckwheat by means of protein purification, subsequent cDNA isolation, and characterization of recombinant enzymes expressed in both *E. coli* and tobacco cells. We also confirmed the expression of the *FeF3G6″RhaT* gene and accumulation of rutin in several organs of buckwheat. These results indicated that rutin biosynthesis begins during the early development stages of rutin-accumulating organs, such as flowers and leaves, and proceeds throughout maturation.

Author Contribution Statement

EK, MS, and GT conceived and designed the research. EK, SO, YM, HS, and GT performed experiments and analyzed data. MS and GT wrote the manuscript. All authors read and approved the manuscript.

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Disclosure statement

The authors declare that they have no conflict of interest.

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736

737 **Table 1**

738 Purification of rhamnosyltransferase from common buckwheat cotyledons

Purification Step	Protein (mg)	Total activity (nkat)	Specific activity (nkat mg_protein^{-1})	Purity (-fold)	Yield (%)
Crude enzyme	592	1908	3.2	1.0	100
$(NH_4)_2SO_4$	410	1599	3.9	1.2	83.8
Ultracentrifuge	382	1758	4.6	1.4	92.1
Phenyl Sepharose	83.3	757	9.1	2.8	39.7
Sephadex G-100	38.4	543	14	4.4	28.5
DEAE Sepharose	9.54	304	32	9.9	15.9
Reactive Green19	0.50	40.0	80	25	2.1
Mono Q	0.049	17.1	345	107	0.9
Mono Q Fr. 30 [a]	0.020	3.7	184	57	0.2

739

740 [a]Minor peak of rhamnosyltransferase activity detected after separation with Mono Q

741 chromatography (fraction 30, Figure S3).

742

743

744 **Table 2**

745 Substrate specificity of the recombinant FeF3G6″RhaT

Substrate	Activity (nkat mg_protein^{-1})
Flavonoid glycosides	
quercetin 3-*O*-glucoside	15.5 ± 0.95
quercetin 3-*O*-galactoside	14.2 ± 0.21
keampferol 3-*O*-glucoside	15.7 ± 1.3
3-hydroxyflavone glucoside	0.9 ± 0.01
quercetin 7-*O*-glucoside	0.02 ± 0.001
keampferol 7-*O*-glucoside	0.01 ± 0.001
7-hydroxyflavone glucoside	0.01 ± 0.001
keampferol 3,7-di-*O*-glucoside	n.d.[a]
phloridzin	5.0 ± 0.03
daidzin	n.d.
Other glucosides	
2-naphthol glucoside	2.5 ± 0.06
1-naphthol glucoside	1.7 ± 0.03
4-methylumbelliferone glucoside	n.d.
Flavonol aglycon	
quercetin	n.d.
keampferol	n.d.

746 [a]n.d.: not detected. Data indicate the average ± SD (n = 3).

747

748 **Table 3**

749 Flavonoid contents in the organs of common buckwheat used in this study.

	rutin (μmol g FW^{-1} [a])	quercitrin (μmol g FW^{-1})	*C*-glucosylflavones (μmol g FW^{-1})
Floral bud	4.23 ± 0.48	0.49 ± 0.07	n.d.[b]
Flower	12.3 ± 1.58	4.09 ± 0.85	n.d.
Immature seed	2.60 ± 1.04	0.71 ± 0.31	1.02 ± 0.37
Leaf bud	1.23 ± 0.37	n.d.	n.d.
Young leaf	1.01 ± 0.77	n.d.	n.d.
Matured leaf	3.57 ± 2.97	n.d.	n.d.
Cotyledon, 3 days	1.17 ± 0.39	n.d.	9.94 ± 1.05
Cotyledon, 2 weeks	0.53 ± 0.12	n.d.	3.90 ± 0.81
Hypocotyl, 3 days	0.30 ± 0.12	0.00 ± 0.01	n.d.
Stem	0.46 ± 0.29	n.d.	n.d.
Young root	0.02 ± 0.01	n.d.	n.d.
Seed	0.17 ± 0.03	n.d.	0.07 ± 0.04

750

751 Methanol extracts of each organ were analyzed by HPLC (Figure S7), and the
752 calculated amounts of rutin, quercitrin, and *C*-glucosylflavones (sum of orientin,
753 isoorientin, vitexin, and isovitexin) with at least three biological replicates (average ±
754 SD) are shown. [a]FW: fresh weight; [b]n.d.: not detected.

755

Figure captions

Figure 1. Glycosylation of quercetin into rutin in buckwheat.

Flavonol 3-*O*-glucosyltransferase (F3GT), which converts quercetin into isoquercitrin (quercetin 3-*O*-glucoside), and flavonol 3-*O*-glucoside 6″-*O*-rhamnosyltransferase (F3G6″RhaT), which converts isoquercitrin into rutin (quercetin 3-*O*-rutinoside) are involved in the reaction.

Figure 2. SDS-PAGE analysis of the purification of common buckwheat rhamnosyltransferase.

Proteins from each purification of common buckwheat rhamnosyltransferase were separated on 10% SDS-PAGE. Lanes 1–6, 10 μg each of crude extract, ammonium sulfate precipitation, ultracentrifugation, Phenyl Sepharose CL-4B, Sephadex G-100, and DEAE Sepharose CL-6B, respectively; lane 7, 4 μg of Reactive Green19 agarose; lane 8, 2.5 μg of Mono-Q fraction; lane M, standard proteins (unstained protein molecular weight marker, Pierce). The gel was stained with Coomassie brilliant blue R-250. Arrowheads indicate purified rhamnosyltransferase.

Figure 3. HPLC-MS analysis of the recombinant FeF3G6″RhaT reaction products from quercetin 3-*O*-glucoside.

Each panel shows a chromatogram, with the following conditions: the reactions were incubated for 0 and 10 min with UDP-rhamnose (**A, B**) and for 10 min with UDP-glucose (**C**); standard compounds of rutin (**D**). HPLC analysis was performed using a 50 mm ODS column as described in the Materials and Methods section. The eluates were monitored at 350 nm using a diode array detector. The negative

780 electron-splay ionization (ES^-) MS spectra corresponding to the substrate (peak 2) and
781 the product (peak 1) are shown. The retention time of MS peaks was delayed by about
782 0.08 min compared with that of the diode array. Peak identification: 1, rutin; 2,
783 isoquercitrin (quercetin 3-*O*-glucoside).
784
785 **Figure 4.** Quantitative reverse transcription (qRT)-PCR analyses of *FeF3G6″RhaT* (**A**),
786 *FeCHS* (**B**), and *FeCHI* (**C**) in several organs of common buckwheat.
787 qRT-PCR analyses were performed using total RNA extracted from flowers [floral bud
788 (FLB); flowering stage (FL)], immature seed (ImS), leaves [leaf bud (LB); primary leaf
789 after development (YL); matured leaf (ML)], cotyledons [2–3 days (Cot 3d); 2 weeks
790 (Cot 2wk) after sowing], hypocotyl [2–3 days after sowing (Hyp 3d)], stem [red part
791 near the root (St)], and young root from seedling (YR). Transcript levels were estimated
792 via the ddCt method based on the second derivative maximum and are shown relative to
793 that of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), with three biological
794 replicates (average ± SD). Statistical analyses were performed with Tukey's test;
795 different letters above the error bars indicate significant differences ($p < 0.05$).
796
797 **Figure 5.** Heterologous expression of *FeF3G6″RhaT* in tobacco BY-2 cells and
798 confirmation of rhamnosyltransferase activity.
799 (**A)** HPLC of methanol extracts of wild-type (WT) and *FeF3G6″RhaT*-overexpressing
800 BY-2 cells (RhaT1-4 and RhaT3-3) treated with quercetin. The eluates were monitored
801 at 350 nm using a diode array detector. Peak identification: 1, rutin; 2, quercetin
802 3-*O*-glucoside; 3, quercetin malonylglucoside; 4, mono-methylated rutin. (**B)** The
803 negative electron-spray ionization (ES^-) MS spectra of each peak observed in (**A**). The

retention time of MS peaks was delayed by about 0.08 min over that of the diode array. (**C)** RT-PCR analysis of *FeF3G6″RhaT* mRNA accumulation in WT and transformed BY-2 cells. A 515-bp cDNA fragment was expected for *FeF3G6″RhaT* mRNA, 231-bp was expected for tobacco actin mRNA.

Figure 6. Molecular phylogenetic tree inferred from the deduced amino acid sequences of FeF3G6″RhaT and related glycosyltransferases.

