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Anthocyanin Accumulation and Expression of Anthocyanin Biosynthetic Genes in Radish (*Raphanus sativus*)

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Supporting Information

ABSTRACT: Radish [*Raphanus sativus* (Rs)] is an important dietary vegetable in Asian countries, especially China, Japan, and Korea. To elucidate the molecular mechanisms of anthocyanin accumulation in radish, the gene expression of enzymes directly involved in anthocyanin biosynthesis was analyzed. These genes include phenylalanine ammonia lyase (*PAL*), cinnamate 4-hydroxylase (*C4H*), 4-coumarate−CoA ligase (*4CL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol reductase (*DFR*), and anthocyanidin synthase (*ANS*). *RsDFR* and *RsANS* were found to accumulate in the flesh or skin of two radish cultivars (Man Tang Hong and Hong Feng No.1). Radish skin contained higher *CHS*, *CHI*, and *F3H* transcript levels than radish flesh in all three cultivars. In the red radish, 16 anthocyanins were separated and identified by high-performance liquid chromatography (HPLC) and elctrospray ionization−tandem mass spectrometry (ESI−MS/MS). Some of them were acylated with coumaroyl, malonoyl, feruoyl, and caffeoyl moieties. Furthermore (−)-epicatechin and ferulic acid were also identified in the three cultivars.

KEYWORDS: Anthocyanin, radish, *Raphanus sativus*, gene expression, HPLC-ESI-MS/MS, phenolic compound

INTRODUCTION

Anthocyanins, pigments found naturally in many plants, are thought to protect plant tissues or senescing autumn leaves against damaging photo-oxidative effects[1] and UV irradiation and to promote pollination or facilitate seed distribution.[2] Some vegetables, including red cabbage, red potato, and red radish, contain high anthocyanin levels.[3]

Radish (*Raphanus sativus*) belongs to the Brassicaceae family. It is an edible root vegetable cultivated and consumed worldwide. Radish varieties differ in size, color, and cultivation requirements.[4,5] Anthocyanins extracted from red radish are widely used as natural food-coloring agents because of their high stability and other characteristics similar to those of synthetic Food Red No. 40.[6] Red-radish anthocyanins can be distinguished from those in other plants by their aglycon structures: red radish contains mainly pelargonidin, whereas other plants contain cyanidin or delphinidin as their respective aglycons.[7,8]

A multienzymatic pathway, the phenylpropanoid pathway, is responsible for anthocyanin production in radishes. This pathway also synthesizes many secondary metabolites, including flavonoids, which are important for various aspects of plant growth and development, including pigment production, photoprotection, and disease resistance.[9,10] The first enzyme in this pathway, phenylalanine ammonia lyase (PAL), converts L-phenylalanine to *trans*-cinnamic acid. The second enzyme, cinnamate 4-hydroxylase (C4H), catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid.[11] Next, 4-coumarate−CoA ligase (4CL) produces ester 4-coumaroyl-CoA, which is a precursor for various phenylpropanoid biosynthetic derivatives, including lignins and flavonoids. Subsequently, chalcone synthase (CHS) catalyzes the production of a tetrahydroxychalcone, the precursor for all flavonoids. This reaction, which is unique in higher plants, is the first step in the flavonoid biosynthetic pathway. Afterward, chalcone isomerase (CHI) catalyzes conversion of the chalcone to naringenin, which is subsequently converted to dihydrokaempferol by flavanone 3-hydroxylase (F3H). Dihydroflavonol 4-reductase (DFR) is the enzyme responsible for anthocyanidin production. Anthocyanidin synthase (ANS) catalyzes the conversion of leucopelargonidin to pelargonidin.[2,12−15]

Partial cDNA sequences of *RsC4H* and *Rs4CL* (Genbank accession numbers HQ642568 and HQ641569, respectively) were cloned in the present study. To compare the gene expression of anthocyanin-synthesizing enzymes in the skin or flesh of three radish cultivars (Seo Ho, Man Tang Hong, and Hong Feng No.1), these and some previously isolated clones (i.e., *RsPAL*, *RsCHS*, *RsCHI*, *RsF3H*, *RsDFR*, and *RsANS*)[16,17] from *R. sativus* were analyzed. Moreover, anthocyanins and phenolic compounds extracted from the skin or flesh of Chinese red-radish with three cultivars were quantified.

