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ARTICLE

# Differential Expression of Flavonoid Biosynthesis Genes and Accumulation of Phenolic Compounds in Common Buckwheat (*Fagopyrum esculentum*)

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Common buckwheat (*Fagopyrum esculentum*) is a short-season grain crop that is a source of rutin and other phenolic compounds. In this study, we isolated the cDNAs of 11 *F. esculentum* enzymes in the flavonoid biosynthesis pathway, namely, phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL) 1 and 2, chalcone synthase (CHS), chalcone isomerase (CHI), flavone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), flavonol synthase (FLS) 1 and 2, and anthocyanidin synthase (ANS). Quantitative real-time polymerase chain reaction analysis showed that these genes were most highly expressed in the stems and roots. However, high performance liquid chromatography analysis indicated that their flavonoid products, such as rutin and catechin, accumulated in the flowers and leaves. These results suggested that flavonoids may be transported within *F. esculentum*. In addition, light and dark growth conditions affected the expression levels of the biosynthesis genes and accumulation of phenolic compounds in *F. esculentum* sprouts.

**KEYWORDS: Common buckwheat; *Fagopyrum esculentum*; flavonoids; rutin; biosynthetic pathways; gene expression**

## INTRODUCTION

The seeds of common buckwheat (*Fagopyrum esculentum* Moench), a dicotyledon, are used to make many foods, such as pancakes, bread, noodles, and tea. Buckwheat is also a rich source of vitamins, essential amino acids, and phenolic compounds. In particular, it is an important industrial source of rutin (quercetin-3-rutinoside), a flavonol glycoside that protects plants from ultraviolet (UV) radiation and diseases (*1*, *2*). In addition, rutin is an antioxidant that has many pharmacologically useful properties, such as anti-inflammatory, anticarcinogenic, antithrombotic, cytoprotective, and vasoprotective effects, in humans (*3*, *4*).

The phenylpropanoid pathway (**Figure 1**) synthesizes a large array of secondary metabolites, including flavonoids, which are important for many aspects of plant growth and development, such as pigment production, photoprotection, and disease resistance (*5*, *6*). The first enzyme of the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL), catalyzes the conversion of L-phenylalanine to *trans*-cinnamic acid. Then, the second enzyme of the phenylpropanoid pathway, cinnamate 4-hydroxylase (C4H), catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid (*7*). Next, 4-coumarate:CoA ligase (4CL) converts *p*-coumarate to its coenzyme-A ester, which is a precursor for various phenylpropanoid biosynthetic derivatives, including lignins and flavonoids. Subsequently, chalcone synthase (CHS) catalyzes the production of a tetrahydroxychalcone that is the precursor for all flavonoids. This step is the first dedicated reaction of the flavonoid biosynthesis pathway in higher plants. Afterward, chalcone isomerase (CHI) catalyzes the conversion of chalcone to naringenin, which is then converted to dihydrokaempferol and dihydroquercetin by flavone 3-hydroxylase (F3H) and flavonoid 3′-hydroxylase (F3′H), respectively. At this point, the pathway branches and has two possible outcomes (**Figure 1**). In one branch, anthocyanidin synthase (ANS) catalyzes the conversion of leucoanthocyanidin to anthocyanidin (*8*−*13*). In the other branch, flavonol synthase (FLS) converts dihydroflavonols, such as dihydrokaempferol and dihydroquercetin, to flavonols, such as kaempferol and quercetin, respectively (*14*).

The genes that encode flavonoid biosynthesis enzymes have been extensively characterized in maize, petunia, snapdragon, and *Arabidopsis* (*6*). In this study, we used rapid analysis of cDNA ends (RACE) polymerase chain reaction (PCR) to isolate the full-length cDNAs of *F. esculentum PAL* (*FePAL*), *FeCHS*, *FeCHI*,

