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Phenolic compounds and antioxidant properties of breeding lines between the white and black rice

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ABSTRACT

Advanced breeding lines made from the cross between the black and white rice as parents were collected to evaluate phenolic levels and antioxidant properties. No free phenolic acid was found in the soluble fraction, while *p*-coumaric acid, ferulic acid, isoferulic acid and vanillic acid were identified in insoluble bound fractions. Of noteworthy, is isoferulic acid which has rarely been reported to occur in cereal grains. Phenolic dehydrodimers were only observed in the insoluble bound fractions, which mainly consisted of 8-5′-coupled diferulic acids and 5-5′-coupled diferulic acids. Cyanidin 3-glucoside, peonidin 3-glucoside and cyanidin occurred in black and some light-purple rice samples. The breeding line YF53 has the highest total phenolic content (23.3 mg ferulic acid equiv./g), total anthocyanin content (2.07 mg cyanidin-3-glu equiv./g), and antioxidant activities. The results indicate that it is possible to develop advanced breeding lines for improvement of the phenolic profiles and antioxidant capacity with high yield.

1. Introduction

Rice (*Oryza sativa* L.) is the world's most important food crop and responsible for feeding approximately one-third of Earth's population. It is the dietary staple food in many Asian countries (Shen, Jin, Xiao, Lu, & Bao, 2009). Rice yields increased dramatically in China which contributes to about 31% of the world's rice production, due to the introduction of hybrid rice varieties (Li, Salas, DeAngelo, & Rose, 2006). Recently, many attempts have been made to develop better rice varieties, rich in certain functional compounds exhibiting antioxidant activities. Phenolics have been reported as the major hydrophilic antioxidants in rice while carotenoids, tocopherol, and gamma-oryzanols as the principle lipophilic antioxidative constituents (Min, McClung, & Chen, 2011). These substances have associated with the prevention of cardiovascular diseases, certain type of cancer and other diseases related to aging, thanks to their antioxidant properties (Kim, Tsao, Yang, & Cui, 2006; Shen et al., 2009). Thus, some type of rice varieties may be bred which has not only higher yield but also better quality containing increased levels of bioactive compounds.

Rice grain has a bark-like, protective hull, beneath which are the endosperm, bran and germ. Polishing of the dehulled rice to obtain milled rice, the form that is generally consumed, leads to loss of most of the nutritional components of the rice grain that are mostly deposited in the bran. Most rice varieties that are planted and consumed throughout the world have white pericarp. Therefore, more attentions should be paid to develop rice varieties with coloured pericarp or coloured rice bran layer (Nam et al., 2006; Qiu, Liu, & Beta, 2009). It has been shown that consumption of coloured rice causes decrease of oxidative stress and simultaneous increase of antioxidant capacity in the tested models (Hu, Zawistowski, Wenhua, & Kitts, 2003).

In China, attention has been paid to black rice that has an incredibly rich history known as "Forbidden Rice", because it was only reserved for the Emperor's consumption. Black rice has been characterised by the accumulation of phenolic acids, flavonoids and anthocyanins exhibiting antioxidant activities (Kaneda, Kubo, & Sakurai, 2006). However, black rice yield is lower than that of white rice which does not contain anthocyanins. Therefore, the novel rice varieties with high yield and good quality are expected to be bred through the hybrid between the white and black rice. Recently, 15 breeding lines with high yields have been bred through hybrid between the white (II32 B) and black (Yunanheixiannuo)

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rice as the parent. Their phenotypes in rice bran layer are easy to identify difference with colour shades, including black, light-purple and white. However, their functional properties and constitute of bioactive compounds need to be further studied.

The objective of this study was to investigate and compare phenolic compounds and antioxidant abilities among 15 offspring samples deriving from a cross between black and white rice. This study will be helpful for rice breeders to screen the hybrid samples with functional properties through comparative evaluation of phytochemicals profiles and antioxidant abilities among the earlier breeding line samples.

2. Materials and methods

2.1. Samples

A white rice (II32 B, YF43), a black rice (Yunanheixiannuo, a waxy rice, YF68) and 15 breeding lines derived from the cross between II32 B (YF43) and Yunanheixiannuo (YF68) were used in this study. The dehulled and unpolished grains of 15 breeding lines and the parent samples could be divided into three classes according to their colour shades: white (YF43, YF45, YF47, YF50, YF55, YF56), light-purple (YF44, YF49, YF62, YF63, YF67) and black (YF46, YF53, YF54, YF57, YF64, YF68) (Supplementary Fig. 1).

2.2. Chemicals

Folin–Ciocalteu reagent, DPPH, and ferulic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). HPLC grade acetone and methanol were used in the extraction and fractionation. Phenolic acids standards (gallic acid, protocatechuic acid, *p*-hydroxylbenoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, isoferulic acid, *o*-coumaric acid), and anthocyanin and anthocyanidin standards (cyanidin chloride, delphinidin chloride, malvidin chloride, kuromanin chloride, callistephin chloride, idaein chloride, keracyanin chloride, cyanin chloride) were purchased from Sigma–Aldrich Chemical Co. Peonidin 3-*O*-glucoside chloride were purchased from Polyphenols Laboratories AS (Sandnes, Norway). MS grade acetonitrile, methanol and acetic acid were used in HPLC–MS/MS analysis. All of the HPLC grade and MS grade solvents were purchased from Sigma–Aldrich Chemical Co.