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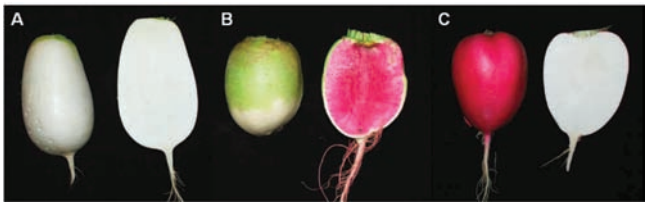

Figure 1. Photographs of the 3 radish cultivars, (A) Seo Ho, (B) Man Tang Hong, and (C) Hong Feng No. 1, used in this study.

MATERIALS AND METHODS

Plant Materials. Three radish cultivars (Seo Ho, Man Tang Hong, and Hong Feng No.1) were grown in a greenhouse at the experimental farm of Rural Development Administration (RDA; Suwon, Korea) in 2009. The plants were harvested when they matured (at 14 to 18 weeks). The color of skin and flesh of Seo Ho is white. Man Tang Hong has white and green colored skin and red interior flesh. Hong Feng No.1 has red colored skin and white interior flesh at maturity (Figure 1A−C). Prior to experiments, radishes were manually peeled, and the epidermal tissues (skin) and the flesh were cut into small cubes. The samples were then freeze-dried at −80 °C for at least 48 h and then ground into a fine powder using a mortar and pestle until high performance liquid chromatography (HPLC) analysis.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction. The skin and flesh samples of each radish were ground with a mortar and pestle under liquid nitrogen. Total RNA was isolated from the skin and flesh samples of each radish cultivar by using the RNeasy Plant Mini Kit (Qiagen; Valencia, CA). For the quantitative real-time polymerase chain reaction (qRT-PCR), 1 μg of total RNA was reverse transcribed using Superscript II First Strand Synthesis Kit (Invitrogen; Carlsbad, CA) and an oligo$(dT)_{20}$ primer.

The primers for radish *C4H* and *4CL* were designed from the conserved sequences of known orthologous sequences (Table S1 in Supporting Information). Then, the PCR products were subcloned into a T-Blunt vector (SolGent, Daejeon, Korea) and sequenced at the National Instrumentation Center for Environmental Management (NICEM; Seoul National University, Korea). The PCR products for C4H and 4CL were obtained from cDNAs of *R. sativus* Seo Ho. The PCR protocol was as follows: 1 cycle of 5 min at 95 °C and 30 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C; and elongation for 30 s at 72 °C. Since the cDNA sequences of radish *RsPAL*, *RsCHS*, *RsCHI*, *RsF3H*, *RsDFR*, *RsANS*, and *RsMYB* have been previously cloned in the database (Genbank accession numbers AB087212, AF031922, AF031921, AB087211, AB087210, AB087206, and DQ538391, respectively), gene-specific primer sets were designed from the cDNA sequences using the Primer3 program, version 0.4.0 (http://frodo.wi.mit.edu/primer3), which helped check primers sequences for self-complementarity or the potential to make secondary structures like hairpins (Table S1 in Supporting Information). Primers were confirmed to be specific for the target genes by electrophoresis of PCR reactions and melting curve analysis of PCR products. Gene expression was normalized to that of the 26S ribosomal gene (Genbank accession number AY366932) as a house-keeping gene.[18] Real-time PCR reactions were performed in 20 μL reaction volumes containing 0.4 μM of each primer and 1× SYBR Green Realtime PCR Master Mix (Toyobo; Osaka, Japan) on a MiniOpticon system (Bio-Rad Laboratories; Hercules, CA). PCR amplification was performed as recommended by the manufacturer with modified cycling parameters. The PCR protocol was as follows: 1 cycle of 5 min at 95 °C and 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. qRT-PCR experiments were performed in triplicate for each sample.

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) and modified by Bioedit Phylodraw software.