A molecular phylogenetic tree was constructed by the neighbor-joining method using MEGA7 software [33] based on the deduced amino acid sequences of UGTs related to the formation of rhamnosides and disaccharides. Bar indicates 0.1 amino acid substitutions per site. Abbreviations and Genbank accession numbers of UGTs are as follows: FeF3G6″RhaT (LC312144), LeABRT2 (LC131336), LeABRT4 (LC131337), PhRT (X71059), Cm1,2RhaT (AY048882), Cm1,6RhaT (LC057678), Cs1,6RhaT (DQ119035), GmF3G2″GT (LC017844), GmSGT3 (AB473731), CaUGT3 (AB443870), GjUGT9 (AB555739), SiSG6′GT (AB333799), BpUGAT (AB190262), IpA3G2″GT (AB192315), GmF3G6″RT (AB828193), GmF3G6″GT (LC126028), AtA3RhaT (NM_102790), AtA3G2″GT (NM_124780), AtF3G2″XylT (NM_124785), AtF7RhaT (NM_100480). Detailed information about the UGTs is shown in Table S2.

Graphical abstract caption

FeF3G6″RhaT catalyzes rutin biosynthesis in buckwheat, which is expressed in rutin-accumulating organs during early developmental stages.

Supplementary Information

Table S1. PCR primers used in this study.

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Figure S3. Purification of buckwheat rhamnosyltransferase on Mono Q anion exchange chromatography.

Figure S4. Nucleotides and deduced amino acid sequence of FeF3G6″RhaT.

Figure S5. Properties of recombinant FeF3G6″RhaT.

Figure S6. Structures of the substrates used in this study.

Figure S7. Distribution of flavonoids in the organs of common buckwheat.

Figure S8. Images of common buckwheat organs used in this study.

852

853 **Figure S9.** Comparison of the DNA sequences of FeF3G6″RhaT and related sequences

854 found in the draft genome database of common buckwheat.

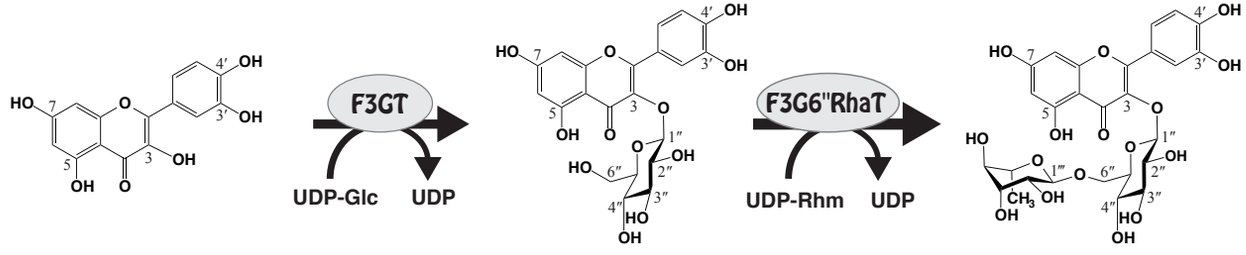
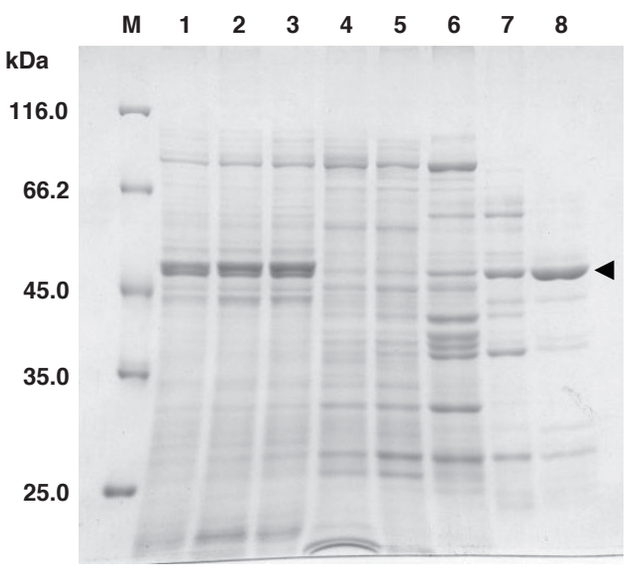


Figure 1. Koja et al.



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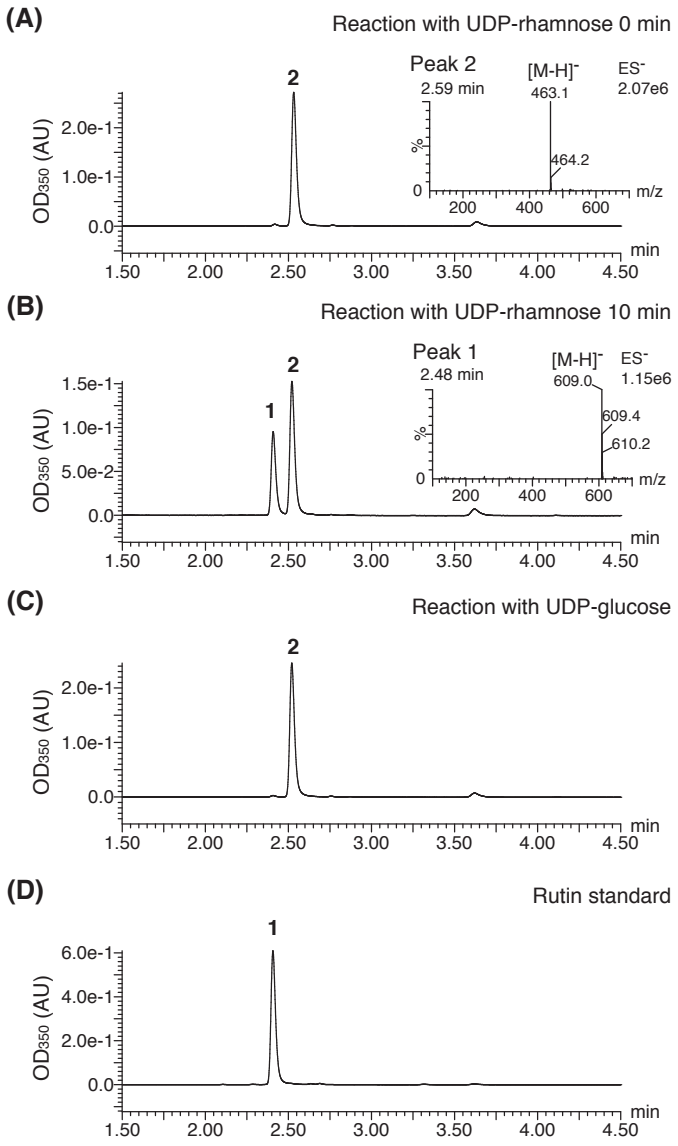

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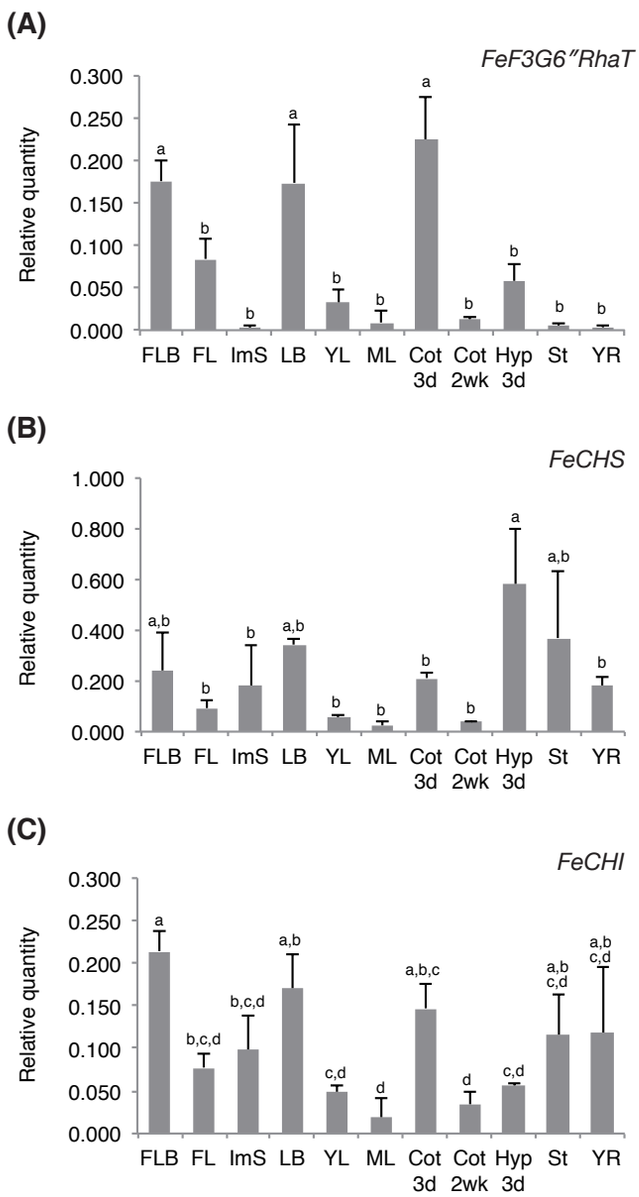