2.3. Extraction of free fractions

Rice flour (2 g) was extracted twice with 80% methanol at a ratio of 1:20 (w/v) for the soluble free phenolic compounds. Each time, the mixture was kept on a mechanical shaker (Thermo/Lab-Line/Barnstead MAX Q 4000, Artisian Scientific, Champaign, IL, USA) for 1 h at room temperature. After centrifuging (Model 2C5C, MANDEL, Guelph, ON) at 1430×*g* for 5 min, the supernatants obtained from each time were combined and concentrated to dryness by using a rotary evaporator (Bochi R-205, Flawil, Switzerland) at 35 °C. The dried methanol extract was redissolved in 5 mL of 50% methanol and the extracts were used as the soluble free fractions on analysis of phenolic compounds.

2.4. Extraction of insoluble bound fractions

The residue above obtained was washed with 40 mL of distilled water to eliminate organic solvent, and then filtered through a Whatman No. 1 filter paper. After drying in a hood at room temperature, the dried residue was hydrolysed with 40 mL of 4 M NaOH on a shaker (Thermo/Lab-Line/Barnstead MAX Q 4000, Artisian Scientific, Champaign, IL, USA) under nitrogen gas for 4 h. After digestion, the solution was adjusted to a pH 1.5–2.0 with 6 M HCl and then extracted with 70 mL of ethyl acetate for three times. The combined ethyl acetate fractions were evaporated to dryness and reconstituted in 5 mL of 50% methanol. The extracts were used as the insoluble bound fractions on analysis of phenolic compounds.

2.5. Extraction of anthocyanins

Extraction of anthocyanins was accomplished according to a modification of the methods reported in the literature (Hosseinian, Li, & Beta, 2008). Briefly, methanol acidified with HCl (1 N) (ratio 85:15, v/v) was added to rice flours (2 g) (sample to solvent ratio of 1:8) and the pH adjusted to 1.0. After shaking at 1800 rpm for 45 min, the supernatant was separated from the pellet by centrifuging at 5000*g*. The supernatant was evaporated to dryness at 40 °C and reconstituted in methanol (5 ml).

2.6. Colour determination

A Minolta spectrophotometer CM-3500d colorimeter (Minolta Co., Ltd., Osaka, Japan) with Spectra Magic version 3.6 software was used to measure the colour of rice samples. The colour was expressed using the L, a^*, and b^* colour space coordinates, where L represents lightness, $+a^*$ redness, $-a^*$ greenness, $+b^*$ yellowness, and $-b^*$ blueness.

2.7. Measurement of total phenolic content (TPC)

The TPC of crude extracts was evaluated by using modifications of the Folin–Ciocalteu method (Singleton & Rossi, 1965). Briefly, 200 μL of the appropriate dilutions of crude extracts was reacted with 1.8 mL of 10-fold diluted Folin–Ciocalteu reagent, which was freshly made. The mixture was then neutralised with 1.8 mL of sodium carbonate (60 g/L). The absorbance was measured at 725 nm after 90 min of reaction at room temperature. Ferulic acid was used as the standard. Results were expressed as mg of ferulic acid equivalents (FAE) per gram of rice (dry weight basis).

2.8. Determination of total anthocyanin content (TAC)

The total anthocyanins content was determined according to a pH-differential method previously described by Al-Farsi, Alasalvar, Morris, Baron, and Shahidi (2005) and modified by Li and Beta (2011). Briefly, 1 mL of anthocyanin extract was respectively diluted with pH 1.0 buffer and pH 4.5 buffer to a 25-mL final volume. The absorption of the diluted sample was measured at 510 nm and 700 nm. The results were expressed as mg of cyanidin 3-glucoside (cy 3-glu) equivalents per gram of rice (dry weight basis).

2.9. Determination of DPPH radical scavenging activity

This assay was based on the method of Brand-Williams, Cuvelier, and Berset (1995) as modified by Li, Pickard, and Beta (2007). Briefly, 200 μL of crude extract (or fraction) was added to 3.8 mL of 60 μM DPPH radical solution, which was freshly made. After 60 min of incubation at room temperature, the absorbance at 515 nm was measured. DPPH free radical scavenging activities of crude extracts were expressed as μM of trolox equivalents (TE) per gram of rice (dry weight basis) using a standard curve of trolox.

2.10. Evaluation of oxygen radical absorbance capacity (ORAC)

The ORAC assay was based on the method described by Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002) and modified by Li et al. (2007). A Precision 2000 automated microplate pipetting

system (BIO-TEK Instruments, Inc., Winooski, VT) was used for plate-to-plate transfer of solutions. An FLx 800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by software KC4 3.0 (version 29) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. The final assay solution contained 120 μL of fluorescence working solution, 60 μL of 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH), and 20 μL of appropriately diluted sample. The fluorescence of the mixture solution was recorded every minute for a total of 50 min. The ORAC values were expressed as micromoles of trolox equivalents (TE) per gram of rice (dry weight basis).

