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ARTICLE

Variation and Correlation Analysis of Flavonoids and Carotenoids in Korean Pigmented Rice (*Oryza sativa* L.) Cultivars

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Flavonoids and carotenoids of pigmented rice (*Oryza sativa* L.), including five black cultivars and two red cultivars, from Korea were characterized to determine the diversity among the phytochemicals and to analyze the relationships among their contents. Black cultivars were higher in flavonoids and carotenoids than the red and white cultivars. The profiles of eight phytochemicals identified from the rice grains were subjected to principal component analysis (PCA) to evaluate the differences among cultivars. PCA could fully distinguish between these cultivars. The Heugjinjubyeo (BR-1) and Heugseolbyeo (BR-2) cultivars were separated from the others based on flavonoid and carotenoid concentrations. Flavonoid contents had a positive correlation with carotenoid contents among all rice grains. The BR-1 and BR-2 cultivars appear to be good candidates for future breeding programs because they have simultaneously high flavonoid and carotenoid contents.

KEYWORDS: Anthocyanin; flavonoid; carotenoid; principal component analysis; rice

INTRODUCTION

Rice is a staple food for nearly half of the world's population. In recent decades, genetic improvement in rice grain quality has become important in rice breeding and considerable progress has been made in breeding for quality. Several successful examples of biofortification of rice to improve its nutritional quality and combat nutritional deficiencies via a transgenic engineering approach have been reported. Ye et al. introduced the β-carotene synthesis pathway into rice endosperm by genetic engineering to obtain golden rice that produces 0.16 mg/100 g of β-carotene in the grains (*1*). Storozhenko et al. reported on the biofortification of folate content in rice grains by overexpression of two genes encoding GTP cyclohydrolase I and aminodeoxychorismate synthase (*2*). Recently, the α' and β subunits of soybean β-conglycinin were expressed in rice seeds to improve the nutritional and physiological properties of rice as a food (*3*). However, few reports have described the biofortification of rice grains to improve nutritional quality by conventional breeding (*4*). As the first step toward achieving this goal, investigating diversity in phytochemicals among rice varieties is necessary to find a way of enriching these compositions through breeding.

Some pigmented rice cultivars contain phytochemicals that are responsible for their color. Generally, these colored compounds or pigments fall into several large groups, such as chlorophylls, carotenoids, and flavonoids. The genotypic diversity of some phytochemicals in rice grains has been characterized. For example, Shen et al. reported a wide range of total flavonoid contents and antioxidant capacity in rice grains (*5*). Jiang et al. reported the mineral contents and their correlations with other quality traits of rice (*6*). However, individual flavonoids and carotenoids have not attracted as much attention as other components in rice grains and the phytochemicals in other cereals, fruits, and vegetables (*7*, *8*).

Anthocyanins, a group of reddish purple, water-soluble flavonoids that are the primary pigments in the red and black grains, have been widely identified and characterized in cereal grains (*9*, *10*). The major components of anthocyanidins in pigmented rice are cyanidin-3-*O*-β-glucoside and peonidin-3-*O*-β-glucoside. However, few studies have reported on the characterization of other flavonoids, such as flavonols and flavones, in pigmented rice (*11*).

Carotenoids play essential roles in plants, e.g., photoprotective functions during photosynthesis (*12*). Anthocyanins in plants also function as photoprotectants (*13*). Nagira et al. suggested that changes in the levels of endogenous abscisic acid (ABA), the metabolic end product of carotenoid, may play an important role in the induction of anthocyanin synthesis in regenerated torenia shoots (*14*). However, no previous reports have described the correlations between these pigments, and their contents or compositions in Korean pigmented rice have not been determined.

The present study was performed to determine the flavonoids and carotenoids in black, red, and white rice grains and to analyze the relationships among their contents. The results of this study could provide rice breeders and eventually commercial rice growers new opportunities to promote the production of rice with enhanced levels of bioactive compounds.

