Anthocyanin and Isoflavone Contents in Korean Black Soybean Landraces and Their Antioxidant Activities

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ABSTRACT Anthocyanin and isoflavone contents and antioxidant activities of 56 Korean black soybean landraces were examined in this study. Total isoflavone content of 56 Korean black soybean landraces ranged from 43.8 to 347.5 mg/100 g. Total anthocyanin content ranged from 19.8 to 1,420.4 mg/100 g, with cyaniding-3-glucoside being the most abundant anthocyanin in all landraces, ranging from 12.2 to 1,207.3 mg/100 g. Antioxidant activities based on 2,2-diphenyl-1-picrylhydrazyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ferric-reducing antioxidant power, and total polyphenol content assays showed wide variations, ranging from 17.1 to 76.6 (IC₅₀), 53.5 to 127.7 (IC₅₀), 5.4 to 34.2 mg ascorbic acid/g, and 9.7 to 36.8 mg gallic acid equivalents/g, respectively. Using relative antioxidant capacity index, IT156132 sample had the highest antioxidant activity. In clustering analysis, the 56 Korean black soybean landraces were classified into five groups. Group I consisted of four landraces with high isoflavone contents and antioxidant activities.

Keywords Antioxidant activity, Anthocyanin, Korean black soybean landraces, Isoflavone

INTRODUCTION

Various kinds of soybeans (*Glycine max* L. Merr.) are cultivated in East Asia as diverse food sources after adapting in different environments for a long time (Cho *et al.* 2008). Previous studies have reported that Korean soybean landraces have large genetic variations (Kwon *et al.* 1974; Perry and McIntosh 1991; Song *et al.* 1991; Yoon 2003). Many countries such as America, Canada, China, and Japan have developed various soybean cultivars with traits such as high yield, disease resistance, and tolerance of environment stress using Korean soybean landraces (Lee 2003).

Soybean is a well-known food staple that contains starch, dietary fiber, protein, lipids, and essential minerals as well as beneficial secondary metabolites (Bellaloui 2012). Recently, black soybeans have been found to contain high contents of γ -tocopherol, isoflavones,

flavonoids, and anthocyanins with biological activity (Correa *et al.* 2010). Antioxidant properties resulting from free radical scavenging effects and total phenolic compounds have been found to be higher in black soybeans compared to yellow soybeans (Dajanta *et al.* 2013).

Anthocyanins are secondary metabolites and water-soluble pigments that are responsible for the red, purple, or blue coloration of many fruits, vegetables, and cereal grains (Kim *et al.* 2012). They play various important roles in plants, such as attracting animals participating in pollination and seed disposal, repelling harmful insects, and protecting plants against damages caused by ultraviolet light (Shin *et al.* 2009). The black color in soybean is ascribed to an accumulation of anthocyanins in its epidermis palisade layer (Todd and Vodkin 1993). Three main anthocyanins (delphinidin-3-O- β -D-glucoside [D3G], cyanidin 3-O- β -D-glucoside [C3G], and petunidin-3-O-

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β -D-glucoside [Pt3G]) have been detected in the seed coat of black soybean (Choung *et al.* 2001).

Consumption of isoflavones is associated with benefits to human health, such as decreased risk of heart disease, reduced menopausal symptoms, and reduced risk of some hormone-related cancers (Yao *et al.* 2004). Isoflavones in soybeans are mainly aglycones (i.e., daidzein, genistein, glycitein), β -glucosides (i.e., daidzin, genistin, glycitin), malonyl- β -glucosides (i.e., 6''-O-malonyldaidzin, 6''-O-malonylgenistin, 6''-O-malonylglycitin), and acetyl- β -glucosides (i.e., 6''-O-acetyldaidzin, 6''-O-acetylgenistin, 6''-O-acetylglycitin) (Lee and Lee 2009). Aglycones are flavonoid molecules lacking any attached sugars or other modifiers. Among different forms of isoflavones, aglycones are especially important because they are readily bioavailable to humans (Lee and Lee 2009).