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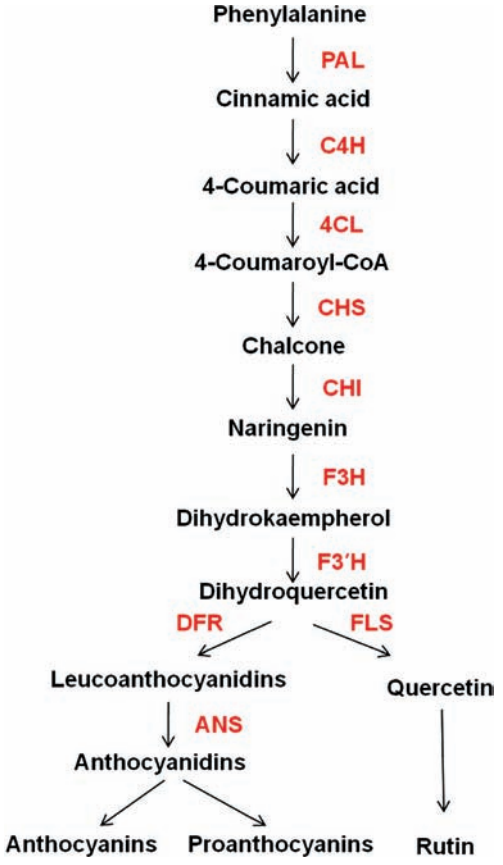


**Figure 1.** Flavonoid biosynthesis pathway in *F. esculentum*. Enzyme genes that have been cloned are indicated in red. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL1 and 4CL2, 4-coumaroyl CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase, F3H, flavone 3-hydroxylase, F3′H, flavonoid 3′-hydroxylase; ANS, anthocyanidin synthase.

*FeF3H*, and *FeANS* (GenBank accession numbers HM149783, HM149787, HM149788, HM149789, and HM149791, respectively) and partial cDNAs of *FeC4H*, *Fe4CL1*, *Fe4CL2*, *FeF3′H*, *FeFLS1*, and *FeFLS2* (GenBank accession numbers HM149784, HM149785, HM149786, HM149790, HM357804, and HM357805, respectively). Then we compared the expression levels of these genes, along with that of *F. esculentum* dihydroflavonol-4-reductase (DFR), which was previously isolated, in different organs of *F. esculentum* and in sprouts grown under light or dark conditions. Finally, we examined the effect of different growth conditions on the accumulation of phenolic compounds in the sprouts.

## MATERIALS AND METHODS

**Plant Materials and Growth Conditions.** The seeds of common buckwheat (*F. esculentum* cv. Suwon-1) were germinated in a growth chamber at 25 °C with approximately 60% humidity for 10 days in the light/dark (16/8 h). The germinated seedlings were transferred to a greenhouse (25 °C, 50% humidity) and grown until they flowered. Then, plant samples were collected from different organs (e.g., roots, stems, leaves, and inflorescences). For experiments with sprouts, the seeds were surface-sterilized by soaking them in 50% sodium hypochlorite (NaClO) for 10 min, and then they were germinated in a growth chamber under light or dark conditions 2−12 days after sowing (DAS). Plant samples, nongerminated seeds, and seedlings were frozen at −80 °C and lypophilized.

**RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction.** Total RNA was isolated from *F. esculentum* organs and seedlings by using the RNeasy Plant Mini Kit (Qiagen; Valencia, CA, USA). For quantitative real-time polymerase chain reaction (qRT-PCR), 1 $\mu$g of total RNA was reverse transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen; Carlsbad, CA, USA) and an oligo(dT)$_{20}$ primer.

Gene-specific primer sets were designed by using the conserved sequences of known orthologous sequences (Supplementary Table S1 in the Supporting Information). Gene expression was normalized to that of the histone H3 gene as a housekeeping gene (*15*). Real-time PCR reactions were performed in triplicate on a MiniOpticon system (Bio-Rad Laboratories; Hercules, CA) with the Quantitect SYBR Green PCR Kit (Qiagen). The PCR protocol was as follows: denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 15 s at 56 °C, and elongation for 20 s at 72 °C.

**Cloning of cDNAs Encoding Flavonoid Biosynthesis Enzymes.** The GeneRacer kit (Invitrogen) was used to synthesize single-stranded cDNA. Then, the PCR products were subcloned into a TOPO TA vector (Invitrogen) and sequenced at the National Instrumentation Center for Environmental Management (NICEM; Seoul National University, Korea). The sequence data were used to design new primer pairs for RACE PCR (Supplementary Table S1 in the Supporting Information). All PCRs were initiated with the hot start method by using the RACE cDNA template, and the products were subcloned into TOPO TA vectors and sequenced as described above. The cDNA of *F. esculentum DFR* (*FeDFR*) was isolated previously (GenBank accession number: GU169469).