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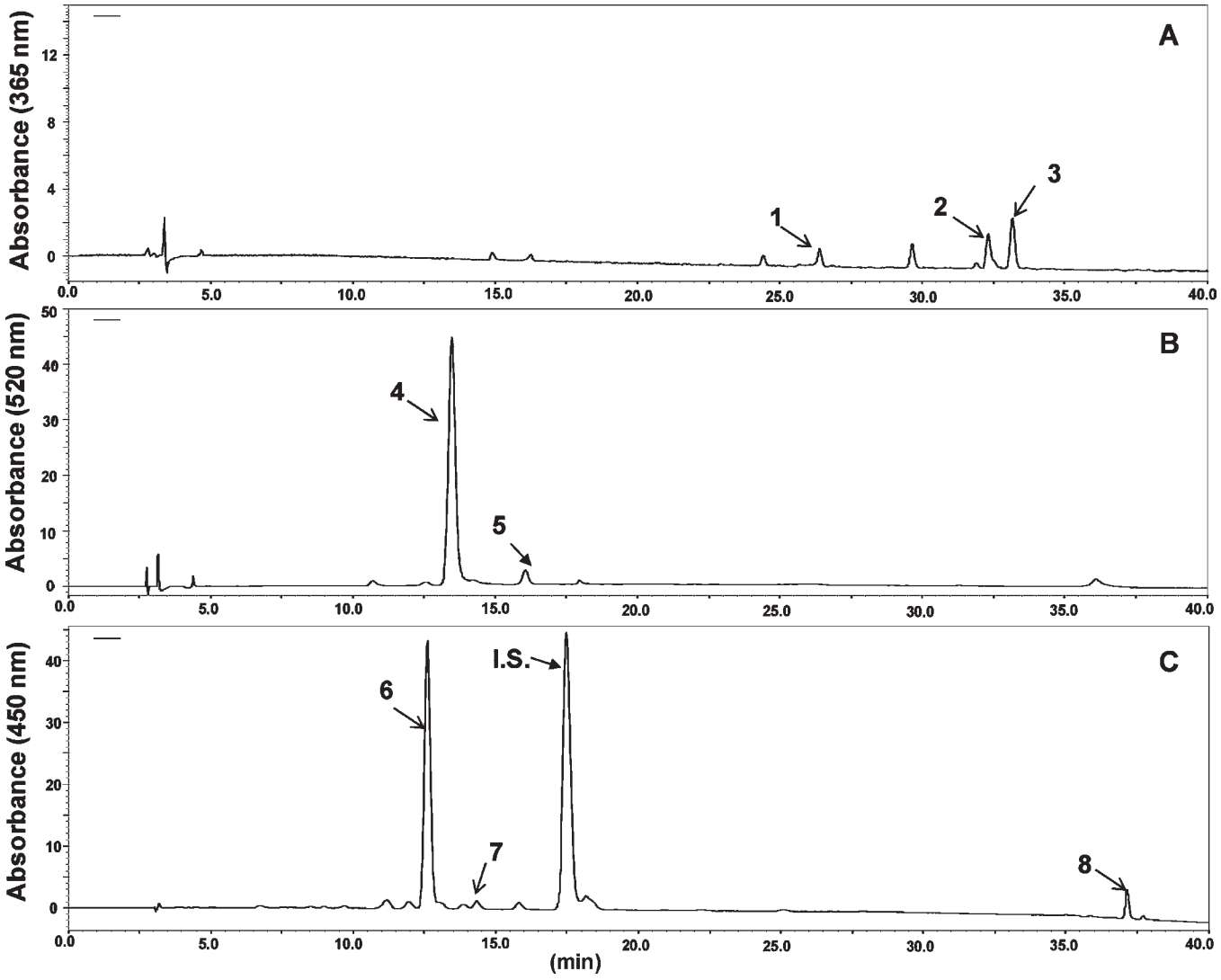

Figure 1. HPLC chromatograms of (A) flavonoid aglycones, (B) anthocyanins, and (C) carotenoids in rice seed (BR-1). The peaks correspond to the following: 1, quercetin; 2, apigenin; 3, kaempferol; 4, cyanidin-3-*O*-glucoside; 5, peonidin-3-*O*-glucoside; 6, lutein, 7, zeaxanthin; 8, β-carotene; and IS, internal standard (β-apo-8′-carotenal).