Antioxidant compounds have received attention from natural-product consumers and researchers due to their pharmacological properties. Antioxidants can lower oxidative stress caused by reactive oxygen species (Nordberg and Arnér 2001). There is an increasing interest in natural antioxidant products for use as medicines and food additives (Mossi *et al.* 2004; Willcox *et al.* 2004). Phytochemicals such as polyphenols and carotenoids are important because they contribute to human health with multiple biological effects such as antioxidant, antimutagenic, anticarcinogenic, and cytoprotective activities (Ajila and Prasada Rao 2008).

In this study, 56 Korean black soybean landraces were analyzed to determine anthocyanins and isoflavones contents and antioxidant activities. In addition, the relation among anthocyanins, isoflavones, and antioxidant activity of samples was tested. Furthermore, black soybean landraces with high anthocyanin and isoflavone contents and antioxidant activities were identified.

MATERIALS AND METHODS

Plant materials

Fifty-six Korean black soybean landraces were obtained from the National Agrobiodiversity Center of the Rural Development Administration, Korea (<http://genebank.rda.go.kr>).

go.kr).

Analysis of anthocyanins in soybean seeds

One-hundred mg of each Korean black soybean landrace was mixed with 15 ml of 1% HCl in 99% MeOH for 24 hours at 4°C in the dark. After centrifugation at 13,000 rpm for 10 minutes, each specimen was filtered through a 0.45 μ m syringe filter and analyzed with Agilent 1260 Infinity HPLC system (Agilent Technol., Santa Clara, CA, USA). The analysis was performed using Waters XSelect HSS Cyano XP column (2.5 μ m, 2.1 $\times$ 75 mm; Waters, Milford, MA, USA). HPLC conditions were as follows: solvent A, 0.1% TFA/H₂O; solvent B, 0.1% TFA/CH₃CN; gradient, 5% (B) for 0.3 minutes, 20% (B) for 6.0 minutes, 95% (B) for 8.0 minutes, and 5% (B) for 10 minutes; column temperature, 40°C; and flow rate, 0.5 ml/min. The filter detector was set at 520 nm.

Analysis of isoflavones in soybean seeds

One-hundred milligram of each sample was added to 2 ml of 80% MeOH and sonicated for 1 hour. The sample in each tube was hydrolyzed using 150 μ l of 2 N NaOH. After mixing for 10 minutes, the solution was neutralized with 50 μ l of glacial acetic acid. The sample was centrifuged at 3,000 rpm for 5 minutes. Supernatant was collected and filtered using a 0.45 μ m syringe filter prior to analysis by with Agilent 1260 Infinity HPLC system (Agilent Technol.). The analysis was performed using a Proshell 120 SB-C15 column (2.7 μ m, 2.1 $\times$ 50 mm; Agilent Technol.). HPLC conditions were as follows: solvent A, 0.1% TFA/H₂O; solvent B, 0.1%TFA/CH₃CN; gradient, 10% (B) for 0.35 minutes, 10% to 30% (B) in 3.96 minutes, hold at 30% (B) for 0.36 minutes, re-equilibrate at 10% (B) for 1.8 minutes; column temperature, 30°C; and flow rate, 0.58 ml/min. The filter detector was set at 254 nm.

Extraction for antioxidant activities

One-hundred milligram of each soybean powder was added to 1 ml of 80% EtOH. The mixture was sonicated in an ultrasonic bath for 3 hours at room temperature. The suspension was centrifuged at 13,000 rpm for 10 minutes. The clear supernatant was then used for 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzo-

thiazoline-6-sulphonic acid) (ABTS), ferric-reducing antioxidant power (FRAP), and total polyphenol content (TPC) assays.

DPPH assay

DPPH radical-scavenging activities of extracts were assessed using published method with slight modifications (Lee and Lee 2004). Briefly, DPPH solution (150 μ l; 150 μ M in anhydrous EtOH) was added to 100 μ l of sample solution. The mixture was shaken vigorously and left to stand at 25°C in the dark for 30 minutes. Absorbance at 517 nm was measured using a spectrophotometer (Epoch; Bio-Tek, Winooski, VT, USA). Results were expressed as IC50 and compared to ascorbic acid standard.

ABTS assay

ABTS radical-scavenging activity was estimated using previously described method with modifications (Re *et al.* 1999). Briefly, ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulfate followed by an overnight incubation in the dark at room temperature. The ABTS radical cation solution was diluted with methanol (MeOH) to obtain an absorbance of 0.7 ± 0.02 at 735 nm. The diluted ABTS radical cation solution (190 μ l) was added to 10 μ l of sample solution. After 6 minutes of incubation, absorbance at 735 nm was determined using a spectrophotometer. Results were expressed as IC50 and compared to ascorbic acid standard.

