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Comparative analysis of phenolic acid profiles of rice grown under different regions using multivariate analysis

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Abstract

This study was conducted to determine the content of phenolic acids in various rice cultivars and to evaluate the impact of genotype versus environmental influence on the phenolic acid profiles of rice grains. Three forms of phenolic acids (free, esterified, and insoluble-bound forms) were identified using gas chromatography–time-of-flight mass spectrometry (GC-TOFMS) in samples of eight Korean rice cultivars (*Oryza sativa* L.) grown together at two different locations. The phenolic acid profiles were subjected to data mining processes, including principal components analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA). The results of OPLS-DA showed clear discrimination between the rice samples based on their growing locations rather than by their genotypes. The major components that contributed to the separation between the two regions were sinapic and ferulic acids in both free and bound forms. These results suggest that the phenolic acid composition in rice grains is determined by environmental factors such as growing condition rather than by genetic factors. This study illustrates the utility of metabolite profiling, combined with chemometrics, as a tool for identifying metabolic differences between crop samples from different regions of cultivation.

Keywords: gas chromatography; OPLS-DA; *Oryza sativa*; PCA; phenolic acid; PLS-DA.
Abbreviations: GC-TOFMS_gas chromatography–time-of-flight mass spectrometry; MTBSTFA_*N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluroacetamide; PCA_principal components analysis; PLS-DA_partial least-squares discriminant analysis; TBDMCS_*tert*-butyldimethylchlorosilane.

Introduction

Phenolic compounds that possess an aromatic ring bearing one or more hydroxyl substituents constitute a large class of plant secondary metabolites that includes many different families of aromatic metabolites such as phenylpropanoids, flavonoids, phenolic acids, tannins, and lignins. These various substances are essential for growth and reproduction in plants, and known to play multifunctional roles in plant defense mechanisms against injuries caused by insects, pathogens, and environmental stresses (Dercks et al., 1990; Horax et al., 2005; Mandal et al., 2010). Recent interest in phenolic acids has increased greatly because of their radical scavenging activity, which is determined by their hydrogen atom donating ability. Many studies have shown a wide range of physiological properties of these compounds such as antioxidant, antiallergenic, antimicrobial, cardioprotective, and vasodilatory properties (Pupponen-Pimiä et al., 2001; Vichapong et al., 2010). Thus, interest is growing in investigating the phenolic acid profiles of plant-derived foods including fruits, vegetables, and cereal grains. Phenolic acids are produced in plants via shikimic acid through the phenylpropanoid pathway. Naturally occurring phenolic acids contain two distinguishing constitutive carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures. The most commonly encountered hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic acids. Hydroxybenzoic acids consist mainly of *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids (Qiu et al., 2010). These compounds exist in plants in three forms: free acids, esters, or acetal bonds to structural components (e.g., cellulose, lignin), smaller organic molecules (e.g., glucose, quinic, or maleic acids), or other natural products (e.g., flavonoids, terpenes; Robbins, 2003). This diversity in structures is one of the difficulties in determining phenolic acids in aromatic plants, both qualitatively and quantitatively. Recently, we determined the phenolic acid content in colored rice grains using *tert*-butyldimethylsilyl (TBDMS) derivatization and the gas chromatography–mass spectrometry (GC-MS) technique in which compounds were analyzed as two forms, free acids and esters (Park et al., 2012). However, the content and composition of the three forms of phenolic acids in rice grains of various varieties and from different growing regions had not been evaluated. Biochemical profiling coupled with chemometrics such as principal components analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal PLS-DA (OPLS-DA) allows for sample

Table 1. Environmental conditions at the growing locations in Korea.

Location	Soil pH[1)]	Temp (°C)[2)]			Rainfall (mm)[2)]
		Min	Max	Average	
Gunwi	6.5	17.9	28.6	22.7	5.6
Suwon	6.3	20.6	28.8	24.3	11.0

[1)]Value before planting. [2)]Average per location from planting to harvest.