MATERIALS AND METHODS

Samples and Chemicals. Five cultivars of black rice (Heugjinjubyeo, BR-1; Heugseolbyeo, BR-2; Josengheugchalbyeo, BR-3; Heugnambyeo, BR-4; and Heughyangbyeo, BR-5), two cultivars of red rice (Hongjinjubyeo, RR-1; and Jeogjinjubyeo, RR-2), and one cultivar of white rice (Hwasungbyeo, WR-1) were used in this study. All of the rice grains were harvested at the National Institute of Crop Science farm in 2008. They were manually hulled and ground to obtain a fine powder using a cyclone mixer mill (HMF-590, Hanil, Seoul, Korea) and a mortar and pestle. The milled rice powders were kept at −80 °C before extraction. Apigenin, kaempferol, quercetin, and β-apo-8′-carotenal were purchased from Sigma Chemical Co. (St. Louis, MO). Lutein, β-carotene, and zeaxanthin were obtained from CaroteNature (Lupsingen, Switzerland). Cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were purchased from Extrasynthese (Genay, France).

Extraction and Analysis of Flavonoids. Extraction, separation, and measurement of flavonoid aglycones by high-performance liquid chromatography (HPLC) were performed as described by our group (*15*). Flavonoids were released and hydrolyzed from the powdered rice (100 mg, approximately five grains) by adding 1.2 mL of 50% MeOH containing 1.2 M HCl at 80 °C in a water bath for 2 h. The crude suspensions were centrifuged at 10000*g* at 4 °C for 5 min. The crude extracts were passed through 0.22 μm Teflon polytetrafluoroethylene (PTFE) syringe filters and injected directly into the HPLC column. Flavonoid aglycones were separated on a C_{18} column (250 × 4.6 mm, 5 μm, Inertsil ODS-3, GL Sciences, Tokyo, Japan) by HPLC (Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector. Elution was performed using a binary gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) according to the following program: 0 min, 95% A/5% B; 30 min, 60% A/40% B; 45 min, 50% A/50% B; 50 min, 0% A/100% B; 60 min, 0% A/100% B; 62 min, 95% A/5% B; and 70 min, 95% A/5% B. The flow rate was 1.0 mL/min, and the column temperature was 40 °C. The ultraviolet−visible (UV−vis) detector wavelength was set at 364 nm. For quantification purposes, a mixture of three flavonoid standards (apigenin, kaempferol, and quercetin) was used to create a calibration curve of the individual flavonoids. For identification of kaempferol and quercetin in WR-1 cultivar, the extracts were separated on a Waters (Milford, MA) symmetry C_{18} (150 × 2.1 mm, 5 μm) column using a HPLC system. The elution buffer and column temperature were the same as described above. The following elution program was applied: 0 min, 100% A/0% B; 25 min, 0% A/100% B; 35 min, 0% A/100% B; 37 min, 100% A/0% B; and 47 min, 100% A/0% B. The flow rate was 0.2 mL/min. The eluate was diverted to a quadrupole mass spectrometer (LC/MS2010A, Shimadzu) equipped with a negative electrospray ionization source. The spray voltage was set to 4.5 kV, and the capillary temperature was set to 250 °C.

Extraction of anthocyanin was performed according to a slightly modification of the method by Abdel-Aal et al. (*9*). A total of 50 mg of sample was extracted with 0.8 mL of 85% methanol acidified with 1.0 N HCl followed by sonication for 1 min. The extracts were then incubated at

38 °C for 30 min with a mixing frequency of 500 rpm using a Thermomixer compact (Eppendorf, Hamburg, Germany). The crude suspension was centrifuged at 10000*g* at 4 °C for 5 min, and the crude extract was passed through a 0.22 μm Teflon PTFE syringe filter before HPLC analysis. Anthocyanin was separated on a C_{18} column (250 × 4.6 mm, 5 μm, Inertsil ODS-3, GL Sciences, Tokyo, Japan) by HPLC as described above. Elution was performed using a binary gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) according to the following program: 0 min, 95% A/5% B; 40 min, 50% A/50% B; 42 min, 0% A/100% B; 52 min, 0% A/100% B; 54 min, 95% A/5% B; and 64 min, 95% A/5% B. The flow rate was 1.0 mL/min, and the column temperature was 40 °C. The UV−vis detector wavelength was set at 520 nm. The anthocyanin contents were calculated by HPLC peak areas compared to external standard calibration curves. The linear equations and regression coefficients for cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were $y = 12100x - 934.5$, with $r = 0.9967$, and $y = 22981x - 3972$, with $r = 0.9999$, respectively.