FRAP

The reducing power of the 56 Korean black soybean landraces was determined using published method with slight modifications (Yen and Duh 1993). Briefly, 0.1 ml aliquot of the extract was mixed with a 0.5 ml phosphate buffer (0.2 M, pH 6.6) containing 1% K₃Fe(CN)₆. The mixture was incubated at 50°C for 20 minutes. After centrifugation at 200 g for 10 minutes, the upper layer (10 μ l) was mixed with 390 μ l of 1% ferric chloride. The absorbance was monitored at 700 nm using a spectrophotometer.

TPC assay

TPC was measured using modified Folin-Ciocalteu method (Waterhouse 2003). Folin-Ciocalteu reagent (100

μ l) was added to 100 μ l of sample solution and reacted at room temperature for 3 minutes. After adding 100 μ l of 2% sodium carbonate, the mixture was incubated at room temperature for 30 minutes. Absorbance was measured at 750 nm on a spectrophotometer using distilled water as the blank. Total phenolic content was reported as milligrams of gallic acid equivalents (GAE) per gram of dry weight sample (mg GAE mg⁻¹ dry seed).

Data analysis

Duncan's multiple-range test and correlation analysis were used to determine differences among the 56 Korean black soybean landraces using IBM SPSS Statistics ver. 20 (IBM Co., Armonk, NY, USA). Cluster analysis was performed using R statistical software environment (<http://www.r-project.org>). Software PAST3 was used for principal component analyses (PCA) (Hammer *et al.* 2001). Statistical significance was considered when *P*-value was less than 0.05.

RESULTS

Isoflavone content

Total and individual isoflavone contents of the 56 Korean black soybean landraces are summarized in Table 1 and Supplementary Table 1. Composite values for six isoflavones (namely daidzin, genistin, glycitin, daidzein, genistein, and glycitein) were analyzed and expressed as total isoflavone content. Total isoflavone contents (TIC) of Korean black soybean landraces ranged from 43.8 to 347.5 mg/100 g. Among soybean landraces, sample IT177372 had the highest TIC content (347.5 ± 2.5 mg/100 g). The content of isoflavone glycosies ranged from 39.1 to 335.5 mg/100 g. Among isoflavone glycosides, daidzin, glycitin, and genistin contents ranged from 12.3 to 153.5, 2.4 to 35.5, and 20.8 to 146.6 mg/100 g, respectively, while daidzein, glycitein and genistein contents ranged from 0.3 to 9.2, 0.7 to 14.9, and 0.2 to 3.1 mg/100 g, respectively.

Anthocyanin content

The following three anthocyanins, D3G, C3G, and Pt3G, were detected in 56 Korean black soybean landraces

Table 1. Descriptive statistics of anthocyanins, isoflavones, and antioxidant activities of the 56 Korean black soybean landraces used in this study.

Variable	Subtotal	Range (minimum-maximum)	Mean±standard deviation	Skewness	Kurtosis
Isoflavone content (mg/100 g)	Daidzin	12.3-153.5	55.5±23.3	1.37	5.08
	Glycitin	2.4-35.5	16.4±7.3	0.62	0.81
	Genistin	20.8-146.6	70.8±26.0	0.37	0.38
	Glycosides	39.1-335.5	142.7±51.9	0.69	2.82
	Daidzein	0.3-9.2	2.6±2.0	1.43	2.30
	Glycitein	0.7-14.9	3.5±2.9	2.70	7.65
	Genistein	0.2-3.1	1.2±0.8	1.12	0.37
	Aglycones	1.7-18.8	7.3±3.8	1.19	1.55
	TIC ²⁾	43.8-347.5	149.9±53.7	0.61	2.70
Anthocyanin content (mg/100 g)	D3G	0.0-216.4	63.9±44.8	1.22	2.14
	C3G	12.2-1207.3	333.6±295.6	1.19	0.84
	Pt3G	0.0-42.8	12.0±10.0	1.39	1.61
	TAC	19.8-1420.4	433.5±335.2	1.08	0.71
Antioxidant activity	DPPH (IC50)	17.1-76.6	59.3±15.6	-1.18	0.62
	ABTS (IC50)	53.5-127.7	75.1±20.3	1.17	0.57
	FRAP (mg ASC/g)	5.4-34.2	15.5±6.9	0.48	-0.44
	TPC (mg GAE/g)	9.7-36.8	20.0±6.5	0.48	-0.47