**Sequence Analyses.** Sequence similarities were calculated with the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments were constructed using the MultAlin program (http://bioinfo.genotoul.fr/multalin/multalin.html).

**High Performance Liquid Chromatography Analysis.** Prior to high performance liquid chromatography (HPLC) analysis, *F. esculentum* organs were freeze-dried at −80 °C for at least 48 h, and then ground into a fine powder using a mortar and pestle. For the quantification of phenolic compounds, the powdered organs (50 mg) were extracted with 2 mL of 80% (v/v) ethanol at 60 °C for 1 h. After centrifuging the extract, the supernatant was filtered with a 0.45 $\mu$m Acrodisc syringe filter (Pall Corp.; Port Washington, NY), and then analyzed by HPLC.

HPLC analysis was performed with a $C_{18}$ column (250 × 4.6 mm, 5 $\mu$m; RStech; Daejeon, Korea). The mobile phase consisted of methanol and water:acetic acid (98:2 v/v), and the column was maintained at 30 °C. The flow rate was maintained at 1.0 mL·min$^{-1}$, the injection volume was 20 $\mu$L, and the detection wavelength was 280 nm. The concentrations of phenylpropanoid compounds were determined by using a standard curve. All samples were analyzed in triplicate.

**Statistical Analysis.** Each result shown in the figure or table was the mean of three replicated treatments. The significant differences between treatments were statistically evaluated by standard deviation.

## RESULTS AND DISCUSSION

**Cloning and Sequence Analysis of Flavonoid Biosynthesis Genes from *F. esculentum*.** The amino acid sequences encoded by full-length cDNAs for *FePAL*, *FeCHS*, *FeF3H*, *FeCHI*, and *FeANS* and partial cDNAs for *FeC4H*, *Fe4CL1*, *Fe4CL2*, *FeF3′H*, *FeFLS1*, and *FeFLS2* were aligned and compared with orthologous sequences from other plants (Supplementary Figures 1 and 2 in the Supporting Information). The 2401 bp *FePAL* cDNA contained a 2112 open reading frame (ORF) that encoded a 704 amino acid protein. A BLAST analysis showed that this protein shared 80−83% identity and 89−91% similarity with PAL from *Arabidopsis thaliana*, *Vitis vinifera*, *Astragalus membranaceus*, *Camellia sinensis*, *Lactuca sativa*, *Populus trichocarpa*, and *Glycine max*. Similarly, the 1449 bp *FeCHS* cDNA contained a 1179 bp ORF that encoded a 393 amino acid protein. This protein shared 84−87% identity and 93−94% similarity with CHS from *Ageratina adenophora*, *Garcinia mangostana*, *P. trichocarpa*, *Citrus sinensis*, and *V. vinifera* and 93% identity and 98% similarity with *Polygonum cuspidatum* CHS. The 970 bp *FeCHI* cDNA contained a 771 bp ORF that encoded a 256 amino acid protein. This protein shared 56−64% identity and 72−83% similarity with CHI from *Citrus sinensis*, *Gossypium hirsutum*, *P. trichocarpa*, *V. labrusca*, and *A. thaliana*. The 1343 bp *FeF3H* cDNA contained