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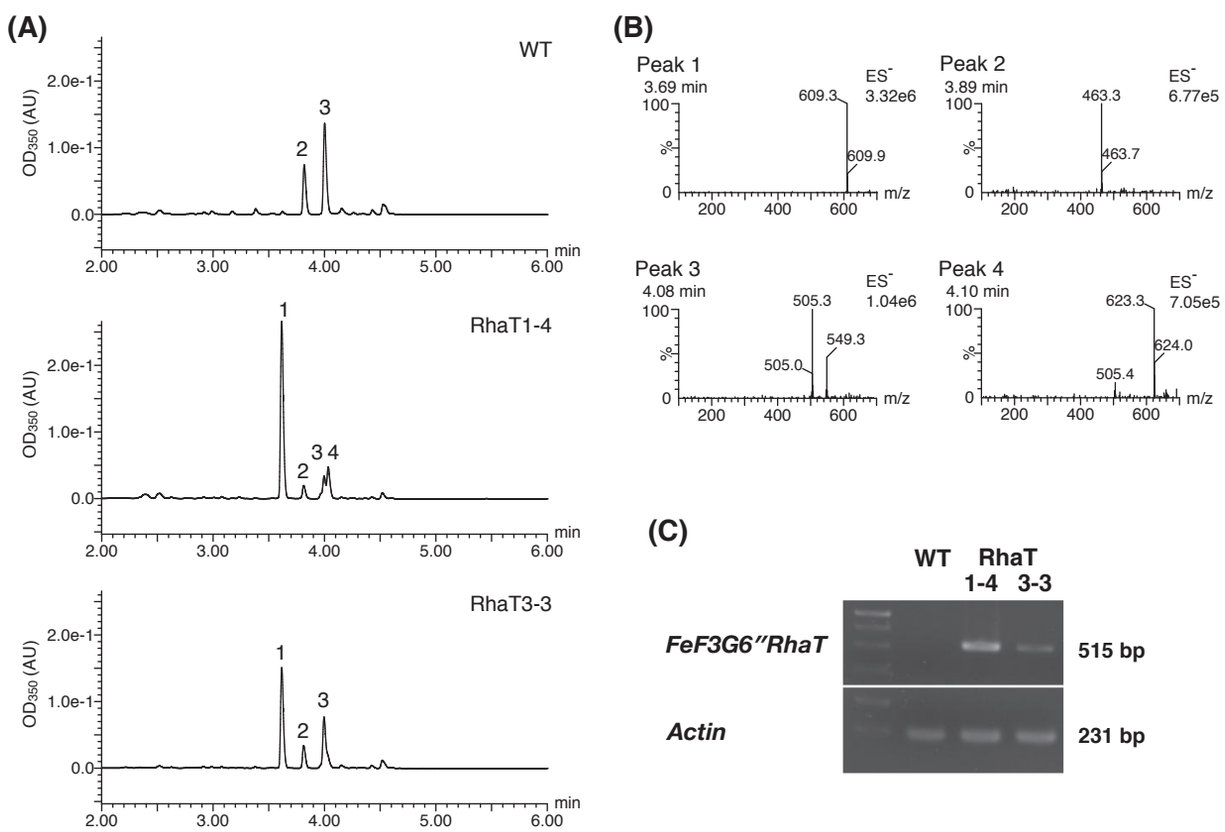

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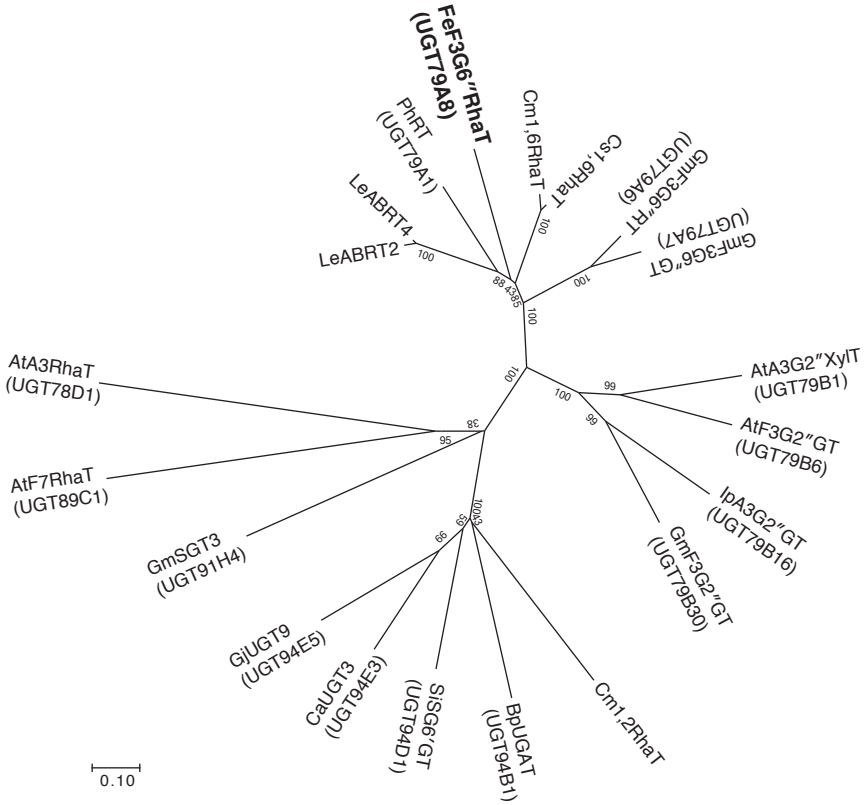

Figure 6. Koja et al.

Supplementary Information

for

Identification and characterization of a rhamnosyltransferase involved in rutin biosynthesis in *Fagopyrum esculentum* (common buckwheat)

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[*]Corresponding author. e-mail : gtagtag@shinshu-u.ac.jp

Table S1. PCR primers used in this study.

Primer	Sequence
SFF-Fw1	5'-TCTTTCTTYTCNGCNCCNGGNAACAT-3'
GDQ-Rv1	5'-TTRGARTTNAARAAYTGRTCNCC-3'
M13-FL	5'-GTTTTCCCAGTCACGACGTTGTA-3'
M13-Rv	5'-CAGGAAACAGCTATGACCAT-3'
FeRhaT-F1	5'-cacccatatgATGGGAACCCAAGCAAAC-3'
FeRhaT-R1	5'-ctcgagTTAATTACCAACCAAAGCCTTAAC-3'
FeRhaT-F2	5'-caccATGGGAACCCAAGCAAAC-3'
FeRhaT-324F	5'-CACCCAACTCAAACCCGA-3'
FeRhaT-548F	5'-AAGACATTCCAGGCGAAGGAC-3'
FeRhaT-653R	5'-CGATTGCATCACATCCTTGG-3'
FeRhaT-838R	5'-CGGATCCGAAGGAGCATAG-3'
FeGAPDH-543F	5'-CGTTGAGGGCCTAATGACCA-3'
FeGAPDH-689R	5'-TTGCCAACAGCCTTAGCAGC-3'
FeCHI-215F	5'-TCACGGCGATCGGAATTTAC-3'
FeCHI-343R	5'-CGAATTGACCGGTGACAACA-3'
FeCHS-221F	5'-CAAGCGCATGTGTGACAAGTC-3'
FeCHS-323R	5'-GGATGGAGCCATGTAAGCACA-3'
AtRhm2-Fw	5'-ggaattcATGGATGATACTACGTATAAGCC-3'
AtRhm2-Rv	5'-ccgctcgagGGTTCTCTTGTTTGGTTCA-3'
35S-1	5'-GCTCCTACAAATGCCATCA-3'
NtActin-Fw	5'-GATTGGAATGGAATGGAAGCTG-3'
NtActin-Rv	5'-CCTCCAATCCAAACACT-3'

Table S2. List of uridine diphosphate (UDP)-sugar dependent glycosyltransferases (UGTs) used in the phylogenetic analysis.