²⁾TIC: total isoflavone content, D3G: delphinidin-3-O-β-D-glucoside, C3G: cyanidin 3-O-β-D-glucoside, Pt3G: petunidin-3-O-β-D-glucoside, TAC: total anthocyanin content, DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), FRAP: ferric-reducing antioxidant power, ASC: ascorbic acid, TPC: total polyphenol content, GAE: gallic acid equivalents.

(Table 1 and Supplementary Table 1). Total anthocyanin content (TAC) ranged from 19.8 to 1,420.4 mg/100 g. Among soybean landraces, sample IT177257 had the highest TAC content (1,420.4±1.8 mg/g), while sample IT112847 had the lowest TAC content (19.8±2.1 mg/g). C3G was the main component in anthocyanin (ranging from 12.2 to 1,207.3 mg/100 g). D3G and Pt3G contents ranged from 0.0 to 216.4 mg/100 g and 0.0 to 42.8 mg/100 g, respectively.

Antioxidant activity

DPPH, ABTS, FRAP, and TPC were used to determine the antioxidant activities of the 56 Korean black soybean landraces. Results are summarized in Table 1 and Supplementary Table 1. DPPH radical-scavenging activities of soybean landraces ranged from 17.1 to 76.6 (IC50). Sample IT156132 had the highest DPPH radical-scavenging activity (17.1±8.2, IC50). The ABTS antioxidant activities of soybean landraces ranged from 53.5 to 127.7 (IC50). Sample IT177485 and IT177530 had the highest ABTS-

scavenging activity (53.5±0.1, IC50). FRAP of soybean landraces ranged from 5.4 to 34.2 mg ascorbic acid (ASC)/g. Sample IT177253 had the highest FRAP (34.2±1.5 mg ASC/g) while IT177430 had the lowest FRAP (5.4±0.2 mg ASC/g). TPC of soybean landraces ranged from 9.7 to 36.8 mg GAE/g. Sample IT177370 had the highest TPC (36.8±3.4 mg GAE/g) while IT177430 had the lowest TPC (9.7±0.2 mg GAE/g). The integration of antioxidant capacity results derived from different chemical methods allowed us to calculate the relative antioxidant capacity index (RACI). Results are shown in Fig. 1. It was found that IT156132 had the highest RACI (2.12), followed by IT177485 (1.37), IT177370 (1.34), and IT177253 (1.31), with IT177430 having lowest value of RACI (-1.30).

Correlation analysis

The correlations among anthocyanins, isoflavones, and antioxidant activities were analyzed. Results are shown in Table 2. There was no significant correlation

Fig. 1. Relative antioxidant capacity index (RACI) of 56 Korean black soybean landraces.

Table 2. Correlations among anthocyanins, isoflavones, and antioxidant activities of the 56 Korean black soybean landraces.