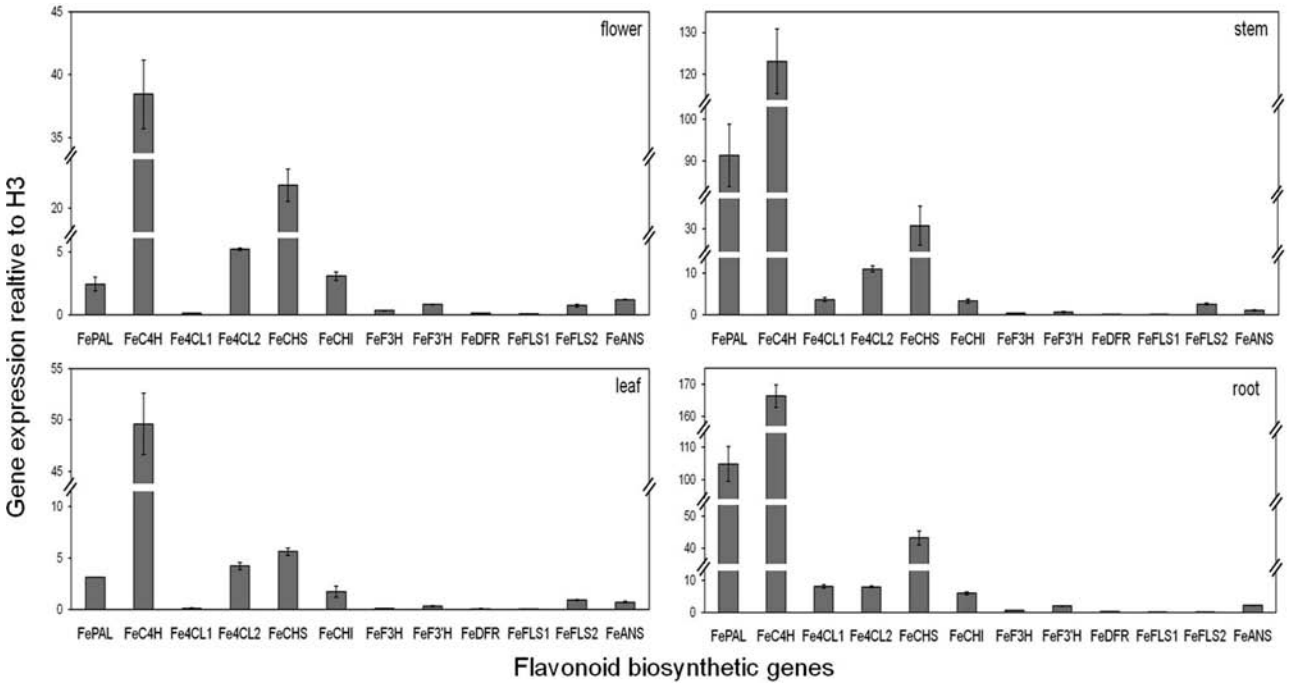


**Figure 2.** Expression levels of flavonoid biosynthesis genes in different organs of *F. esculentum*. The expression level of each gene is relative to that of the constitutively expressed histone H3 gene. Each value is the mean of three replicates ± SD.

a 1104 bp ORF that encoded a 367 amino acid protein. This protein shared 79−85% identity and 87−91% similarity with F3H from *Gossypium hirsutum*, *Dimocarpus longan*, *Garcinia mangostana*, *C. sinensis*, *V. vinifera*, *Rubus coreanus*, *Pyrus communis*, *G. max*, and *Actinidia chinensis*. Finally, the 1593 bp *FeANS* cDNA contained a 1077 bp ORF that encoded a 358 amino acid protein. This protein shared 78−84% identity and 89−92% similarity with ANS from *Spinacia oleracea*, *V. vinifera*, *Solanum melongena*, *Pyrus communis*, *Gypsophila elegans*, *C. sinensis*, and *Gossypium hirsutum*.

**Expression of Flavonoid Biosynthesis Genes in Different Organs of *F. esculentum*.** To investigate the control of flavonoid biosynthesis in *F. esculentum*, we examined the expression levels of flavonoid biosynthesis genes in different organs and sprouts of *F. esculentum*. The expression levels of *FePAL*, *FeC4H*, *Fe4CL1*, *Fe4CL2*, *FeCHS*, *FeCHI*, *FeF3H*, *FeF3′H*, *FeDFR*, *FeFLS1*, *FeFLS2,* and *FeANS* are shown in **Figure 2**. Although the gene transcripts for all of these enzymes were expressed in every organ of *F. esculentum*, the expression levels were the highest in the stems and roots (Supplementary Figure 3 in the Supporting Information). In particular, the expression level of *FePAL* in the stems and roots was higher than in the flowers and leaves (**Figure 2**). In contrast to *C4H*, which was strongly expressed in all organs, *FeF3H*, *FeF3′H*, *FeDFR*, *FeFLS1, FeFLS2,* and *FeANS* were expressed at low levels in all organs. In addition, *FeFLS2* was expressed at very low levels in the roots, unlike *FeFLS1*.

The expression levels of flavonoid biosynthesis genes also were investigated in nongerminated seeds and sprouts grown under light or dark conditions (**Figure 3**). The expression levels of all of the genes examined in this study, except *FePAL* and *Fe4CL1*, in sprouts grown under either light or dark conditions peaked within 4 DAS, and then gradually decreased. For *FePAL*, the expression level in sprouts grown under either light or dark conditions was the highest 12 and 8 DAS, respectively. Similarly, for *Fe4CL1*, the expression level in light or dark conditions was the highest 10 and 12 DAS, respectively. However, the expression of *FeC4H* was exceptional because it was the highest 2 DAS but did not decrease as much as the expression of the other genes.