Name	Function	Organism	Accession No.[a]
FeF3G6″RhaT (UGT79A8)	Flavonol 3-*O*-glucoside 6″-*O*-glucosyltransferase	*Fagopyrum esculentum*	LC312144
LeABRT2	Delphinidin 3-*O*-glucoside 6″-*O*-rhamnosyltransferase	*Lobelia erinus*	LC131336
LeABRT4	Delphinidin 3-*O*-glucoside 6″-*O*-rhamnosyltransferase	*Lobelia erinus*	LC131337
PhRT (UGT79A1)	Anthocyanidin 3-*O*-glucoside 6″-*O*-rhamnosyltransferase	*Petunia x hybrida*	X71059
Cm1,2RhaT	Flavanone 7-*O*-glucoside 2″-*O*-rhamnosyltransferase	*Citrus maxima*	AY048882
Cm1,6RhaT	Flavanone 7-*O*-glucoside 6″-*O*-rhamnosyltransferase	*Citrus maxima*	LC057678
Cs1,6RhaT	Flavanone 7-*O*-glucoside 6″-*O*-rhamnosyltransferase	*Citrus sinensis*	DQ119035
GmF3G2″GT (UGT79B30)	Flavonol 3-*O*-glucoside 2″-*O*-glucosyltransferase	*Glycine max*	LC017844
GmSGT3 (UGT91H4)	Soyasaponin III 2″-*O*-rhamnosyltransferase	*Glycine max*	AB473731
CaUGT3 (UGT94E3)	Flavonoid glucoside 6″-*O*-glucosyltransferase	*Catharanthus roseus*	AB443870
GjUGT9 (UGT94E5)	Crocetin glucoside 6′-*O*-glucosyltransferase	*Gardenia jasminoides*	AB555739
SiSG6′GT (UGT89D1)	Sesaminol 2-*O*-glucoside 6′-*O*-glucosyltransferase	*Sesamum indicum*	AB333799
BpUGAT (UGT94B1)	Anthocyanin 3-*O*-glucoside 2″-*O*-glucuronosyltransferase	*Bellis perennis*	AB190262
IpA3G2″GT (UGT79B16)	Anthocyanidin 3-*O*-glucoside 2″-*O*-glucosyltransferase	*Ipomoea purpurea*	AB192315
GmF3G6″RT (UGT79A6)	Flavonol 3-*O*-glucoside 6″-*O*-rhamnosyltransferase	*Glycine max*	AB828193
GmF3G6″GT (UGT79A7)	Flavonol 3-*O*-glucoside 6″-*O*-glucosyltransferase	*Glycine max*	LC126028
AtA3RhaT (UGT78D1)	Anthocyanin 3-*O*-rhamnosyltransferase	*Arabidopsis thaliana*	NM_102790
AtF3G2″GT (UGT79B6)	Flavonoid 3-*O*-glucoside 2″-*O*-glucosyltransferase	*Arabidopsis thaliana*	NM_124780
AtF3G2″XylT (UGT79B1)	Anthocyanin 3-*O*-glucoside 2″-*O*-xylosyl-transferase	*Arabidopsis thaliana*	NM_124785
AtF7RhaT (UGT89C1)	Flavonol 7-*O*-rhamnosyltransferase	*Arabidopsis thaliana*	NM_100480

[a] Accession No. for gene sequences.

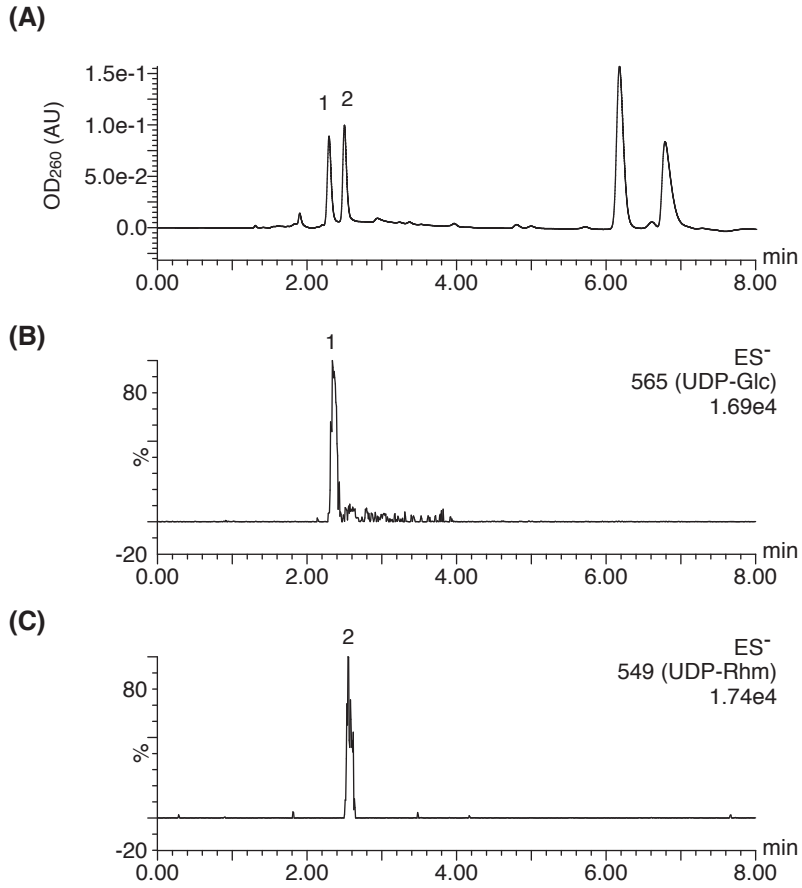


Figure S1. HPLC analysis of the UDP-rhamnose solution synthesized by the enzyme reaction. UDP-rhamnose was synthesized from UDP-glucose via an enzymatic reaction using Arabidopsis Rhm2 and was analyzed by HPLC as described in the Materials and Methods Section. The eluates were monitored at 260 nm using a diode array detector (A), the negative electron-spray ionization (ES^-) MS at m/z = 565 for UDP-glucose (B), and at m/z = 549 for UDP-rhamnose (C). The retention time of the MS peaks was delayed by about 0.08 min over that of the diode array. Peak identification: 1, UDP-glucose; 2, UDP-rhamnose.

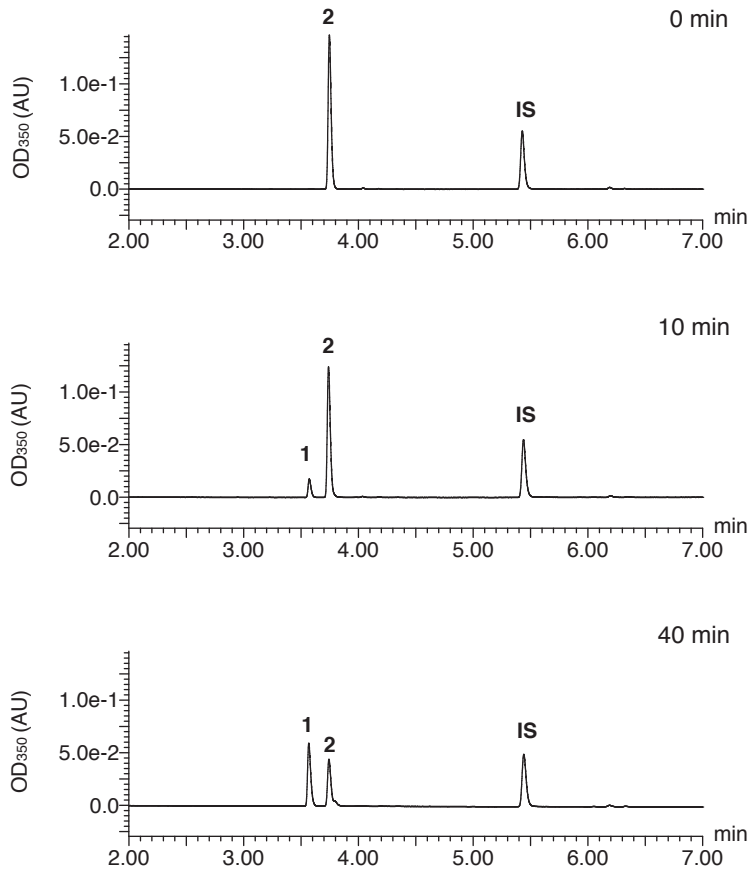


Figure S2. Rhamnosyltransferase activity in the cell-free extract of buckwheat etiolated cotyledon. Cell-free extract of buckwheat etiolated cotyledon was incubated with isoquercitrin (quercetin 3-*O*-glucoside) and UDP-rhamnose solution at 30 °C for 0, 10, and 40 min, and analyzed by HPLC with a 100 mm ODS column as described in the Materials and Methods Section. The eluates were monitored at 350 nm using a diode array detector. Peak identification: 1, rutin; 2, isoquercitrin; IS, quercetin added to the samples as an internal standard.