2.11. Phenolic acids and dimers analysis by HPLC–DAD–Q-TOF–MS/MS

An HPLC (Waters 2695) equipped with a photodiode array detector (PDA) (Waters 996) and autosampler (717 plus, Waters) coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA) was employed for HPLC and mass spectrometric analyses (LC–MS/MS). A 250 mm × 4.6 mm, 5 μm RP 18 column (Shim-pack HRC-ODS, SHIMADZU Corp., Kyoto, Japan) was used for separation. During the HPLC–MS analysis, 10 μL of sample was loaded and injected by an autosampler and eluted through the column with a gradient mobile phase consisting of A (water containing 0.1% acetic acid) and B (methanol containing 0.1% acetic acid) with the flow rate of 1 mL/min. A 70 min linear gradient was programmed as follows: 0–11 min, 9–14% B; 11–14 min, 14–15% B; 14–17 min, 15% B; 17–24 min, 15–16.5% B; 24–28 min, 16.5–19% B; 28–30 min, 19–25% B; 30–36 min, 25–26% B; 36–38 min, 26–28% B; 38–41 min, 28–35% B; 41–46 min, 35–40% B; 46–48 min, 40–48% B; 48–53 min, 48–53% B; 53–70 min, 53–70% B. Phenolic acids were identified by comparing retention time and spectral matching with standards, and confirmed by HPLC–Q-TOF–MS/MS. The quantification of phenolic acids was based on the area of the peak at a wavelength of 280 nm using external calibration curves. The Q-TOF–MS was calibrated using sodium iodide for the negative mode through the mass range of 100–1000. A resolution of 5000 was achieved. Full mass spectra were recorded in negative mode by using a capillary voltage of 1.45 kV and a cone voltage of 45 V. The flow rates of desolvation gas (N_2) and cone gas (He) were 900 and 45 L/h, respectively. The desolvation gas temperature and the ion source temperature were set at 350 and 150 °C, respectively. The MS/MS spectra were acquired by using a collision energy of 30 V.

The response factors (RF) of ferulic acid dehydrodimers against trans-cinnamic acids were summarised by Waldron, Parr, Ng, and Ralph (1996) as follows: RF (8-8′ DFA) = 0.17, RF (5-5′ DFA) = 0.21, and RF (8-5′ DFA) = 0.12.

2.12. Anthocyanins analysis by HPLC–DAD–Q-TOF–MS/MS

Analysis was performed with a Gemini 5l C18 110A column (150 mm × 4.60 mm) (Phenomenex, Torrance, CA). A gradient of solvent A (1% acetic acid in water) and solvent B (1% acetic acid in methanol) was used for 40 min at a flow rate of 0.5 mL/min. The gradient was as follows: 0–5 min, 0% B; 5–10 min, 0–5% B; 10–15 min, 5–8% B;15–20 min, 8–13% B; 20–30 min, 13–17% B; 30–35 min, 17–30% B; 35–40 min, 30–40% B. Detector was set at 520 nm. Anthocyanins were identified by comparing retention time and spectral matching with standards, and confirmed by HPLC–Q-TOF–MS/MS. The quantification of anthocyanins was based on the area of the peak at a wavelength of 520 nm using external calibration curve. The calibration and conditions for Q-TOF–MS were the same as described above for phenolic acids and dimers.

2.13. Statistical analysis

In the above assays, all of the samples were extracted and analysed in triplicate. The results were reported as mean ± standard deviation (SD). Data were analysed by Duncan's multiple range test of one-way ANOVA using SPSS version 13.0 for Windows. Least significant differences (LSD) at $p < 0.05$ were tested to assess significant difference among samples.

3. Results and discussion

3.1. Rice grain colour

The colour characteristics in terms of Hunterlab values can be useful in phenotypically segregating pigmented or coloured cereal grains. Table 1 shows that the L value, which measures lightness, ranged from 20.78 to 23.81, from 36.69 to 41.50, and from 63.35 to 67.70 for the black rice, light-purple rice, white rice samples, respectively. The a^* value, which measures redness, ranged from 2.93 to 4.09, from 7.31 to 10.13, and from 2.60 to 3.59 for the black, light-purple, and white rice samples, respectively. The b^* value, which measures yellowness, ranged from 2.19 to 4.67, from 15.32 to 20.48, and from 20.75 to 22.45 for the black, light-purple, and white rice samples, respectively.

The L and b^* values of white rice samples were significantly higher than those of the light-purple and black samples at $p < 0.05$ indicating that white rice had higher lightness and yellowness than light-purple and black rice. The a^* values of light-purple rice samples were significantly higher than those of the white and black samples at $p < 0.05$. There was no significant difference in redness between the white and black samples. Similar results were found using barely where the purple barley grain had the highest a^* value among black and yellow barley grain (Bellido & Beta, 2009).

3.2. Total phenolic content (TPC)

Phenolic compounds may directly contribute to antioxidant activities; therefore, total phenolic content (TPC) in cereal grains was measured. The TPC was expressed as milligrams of ferulic acid equivalents (FAE) per gram of rice (Table 2). TPC ranged from 3.58 to 16.74 mg FAE/ g rice in soluble fractions. Black rice had the highest TPC in the soluble fractions among all the rice samples. Insoluble bound fractions had TPC ranging from 0.75 to 4.79 mg FAE/ g rice, with light-purple rice exhibiting the highest contents compared to the black and white samples.