**Content of Phenolic Compounds in Different Organs of *F. esculentum*.** The amounts of several phenolic compounds, namely, gallic acid, (−)-epigallocatechin, chlorogenic acid, (−)-catechin hydrate, caffeic acid, (−)-epicatechin, rutin, ferulic acid, and quercetin, in different organs of *F. esculentum* are shown in **Table 1**. The contents of all of these compounds, except (−)-epigallocatechin and quercetin, were several-fold higher in the flowers than in the other organs. In the roots, most of the phenolic compounds were only present at very low concentrations, and quercetin and ferulic acid were not detected at all. Quercetin also was not found in the leaves or stems. Rutin was most abundant in the flowers (6809 mg/100 mg dry weight (DW)), and was 1000 times more concentrated in the flowers than in the roots (6.25 mg/100 mg DW) (**Table 1**). These results are consistent with previous studies that show that the amount of rutin in buckwheat flowers peaks at the full flowering stage (*16*, *17*). Furthermore, these results demonstrated that the flowers are the richest source of rutin in buckwheat.

In addition, (−)-epigallocatechin, catechin hydrate, and epicatechin were detected in the organs. Catechin hydrate and epicatechin were more concentrated in the flowers than in the other organs. However, (−)-epigallocatechin was more abundant in the roots than in the flowers. Catechins have important pharmacological properties in humans, such as antioxidant, anticancer, antihypertensive, antivascular, and anti-inflammatory effects, as well as negative allelopathic effects on neighboring plants (*18*, *19*).

Interestingly, the expression of almost all of the genes examined in this study was higher in the lower parts of the plant, such as the stems and roots, than in the higher parts, such as the flowers and leaves (**Figure 2** and Supplementary Figure 3 in the Supporting Information). In contrast, the concentration of the secondary metabolites that are produced by these genes, such as rutin, was greater in the higher parts than in the lower parts (**Table 1**). This inverse relationship between the expression of flavonoid biosynthesis genes and the accumulation of their products may be due to the transport of flavonoids within *F. esculentum* (*20*, *21*).

**Content of Phenolic Compounds in *F. esculentum* Seeds and Sprouts.** The amounts of several flavonoids, namely, gallic acid,