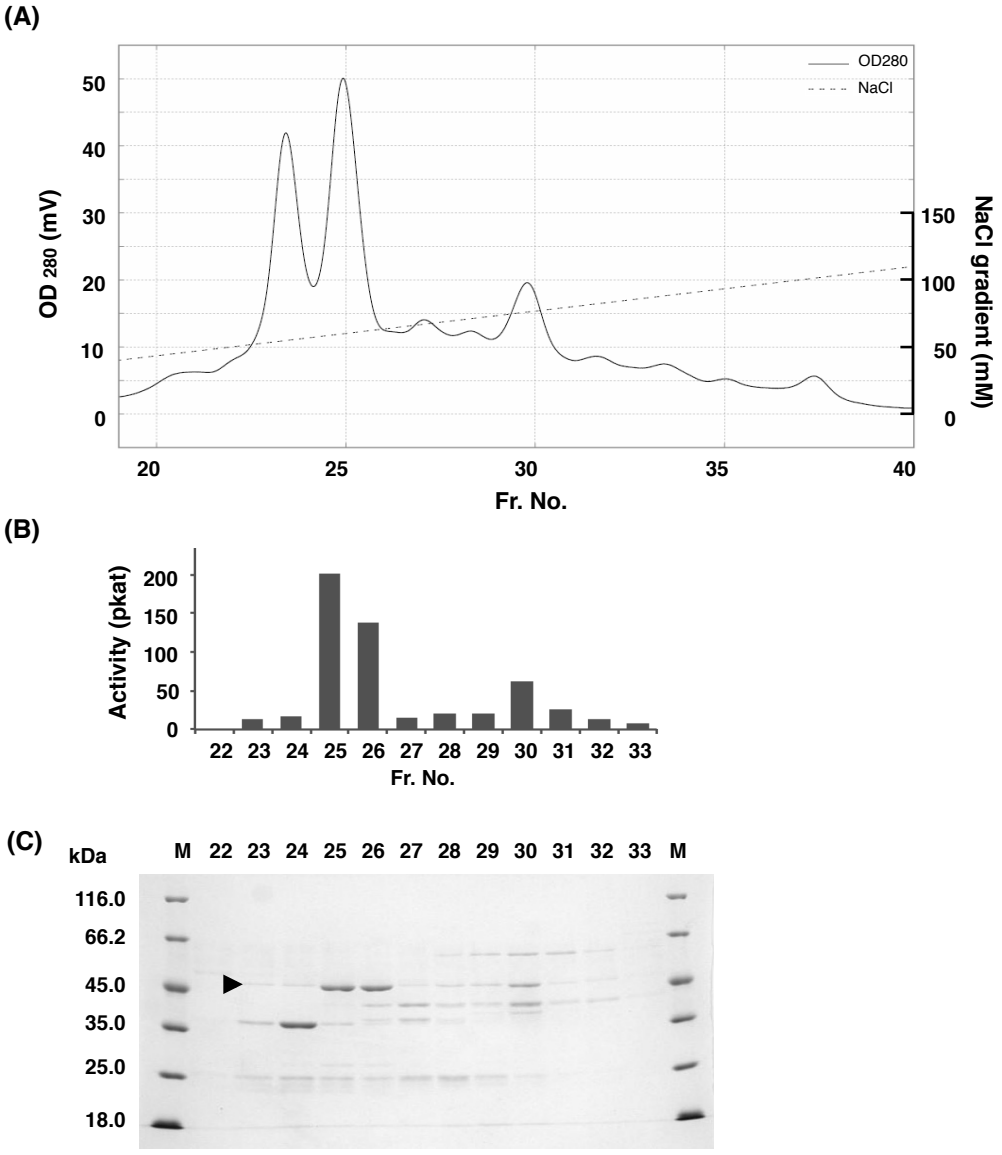


Figure S3. Purification of buckwheat rhamnosyltransferase on Mono Q anion exchange chromatography. (A) Chromatogram of rhamnosyltransferase from buckwheat seedlings on Mono Q. The solid and dashed lines represent the protein concentration (OD_{280}) and NaCl concentration, respectively. (B, C) Rhamnosyltransferase activity (B) and SDS-PAGE analysis (C) of the purified fractions after Mono Q separation, respectively. The numbers indicate the fraction number on Mono Q shown in (A). Arrowhead in (C) indicates the purified rhamnosyltransferase. Lane M: standard proteins.

```
   1  ATGGGAACCCAAGCAAACACAACCGATCTTCACATAGCCGTATTTCCCTACTTCGCCTTCGGCCACATCAACCCATTCGTTCACATCTCA
        M  G  T  Q  A  N  T  T  D  L  H  I  A  V  F  P  Y  F  A  F  G  H  I  N  P  F  V  H  I  S

  91  AACAAGCTCGCCTCCCATGGAATCAAGATCTCCTTCTTCTCCGCTCCAGGGAACATCCCAAGAATCAAATCGTCACTTTCCACCTCACCC
        N  K  L  A  S  H  G  I  K  I  S  F  F  S  A  P  G  N  I  P  R  I  K  S  S  L  S  T  S  P

 181  TTGATCTCAATCGTACCGCTCACCTTCCCCCACGTCGACGGCCTCCCCGCCGGCTTCGAAAGCACTGCTGACATCACTCCCGCCATTGCT
        L  I  S  I  V  P  L  T  F  P  H  V  D  G  L  P  A  G  F  E  S  T  A  D  I  T  P  A  I  A

 271  GAGCTTCTCAAGGTCGCTCTTGACAAAATGCAGCCTCAAATTCGTTCTCTGCTCACCCAACTCAAACCCGACGTCGTTTTCTTCGACTTC
        E  L  L  K  V  A  L  D  K  M  Q  P  Q  I  R  S  L  L  T  Q  L  K  P  D  V  V  F  F  D  F

 361  GCTCAGAATTGGATCCCTTCTCTTGCCTCCGAACTTGGGATTAAGACTGTTATGTTTTCCGTCTTCTCTCTTATCTCCAACTCTTATTTA
        A  Q  N  W  I  P  S  L  A  S  E  L  G  I  K  T  V  M  F  S  V  F  S  L  I  S  N  S  Y  L

 451  ATGACGCCGGCGAGACTTTCCTCCGACGAGATTCCGACCATTGAAGAGCTCAAGAAACCTCCTCAAGGCTATCCCAACCCCGACCTCTCC
        M  T  P  A  R  L  S  S  D  E  I  P  T  I  E  E  L  K  K  P  P  Q  G  Y  P  N  P  D  L  S

 541  CTGAAGACATTCCAGGCGAAGGACTTGTTGTATCCGTTCAGACGGTTCAACGGCGGTCCATCGGCGCTGGAGCGGAACTACGCTGGAATC
        L  K  T  F  Q  A  K  D  L  L  Y  P  F  R  R  F  N  G  G  P  S  A  L  E  R  N  Y  A  G  I

 631  CAAGGATGTGATGCAATCGCTTACAAGTCTTGTCACGAGATGGAAGGTCCATACTGGAGCTACTTCAAGAAAGTCATCGGAAAGCCAATC
        Q  G  C  D  A  I  A  Y  K  S  C  H  E  M  E  G  P  Y  W  S  Y  F  K  K  V  I  G  K  P  I

 721  ATAATGGCCGGAATTCCGATCCCGGAAACGTCTTCCTCCGGCGACCTCGACAGCAACTGGGCAACATGGCTAGCAAAATTCCCACCAAAA
        I  M  A  G  I  P  I  P  E  T  S  S  S  G  D  L  D  S  N  W  A  T  W  L  A  K  F  P  P  K

 811  TCAGTTACTCTATGCTCCTTCGGATCCGAAACGTTTCTCACCGACGTCCAAGTCCAAGAGCTTGCTCTTGGACTTGAACTCACAGAGCTT
        S  V  T  L  C  S  F  G  S  E  T  F  L  T  D  V  Q  V  Q  E  L  A  L  G  L  E  L  T  E  L

 901  CCATTTCTAATGGTACTGAGCTCCAATGGCTTCGATCAAGAAAGACTGAACAAAATCCTCCCTGAGGGGTTCCTGGAGCGGGTTAAAGAT
        P  F  L  M  V  L  S  S  N  G  F  D  Q  E  R  L  N  K  I  L  P  E  G  F  L  E  R  V  K  D

 991  AGAGGCTTGATTCATATCGGTTGGGTGCCACAGCAGAAGATTATGGCTCATGAGAATGTGGGTTGTTATGTTAATCATGCTGGGTTTGGA
        R  G  L  I  H  I  G  W  V  P  Q  Q  K  I  M  A  H  E  N  V  G  C  Y  V  N  H  A  G  F  G

1081  TCTGTGATTGAAGCCATTGTTACTGATTGTCAGCTGGTTTTGCTTCCATTTAAAGGCGACCAGTTCTTGAACTCGAAGCTGTTGAGTCTG
        S  V  I  E  A  I  V  T  D  C  Q  L  V  L  L  P  F  K  G  D  Q  F  L  N  S  K  L  L  S  L

1171  GACATGAAGGTTGGGGTGGAGGTAAATAGGAGAGATGAAGATGGGCATTTTGGGAAAGAGGATATATTTGAGGCAGTGAGGATTGTTACA
        D  M  K  V  G  V  E  V  N  R  R  D  E  D  G  H  F  G  K  E  D  I  F  E  A  V  R  I  V  T

1261  GTGGATGGTGATAAAGAGCCTGGGAAGAAGATTAGAGGTAATCTTGTGAAGTGGAAGGAGTTGCTGATGAACAAAGAGTTTGAAGAGAAG
        V  D  G  D  K  E  P  G  K  K  I  R  G  N  L  V  K  W  K  E  L  L  M  N  K  E  F  E  E  K

1351  TATGTTCTTGAATTGGTTAAGGAAGTTAAGGCTTTGGTTGGTAATTAA   1398
        Y  V  L  E  L  V  K  E  V  K  A  L  V  G  N  *
```

Figure S4. Nucleotides and deduced amino acid sequence of *FeF3G6"RhaT*.