Chemicals. Seven phenolic compounds, such as chlorogenic acid, caffeic acid, (−)epicatechin, *p*-coumaric acid, benzoic acid, quercetin, and kaempferol, were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Two anthocyanins (cyanidin-3-*O*-glucoside chloride and pelargonidin-3-*O*-glucoside chloride) for external standards were purchased from Extrasynthèse (Genay, France). HPLC-grade acetonitrile (CH_3CN) and methanol (MeOH) were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Formic acid and acetic acid were provided from Kanto Chemical Co., Inc. (Tokyo, Japan) and Jun sei Chemical Co., Ltd.(Kyoto, Japan), respectively.

Extraction and High-Performance Liquid Chromatography (HPLC) Analysis of Anthocyanin. A 100 mg portion of the lyophilized powder was transferred to a 2 mL Eppendorf tube, to which was added 2 mL of water/formic acid (95/5, v/v). The solution was vigorously vortexed for 5 min, sonicated for 20 min, and centrifuged at 8,000 rpm for 15 min. The extract was filtered through a 0.45 μm PTFE syringe filter (Advantec DISMIC-13_{HP}, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was then analyzed by an Agilent Technologies 1200 Series HPLC (Agilent Technologies, Palo Alto, CA), equipped with an Agilent 1200 HPLC variable wavelength detector. The data were recorded with a COMPAQ Presario CQ3100KL computer (Compaq Computer Corporation, Houston, Tex) and analyzed by Agilent 1200 HPLC ChemStation software.

The chromatographic separation was performed on a Synergy 4 μm Polar-RP 80A (150 × 2 mm, i.d.) column with a Security Guard AQ C18 (4 × 3 mm, i.d.) both purchased from Phenomenex (Torrance, CA). Detection was made at a wavelength of 520 nm, and the column oven temperature was set at 40 °C. The injection sling was 10 μL. The solvent system was delivered at a rate of 0.4 mL/min and consisted of a mixture of (A) water/formic acid (95:5, v/v) and (B) acetonitrile/formic acid (95:5, v/v). The gradient program largely modified from that previously published[19,20] is described as follows: 0−4 min, 15−18% B; 4−8 min, 18% B; 8−13 min, 18−20% B; 13−18 min, 20% B; 18−25 min, 20−26% B; 25−28 min, 26% B; 28−30 min, 26−28% B; 30−33 min, 28% B; 33−35 min, 28−30% B; 35−38 min, 30−40% B; 38−43 min, 40% B; 43−45 min, 40−15% B; 45−55 min, 15% B. Quantification of the different anthocyanins was based on peak areas and calculated as equivalents of two representative standard compounds. All contents were expressed as milligram per g dry weight.

LC/ESI-MS/MS Analysis for Qualitative of Anthocyanins. An API 4000 Q TRAP tandem mass spectrometer (Applied Biosystems, Foster City, CA), equipped with an Agilent 1200 series HPLC system (Agilent Technologies) and an electrospray ionization−tandem mass spectrometry (ESI−MS/MS) source in positive ion mode ($M + H^+$), was used to identify the anthocyanins in red radish (Hong Feng skin). The HPLC conditions were the same as those described above, but the mobile phase was only changed to (A) water/formic acid (99:1, v/v) and (B) acetonitrile/formic acid (99:9, v/v) because of low pH of less than 2.0. The analytical conditions of mass spectrometry were described in detail as follows: range, start (100 amu), stop (1,300 amu), and scan time (4.8 s); curtain gas, 20 psi (N2); heating gas temperature, 550 °C; nebulizing gas, 50 psi; heating gas, 50 psi; ion spray voltage, 5500 V; declustering potential, 100 V; entrance potential, 10 V.