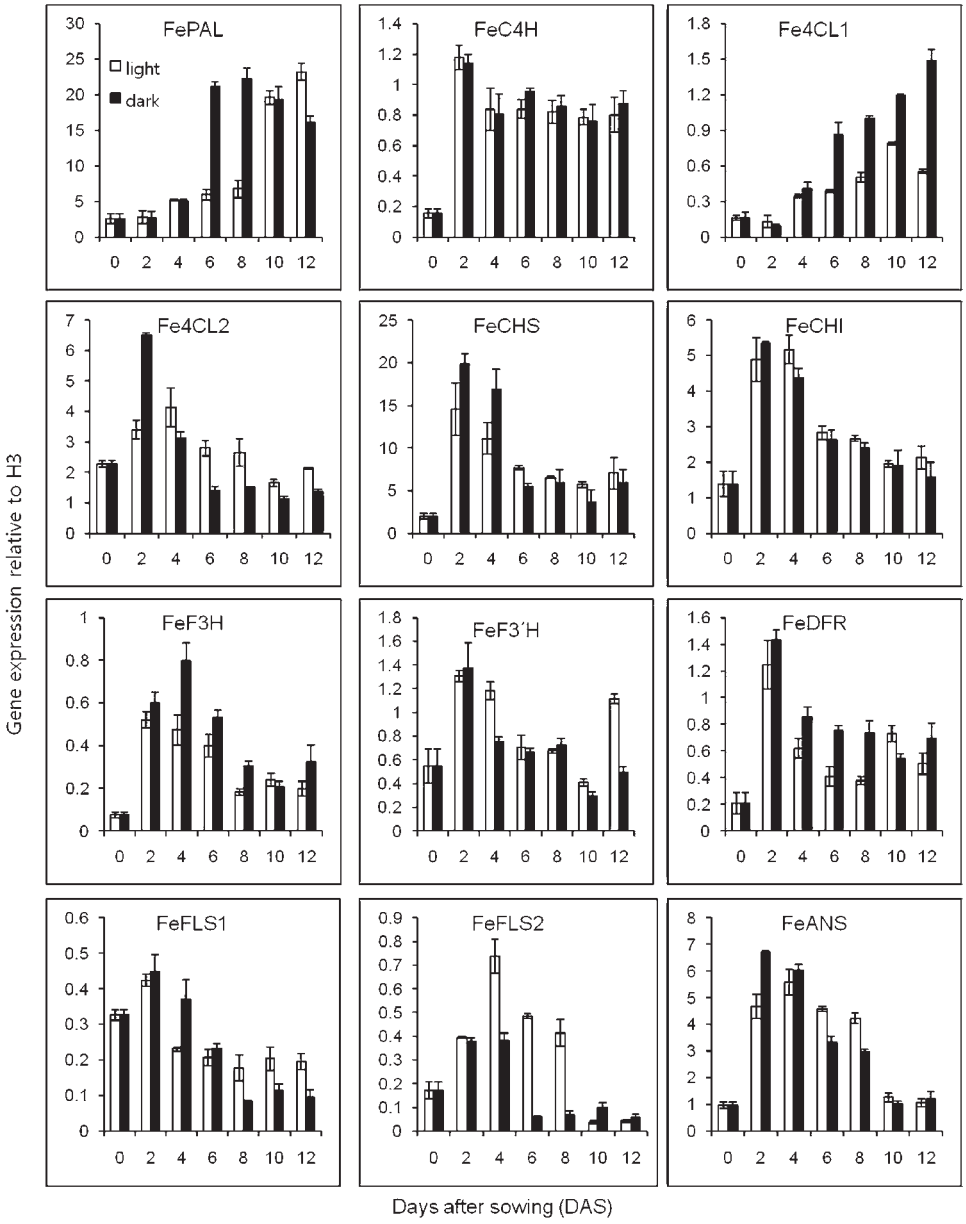


**Figure 3.** Expression levels of flavonoid biosynthesis genes in nongerminated seeds (0 days after sowing, DAS) and sprouts (2−12 DAS) of *F. esculentum* grown under light or dark conditions. The expression level of each gene is relative to that of the constitutively expressed histone H3 gene.

**Table 1.** Content of Phenolic Compounds (mg/100 mg Dry Weight (DW)) in Different Organs of *F. esculentum*[a]

| compound | flowers | leaves | stems | roots |
|---|---|---|---|---|
| gallic acid | 8.41 ± 1.15 | 5.37 ± 1.51 | 2.60 ± 0.09 | 2.13 ± 0.02 |
| (−)-epigallocatechin | 199.28 ± 20.08 | 309.26 ± 19.03 | 10.38 ± 1.62 | 16.07 ± 0.06 |
| chlorogenic acid | 907.65 ± 15.10 | 13.07 ± 2.18 | 32.54 ± 2.03 | 6.43 ± 0.10 |
| (−)-catechin hydrate | 137.82 ± 9.21 | 3.02 ± 0.21 | 1.73 ± 0.03 | 21.07 ± 2.21 |
| caffeic acid | 3.46 ± 0.07 | 2.38 ± 0.07 | 2.59 ± 0.02 | 2.69 ± 0.02 |
| (−)-epicatechin | 536.48 ± 14.20 | 103.20 ± 5.72 | 28.42 ± 1.61 | 17.71 ± 2.11 |
| quercetin | 1.37 ± 0.01 | | | 4.56 ± 0.05 |
| ferulic acid | | 2.07 ± 0.03 | 22.51 ± 0.87 | |
| rutin | 6809.37 ± 147.23 | 5524.71 ± 236.30 | 294.25 ± 7.34 | 6.25 ± 0.04 |

[a] Results are expressed as mean ± SD ($n$ = 3).

catechin, chlorogenic acid, 4-hydoxy-3-methoxy benzoic acid, caffeic acid, epicatechin, *p*-coumaric acid, ferulic acid, and rutin, in the nongerminated seeds and sprouts of *F. esculentum* are shown in **Figure 4** and Supplementary Table S2 in the Supporting Information. In nongerminated seeds, all of these flavonoids except gallic acid were present at very low levels; however, in sprouts grown under light or dark conditions, the levels of these flavonoids increased significantly. This result agreed with a previous study that showed that the increase of flavonoids during sprouts growth 6−10 DAS of common buckwheat (*22*).