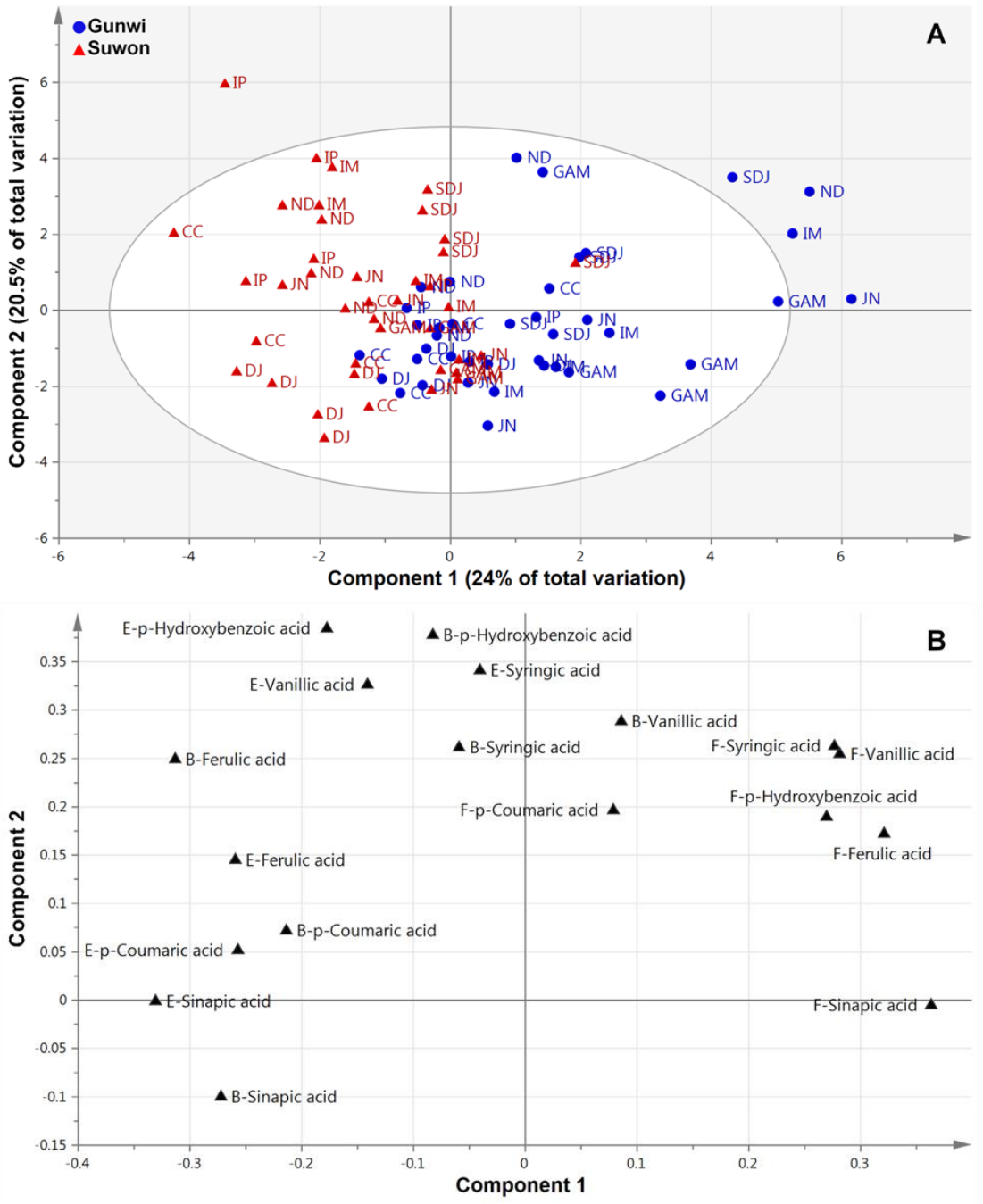

Fig 1. Score (A) and loading (B) plots of principal components 1 and 2 of PCA results obtained from data on the phenolic acids of eight rice cultivars (Chucheong, CC; Dongjin, DJ; Goami, GAM; Ilmi, IM; Ilpum, IP; Junam, JN; Nakdong, ND; Shindongjin, SDJ). Rice samples cultivated in Gunwi and Suwon are indicated with the blue circle or red triangle, respectively. F, E, and B indicate free, esterified, and insoluble-bound phenolic acids, respectively.

classification of diverse biological status, origin, or quality. Recently, metabolomics using diverse analytical instruments such as GC-MS and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy have been applied to determine the geographical origin of various kinds of food, including beef (Jung et al., 2010), kimchi (Kim et al., 2012), maize (Frank et al., 2012), cabbage (Kim et al., 2013a), and mungbean (Kim et al., 2013c). Therefore, this study aimed to determine the content of phenolic acids in three forms (free, esterified, and insoluble-bound forms) occurring in various rice cultivars grown in different locations, and to identify the specific chemical composition of rice samples depending on geographic origin or genotypes, using GC-MS combined with chemometrics. This study is the first to demonstrate the impact of genotype versus environmental influence on the phenolic acid profiles of rice grains. For a comparative investigation on the impact of genotypes versus environmental influence on phenolic acid profiles, eight Korean rice cultivars were grown together under two different environmental conditions. The compositional data obtained were then subjected to multivariate statistical analyses (i.e., PCA, PLS-DA, OPLS-DA) to determine the variation in phenolic acid profiles.