TPC in soluble fractions were higher than insoluble bound fractions. Thus phenolic compounds of breeding lines mainly occurred in soluble free fractions except for YF45 (white rice) and YF67 (light-purple) which had higher insoluble bound TPC (4.45 and 4.79 mg/g, respectively) than those of soluble fractions (Table 2). It could be due to some important genes that control the linkage between phenolics and lignins which segregated and assorted resulting in different offsprings of rice hybrid. Furthermore, the TPC of soluble fractions of black rice was significantly higher than that in light-purple and white rice. The TPC of soluble fractions of black rice YF53 was the highest among all fractions in the 17 samples, which showed that hybridisation could enhance the total soluble phenolic content (Table 2).

3.3. Composition of phenolic acids and dimers

Table 3 shows the phenolic acids present in insoluble bound fractions of the rice samples. No phenolic acid was detected in soluble free fractions at the given concentration because only trace

Table 1
Colour determination, total anthocyanin content (TAC) and anthocyanin content of rice samples.[A]

	Colour parameters			Anthocyanin content			
	L	a^*	b^*	TAC (mgcyanidin-3-glu equiv./g)	Cyanidin-3-glu (mg/g)	Peonidin-3-glu (mgcyanidin-3-glu equiv./g)	Cyanidin content (mgcyanidin-3-glu equiv./g)
White							
YF43 (parent)	63.75 ± 0.11^{d}	3.54 ± 0.10^{f}	20.75 ± 0.11^{c}	0.02 ± 0.01^{j}	nd	nd	nd
YF45	65.50 ± 0.19^{b}	3.14 ± 0.04^{h}	21.62 ± 0.19^{b}	0.09 ± 0.02^{h}	nd	nd	nd
YF47	63.35 ± 0.31^{d}	3.59 ± 0.05^{f}	21.78 ± 0.14^{b}	0.04 ± 0.04^{k}	nd	nd	nd
YF50	67.70 ± 0.37	2.60 ± 0.06^{i}	22.45 ± 0.23^{a}	0.04 ± 0.02^{i}	nd	nd	nd
YF55	65.05 ± 0.07^{c}	3.13 ± 0.10^{h}	22.16 ± 0.07^{a}	0.02 ± 0.00^{j}	nd	nd	nd
YF56	65.01 ± 0.22^{c}	3.40 ± 0.02^{g}	20.98 ± 0.05^{c}	0.03 ± 0.02^{i}	nd	nd	nd
Light-purple							
YF44	38.10 ± 0.29^{g}	9.72 ± 0.13^{b}	18.13 ± 0.04^{f}	0.08 ± 0.02^{h}	nd	nd	nd
YF49	40.20 ± 0.29^{f}	9.07 ± 0.08^{b}	19.04 ± 0.17^{d}	0.29 ± 0.01^{f}	0.273 ± 0.002^{e}	0.104 ± 0.005^{d}	0.065 ± 0.003^{f}
YF62	36.69 ± 0.15^{h}	10.12 ± 0.06^{a}	18.52 ± 0.15^{e}	0.21 ± 0.00^{g}	0.256 ± 0.001^{e}	0.099 ± 0.000^{d}	0.047 ± 0.00^{g}
YF63	38.44 ± 0.39^{g}	7.32 ± 0.02^{c}	15.32 ± 0.07^{g}	0.23 ± 0.04^{g}	0.261 ± 0.002^{e}	0.073 ± 0.003^{e}	0.038 ± 0.006^{g}
YF67	41.50 ± 0.09^{e}	10.13 ± 0.11^{a}	20.48 ± 0.16^{c}	0.03 ± 0.01^{i}	nd	nd	nd
Black							
YF46	22.01 ± 0.17^{k}	4.09 ± 0.08^{d}	3.46 ± 0.25^{ij}	1.76 ± 0.02^{b}	0.594 ± 0.001^{c}	0.146 ± 0.000^{b}	0.130 ± 0.001^{b}
YF53	23.81 ± 0.07^{i}	3.80 ± 0.02^{ef}	3.33 ± 0.07^{j}	2.07 ± 0.11^{a}	0.792 ± 0.07^{a}	0.161 ± 0.001^{a}	0.181 ± 0.006^{a}
YF54	23.31 ± 0.15^{j}	3.58 ± 0.03^{f}	3.67 ± 0.16^{i}	1.56 ± 0.03^{d}	0.590 ± 0.01^{c}	0.131 ± 0.000^{c}	0.135 ± 0.001^{b}
YF57	22.36 ± 0.14^{k}	2.93 ± 0.22^{h}	2.19 ± 0.14^{k}	1.53 ± 0.01^{de}	0.585 ± 0.04^{c}	0.128 ± 0.000^{c}	0.095 ± 0.000^{e}
YF64	23.70 ± 0.17^{ij}	3.86 ± 0.05^{e}	4.67 ± 0.17^{h}	1.47 ± 0.09^{e}	0.564 ± 0.03^{d}	0.107 ± 0.002^{d}	0.127 ± 0.003^{c}
YF68 (parent)	20.78 ± 0.18^{l}	3.69 ± 0.15^{f}	2.30 ± 0.08^{k}	1.66 ± 0.07^{c}	0.638 ± 0.14^{b}	0.148 ± 0.002^{b}	0.103 ± 0.002^{d}

[A] Values in each column with the same superscript are not different ($p > 0.05$); nd = not detected.