Variable	DPPH ^(b)	ABTS ^(c)	FRAP	TPC	Daidzin	Glycitin	Genistin	Glycosides	Daidzein	Glycitein	Genistein	Aglycones	TIC	D3G	C3G	Pt3G	TAC
ABTS	0.127																
FRAP	-0.241	-0.792**															
TPC	-0.294*	-0.727**	0.838**														
Daidzin	-0.089	0.070	0.089	0.078													
Glycitin	-0.111	0.120	0.071	0.049	0.872**												
Genistin	-0.121	0.033	0.024	-0.078	0.738**	0.594**											
Glycosides	-0.116	0.065	0.062	0.003	0.942**	0.831**	0.917**										
Daidzein	-0.007	0.111	0.038	-0.074	0.466**	0.418**	0.625**	0.582**									
Glycitein	0.080	0.039	-0.208	-0.156	0.146	-0.006	0.130	0.130	-0.112								
Genistein	0.082	-0.117	-0.050	0.084	0.185	0.004	0.330*	0.249	-0.006	0.564**							
Aglycones	0.077	0.067	-0.155	-0.142	0.391**	0.211	0.490**	0.451**	0.425**	0.834**	0.646**						
TIC	-0.107	0.068	0.049	-0.007	0.938**	0.817**	0.920**	0.998**	0.592**	0.185	0.287*	0.507**					
D3G	-0.267*	-0.105	0.175	0.176	0.142	0.121	0.100	0.131	-0.032	-0.198	-0.131	-0.199	0.113				
C3G	-0.076	-0.053	0.052	0.057	0.207	0.256	0.194	0.226	-0.095	-0.092	-0.024	-0.127	0.210	0.314*			
Pt3G	-0.266*	-0.023	0.109	0.059	0.162	0.188	0.129	0.164	0.087	-0.234	-0.258	-0.194	0.145	0.752**	-0.003		
TAC	-0.289*	-0.207	0.261	0.234	0.188	0.173	0.153	0.185	-0.001	-0.223	-0.159	-0.210	0.164	0.835**	0.361**	0.665**	
RACI	-0.502**	-0.830**	0.916**	0.891**	0.051	0.013	0.030	0.040	-0.020	-0.133	0.035	-0.109	0.031	0.214	0.022	0.162	0.296*

* $P < 0.05$, ** $P < 0.01$.

²⁾DPPH and ABTS: IC50.

³⁾DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), FRAP: ferric-reducing antioxidant power, TPC: total polyphenol content, TIC: total isoflavone content, D3G: delphinidin-3-O- β -D-glucoside, C3G: cyanidin 3-O- β -D-glucoside, Pt3G: petunidin-3-O- β -D-glucoside, TAC: total anthocyanin content, RACI: relative antioxidant capacity index.

between anthocyanins and isoflavones or between antioxidant activities and isoflavones. DPPH (IC50) showed negative correlations with TPC ($r = -0.294$, $P < 0.05$), D3G ($r = -0.267$, $P < 0.05$), Pt3G ($r = -0.266$, $P < 0.05$), and TAC ($r = -0.289$, $P < 0.05$). ABTS (IC50) only showed negative correlation with the following two antioxidant activities: FRAP ($r = -0.792$, $P < 0.01$) and TPC ($r = -0.727$, $P < 0.01$). FRAP showed a positive

correlation with TPC ($r = 0.838$, $P < 0.01$). RACI showed negative correlations with DPPH ($r = -0.502$, $P < 0.01$) and ABTS ($r = -0.830$, $P < 0.01$). However, it had positive correlations with FRAP ($r = 0.916$, $P < 0.01$), TPC ($r = 0.891$, $P < 0.01$), and TAC ($r = 0.296$, $P < 0.05$).

Principal component analyses (PCA) analysis

PCA analysis was performed for anthocyanins iso-

flavones. Results of PCA analysis are shown in Table 3. The cumulative variance of the first five axes with Eigen value of > 1.0 was 84.0%. The first PC was more related to isoflavone glycosides, daidzin, glycitin, and genistin than TIC. In second principal component, the antioxidant activities (ABTS, FRAP, TPC) and RACI were more related traits except DPPH. The third principal component exhibited positive effects on isoflavones (aglycones, daidzein, glycitein, and genistein) and ABTS. However, it had negative effects on Pt3G and TAC. The fourth principal component was also more related to isoflavones (aglycones, daidzein, glycitein, and genistein) and TAC. The fifth principal component exhibited positive effect on C3G but negative effect on daidzein, Pt3G, and DPPH. The distribution of Korean black soybean landraces in PCA analysis is shown in Fig. 2. After placing an ellipse around the data with 95% confidence interval using Hotelling's T2 statistic, it was possible to observe all soybean landraces

except two (IT177485 and IT177372). IT177485 showed the lowest content of isoflavone aglycones (daidzein, 0.3 ± 0.0 mg/100 g; glycitein, 0.7 ± 0.1 mg/100 g; total aglycones, 1.7 ± 0.2 mg/100 g), while IT177372 had the highest levels of isoflavone glycosides (daidzin, 153.5 ± 1.4 mg/100 g; glycitein, 35.4 ± 0.3 mg/100 g; genistein, 146.6 ± 0.2 mg/100 g; total glycoside, 335.5 ± 1.6 mg/100 g).