Results and Discussion

Assessment of phenolic acid profiles by PCA

PCA is the most common chemometric tool for extracting and rationalizing information from any multivariate description of a biological system (Ramadan et al., 2006), which has been used to assess differences between plant varieties or genetically modified (GM) plants and their non-GM counterparts at the metabolome level (Kim et al., 2013d; Park et al., 2013b). The quantitative data for the three forms of phenolic acids were subjected to PCA to identify differences in phenolic acid profiles among cultivars. The two highest-ranking components accounted for 44.5% of the total variation within the data set, which revealed no clear separation among the eight rice cultivars. However, a small indication of separation between Gunwi and Suwon samples was indicated by principal component 1, accounting for 24% of the total variation (Fig. 1A). The major components responsible for separation between the two growing locations were determined by analyzing the corresponding loading plots. Variation was mainly attributable to three forms of phenolic acids, of which the loading was positive for all free phenolics and negative for all esterified and bound phenolics, with the exception of bound vanillic acid (Fig. 1B). The loading plot indicated that the six phenolic acids in free form existed at higher levels in rice samples grown in Gunwi than in those grown in Suwon, and most esterified and bound phenolics were lower in samples grown in Gunwi than in those grown in Suwon. Previously, Mpofu et al. (2006) showed large variation in phenolic acid concentrations of six wheat genotypes grown at four locations in Canada, and revealed that environmental effects were greater than genotypic effects, and neither growing temperature nor rainfall from anthesis to maturity was thought to be responsible for the variation. Fernanez-Orozco et al. (2010) determined the phenolic acid content and composition as three forms (free, soluble conjugated, and bound phenolics) in 26 wheat genotypes grown at four locations. In that study, the samples grown in Hungary over three successive years showed a significant correlation of temperature with the contents of free phenolic acids, but no significant correlation were found between total phenolic acid content and precipitation or temperature. In the present study, although the environmental variables, soil pH, temperature, and rainfall, were presented (Table 1), more research is required to investigate the cause of environmental effects for the content and composition of phenolic acid in rice.

Difference analysis of phenolic acid profiles by PLS-DA and OPLS-DA

PLS-DA is a supervised pattern recognition method that separates groups of observations by rotating the PCA (Kim et al., 2013b). In particular, PLS-DA is preferred to PCA for sample discrimination because the dimension reduction provided by PLS is guided explicitly by among-group variability, here being rice varieties or growing locations, where PCA was only capable of identifying gross variability directions and incapable of distinguishing “among-group” or “within-group” variability (Barker and Rayens, 2003; Brereton, 2009). Therefore, quantitative data for the phenolic acids were subjected to PLS-DA to identify differences in the metabolite profiles between the growing locations (Fig. 2A) or varieties (Fig. 2B). The PLS-DA results revealed differences among the rice samples according to their growing locations, but clustering by genotype was observed only in ‘Chucheong’ and ‘Dongjin’ cultivars. The quality of the model was described using the Q^2 value that is defined as the proportion of variance in the data predictable by the model and indicates the predictability. If $Q^2 > 0.5$, the model is considered to have good predictive ability (Eriksson et al., 2001). The models had a Q^2 of 0.235 for the variety and Q^2 of 0.542 for the growing location. These results suggest that the phenolic acid content of rice grains was more affected by environmental factors such as growing condition than by genetic factors, which is in agreement with the results of recent studies investigating the impact of genotypic difference versus environmental influence on the metabolite profiles of maize and cabbage (Skogerson et al., 2010; Frank et al., 2012; Kim et al., 2013a). The results of comparative analyses using metabolite data from the samples grown in different locations indicated that the environmental impacts on the metabolic phenotype of maize and cabbage were far more pronounced than the influences of genotypic difference. The differences in metabolic composition caused by environmental influences were previously demonstrated by metabolite profiling of beef and kimchi in which the samples from different countries could be discriminated by geographical origin (Jung et al., 2010; Kim et al., 2012). This study is the first to demonstrate the relative contribution of genotype and environmental conditions to the phenolic acid profiles of rice genotypes grown in different locations. OPLS-DA was introduced as an improvement of the PLS-DA method to discriminate between two groups (classes) using multivariate data. In OPLS-DA, a regression model is calculated between the multivariate data and a response variable that only contains class information (Westerhuis et al., 2010). Like PLS-DA, OPLS-DA is a supervised pattern recognition technique, but has improved predictive quality because the structured noise is modeled separately (Want et al., 2007). The OPLS-DA results revealed a clear separation between rice samples on the basis of growing location (Fig. 3A). The models had a Q^2 of 0.605. The contribution of variables in the projection could be explained using variables important in the projection (VIP) scores. VIP is a weighted sum of squares of the PLS weight, and a value greater than 1 is generally used as a criterion to identify the variables most important to the model (Kim et al., 2013b). Among the phenolic acids analyzed, seven compounds had a significant VIP value (>1) in which sinapic acid in free form was the most important for creating a prediction of rice classification (Fig. 3B).