amounts of soluble phenolic acids were obtained using 80% methanol extraction, and possibly low detection limit was an additional factor (Fig. 1). Only minute quantities were measured as free phenolic acids in wild rice (*Zizania aquatica* L.) (Qiu, Liu, & Beta, 2010). In rice grains, *p*-coumaric acid, ferulic acid, isoferulic acid and vanillic acid occurred in insoluble bound fractions (Fig. 1 and Table 3). The results further confirmed that phenolic acids of cereal grains are mainly present in the bound form, which esterified to the cell wall. These bound phenolic acids were subjected to alkaline hydrolysis and converted from a bound form to a free monomer form. Among the insoluble bound fractions, the mean ferulic acid content was the highest (140.23 μg/g) nearly constituting up to 65% of total phenolic acids, followed by *p*-coumaric acid and isoferulic acid. Similar results that ferulic acid was the dominant phenolic acid in wheat, wild rice and barley have been reported (Kim et al., 2006; Qiu et al., 2010). These compounds mainly occurred in the esterified form with arabinose or short oligoarabinoxylan (Singleton & Rossi, 1965). For insoluble bound fractions, total content of three phenolic acids (ferulic, *p*-coumaric and isoferulic) of the white samples were significantly higher than those of the light-purple and black rice ($p < 0.05$). Hence the levels of phenolic acids in rice samples are dependent on the genotype of the varieties.

In the present study, the peak at retention time 45.37 min had the same molecular weight (193) as ferulic acid according to HPLC–DAD–MS, but the retention time was different from that of ferulic acid which was at 42.82 min. Some researchers reported it as *trans/cis* isomers of ferulic acid in wild rice, wheat and barley (Bunzel, Allerdings, Sinwell, Ralph, & Steinhart, 2002; Doberstein & Bunzel, 2010) besides ferulic acid. Moreover, the retention time

Table 2
Total phenolic content (TPC) of soluble and insoluble fractions of rice samples.[A]

	Total phenolic content (mg ferulic acid equiv./g rice)		Total content of two fractions (mg ferulic acid equiv./g rice)
	Soluble fraction	Insoluble bound fraction	
White			
YF43 (parent)	4.21 ± 0.08^{efg}	1.90 ± 0.12^{gh}	6.11
YF45	4.05 ± 0.11^{efg}	4.45 ± 0.28^{b}	8.50
YF47	3.58 ± 0.15^{g}	1.90 ± 0.07^{gh}	5.48
YF50	4.04 ± 0.06^{efg}	2.38 ± 0.10^{f}	6.42
YF55	3.96 ± 0.32^{fg}	2.47 ± 0.22^{f}	6.43
YF56	3.62 ± 0.12^{g}	2.32 ± 0.14^{f}	5.94
Light-purple			
YF44	4.74 ± 0.07^{e}	1.38 ± 0.14^{ij}	6.12
YF49	4.85 ± 0.17^{e}	3.13 ± 0.18^{e}	7.98
YF62	5.79 ± 0.52^{d}	4.16 ± 0.18^{bc}	9.95
YF63	4.74 ± 0.49^{e}	2.18 ± 0.07^{fg}	6.92
YF67	4.32 ± 0.23^{ef}	4.79 ± 0.31^{a}	9.11
Black			
YF46	14.59 ± 0.70^{b}	1.25 ± 0.09^{j}	15.84
YF53	16.74 ± 0.89^{a}	4.11 ± 0.31^{c}	20.85
YF54	14.22 ± 0.44^{b}	0.75 ± 0.16^{k}	14.97
YF57	14.55 ± 0.15^{b}	1.62 ± 0.10^{hi}	16.17
YF64	11.19 ± 0.18^{c}	3.53 ± 0.22^{d}	14.72
YF68 (parent)	16.10 ± 0.18^{a}	3.95 ± 0.26^{c}	20.05

[A] Values in each column with the same superscript are not different ($p > 0.05$).