Clustering analysis

The 56 Korean black soybean landraces were classified into five clusters according to their anthocyanins, isoflavones, and antioxidant activities (Table 4 and Fig. 3). There was no significant difference in D3G, Pt3G, or DPPH among the five clusters. Cluster I contained four landraces (IT177372, IT177349, IT177253, and IT177309). They had high contents of daidzin, glycitin, genistin, total isoflavone glycosides, TIC, and C3G. Cluster II contained seven landraces with high glycitein and total isoflavone

Table 3. Eigen value, percent of total variation, and component matrix for the principal component axes.

Principal components	PC ²⁾ 1	PC2	PC3	PC4	PC5
Eigen value	5.35	4.22	2.71	1.60	1.24
% of variance	29.7	23.5	15.1	8.9	6.9
Cumulative %	29.7	53.2	68.3	77.1	84.0
Component matrix					
Daidzin	0.398	-0.010	-0.031	-0.109	0.094
Glycitin	0.351	0.001	-0.115	-0.213	0.111
Genistin	0.390	-0.042	0.011	-0.032	-0.050
Glycosides	0.424	-0.026	-0.024	-0.095	0.033
Daidzein	0.268	-0.054	-0.009	-0.267	-0.374
Glycitein	0.090	-0.204	0.315	0.475	0.063
Genistein	0.132	-0.114	0.354	0.356	0.174
Aglycones	0.234	-0.211	0.314	0.305	-0.107
TIC	0.426	-0.040	-0.001	-0.070	0.024
D3G	0.089	0.261	-0.339	0.383	0.047
C3G	0.100	0.081	-0.174	0.038	0.702
Pt3G	0.096	0.218	-0.355	0.285	-0.315
TAC	0.110	0.290	-0.309	0.315	0.090
DPPH	0.094	0.217	0.049	0.219	-0.408
ABTS	-0.017	0.370	0.309	-0.044	0.066
FRAP	0.045	0.408	0.238	-0.160	0.035
TPC	0.024	0.390	0.263	-0.084	0.104
RACI	0.045	0.430	0.266	-0.021	-0.063

²⁾PC: principal component, TIC: total isoflavone content, D3G: delphinidin-3-O- β -D-glucoside, C3G: cyanidin 3-O- β -D-glucoside, Pt3G: petunidin-3-O- β -D-glucoside, TAC: total anthocyanin content, DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), FRAP: ferric-reducing antioxidant power, TPC: total polyphenol content, RACI: relative antioxidant capacity index.

Fig. 2. Two-dimensional scatter diagram of principal component analysis of 56 Korean black soybean landraces based on anthocyanins, isoflavones, and antioxidant activities.

aglycones but low C3G and TAC. Cluster III and IV consisted of 13 and 22 soybean landraces, respectively. They had high TAC. Cluster V comprised 10 landraces with low daidzin, glycitin, genistin, total isoflavon glycosides, daidzein, total isoflavone aglycones, and TIC. In antioxidant activities, Cluster III showed low ABTS while cluster IV had high ABTS. In FRAP, TPC, and RACI, Cluster I, III, and V showed higher antioxidant activities compared to Cluster II or IV.

DISCUSSION

Black soybean (*Glycine max* L.) has been widely utilized as a health food and herbal material in Oriental medicine for hundreds of years (Xu and Chang 2008a). For centuries, farming communities have continuously contributed to the evolution, enrichment, and maintenance of landrace diversity on-farm (Brush 1995; Jarvis *et al.* 2008). However, little has been done to understand the landrace diversity or to improve these landraces (Sthapit and Rao 2009). Sthapit and Rao (2009) have suggested that landraces can be effectively improved by simple trait

selection if these landraces could offer sufficient natural variations in the population. Our results revealed that the 56 Korean black soybean landraces had different compositions in anthocyanin and isoflavone (Table 1).