Table 2. Contents (micromoles per gram on dry weight basis) of free phenolic acids in brown rice.

Location	Cultivar	*p*-OH	Van	Syr	Cou	Fer	Sin	Sum
Gunwi	Chucheong	5.85±0.08g	5.80±0.15cde	5.60±0.04def	12.78±3.19f	19.82±1.98abcd	7.23±0.14cdef	57.08±4.07
	Dongjin	6.21±0.14f	5.57±0.15de	5.65±0.06cde	18.40±2.58abcd	18.73±07bcde	7.46±0.16ab	62.16±3.91
	Goami	7.01±0.35a	6.17±0.22ab	5.84±0.08a	13.58±4.43ef	20.31±1.67abcd	7.55±0.16a	60.47±5.45
	Ilmi	6.42±0.23cdef	6.17±0.25ab	5.76±0.08ab	14.70±4.08def	23.28±7.10a	7.44±0.12ab	63.76±10.11
	Ilpum	6.30±0.14ef	5.83±0.13cde	5.64±0.03cdef	15.04±3.52cdef	19.92±1.47abcd	7.29±0.10bcde	60.03±3.39
	Junam	6.54±0.23bcde	5.94±0.23bcd	5.61±0.22def	19.47±4.78a	21.56±3.42ab	7.40±0.29abc	66.61±8.82
	Nakdong	6.63±0.42bcd	6.14±0.24ab	5.80±0.12ab	19.29±2.85ab	21.32±3.06abc	7.35±0.18bcd	66.53±6.33
	Shindongjin	6.78±0.32ab	6.13±0.21ab	5.84±0.09a	18.67±1.99abc	21.80±1.72ab	7.38±0.11abcd	66.59±3.84
Suwon	Chucheong	5.57±0.08g	5.71±0.08de	5.56±0.02ef	15.07±0.40cdef	19.27±0.79bcde	7.10±0.05f	58.46±1.05
	Dongjin	6.29±0.31ef	5.62±0.19e	5.53±0.04f	16.97±0.76abcde	15.81±0.82e	7.22±0.07cdef	57.43±1.36
	Goami	6.74±0.11abc	6.04±0.19abc	5.70±0.05bcd	15.54±0.84bcdef	16..80±0.46de	7.24±0.09cdef	58.07±1.31
	Ilmi	6.68±0.21bcd	6.14±0.18ab	5.74±0.03abc	15.83±0.39abcdef	18.77±0.68bcde	7.16±0.04ef	60.26±0.95
	Ilpum	6.45±0.13bcdef	5.82±0.06cde	5.63±0.03cdef	17.62±0.47abcd	18.21±0.86bcde	7.15±0.07ef	60.90±1.04
	Junam	6.41±0.16def	5.82±0.13cde	5.60±0.03def	18.49±0.75abc	18.04±0.73cde	7.12±0.04ef	61.47±1.14
	Nakdong	6.19±0.15f	5.97±0.16abcd	5.70±0.04bcd	18.94±0.97ab	17.98±0.60cde	7.13±0.07ef	61.91±1.21
	Shindongjin	7.00±0.21a	6.23±0.17a	5.84±0.03a	18.53±1.09abc	21.21±0.77abc	7.20±0.07def	66.01±1.92

Different letters represent significant ($p < 0.05$) differences between means according to ANOVA combined with Duncan's multiple range test. Each value represents the mean ± standard deviation ($n = 5$). *p*-OH=*p*-hydroxybenzoic acid. Van=vanillic acid. Syr=syringic acid. Cou=*p*-coumaric acid. Fer=ferulic acid. Sin=sinapic acid.

Table 3. Contents (micromoles per gram on dry weight basis) of esterified phenolic acids in brown rice.