Extraction and Analysis of Carotenoids. The extraction method used for carotenoid analysis was similar to that described elsewhere (*16*). Briefly, carotenoids were released from the rice samples (0.6 g) by adding 3 mL of ethanol containing 0.1% ascorbic acid (w/v), vortex mixing for 20 s, and placing in a water bath at 85 °C for 5 min. The carotenoid extract was saponified with potassium hydroxide (120 μL, 80%, w/v) at the 85 °C water bath for 10 min. After saponification, samples were placed immediately on ice and cold deionized water (1.5 mL) was added. β-Apo-8′-carotenal (0.05 mL, 25 μg/mL) was added as an internal standard. Carotenoids were extracted twice with hexane (1.5 mL) by centrifugation at 1200*g* to separate the layers. Aliquots of the extracts were dried under a stream of nitrogen and redissolved in 50:50 (v/v) dichloromethane/methanol before analysis by HPLC. The carotenoids were separated on a C_{30} YMC column (250 × 4.6 mm, 3 μm, Waters Corporation, Milford, MA) by HPLC as described above. Chromatograms were generated at 450 nm. Solvent A consisted of methanol/water (92:8, v/v) with 10 mM ammonium acetate. Solvent B consisted of 100% methyl *tert*-butyl ether. Gradient elution was performed at 1 mL/min under the following conditions: 0 min, 83% A/17% B; 23 min, 70% A/30% B; 29 min, 59% A/41% B; 35 min, 30% A/70% B; 40 min, 30% A/70% B; 44 min, 83% A/17% B; and 55 min, 83% A/17% B. For quantification purposes, calibration curves were drawn by plotting at four different concentrations of carotenoid standards according to the peak area ratios with β-apo-8′-carotenal. The responses were linear in the following ranges: 0.03−0.25 μg/mL ($y = 0.1048x + 0.0017$, with $r = 0.9846$), 0.03−0.25 μg/mL ($y = 0.0656x + 0.0010$, with $r = 0.9896$), and 0.03−0.50 μg/mL ($y = 0.3340x + 0.0051$, with $r = 0.9968$) for β-carotene, lutein, and zeaxanthin, respectively.

Statistical Analysis. All analyses were carried out at least in triplicate. Experimental data were analyzed by the analysis of variance (ANOVA), and the significant differences among means were determined by Duncan's multiple range test. Correlation analysis and principal component analysis (PCA) of the results were performed using SAS 9.1 (SAS Institute, Cary, NC). PCA was performed on the basis of the correlation matrix and the calculated eigenvalues and eigenvectors. The principal components with eigenvalues > 1.0 were extracted. The output from PCA consisted of score plots to visualize the contrast between different samples and loading plots to explain the reason for cluster separation. Pearson correlation analysis was carried out among the contents of eight metabolites.

RESULTS AND DISCUSSION

Variation in Flavonoids and Carotenoids in Pigmented Rice. Identification and peak assignment of flavonoids and carotenoids were primarily based on a comparison of their retention time and UV−vis spectrum data to that of standards and guidelines presented previously by Abdel-Aal et al. (*9*), Kim et al. (*15*), and Howe and Tanumihardjo (*16*). Apigenin, kaempferol, quercetin, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, lutein, β-carotene, and zeaxanthin were detected in black rice (**Figure 1**). The total flavonoid and carotenoid levels detected in rice grains are shown in **Figure 2**. The flavonol, carotenoid, and anthocyanin contents varied significantly among black, red, and white grains,

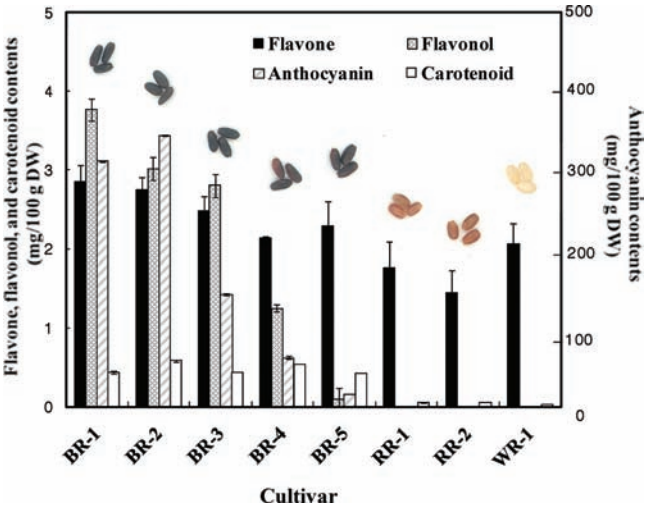

Figure 2. Flavone, flavonol, carotenoid, and anthocyanin contents in eight rice cultivars grown in the Republic of Korea. Photographs show black rice (BR-1, BR-2, BR-3, BR-4, and BR-5), red rice (RR-1 and RR-2), and white rice (WR-1) seeds. The flavone content is apigenin content. The total flavonol content is the sum of quercetin and kaempferol contents. The total carotenoid content is the sum of lutein, zeaxanthin, and β-carotene contents. The total anthocyanin content is the sum of cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside contents. Bars represent mean ± standard error (SE) over three sampling dates.