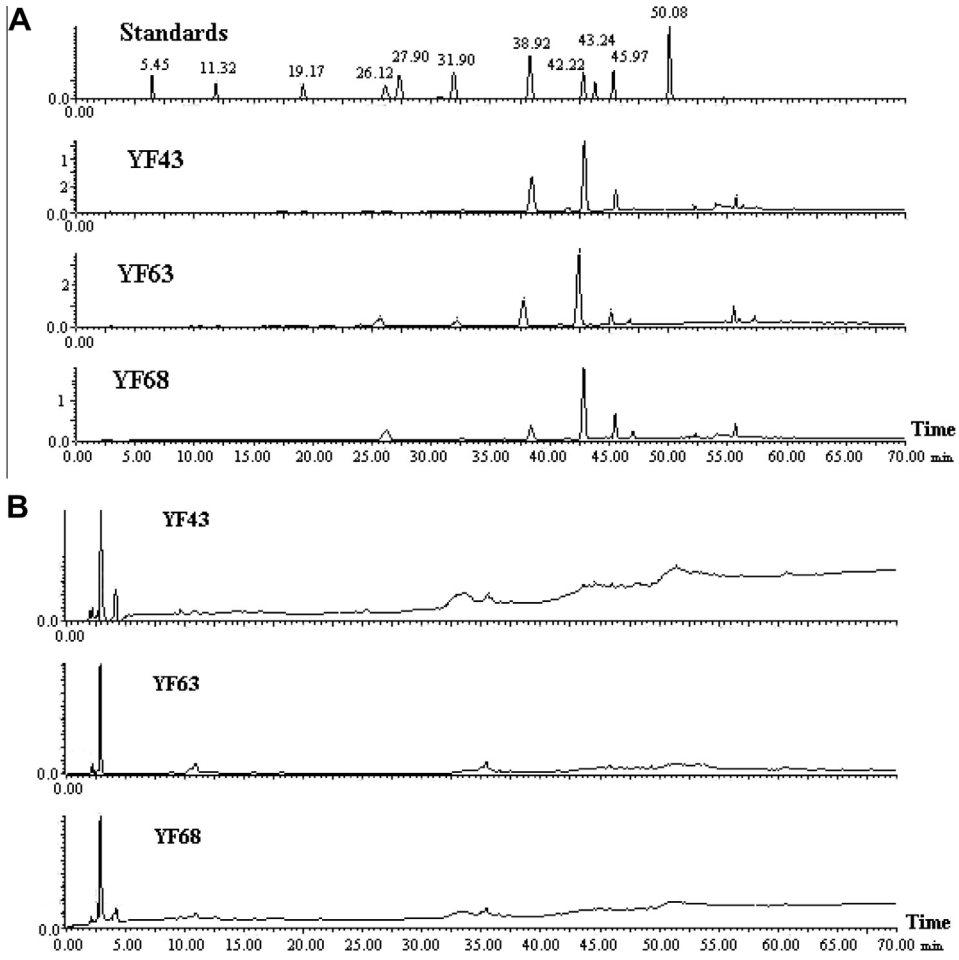


Fig. 1. HPLC chromatograms of phenolic acid detected at the wavelength of 280 nm. (A) Insoluble bound fraction; (B) soluble free fraction.

Table 3
Amounts of phenolic acids and diferulic acids of insoluble bound fractions of hybrid rice samples.[A]

	Ferulic acid (μg/g)	p-Coumaric acid (μg/g)	Isoferulic acid (μg/g)	Vanillic acid (μg/g)	8-8′ DFA (μg/g)	8-5′ DFA benzofuran form (μg/g)	5-5′ DFA (μg/g)
White							
YF43 (parent)	153.79 ± 2.85^{e}	56.74 ± 0.41^{b}	25.89 ± 0.26^{e}	nd	14.59 ± 0.85^{de}	10.14 ± 0.05^{e}	15.58 ± 0.05^{b}
YF45	199.23 ± 0.70^{b}	34.79 ± 0.21^{f}	35.96 ± 0.03^{b}	8.09 ± 0.03^{d}	25.34 ± 0.06^{b}	18.14 ± 0.06^{b}	14.71 ± 0.12^{bc}
YF47	119.37 ± 1.81^{h}	38.78 ± 0.19^{e}	25.33 ± 0.10^{e}	nd	8.14 ± 0.04^{h}	6.67 ± 0.03^{i}	7.44 ± 0.02^{i}
YF50	168.18 ± 2.50^{d}	50.19 ± 0.72^{c}	25.92 ± 0.43^{e}	nd	10.34 ± 0.07^{f}	16.25 ± 0.06^{d}	12.16 ± 0.04^{d}
YF55	147.79 ± 0.02^{f}	46.05 ± 0.11^{d}	24.10 ± 0.19^{f}	nd	9.49 ± 0.06^{g}	7.77 ± 0.09^{h}	8.17 ± 0.04^{h}
YF56	134.52 ± 0.51^{g}	39.35 ± 0.25^{e}	14.95 ± 0.08^{i}	nd	6.68 ± 0.03^{k}	7.45 ± 0.03^{h}	6.94 ± 0.06^{i}
Light-purple							
YF44	64.58 ± 0.40^{j}	14.24 ± 0.21^{i}	24.99 ± 0.28^{ef}	4.17 ± 0.07^{g}	15.94 ± 0.05^{d}	nd	10.96 ± 0.05^{f}
YF49	156.40 ± 0.14^{e}	26.99 ± 0.94^{g}	23.10 ± 0.39^{g}	5.98 ± 0.04^{f}	10.01 ± 0.04^{f}	10.44 ± 0.03^{e}	9.19 ± 0.04^{g}
YF62	207.23 ± 0.73^{b}	32.08 ± 0.09^{f}	28.06 ± 0.18^{d}	15.46 ± 0.08^{b}	8.54 ± 0.08^{h}	17.75 ± 0.06^{c}	11.92 ± 0.03^{e}
YF63	100.70 ± 0.27^{i}	26.49 ± 0.17^{g}	11.41 ± 0.06^{j}	8.01 ± 0.03^{d}	7.78 ± 0.04^{i}	8.09 ± 0.02^{g}	9.98 ± 0.06^{g}
YF67	218.39 ± 1.28^{a}	70.96 ± 0.33^{a}	34.48 ± 0.33^{c}	1.98 ± 0.02^{h}	16.23 ± 0.07^{d}	18.69 ± 0.06^{b}	13.11 ± 0.01^{c}
Black							
YF46	65.36 ± 0.34^{j}	9.09 ± 0.09^{j}	16.61 ± 0.18^{h}	4.68 ± 0.03^{g}	8.68 ± 0.03^{h}	9.13 ± 0.01^{f}	7.10 ± 0.04^{i}
YF53	187.40 ± 1.86^{c}	28.51 ± 0.21^{fg}	15.10 ± 0.24^{i}	12.15 ± 0.06^{c}	26.86 ± 0.03^{a}	20.84 ± 0.03^{a}	12.06 ± 0.05^{d}
YF54	26.09 ± 0.11^{k}	6.57 ± 0.03^{k}	6.30 ± 0.03^{k}	7.56 ± 0.06^{d}	12.55 ± 0.06^{e}	nd	3.34 ± 0.01^{j}
YF57	68.97 ± 0.10^{j}	8.75 ± 0.03^{j}	14.27 ± 0.03^{i}	6.68 ± 0.06^{e}	9.13 ± 0.05^{g}	8.56 ± 0.03^{g}	8.41 ± 0.02^{h}
YF64	141.48 ± 0.68^{g}	23.47 ± 0.10^{h}	44.49 ± 0.16^{a}	16.39 ± 0.04^{b}	24.17 ± 0.09^{c}	18.94 ± 0.03^{b}	12.42 ± 0.02^{d}
YF68 (parent)	215.21 ± 0.21^{a}	27.90 ± 0.11^{g}	45.31 ± 0.34^{a}	19.87 ± 0.02^{a}	24.76 ± 0.03^{c}	20.13 ± 0.02^{a}	16.01 ± 0.02^{a}