Location	Cultivar	*p*-OH	Van	Syr	Cou	Fer	Sin	Sum
Gunwi	Chucheong	34.09±1.22cde	16.31±1.48	6.65±0.32	15.70±3.20	176.99±25.07abcd	151.10±39.67bcdef	400.83±62.17
	Dongjin	26.88±3.20efg	15.12±00.69	6.73±0.54	15.42±6.90	175.14±31.36abcde	161.80±22.09bcde	401.08±47.56
	Goami	24.52±4.46g	15.71±3.14	7.99±3.17	17.36±6.77	117.20±22.81g	83.86±36.23g	266.65±34.98
	Ilmi	31.04±3.19efg	15.65±0.78	7.16±0.76	15.40±5.37	173.86±34.80abcde	136.79±56.10cdef	379.90±87.32
	Ilpum	42.08±2.87b	15.16±0.55	6.51±0.40	17.95±3.66	139.67±8.25efg	139.09±30.65bcdef	360.45±35.65
	Junam	32.34±2.60def	14.74±0.80	5.90±0.32	12.15±7.08	136.22±29.48fg	113.53±8.08efg	314.87±41.39
	Nakdong	44.14±5.05b	15.97±0.36	7.84±0.89	14.03±6.39	177.18±25.97abcd	138.75±49.95bcdef	397.91±76.07
	Shindongjin	39.97±4.11bc	16.48±0.86	7.26±0.54	13.80±4.07	159.79±20.08cdef	108.60±28.32fg	345.90±36.95
Suwon	Chucheong	40.73±7.99bc	16.23±1.88	6.60±1.02	16.90±2.72	207.75±31.70a	175.57±17.65abc	463.78±53.82
	Dongjin	29.57±3.85efg	15.15±0.56	6.63±0.31	18.07±1.91	166.53±15.86bcdef	211.85±24.78a	447.80±34.08
	Goami	26.14±1.01fg	15.00±0.37	6.80±0.36	14.85±2.13	144.33±15.10defg	158.73±29.31bcdef	365.84±42.46
	Ilmi	40.28±7.59bc	16.90±1.64	7.71±1.02	16.87±3.47	208.51±32.98a	167.87±66.51abcd	458.14±104.16
	Ilpum	52.42±10.56a	16.50±1.40	7.22±1.04	17.88±1.24	166.75±9.76bcdef	158.48±32.57bcdef	419.25±45.43
	Junam	39.28±7.65bcd	15.74±1.47	6.26±0.80	19.11±2.30	171.94±20.80bcde	123.16±17.24defg	375.49±44.18
	Nakdong	45.45±6.29ab	16.53±1.21	7.74±0.47	16.27±0.71	200.00±15.85ab	189.73±15.44ab	475.71±35.83
	Shindongjin	46.44±4.28ab	17.04±0.85	7.42±0.36	16.55±3.93	193.88±27.32abc	162.35±14.22bcde	443.68±42.36

Different letters represent significant ($p < 0.05$) differences between means according to ANOVA combined with Duncan's multiple range test. Each value represents the mean ± standard deviation ($n = 5$). *p*-OH=*p*-hydroxybenzoic acid. Van=vanillic acid. Syr=syringic acid. Cou=*p*-coumaric acid. Fer=ferulic acid. Sin=sinapic acid.

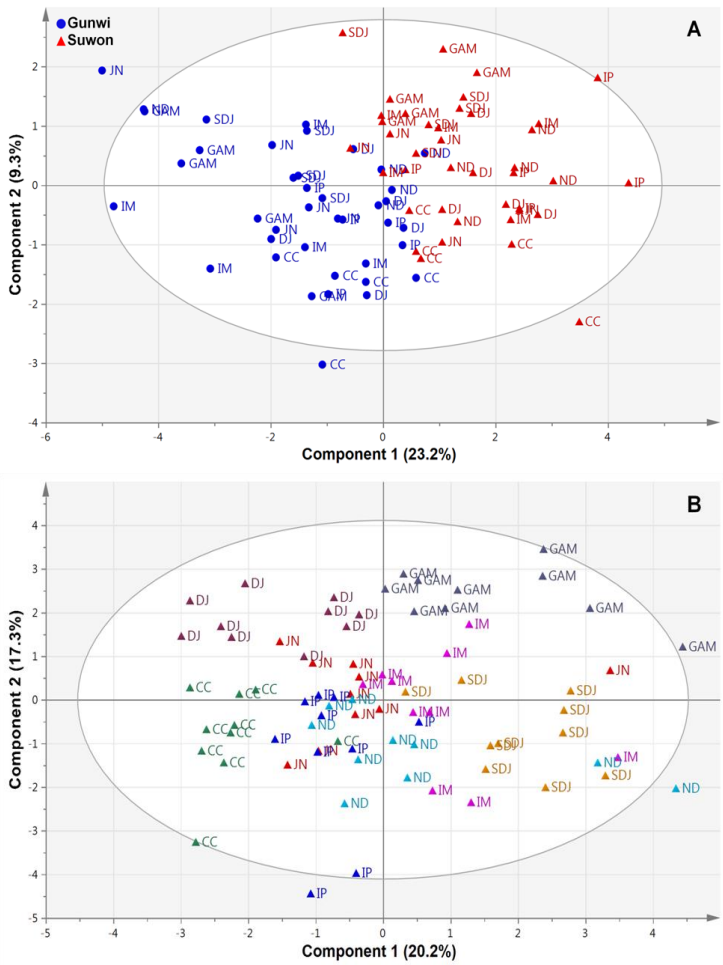

Fig 2. PLS-DA score plots separating samples according to their growing locations (A) and cultivars (B). The results were obtained from data on phenolic acids of eight rice cultivars (Chucheong, CC; Dongjin, DJ; Goami, GAM; Ilmi, IM; Ilpum, IP; Junam, JN; Nakdong, ND; Shindongjin, SDJ).