The peptide sequences obtained by LC-MS/MS analysis of rhamnosyltransferase purified from buckwheat seedlings are highlighted in green.

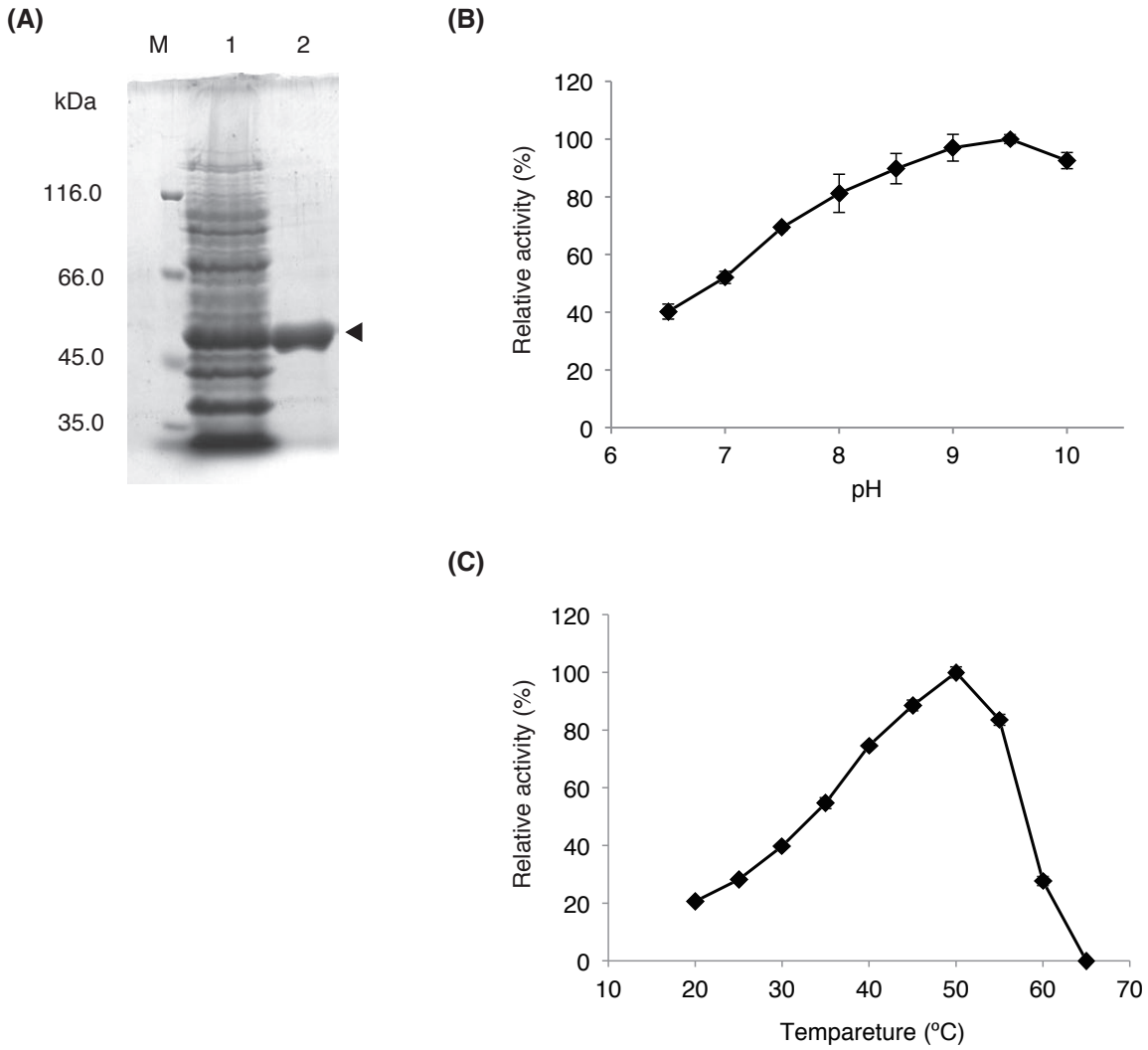
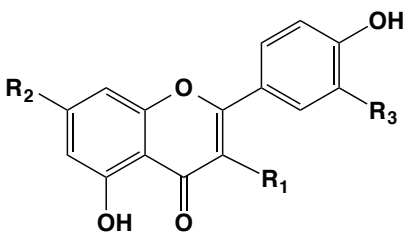


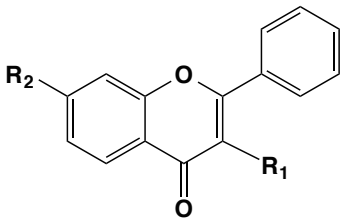
Figure S5. Properties of recombinant FeF3G6″RhaT.

(A) SDS-PAGE analysis of the recombinant FeF3G6″RhaT. Proteins were separated on a 10% SDS-polyacrylamide gel. Lanes 1 and 2, Crude extract and an affinity-purified fraction of the recombinant FeF3G6″RhaT enzyme, respectively; lane M, standard proteins. (B, C) The pH preference (B) and optimal temperature (C) for the FeF3G6″RhaT reaction. The reactions were examined using UDP-rhamnose and quercetin 3-*O*-glucoside as substrates, as described in the Materials and Methods Section. Relative activity is given as the average ± SD (n = 3) with maximum activity levels assumed to be 100%.

Flavones and flavonols

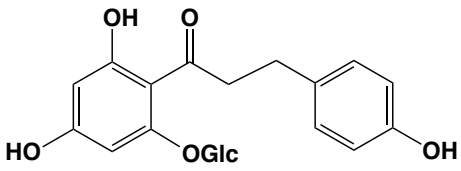


Quercetin	R_1=R_2=R_3=OH
Kaempferol	R_1=R_2=OH, R_3=H
Quercetin 3-*O*-glucoside	R_1=OGlc, R_2=R_3=OH
Quercetin 3-*O*-galactoside	R_1=OGal, R_2=R_3=OH
Kaempferol 3-*O*-glucoside	R_1=OGlc, R_2=OH, R_3=H
Quercetin 7-*O*-glucoside	R_1=R_3=OH, R_2=OGlc
Kaempferol 7-*O*-glucoside	R_1=OH, R_2=OGlc, R_3=H
Kaempferol 3,7-di-*O*-glucoside	R_1=R_2=OGlc, R_3=H

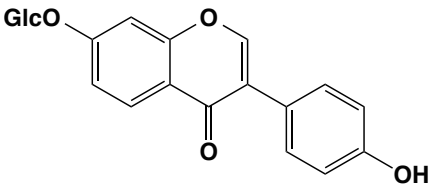


3-Hydroxyflavone glucoside	R_1=OGlc, R_2=H
7-Hydroxyflavone glucoside	R_1=H, R_2=OGlc

Dihydrochalcone and isoflavone

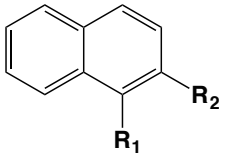


Phloridzin

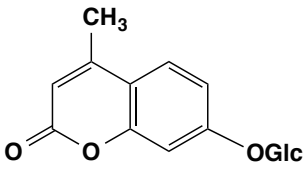


Daidzin

Others



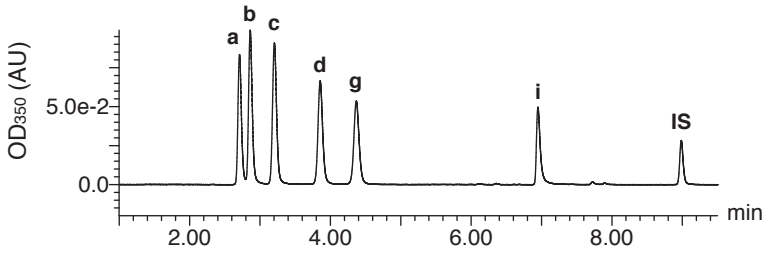
1-Naphthol glucoside	R_1=OGlc, R_2=H
2-Naphthol glucoside	R_1=H, R_2=OGlc



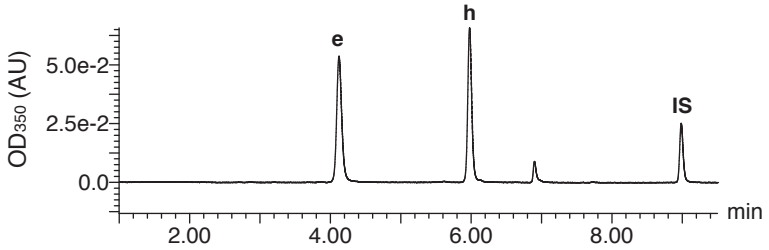
4-Methylumbelliferone glucoside

Figure S6. Structures of the substrates used in this study.