High Performance Liquid Chromatography (HPLC) Analysis for Phenolic Compounds. For the quantification of phenolic compounds, the powdered tissues (100 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 μm Acrodisc syringe filter (Pall Corp.; Port Washington, NY) prior to HPLC analysis. HPLC analysis

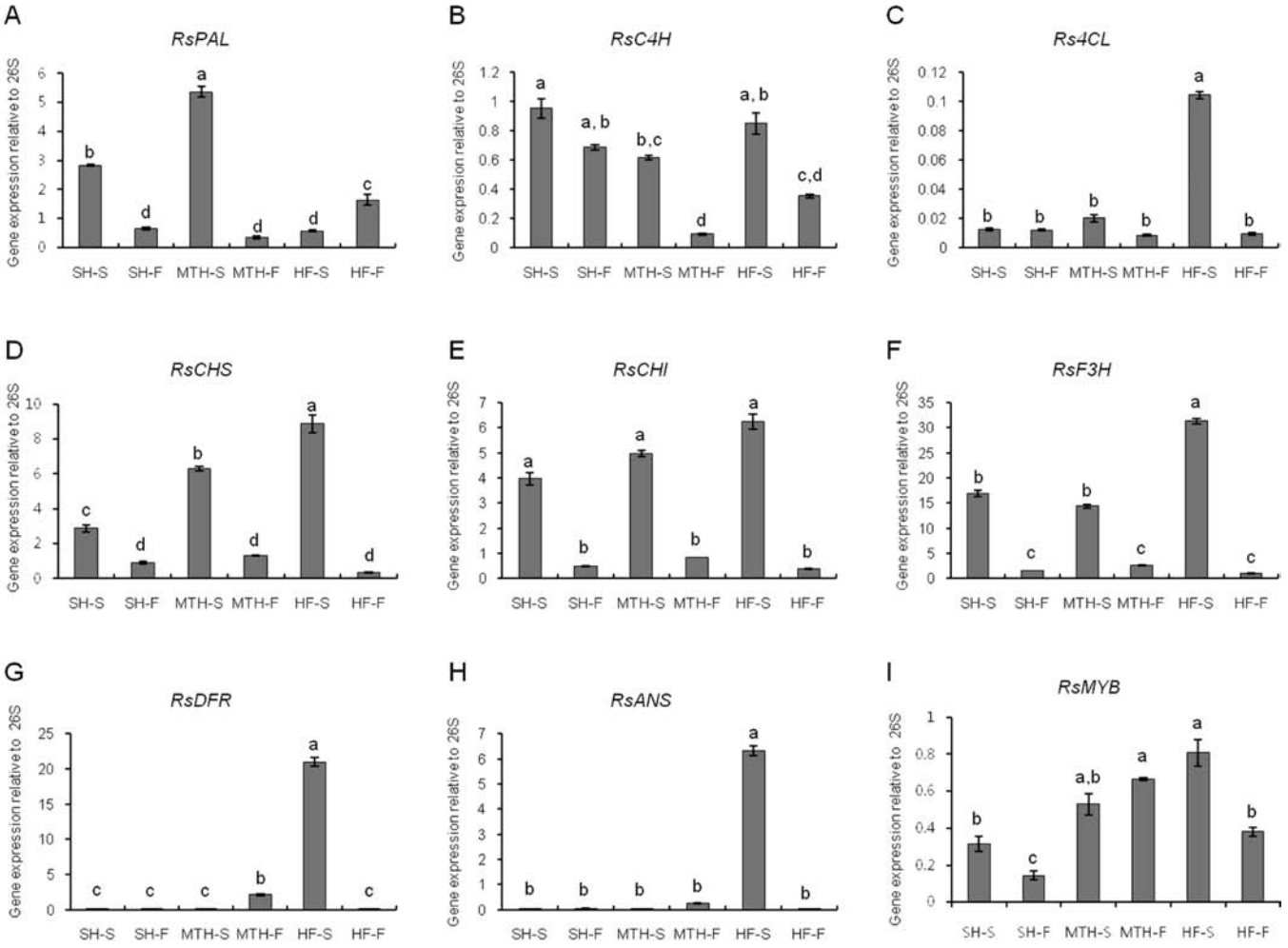

Figure 2. Accumulation of anthocyanin-biosynthetic genes and RsMYB gene in the skin and flesh of 3 radish cultivars. (A) *RsPAL*, (B) *RsC4H*, (C) *Rs4CL*, (D)*RsCHS*, (E) *RsCHI*, (F) *RsF3H*, (G) *RsDFR*, (H) *RsANS*, and (I) RsMYB gene expression in the skin and flesh of 3 radish cultivars. SH-S, Seo Ho skin; SH-F, Seo Ho flesh; MTH-S, Man Tang Hong skin; MTH-F, Man Tang Hong flesh; HF-S, Hong Feng No.1 skin; and HF-F, Hong Feng No.1 flesh. Error bars represent the standard error of the mean ($n = 3$). Statistical significance of the differences between treatments was determined using ANOVA followed by paired-group comparisons. Different letters indicate significance at $P < 0.05$.