[A] Values in each column with the same superscript are not different ($p > 0.05$). nd = not detected.

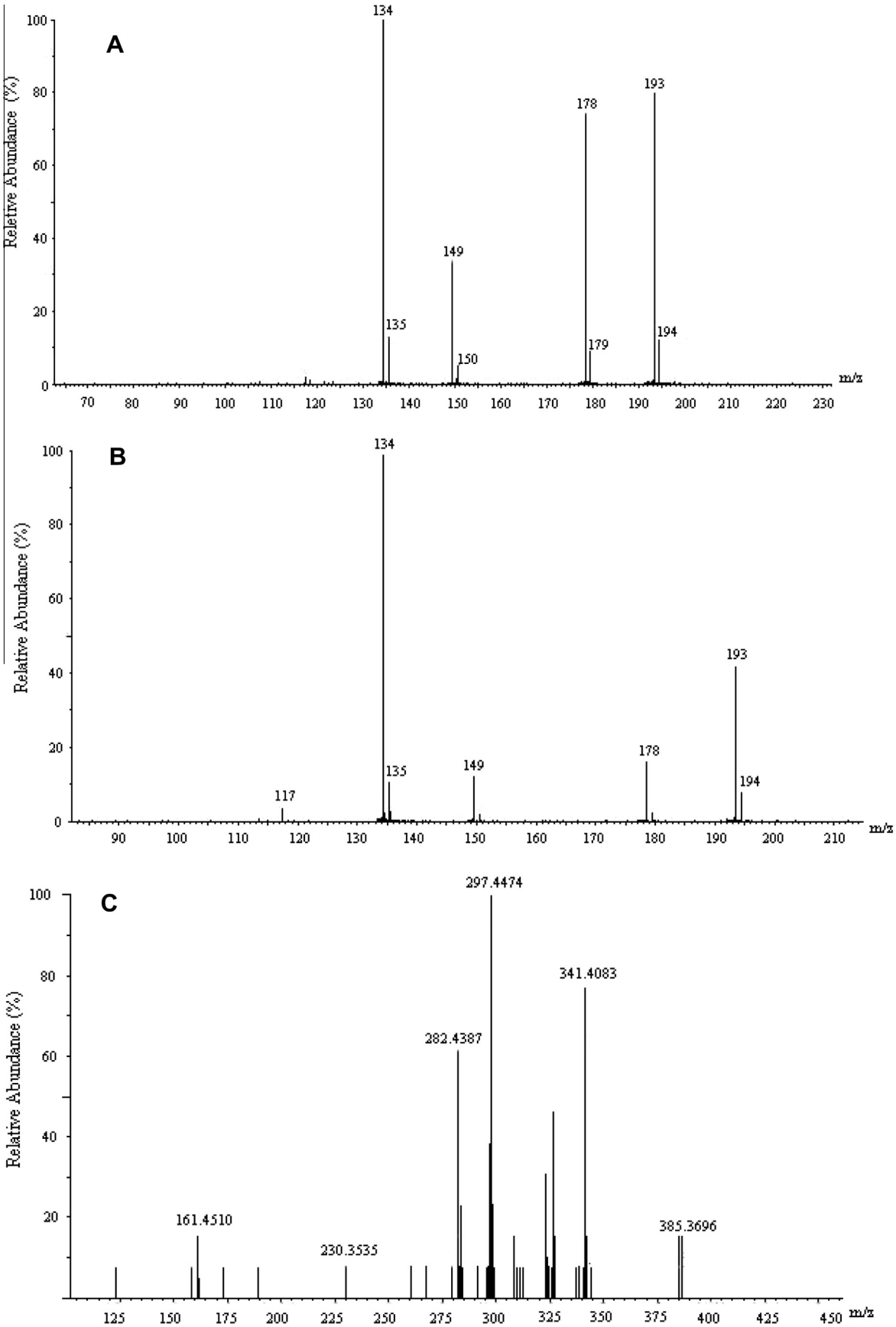
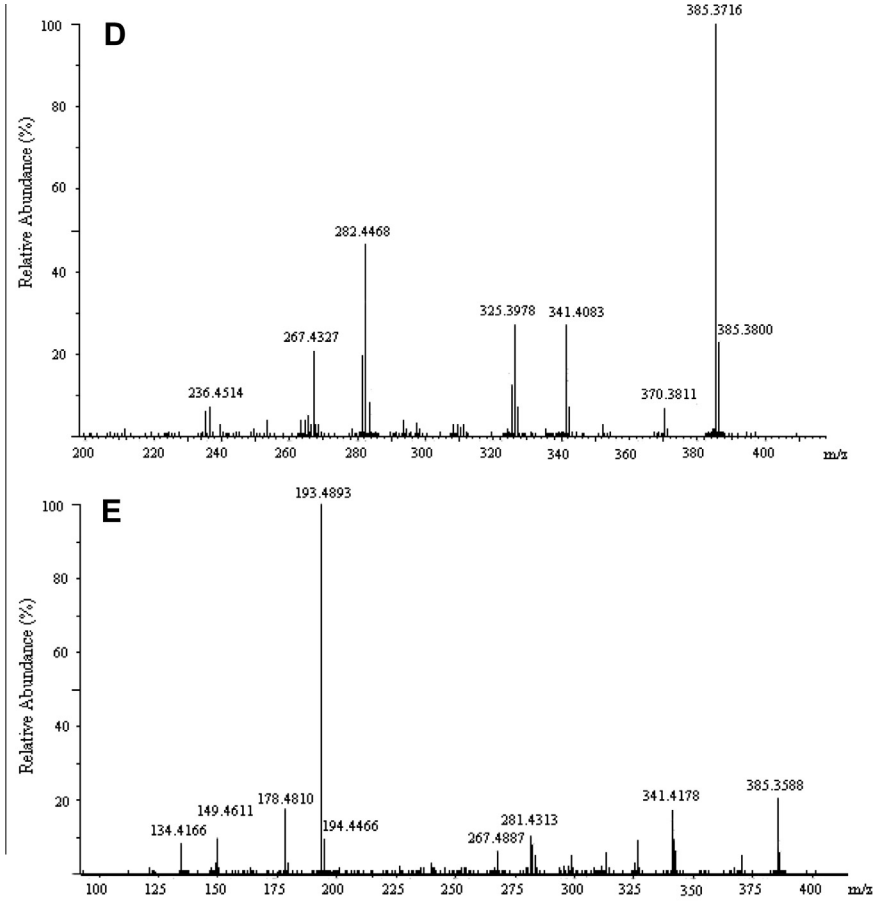