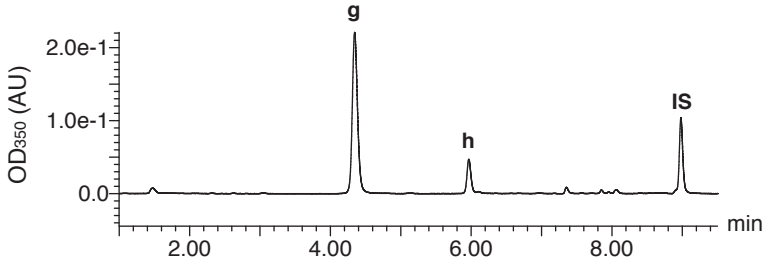
(A) Flavonoid standards 1



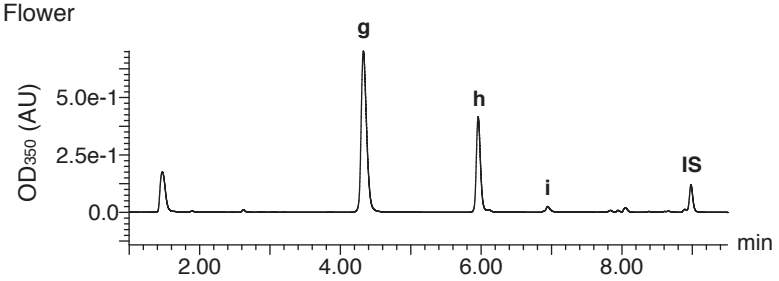
(B) Flavonoid standards 2



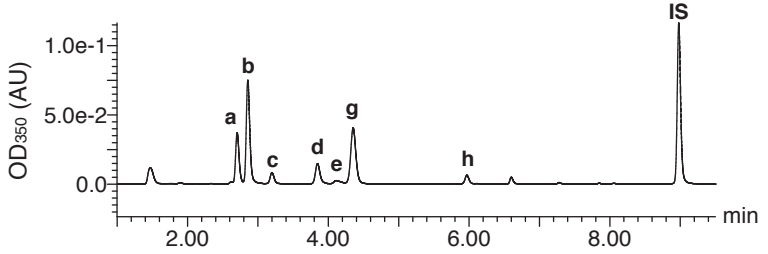
(C) Floral bud



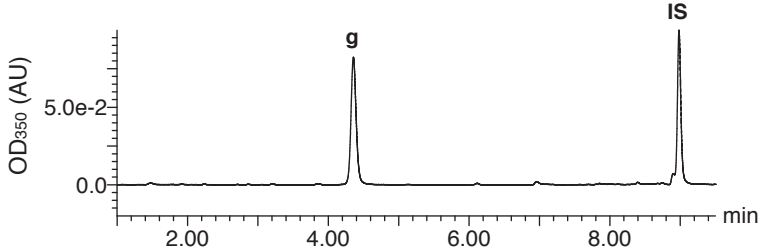
(D) Flower



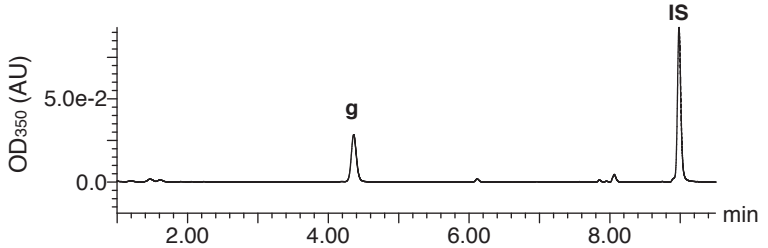
(E) Immature seed



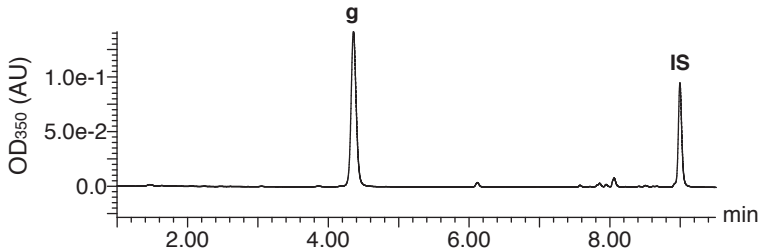
(F) Leaf bud



(G) Young leaf



(H) Matured leaf



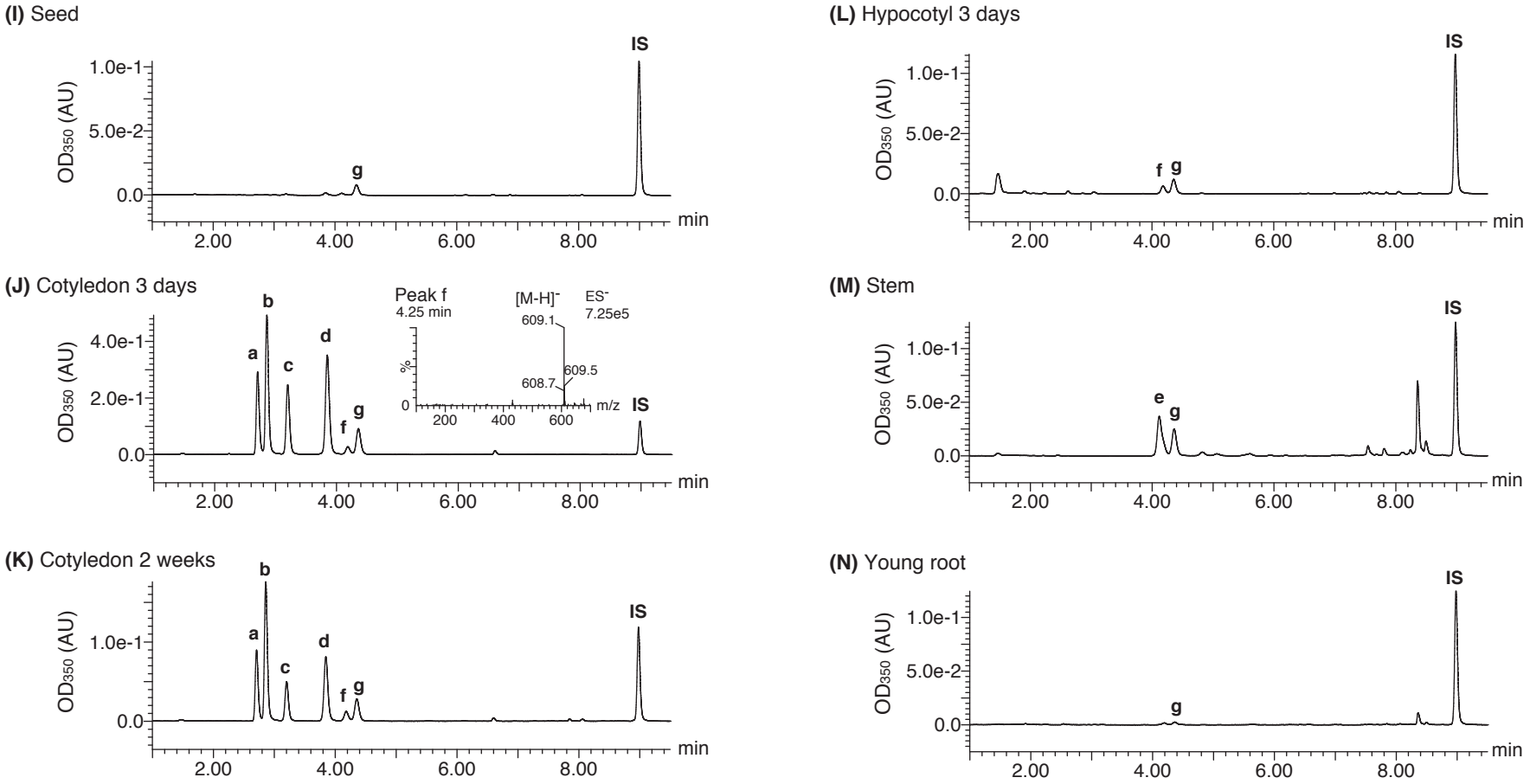


Figure S7. Distribution of flavonoids in the organs of common buckwheat.

Chromatograms reflect the following conditions: (A, B) authentic compounds of flavonoids; methanol extract of the floral bud (C), flower including bud (D), immature seed (E), leaf bud of the primary leaf (F), primary leaf after development (G), matured leaf (H), seed (I), cotyledons [3 days (J) and 2 weeks (K) after sowing], hypocotyl 3 days after sowing (L), stem (red part near the root) (M), and young root from seedling (N). The eluates were detected by measuring the absorbance at 350 nm. The negative electron-splay ionization (ES^-) MS spectra corresponding to the peak f is shown. The retention time of MS peaks was delayed by about 0.08 min compared with that of the diode array. Peak identifications: a, orientin; b, isoorientin; c, vitexin; d, isovitexin; e, quercetin 3-*O*-galactoside; f, a compound predicted to be quercetin 3-*O*-robinobioside; g, rutin; h, quercitrin; i, quercetin; IS, chrysin added to the samples as an internal standard.