was performed with a C_{18} column (250 × 4.6 mm, 5 μm; RStech; Daejeon, Korea). The mobile phase was composed of 0.15% acetic acid (A) and methanol (B). The gradient conditions were as follows: The initial mobile phase composition was 5% B, followed by a linear gradient from 5% to 80% of solvent B in 48 min, then returning to the initial conditions in 5 min, and holding at 5% solvent B for an additional 10 min. The column was maintained at 30 °C, and the flow rate was maintained at 1.0 mL·min^{-1}. The injection volume was 20 μL, and the detection wavelength was 280 nm. The concentrations of phenylpropanoid compounds were determined by using a standard curve. Quantification of the different phenolic compounds were based on peak areas and calculated as equivalents of seven representative standard compounds. All contents were expressed as microgram per 100 mg dry weight.

Statistical Analysis. Data were analyzed using SPSS, version 18.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by pairwise comparisons was performed with posthoc Tukey's honestly significant different (HSD) test, with significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Isolation of Gene Sequences of Anthocyanin-Biosynthetic Enzymes from *R. sativus*. Using conserved sequences of C4H and 4CL, the nucleotide sequences of partial cDNAs for *RsC4H* and *Rs4CL* were obtained and cloned from *R. sativus* Seo Ho. Protein sequence alignment was used to compare these with orthologous sequences from other plants (Figure S1 in Supporting Information). These sequences and previously isolated clones for *RsPAL*, *RsCHS*, *RsCHI*, *RsF3H*, *RsDFR*, and *RsANS* from *R. sativus* were used to compare gene-expression levels of the corresponding enzymes.

Gene Expression of Anthocyanin-Synthesizing Enzymes in the Skin and Flesh of Different Radish Cultivars. To investigate the mechanisms controlling anthocyanin biosynthesis in *R. sativus*, gene-expression levels of anthocyanin-synthesizing enzymes were examined in the skin and flesh of three radish cultivars (Seo Ho, Man Tang Hong, and Hong Feng No.1) (Figure 1A−C). Gene expression levels of *RsPAL*, *RsC4H*, *Rs4CL*, *RsCHS*, *RsCHI*, *RsF3H*, *RsDFR*, and *RsANS* are shown in Figure 2A−H. Although the transcripts for most of these enzymes were expressed in both tissue types (skin and flesh) of the three radish cultivars, *RsDFR* and *RsANS* were not expressed in the skin/flesh of Seo Ho, the skin of Man Tang Hong, or the flesh of Hong Feng No.1. Particularly, *RsCHS*, *RsCHI*, and

Table 1. Anthocyanin Levels (mg/g Dry wt.) in the 3 Radish Cultivars (Seo Ho, Man Tang Hong, and Hong Feng No.1) (n = 3)