while the flavone (apigenin) contents were not markedly different among them ($p = 0.0509$). The carotenoid contents indicated distinct differences depending upon grain color. The mean carotenoid levels were 0.482 mg/100 g in black cultivars, 0.052 mg/100 g in red cultivars, and 0.021 mg/100 g in the white cultivar. The contents of kaempferol and quercetin in red and white cultivars were below the limits of detection in this study. However, from liquid chromatography/mass spectrometry (LC/MS) analysis, kaempferol and quercetin were detected in the white cultivar WR-1 (**Figure 3**).

In black rice grains, the predominant flavonols were kaempferol and quercetin (0.10−3.68 mg/100 g) (**Table 1**). A previous study showed that the kaempferol and quercetin contents in French bean were 1.2 and 3.9 mg/100 g, respectively (*17*). Recently, these compounds were found to act synergistically in the inhibition of cell proliferation in human gut cancer lines (*18*). Furthermore, the level of the vitamin A precursor, β-carotene, was significantly higher in black rice than in red and white rice. While Frei and Becker reported that black rice samples from the Philippines had β-carotene values up to 0.013 mg/100 g (*19*), the β-carotene contents in black varieties in the present study ranged from 0.026 to 0.048 mg/100 g. The rice cultivars showed distinct differences in the lutein content depending upon grain color. The highest average content was found in black cultivars, with values up to 0.643 mg/100 g. Lutein has no function as a vitamin A precursor but is a principal component of the macular pigment in the eyes.

Rice grain quality has now become the primary consideration of rice customers and breeding programs. Biofortification by conventional breeding is one of the strategies to improve the nutritional quality of rice grains. The most promising varieties for future breeding purposes would be those with the highest flavonoid and carotenoid contents. Flavonoid and carotenoid contents clearly differ with grain color, but these components still differ among black rice cultivars (**Table 1**); the black rice cultivar BR-2 had the highest flavonoid and carotenoid contents among those examined in the present study.