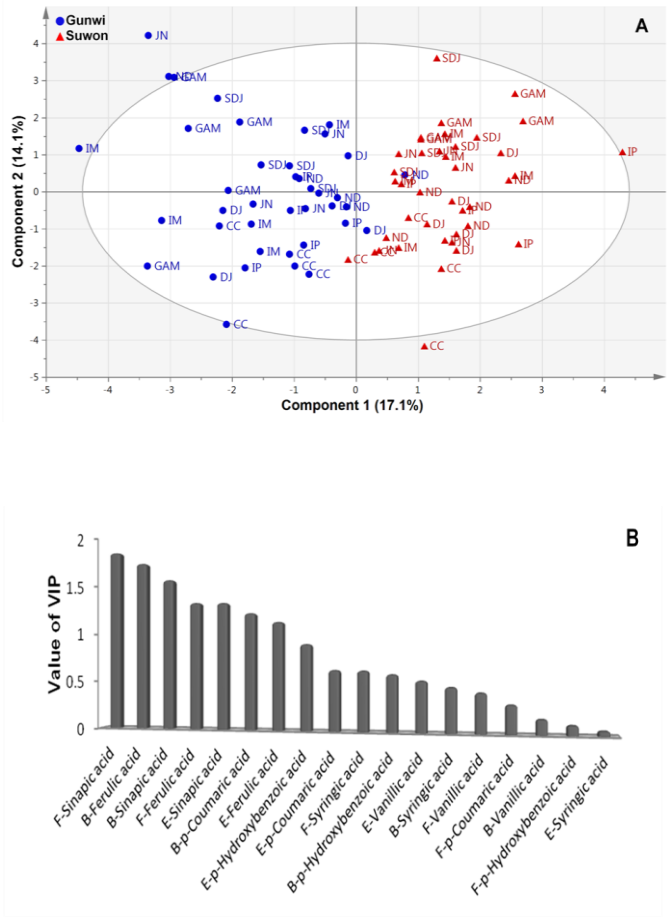

Fig 3. (A) Score plot of principal components 1 and 2 from OPLS-DA results obtained using data on the phenolic acids of eight rice cultivars (Chucheong, CC; Dongjin, DJ; Goami, GAM; Ilmi, IM; Ilpum, IP; Junam, JN; Nakdong, ND; Shindongjin, SDJ). Rice samples cultivated in Gunwi and Suwon are indicated with the blue circle or red triangle, respectively. (B) Variable importance in the prediction (VIP) value of variables from the OPLS-DA. The VIP value indicates the relative influence of each compound to the grouping. F, E, and B indicate free, esterified, and insoluble-bound phenolic acids, respectively.

Phenolic acid profiles of rice grown in different locations

Six types of phenolic acids including *p*-hydroxybenzoic, vanillic, syringic, *p*-coumaric, ferulic, and sinapic acids were detected in three forms in all of the cultivars. The free phenolic acid content of the eight rice cultivars grown in two different locations are shown in Table 2. Predominant free-form compounds were ferulic acid, followed by *p*-coumaric and sinapic acids. Total content of the six free phenolics varied, ranging from 57.08 to 66.61 μmol/g. The esterified phenolic acids were identified from methanol-soluble fractions followed by an alkaline hydrolysis. The major portion of the esterified-form was composed of ferulic and sinapic acids, which accounted for 78.8–88.0% of the total ester content in all varieties (Table 3). This is consistent with the results of a previous study regarding the contents of methanol-soluble phenolics in rice grains (Park et al., 2012), which showed that alkaline hydrolysis of the rice soluble fractions released the major portion of total soluble phenolic acid in which dominant compounds were ferulic and sinapic acids. The total level of soluble phenolic esters was highest in the Suwon-grown 'Nakdong' cultivar (475.71 μmol/g) and lowest in the Gunwi-grown 'Goami' cultivar (266.65 μmol/g).

A noticeable difference between Gunwi- and Suwon-grown rice was observed in the level of soluble-sinapic acid. The values of free-sinapic acid in Gunwi-grown rice was higher than that in Suwon-grown rice, whereas Suwon-grown rice contained relatively higher levels of the esterified-sinapic acid compared to Gunwi-grown rice. These results were consistent with the findings from PCA loading plots (Fig. 1B), indicating that PCA can be used to visualize complex data. Insoluble-bound phenolic acids were extracted by the alkaline hydrolysis of methanol-insoluble residues (Table 4). The total level of insoluble phenolics was over twofold higher than that of soluble-phenolics, which was highest in the Suwon-grown 'Ilpum' cultivar (1424.26 μmol/g) and lowest in the Gunwi-grown 'Ilmi' cultivar (975.43 μmol/g). Among the six phenolic acids analyzed, ferulic acid constituted the largest portion of insoluble phenolics (79.6–84.7%), ranging from 761.45 to 853.19 μmol/g in Gunwi-grown rice and from 898.53 to 1176.95 μmol/g in Suwon-grown rice, which is in agreement with the findings of Zhou et al. (2004), Qiu et al. (2010), and

Table 4. Contents (micromoles per gram on dry weight basis) of insoluble-bound phenolic acids in brown rice.