		radish cultivars					
no.[a]	anthocyanin	SH-S[b]	SH-F	MTH-S	MTH-F	HF-S	HF-F
1	pelargonidin 3-diglucoside-5-glucoside	Nd	Nd	0.01 ± 0.00	0.17 ± 0.02	0.08 ± 0.01	Nd
2	pelargonidin 3-diglucoside-5-(malonyl)glucoside	Nd	Nd	Tr[c]	0.20 ± 0.01	0.24 ± 0.02	Nd
3	pelargonidin 3-(caffeoyl)diglucoside-5-glucoside	Nd	Nd	Nd[d]	0.02 ± 0.00	0.02 ± 0.01	Nd
4	cyanidin 3-(glucosyl)rhamnoside	Nd	Nd	Nd	0.12 ± 0.00	0.14 ± 0.02	Nd
5	pelargonidin 3-(caffecoyl)diglucoside-5-glucoside	Nd	Nd	Nd	Nd	0.04 ± 0.01	Nd
6	pelargonidin 3-(*p*-coumaroyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.37 ± 0.00	0.56 ± 0.04	Nd
7	pelargonidin 3-(caffeoyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.09 ± 0.00	0.36 ± 0.03	Nd
8	pelargonidin 3-(feruloyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.03 ± 0.00	0.02 ± 0.01	Nd
9	pelargonidin 3-(*p*-coumaroyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.29 ± 0.00	0.28 ± 0.02	Nd
10	pelargonidin 3-(*p*-coumaroyl)glucoside-5-glucoside	Nd	Nd	Nd	0.20 ± 0.00	0.07 ± 0.01	Nd
11	pelargonidin 3-(*p*-coumaroyl)(caffecoyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.16 ± 0.01	0.08 ± 0.01	Nd
12	pelargonidin 3-(feruloyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.06 ± 0.00	0.02 ± 0.01	Nd
13	pelargonidin 3-(*p*-coumaroyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	1.37 ± 0.02	1.87 ± 0.08	Nd
14	pelargonidin 3-(feruloyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	0.01 ± 0.00	1.33 ± 0.03	0.61 ± 0.04	Nd
15	pelargonidin 3-(feruloyl) (caffecoyl)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.10 ± 0.01	0.03 ± 0.01	Nd
16	pelargonidin 3-(*p*-coumaroyl)(feruloy)diglucoside-5-(malonyl)glucoside	Nd	Nd	Nd	0.20 ± 0.00	0.04 ± 0.00	Nd
total		Nd	Nd	**0.03 ± 0.00**	**4.72 ± 0.13**	**4.48 ± 0.32**	Nd

[a] no. corresponds to elution order by HPLC analysis in Figure S3, Supporting Information. [b] SH-S, Seo Ho skin; SH-F, Seo Ho flesh; MTH-S, Man Tang Hong skin; MTH-F, Man Tang Hong flesh; HF-S, Hong Feng No.1 skin; and HF-F, Hong Feng No.1 flesh. [c] Tr, trace. [d] Nd, not detected.

RsF3H expression levels were similarly high in the skin, but low in the flesh, of all radish cultivars. In Seo Ho and Man Tang Hong, the levels of *RsPAL*, *RsC4H*, *RsCHS*, *RsCHI*, and *RsF3H* were lower in the flesh than in the skin. However, the levels of *RsDFR* and *RsANS* were higher in the flesh than in the skin. In Hong Feng No.1, expression of all the genes except *RsPAL* was lower in the flesh than in the skin.

In white radish and white tissues of red radish, six genes involved in anthocyanin biosynthesis (except *RsDFR* and *RsANS*) were expressed. Although anthocyanin-biosynthetic genes exist in three radish cultivars, there could be many transcription factors which are involved in the regulation of anthocyanins production.[21] The *Arabidopsis* R2R3-MYB transcription factor, PAP1, was known to regulate the transcription of flavonoid-pathway genes.[22] Previously cloned partial-*RsMYB* was investigated in the same tissues of three radish cultivars (Man Tang Hong, Seo Ho, and Hong Feng No.1). As expected, Man Tang Hong flesh and Hong Feng No.1 skin showed higher gene-expression levels of *RsMYB* than Man Tang Hong skin and Hong Feng No.1 flesh (Figure 2I). *RsMYB* sequences were similar to other transcription factors (e.g., PAP1/2, AtMYB113, and AtMYB114) which are known in the anthocyanin pathway (Figure S2 in Supporting Information).[23] Radish transcription factor, RsMYB, might be correlated with the anthocyanin pathway. *RsMYB* overexpression similar to that of other transcription factors[24] may regulate the transcription of anthocyanin-biosynthetic genes so as to increase anthocyanin production levels.