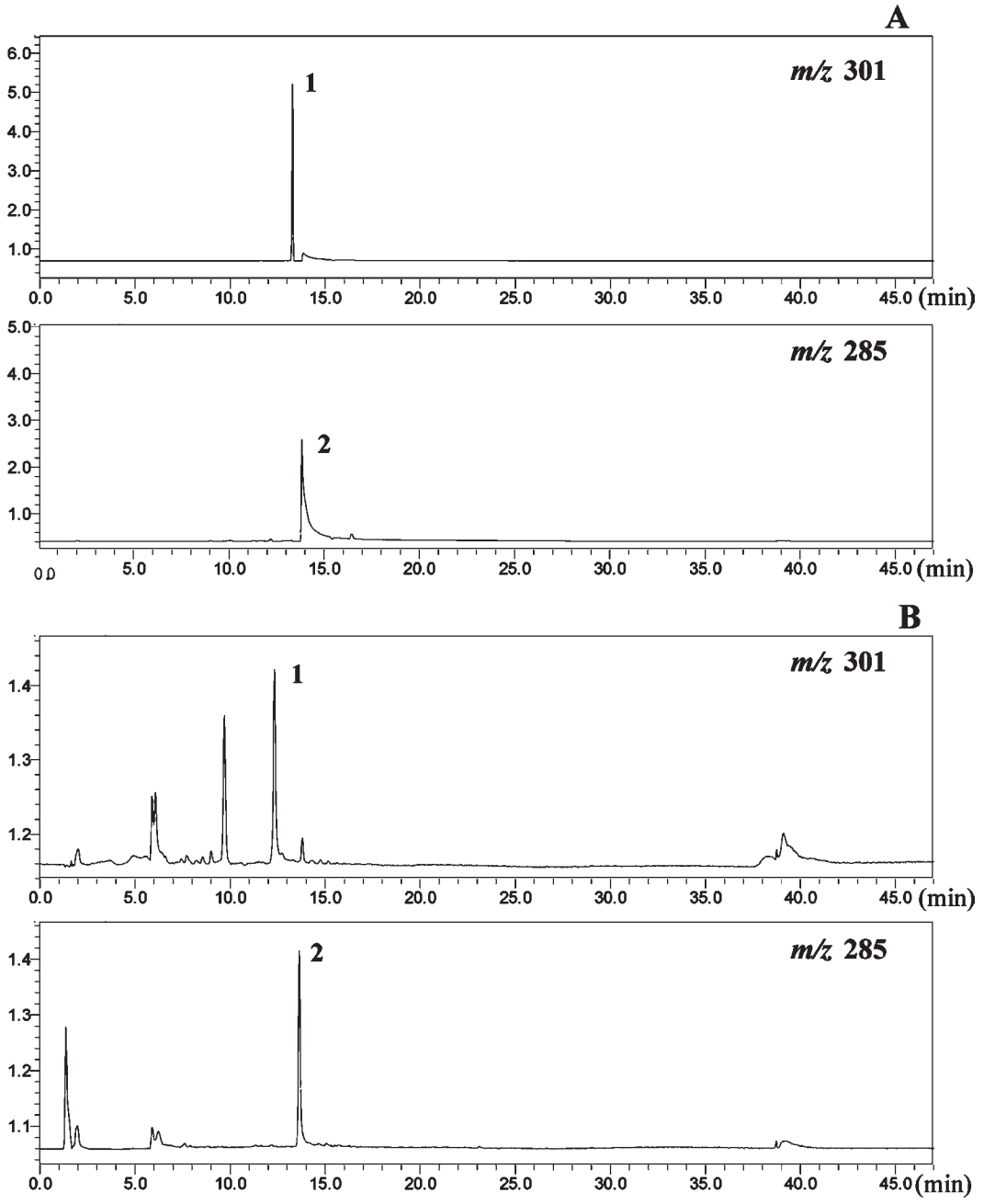

Figure 3. Selective ion chromatograms for *m*/*z* 301 and 285: (A) authentic flavonoid mixtures and (B) flavonoid extracted from rice seed (WR-1). The peaks correspond to the following: 1, quercetin; 2, kaempferol.

Table 1. Content of Flavonoids and Carotenoids in Hulled Rice[a]

	flavonoids (mg/100 g of dry weight)					carotenoids (μg/100 g of dry weight)		
cultivar	quercetin	apigenin	kaempferol	cyanidin[b]	peonidin[c]	lutein	zeaxanthin	β-carotene
BR-1	3.68 ± 0.24 a	2.85 ± 0.37 a	0.59 ± 0.04 a	302.22 ± 28.30 b	9.13 ± 0.49 b	473.76 ± 16.14 c	3.96 ± 2.04 c	21.40 ± 0.66 d
BR-2	2.58 ± 0.30 b	2.75 ± 0.28 ab	0.44 ± 0.04 b	332.64 ± 27.94 a	11.87 ± 0.42 a	642.94 ± 12.69 a	5.37 ± 1.61 c	36.25 ± 2.29 b
BR-3	2.22 ± 0.22 c	2.49 ± 0.32 ac	0.59 ± 0.06 a	139.42 ± 6.09 c	3.80 ± 0.28 c	457.29 ± 29.95 cd	4.38 ± 0.76 c	48.43 ± 0.29 a
BR-4	0.80 ± 0.05 d	2.13 ± 0.04 ad	0.45 ± 0.05 b	60.42 ± 2.90 d	2.30 ± 0.05 d	586.04 ± 30.92 b	8.36 ± 1.63 b	26.43 ± 4.47 c
BR-5	0.10 ± 0.05 e	2.28 ± 0.57 ac	ND[d]	14.42 ± 2.93 e	1.28 ± 0.12 e	441.16 ± 3.33 d	15.68 ± 0.32 a	25.50 ± 1.75 c
RR-1	ND	1.77 ± 0.50 cd	ND	ND	ND	22.73 ± 2.93 e	4.72 ± 1.30 c	6.03 ± 0.88 e
RR-2	ND	1.44 ± 0.47 d	ND	ND	ND	21.83 ± 8.83 e	4.13 ± 2.20 c	6.54 ± 0.79 e
WR-1	ND	2.05 ± 0.26 cd	ND	ND	ND	14.45 ± 0.79 e	2.26 ± 0.08 d	4.73 ± 0.17 f

[a] Each value is the mean of three replications ± standard deviation. Means in each column with similar letters are not significantly different (α = 0.05). [b] Cyanidin = cyanidin-3-*O*-glucoside. [c] Peonidin = peonidin-3-*O*-glucoside. [d] ND = not detectable.