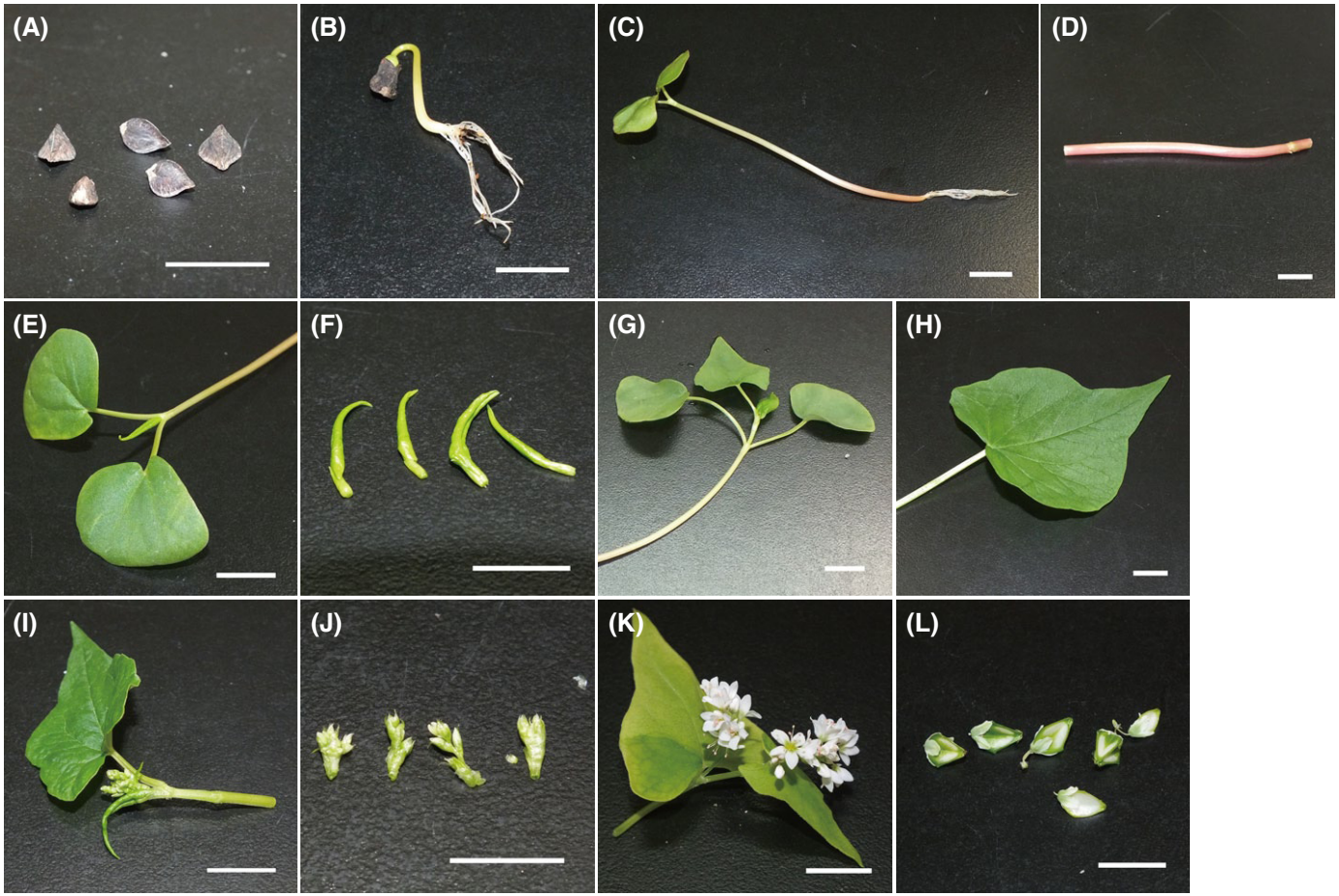


Figure S8. Images of common buckwheat organs used in this study.

The panels show the common buckwheat organs in several developmental stages: seeds (A); 3 day-old seedling for cotyledon and hypocotyl preparation (B); 1-week-old seedling for young root preparation (C); stem (D); 2-week-old seedling for cotyledon and leaf bud (E), and separated leaf buds (F); 3-week-old seedling for primary leaf (G); matured leaf (H); floral bud with leaves (I) and separated floral buds (J); flowering stage including flowers and buds (K); immature seeds (L). White scale bar indicates 1 cm.

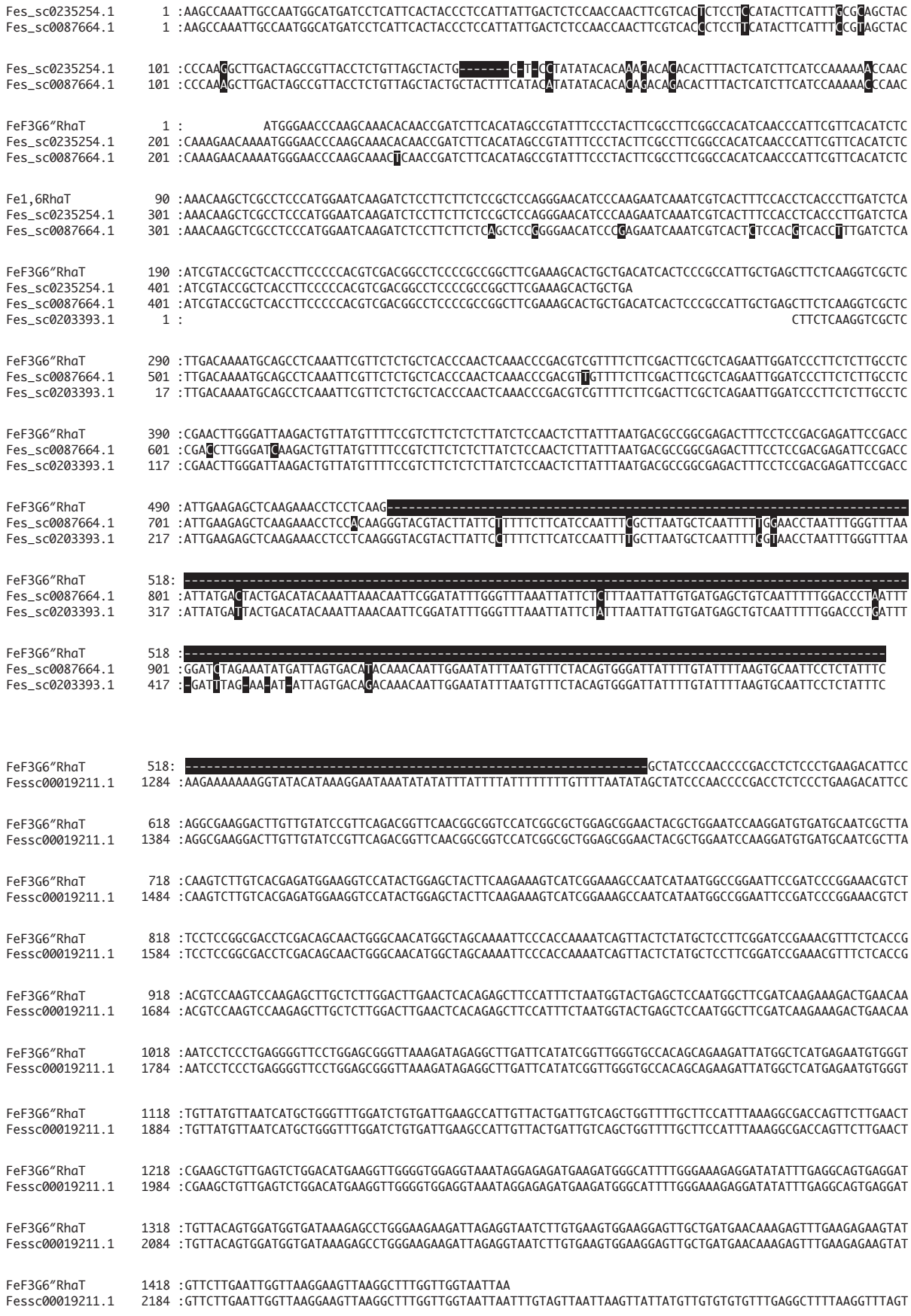


Figure S9. Comparison of the DNA sequences of *FeF3G6"RhaT* and related sequences found in the draft genome database of common buckwheat.

Black shading shows the different nucleotides among the sequences. Fessc00019211.1 is shown only by the relevant part, and any homologous sequence was identified in the omitted 1.3 kb part corresponding to the intron.