Location	Cultivar	*p*-OH	Van	Syr	Cou	Fer	Sin	Sum
Gunwi	Chucheong	27.15±1.93def	24.55±2.25	12.95±1.08abc	82.41±24.99f	840.46±76.63cdef	35.36±4.02f	1022.88±96.81
	Dongjin	25.60±1.54f	22.75±0.86	12.98±0.84abc	110.05±17.38bcde	847.76±119.54cdef	44.92±9.27bcd	1064.06±132.26
	Goami	25.75±1.31f	23.37±1.03	12.62±0.97bcd	114.99±19.27abcd	761.45±113.77f	37.82±4.01def	976.00±133.38
	Ilmi	26.77±1.02def	23.27±1.71	11.57±1.80d	85.43±11.15f	788.07±70.05ef	40.32±7.36cdef	975.43±82.50
	Ilpum	31.68±0.95b	22.38±0.72	12.55±0.56bcd	93.37±16.34def	809.92±105.09ef	42.37±1.42cdef	1012.28±120.46
	Junam	28.51±2.07cde	22.97±1.97	11.73±0.87cd	90.07±11.13ef	818.74±49.28def	34.94±3.60f	1006.95±54.52
	Nakdong	30.90±2.14bc	23.51±0.90	12.55±0.53bcd	96.38±20.65cdef	853.19±175.70cdef	41.89±7.33cdef	1058.42±195.20
	Shindongjin	28.50±2.72cde	23.68±2.42	12.44±1.32bcd	98.80±18.53cdef	810.27±85.80ef	37.00±3.49ef	1010.70±100.58
Suwon	Chucheong	29.05±1.99bcde	22.58±2.74	13.09±0.56ab	90.18±8.76ef	1005.57±129.68bc	43.46±3.80cde	1203.93±139.00
	Dongjin	24.99±1.20f	22.47±0.35	12.62±0.43bcd	119.50±8.58abc	905.20±88.16bcdef	57.23±3.02a	1142.02±100.55
	Goami	26.55±0.86ef	22.81±0.72	12.68±0.27bcd	135.48±6.38a	898.53±73.46bcdef	55.14±2.54a	1151.20±81.35
	Ilmi	29.17±2.48bcde	22.99±1.24	12.39±0.71bcd	101.42±8.52cdef	1034.20±74.57ab	44.55±3.79bcde	1244.72±84.58
	Ilpum	35.06±3.62a	24.92±1.98	13.97±1.30a	127.25±32.36ab	1176.95±270.01a	46.13±9.30bc	1424.26±305.61
	Junam	29.49±1.71bcd	23.13±1.24	11.98±0.45bcd	114.76±14.96abcd	984.49±106.94bcd	38.70±5.83cdef	1202.55±124.10
	Nakdong	31.29±1.62b	23.20±0.97	12.68±0.61bcd	115.54±5.78abcd	951.89±56.43bcde	50.89±2.48ab	1185.50±62.70
	Shindongjin	29.13±0.90bcde	23.04±0.64	12.45±0.43bcd	104.83±5.74bcdef	1001.38±66.99bc	45.51±1.75bc	1216.34±74.85

Different letters represent significant ($p < 0.05$) differences between means according to ANOVA combined with Duncan's multiple range test. Each value represents the mean ± standard deviation ($n = 5$). *p*-OH=*p*-hydroxybenzoic acid. Van=vanillic acid. Syr=syringic acid. Cou=*p*-coumaric acid. Fer=ferulic acid. Sin=sinapic acid.

Sompong et al. (2011). In these studies using various rice varieties from Thailand, China, Sri Lanka, Australia, Canada, and USA, ferulic acid was the most abundant phenolic acid in the insoluble fractions for all the rice samples.

The total level of six phenolic acids including soluble and insoluble phenolics was higher in Suwon-grown rice than that of Gunwi-grown rice for all the rice cultivars analyzed. The precipitation from planting to harvest was two-fold higher in Suwon compared to that in Gunwi (Table 1). This is consistent with the findings of Yu et al. (2003), who demonstrated that irrigated plants had higher contents of phenolic acids in wheat bran. The 'Dongjin' cultivar (1527.31 μmol/g) had the highest phenolic acids among Gunwi-grown rice, and the 'Ilpum' cultivar (1904.43 μmol/g) had the highest phenolic acids among Suwon-grown rice. This is the first study to determine the content of phenolic acids in the three forms occurring in Korean rice cultivars grown in different regions, which provide valuable information regarding future genetic breeding programs for rice containing health beneficial phenolic acids. However, the significant environmental variation must be considered. Further studies are needed to explore the effects of individual environmental factors, such as chemical composition in soil, pathogen, fertilizer, irrigation, solar radiation, etc. as well as temperature and rainfall, on phenolic acid composition.