Antioxidant capacities of plant extracts not only depend on extract composition, but also depend on the conditions of test used (Dudonné *et al.* 2009). In this study, we determined free radical scavenging capacities of Korean black soybean landraces using DPPH, ABTS, FRAP, or TPC assay. These methods have been widely used to determine the antioxidant capacities of plant extracts as they require relatively standard equipment. In addition, they can deliver fast and reproducible results (Dudonné *et al.* 2009). Sun and Tanumihardjo (Sun and Tanumihardjo 2007) have proposed a RACI because each method to measure the antioxidant capacity has its own limitations. Multiple reaction mechanisms and different phase locations are usually involved in the measurement of antioxidant capacity (Xu and Chang 2008a). The key advantage of RACI is that it is a numerical scale that integrates multiple chemical methods, thus allowing comparison of antioxidant capacity for a large number of samples (Sun and Tanumihardjo 2007). Our results showed that the 56

Table 4. Average values±standard deviation of each cluster based on anthocyanins, isoflavones, and antioxidant activities.

Variable	Cluster				
	I (n=4)	II (n=7)	III (n=13)	IV (n=22)	V (n=10)
Isoflavone (mg/100 g)					
Daidzin	112.7±29.1c	57.4±7.2b	57.4±11.3b	56.2±12.5b	27.4±11.5a
Glycitin	32.9±4.2d	13.5±2.6b	17.9±3.9c	17.9±4.4c	6.5±2.8a
Genistin	112.6±23.5c	82.9±21.0b	70.5±12.9b	74.6±20.9b	37.7±16.0a
Glycosides	258.1±55.2c	153.8±29.2b	145.7±15.6b	148.6±29.3b	71.6±28.1a
Daidzein	4.3±1.9b	3.6±3.6b	3.1±1.6b	2.5±1.2b	0.6±0.3a
Glycitein	4.0±0.5a	9.1±5.1b	3.2±1.4a	2.6±0.8a	1.9±1.4a
Genistein	1.2±1.0a	2.1±0.9b	1.4±1.0a	0.9±0.5a	0.8±0.4a
Aglycones	9.5±2.2c	14.7±3.0d	7.7±2.4bc	6.0±1.1b	3.3±1.7a
TIC ²⁾	267.6±57.2c	168.6±26.9b	153.4±16.5b	154.7±29.8b	74.8±29.2a
Anthocyanin (mg/100 g)					
D3G	73.7±39.7ns	27.3±25.8	63.5±29.5	76.1±57.3	59.2±31.3
C3G	694.6±457.3b	177.4±198.8a	223.3±171.7ab	431.1±307.4ab	227.5±218.6ab
Pt3G	14.7±11.1ns	2.9±2.5	12.7±8.1	14.9±12.2	9.8±6.7
TAC	447.2±265.2ab	152±127.8a	542.8±264.9b	520.5±404.7b	291.3±237.7ab
DPPH (IC50)	46.3±17.5ns	64.9±14.5	57.1±18.9	59.4±15.2	63.1±10.3
ABTS (IC50)	69.6±16.5ab	80.9±22.7bc	60.4±4.1a	87.6±20.3c	64.7±15.3ab
FRAP (mg ASC/g)	20.9±10.8b	10.8±5.3a	21.6±3.0b	11.6±4.7a	17.4±6.5b
TPC (mg GAE/g)	25.0±5.0b	14.9±3.4a	25.3±5.3b	16.4±4.8a	22.7±6.6b
RACI	0.6±0.8b	-0.5±0.5a	0.7±0.5b	-0.5±0.7a	0.2±0.7b

Values within a column with different letters were statistically significant ($P<0.05$; Duncan's multiple range test).

²⁾TIC: total isoflavone content, D3G: delphinidin-3-O- β -D-glucoside, C3G: cyanidin 3-O- β -D-glucoside, Pt3G: petunidin-3-O- β -D-glucoside, TAC: total anthocyanin content, DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), FRAP: ferric-reducing antioxidant power, ASC: ascorbic acid, TPC: total polyphenol content, GAE: gallic acid equivalents, RACI: relative antioxidant capacity index, ns: not significant.

Korean black soybean landraces had different rankings in antioxidant capacity (Supplementary Table 1). To compare data obtained by different chemical methods used to evaluate extract antioxidant activity, we used RACI for the 56 Korean black soybean landraces (Fig. 1). RACI results could be used to select black soybeans with higher antioxidant activity. Sample IT156132 had the highest antioxidant activity among the 56 Korean black soybean landraces. Therefore, RACI can be used to develop new breeding materials.