Correlations among the Contents of Eight Phytochemicals in Pigmented Rice. PCA uses a *n*-dimensional vector approach to separate samples on the basis of the cumulative correlation of all component data and then identifies the vector that yields the

greatest separation between samples (*20*). PCA has been used widely in assessing the differences between plant varieties at the metabolome level (*21*). The data obtained for the eight phytochemicals examined were subjected to PCA to outline the differences in phytochemical profiles among cultivars. The results were indicated by plotting the principal component scores. The results indicated that the eigenvalues of the two principal components were all >1.0 and accounted for over 83% of the total variance. The abscissa in **Figure 4** represents the principal component 1 (PC1) score, while the ordinate represents the principal component 2 (PC2) score. PCA in the present study allowed for easy visualization of complex data, and the metabolomes among black, red, and white rice were separated by PC1 and PC2. The PCA results clearly showed the absence of marked variances among samples of the same cultivar. The first principal component, accounting for 66.0% of the total variance, resolved the cultivars according to the total phytochemical contents. The BR-2 cultivar was derived by crossbreeding between Seolgaenbyeo and BR-1 cultivars (*22*). The PCA results showed that the phytochemical profiles of the BR-1 and BR-2 cultivars were less separated than those of other black rice cultivars. Thus, the PCA results indicated the robustness of the present experimental system. Identifying the compounds exhibiting the greatest variance within a population and determining closely related compounds are possible using PCA (*21*). To further investigate the contributors to the principal components, the metabolic loadings in PC1 and PC2 were compared. In PC1, the corresponding loading was positive for all compounds. The variation was mainly attributable to quercetin, cyanidin-3-*O*-glucoside, and peonidin-3-*O*-glucoside, for which the eigenvectors were 0.4072, 0.4000, and 0.3965, respectively. PC2 accounted for an additional 17.7% of the total variance. In PC2, the corresponding loading was positive for carotenoids, such as zeaxanthin, lutein, and β-carotene, and negative for flavonoids, including quercetin, cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, kaempferol, and apigenin. PC2 was directly correlated with zeaxanthin. These results suggested that the reasonable score range of the principal components could be used for excellent sample selection according to the correlations between the original eight variables and these two principal components.

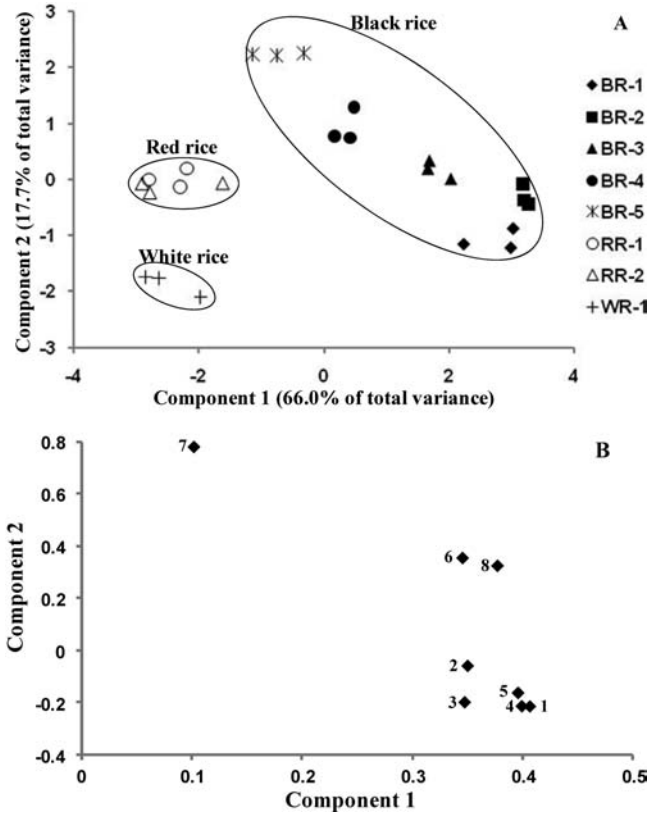

Figure 4. (A) Scores and (B) loading plots of PC1 and PC2 of the PCA results obtained from flavonoid and carotenoid data on eight rice cultivars: 1, quercetin; 2, apigenin; 3, kaempferol; 4, cyanidin-3-*O*-glucoside; 5, peonidin-3-*O*-glucoside; 6, lutein; 7, zeaxanthin; and 8, β-carotene.