The rutin content in sprouts grown under light conditions (556.71 mg/100 g at 12 DAS) was 60% greater than that in those

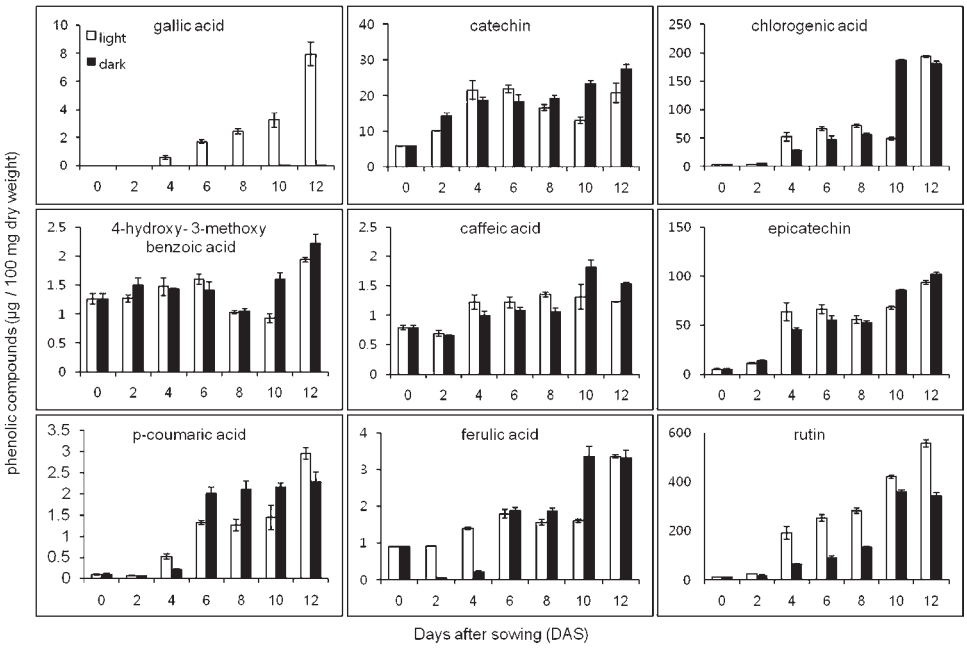


**Figure 4.** Content of phenolic compounds (mg per 100 g dry weight (DW)) in nongerminated seeds (0 days after sowing, DAS) and sprouts (2−12 DAS) of *F. esculentum* grown under light or dark conditions.

grown under dark conditions (343.25 mg/100 g at 12 DAS) (Supplementary Table S2 in the Supporting Information). This result agrees with a previous study that showed that the rutin content of tartary buckwheat seeds depended on growth conditions (*23*). However, light and dark conditions did not significantly affect the amounts of other flavonoids, except gallic acid, in sprouts (**Figure 4**). Interestingly, gallic acid was only found in the sprouts grown under light conditions. Specifically, it gradually increased between 4 and 12 DAS until it reached 7.95 mg/100 g DW at 12 DAS. In buckwheat, gallic acid is an allelochemical (*24*, *25*) and an antimutagen that modulates the gene expression and activity of DNA repair enzymes (*26*). In addition, gallic acid is one of the most potent phenolic antioxidants (*27*).

In this study, we cloned and sequenced the genes encoding flavonoid biosynthesis enzymes in *F. esculentum*, namely, *FePAL*, *FeC4H*, *Fe4CL*, *FeCHS*, *FeCHI*, *FeF3H*, *FeF3′H*, *FeFLS*, and *FeANS*. In addition, the differential expression levels of these genes and the amounts of various phenolic compounds were examined in different organs, sprouts, and seeds. We are currently focusing our research efforts on producing transgenic *F. esculentum* plants to produce medicinally useful compounds.

## ABBREVIATIONS USED

DAS, days after sowing ; RACE, rapid amplification of cDNA ends; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; ANS, anthocyanidin synthase; FLS, flavonol synthase; HPLC, high performance liquid chromatography.

**Supporting Information Available:** Table of primers used for RACE-PCR and real-time PCR, table of data on contents of phenolic compounds, figures depicting protein sequence alignments, and figure depicting accumulation of flavonoid biosynthetic gene transcript in different organs from *F. esculentum*. This material is available free of charge via the Internet at http://pubs.acs.org.

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