The black pigmentation of black soybeans is due to the accumulation of anthocyanins in the epidermis pallade layer of the seed coat (Todd and Vodkin 1993). Anthocyanins are the second most important phytochemicals in black soybean besides isoflavones (Zhang *et al.* 2011). As revealed in previous studies, anthocyanin contents in black soybeans ranged from < 1.0 to 20.4 mg/g (Xu and Chang

2008a; 2008b). Anthocyanins in seed coats play important roles in the protection against oxidative damage (Ramarathnam *et al.* 1989). Among them, C3G was found to be the most abundant anthocyanin in black soybeans. C3G has been reported to possess strong antioxidant activity in antioxidant assays among three anthocyanins isolated from black beans (Tsuda *et al.* 1994). In our study, three anthocyanins (D3G, C3G, and Pt3G) were identified. A wide variation of TAC (from 19.8 to 1,420.4 mg/100 g) was also confirmed in the 56 Korean black soybean landraces (Table 1). Among three detected anthocyanins, C3G was the most abundant anthocyanin in the 56 Korean black soybean landraces. In correlation analysis, TAC showed a positive correlation with RACI, although D3G, C3G, and Pt3G failed to show significant correlation with antioxidant activities.

In soybeans, isoflavones are synthesized through the

Fig. 3. Hierarchical clustering analysis of anthocyanins, isolavones, and antioxidant activities of the 56 Korean black soybean accessions.

DPPH: 2,2-diphenyl-1-picrylhydrazyl, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), FRAP: ferric-reducing antioxidant power, TPC: total polyphenol content, TAC: total anthocyanin content, D3G: delphinidin-3-O- β -D-glucoside, Pt3G: petunidin-3-O- β -D-glucoside, C3G: cyanidin 3-O- β -D-glucoside, TIC: Total isoflavone content.

phenylpropanoid pathway. They are stored in the vacuole as glucosyl- and malonyl-glucose conjugates (Graham 1991). However, their contents vary significantly. They can be affected by both genotype and environmental conditions (Dixon and Paiva 1995; Lee *et al.* 2003). Several researchers have considered isoflavones as major phenolic compounds with concentration in different soybean varieties ranging from 126.1 to 409.2 mg/100 g of soybeans (Wang and Murphy 1994; Carrao-Panizzil and

Kitamura 1995; Tsukamoto *et al.* 1995). The 56 Korean black soybean landraces in this study showed various ranges of isoflavone contents, ranging from 43.8 to 347.5 mg/100 g (Table 1), with glycitein contents ranging from 0.7 to 14.9 mg/100 g. Some previous studies failed to detect glycitein in soybean seeds because it was below the limit of detection (Mujić *et al.* 2011; Sun *et al.* 2011). USDA has reported that glycitein content is ranging from 0.0 to 8.41 mg/100g in 49 soybean mature seeds (Bhagwat *et al.*

2008). Glycitein synthesis is not yet clearly defined. It might have derived from isoliquiritigenin. In a human isoflavone metabolic study, a higher bioavailability of glycitein compared to that of genistein has been demonstrated (Latunde-Dada *et al.* 2001). Genistein could be metabolized into p-ethylphenol, which is not an estrogenic compound (Shutt *et al.* 1970). Song *et al.* (1999) have reported that glycitein has higher bioavailability than genistein in mice. They suggested that glycitein might have been metabolized into compounds with greater estrogenic potency than that of genistein (Song *et al.* 1999). Among 56 Korea black soybean landraces, two accessions showed higher glycitein contents (IT155950, 14.9±1.8 mg/100 g; IT113589, 14.4±1.2 mg/100 g). The two soybean landraces may be used to understand glycitein biosynthesis so that a soybean variety that contains high isoflavone contents can be developed.

In summary, we analyzed anthocyanin and isoflavone contents of 56 Korean black soybean landraces in this study and estimated their antioxidant activities. In addition, we classified the 56 Korean black soybean landraces using PCA analysis and hierarchical clustering analysis. Our results could contribute to more efficient conservation and utilization of black soybean landraces to broaden the genetic bases of commercially grown varieties of soybean. Especially, IT177372, IT177349, IT177253, and IT177309 might be promoted as soy products in daily diet because they have higher contents of healthful factors.

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