To determine the detailed relationships among the contents of the eight metabolites in rice, Pearson's correlation analyses were performed for the accessions (**Table 2**). Among the flavonoids, positive correlations were detected between the contents of flavone and flavonol, between the contents of flavone and anthocyanin, and between the contents of flavonol and anthocyanin. Likewise, significant positive correlations were observed between cyanidin-3-*O*-glucoside and peonidin-3-*O*-glucoside (r = 0.9885) or quercetin (r = 0.9400). These results were consistent with those of previous reports (*23*, *24*). Transcriptional regulators of flavonoid biosynthesis genes induced simultaneous accumulation or reduction of quercetin and anthocyanin.

Examination of the relationships between flavonoids and carotenoids indicated positive associations between all phytochemicals. The contents of functionally related metabolites may show correlations. Although carotenoids differ from flavonoids in that they play essential roles in plants, both carotenoids and flavonoids act as photoprotectants and antioxidants against oxidative damage. Carotenoids are cleaved to xanthoxin (C_{15}) by 9-*cis*-epoxycarotenoid dioxygenase and then converted to ABA via the ABA aldehyde intermediate (*25*). Among the candidates for regulating the induction and accumulation of anthocyanin are plant growth regulators, such as ABA. Although many studies have reported the effects of several types of exogenous plant growth regulators on anthocyanin synthesis (*14*, *26*−*28*), their effects on anthocyanin synthesis are both controversial and complex. However, we postulate that cross talk may occur between flavonoids and carotenoids in rice grains because of the positive correlation between flavonoid and carotenoid contents in rice cultivars.

Table 2. Correlations among Flavonoid and Carotenoid Contents of Hulled Rice

metabolite	quercetin	apigenin	kaempferol	cyanidin	peonidin	lutein	zeaxanthin	β-carotene
quercetin	1.0000							
apigenin	0.7203[a]	1.0000						
kaempferol	0.8937[a]	0.6196[b]	1.0000					
cyanidin[c]	0.9400[a]	0.6935[a]	0.7727[a]	1.0000				
peonidin[d]	0.8977[a]	0.6942[a]	0.7224[a]	0.9885[a]	1.0000			
lutein	0.6800[b]	0.6415[b]	0.7661[a]	0.6889[a]	0.7224[a]	1.0000		
zeaxanthin	0.0029	0.1398	0.0588	0.0265	0.0803	0.5193[b]	1.0000	
β-carotene	0.6525[b]	0.5488[b]	0.7539[a]	0.5660[b]	0.5650[b]	0.8350[a]	0.4858	1.0000

[a] Significant at 0.0001 probability level. [b] Significant at 0.01 probability level. [c] Cyanidin = cyanidin-3-*O*-glucoside. [d] Peonidin = peonidin-3-*O*-glucoside.

In conclusion, the results of this study indicated the diversity of flavonoids and carotenoids in pigmented rice grains and correlations among their contents. PCA was performed to assess overall experimental variation and to test for the presence of differences among the pigmented rice grains; the two black rice cultivars, BR-1 and BR-2, were separated from the others in PC1 and PC2. Metabolic loading in PC1 and PC2 and the results of Pearson correlation analysis indicated correlations between phytochemicals that participate in common or closely related pathways and demonstrated the robustness of the present experimental system.

Consumers are aware of the need for a constant supply of phytochemical-containing plants for antioxidant support and disease prevention. This study provides valuable information regarding future conventional and/or genetic breeding programs for rice containing flavonoids and carotenoids. Black rice containing relatively high levels of flavonols, anthocyanin, and β-carotene should be of high dietary value.

ABBREVIATIONS USED

ABA, abscisic acid; HPLC, high-performance liquid chromatography; PCA, principal component analysis; PC1, principal component 1; PC2, principal component 2; PDA, photodiode array; SE, standard error.

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