Qualitative and Quantitative Analyses of Anthocyanins. A total of 16 anthocyanins were separated and identified by LC−ESI−MS/MS in extracts from Man Tang Hong flesh and Hong Feng No.1 skin (Table 1 and Figure S3 and Table S2 in Supporting Information). To confirm the identity of anthocyanins in the three radish cultivars, MS/MS (m/z) fragmentation patterns were compared with those in the previously published reports.[7,19] As a result, pelargonidins were identified as the major anthocyanidin in the three radish cultivars with a single type of cyanidin. Furthermore, almost all anthocyanin modifications were found to be acylated pelargonidin of anthocyanidins, except for two glycosides, such as pelargonidin 3-diglucoside-5-glucoside and cyanidin 3-(glucosyl) rhamnoside, in this study. Our results concur with previously published findings.[7,25]

Anthocyanin levels were found to be 4.69 and 4.39 mg/g of dry wt. for Man Tang Hong flesh and Hong Feng No.1 skin, respectively (Table 1). These amounts are very similar to or slightly higher than those found in red radish reported in the United States (100.1 mg/100 g fresh wt., 95.6% moisture).[26] However, only 2 types of anthocyanins at extremely low amounts (0.03 mg/g dry wt.) were detected in Man Tang Hong skin. These were not detected in Seo Ho skin, Seo Ho flesh, or Hong Feng No.1 flesh. Moreover, the anthocyanin showing the highest level was pelargonidin 3-(*p*-coumaroyl)diglucoside-5-(malonyl)-glucoside (1.37 and 1.86 mg/g dry wt., respectively) in Man Tang Hong flesh and Hong Feng No.1 skin.

Analysis of Phenolic Compounds. Levels of several phenolic compounds, including benzoic acid, caffeic acid, ferulic acid, chlorogenic acid, quercetin, (−)-epicatechin, and *p*-coumaric acid in the 3 radish cultivars are shown in Figure 3. Although the level of chlorogenic acid (1.72−4.04 μg/100 mg dry wt.) was similar in all three cultivars, it was 2-fold higher in the Seo HO skin than in the Hong Feng No.1 flesh. Among the detected phenolic compounds, caffeic acid was found to comprise 1.61 μg/100 mg dry wt. of the flesh and skin of Man Tang Hong and Hong Feng No.1, respectively. However, the levels of this compound were relatively low in Seo Ho skin, Seo Ho flesh, Man Tang Hong skin, and Hon Feng No.1 flesh, comprising only 0.34−0.9 μg/100 mg dry wt. Because Man Tang Hong flesh and Hong Feng No.1 skin contain red pigments (Figure 1B and C), anthocyanins were found only in their flesh or skin, respectively (Table 1, Figure 3). In addition, (−)-epicatechin was found to be higher in Man Tang Hong flesh and Hong Feng No.1 skin