Materials and Methods

Rice sample preparation

Eight rice cultivars (*Oryza sativa* L. cv. Chucheong, Dongjin, Goami, Ilmi, Ilpum, Junam, Nakdong, and Shindongjin) were planted together at two locations in Korea: Suwon, Gyeonggi-do and Gunwi, Gyeongsangbuk-do. Environmental conditions of these locations are presented in Table 1. The levels of soil pH before planting were very similar in both Gunwi and Suwon fields. The plants were grown side-by-side in one field under the natural conditions and same field management in 2012. The average temperature during the growing and maturing periods was slightly higher in Suwon than in Gunwi, whereas the average rainfall in Suwon was almost two-fold higher compared to that in Gunwi. Five biological replicates per variety were harvested, and the whole grain (rough rice) samples were dried to a final moisture content of 11–14%. Rice samples were manually hulled and ground to obtain a fine powder using a planetary mono mill (Pulverisette 6; Fritsch GmbH, Idar-Oberstein, Germany). The powder was stored at –80°C until analysis.

Phenolic acid extraction

Three forms of phenolic acids (free, esterified, and bound form) were extracted according to the procedure described by Park et al. (2013a), with slight modifications. The powdered samples (0.1 g) were extracted twice by water-based sonication for 5 min at room temperature and incubation at 30°C for 10 min with 1 mL of 85% methanol containing 2 g/L butylated hydroxyanisole (BHA; Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 13,000 rpm for 10 min at 4°C, the combined extracts and residue were analyzed to determine the quantities of soluble (the mixture of free and esterified forms) and insoluble-bound phenolic acids, respectively. Fifty microliters of 3,4,5-trimethoxycinnamic acid (100 μg/mL; Wako Pure Chemical Industries, Osaka, Japan) was added as an internal standard (IS), and the mixture was hydrolyzed with 1 mL 5 N NaOH at 30°C under nitrogen gas for 4 h. Each hydrolyzed sample was adjusted to a pH of 1.5–2.0 with 6 M HCl. With unhydrolyzed soluble fractions (free forms), all extracts were extracted with ethyl acetate and evaporated in a centrifugal concentrator (Eyela, Tokyo, Japan). Derivatization of the extracts and GC-TOFMS procedure was performed according to the method previously reported (Park et al., 2013a). For quantification purposes, a standard stock solution of six phenolics (ferulic, *p*-coumaric, *p*-hydroxybenzoic, sinapic, syringic, and vanillic acids) and 3,4,5-trimethoxycinnamic acid (used as an IS) was prepared in methanol (100 μg/mL). Calibration samples, ranging from 0.01 to 10.0 μg, were prepared by mixing individual stock solutions of the six phenolic acid standards. The level of esterified phenolic acids was calculated from the level of soluble phenolic acids by subtracting the level of free phenolic acids.

Statistical analyses

Experimental data were analyzed using analysis of variance (ANOVA), and significant differences among the means were determined by Duncan's multiple-range test at a 95% confidence level (SAS 9.2; SAS Institute, Cary, NC, USA). Quantification data were subjected to PCA, PLS-DA, and OPLS-DA using BioPAT-SIMCA version 13 (Umetrics, Umeå, Sweden) to evaluate the differences among groups of multivariate data. The data file was scaled with unit variance scaling before all variables were subjected to the PCA, PLS-DA, and OPLS-DA (Park et al., 2013b).

Conclusion

In this study, the compositional differences of the six phenolic acids as free, esterified, and insoluble-bound forms were demonstrated in eight Korean rice cultivars grown together at two different locations. Differentiation of the phenolic acid profile of rice samples was determined using PCA, PLS-DA, and OPLS-DA, and a distinct separation was observed according to their growing location rather than their genotypes. These results suggest that the influences of environmental factors have more impact than genetic background on the phenolic acid composition of rice grains. The contents of individual phenolic acids in different forms varied among rice grains in which the insoluble-bound phenolic acids were the most abundant form, followed by esterified and free forms. The highest level of total phenolic acids was observed in the 'Dongjin' cultivar among Gunwi-grown rice, but the 'Ilpum' cultivar had the highest level of total phenolic acids among Suwon-grown rice. This study suggests that metabolite profiling coupled with chemometric analysis is an efficient tool to identify metabolic differences between rice grains from different regions of cultivation.

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