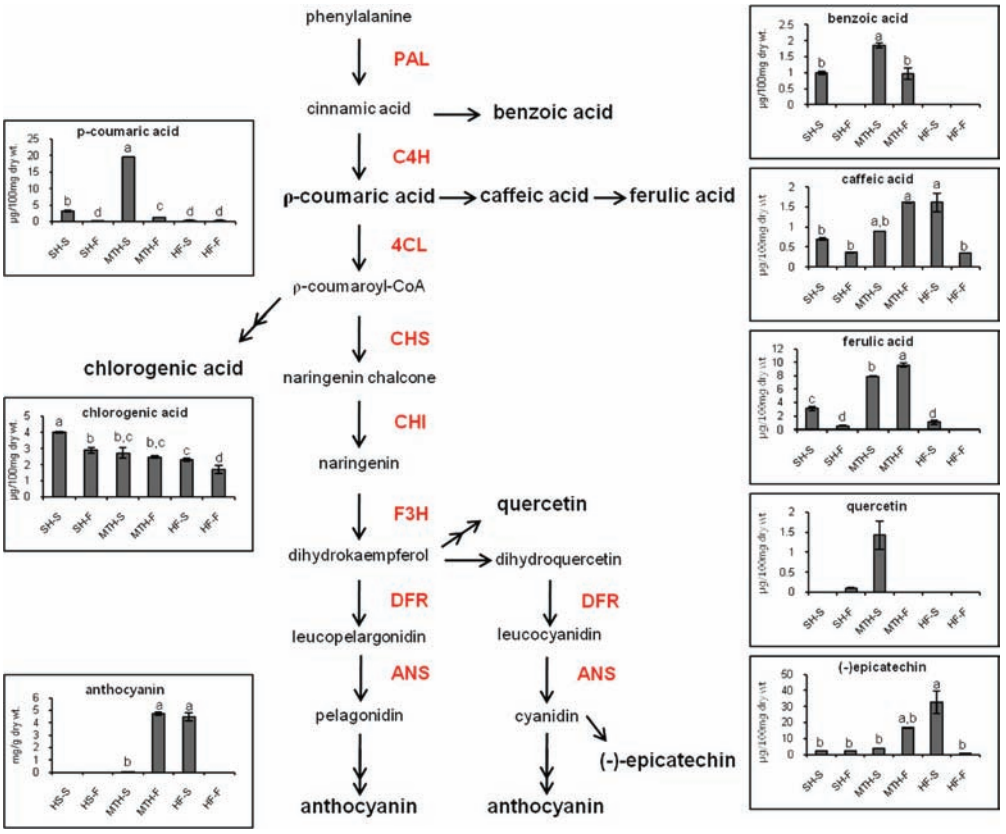

Figure 3. Pathway of anthocyanin biosynthesis and levels of phenolic compounds and anthocyanins in the skin and flesh of 3 radish cultivars. Enzyme genes that have been cloned are indicated in red. SH-S, Seo Ho skin; SH-F, Seo Ho flesh; MTH-S, Man Tang Hong skin; MTH-F, Man Tang Hong flesh; HF-S, Hong Feng No. 1 skin; HF-F, Hong Feng No. 1 flesh; PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate−CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase. Error bars represent the standard error of the mean ($n = 3$). Statistical significance of the differences between treatments was determined using ANOVA, followed by paired-group comparisons. Different letters indicate significance at $P < 0.05$.

(16.92 and 32.57 μg/100 mg dry wt., respectively) than other radish tissues (0.87−3.95 μg/100 mg dry wt.). As shown in Figure 3, (−)-epicatechin and anthocyanin share the same biosynthetic pathway starting from phenylalnine to anthocyanidins (e.g., pelargonidin).[27] The flavanol (−)-epicatechin, an antioxidant found in large quantities in tea and dark chocolate,[28−31] is a precursor for proanthocyanidins.[32,33] Quercetin and *p*-coumaric acid were found mostly in Man Tang Hong skin. However, their levels were low in Man Tang Hong flesh or Hong Feng No.1 skin.

Although the levels of anthocyanins and phenolic compounds were similar between Man Tang Hong flesh and Hong Feng No.1 skin, the expression patterns of related genes differed (Figure 2). Expression levels of *Rs4CL* to *RsANS* were higher in Hong Feng No.1 skin than in other tissues. However, the same genes, including *RsPAL*, showed the lowest levels in Man Tang Hong flesh. These results suggest that the genes described in this study are directly related to anthocyanin production in Hong Feng No.1 skin and Man Tang Hong flesh.

In this study, a total of 16 anthocyanins were identified by LC−ESI−MS/MS in the Chinese red radish. The total anthocyanin content was found to be 4.69 and 4.39 mg/dry wt. in Man Tang Hong flesh and Hong Feng No.1 skin, respectively. Pelargonidins were identified as the major anthocyanidin in the Man Tang Hong flesh and Hong Feng No.1 skin, with a single cyanidin type. To elucidate the molecular basis of anthocyanin accumulation in radish, gene expression of anthocyanin-biosynthetic enzymes (e.g., *PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *DFR*, and *ANS*) were assessed. *RsDFR* and *RsANS* mostly accumulated in Man Tang Hong flesh and Hong Feng No.1 skin. (−)-Epicatechin and caffeic acid were mostly detected in Man Tang Hong flesh and Hong Feng No.1 skin, whereas *p*-coumaric acid and quercetin were found only in Man Tang Hong skin.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. A table of primers used for RACE-PCR and real-time PCR, a table showing data on anthocyanin identification in the Chinese red radish, a figure depicting protein-sequence alignments of *RsC4H* and *Rs4CL*, nucleotide-sequence alignment of RsMYB from *R. sativus* with MYBs from other plants, and a figure depicting HPLC profiles of anthocyanins in the Chinese red radish. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; HPLC, high-performance liquid chromatography; ESI-MS/MS, elctrospray ionization−tandem mass spectrometry; qRT-PCR, quantitative real-time reverse transcription PCR; BLAST, Basic Local Alignment Search Tool.

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