Fig. 2. MS/MS spectra of ferulic acid (A), isoferulic acid (B), 8-8′ diferulic acid (C), 8-5′ benzofuran form diferulic acid (D), 5-5′ diferulic acid (E) and unidentified compound (F).



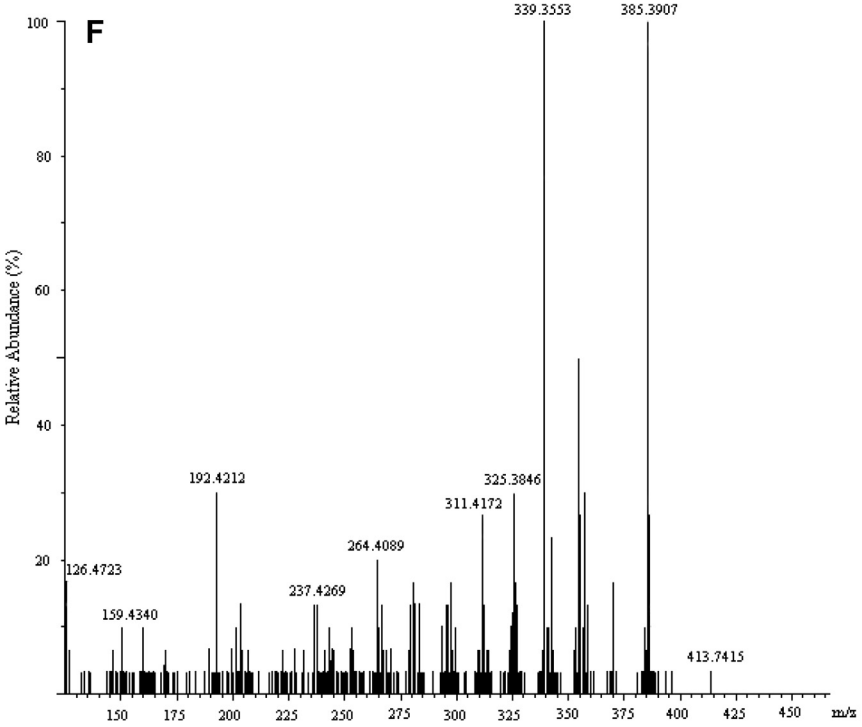


Fig. 2 (*continued*)

of trans-ferulic acid was ahead of that of cis-ferulic acid. Isoferulic acid also shares the same molecular weight and hydrophilic properties as ferulic acid, which caused similar retention time in the chromatogram. By comparing the retention time in the LC chromatogram and MS/MS spectrum between rice samples and isoferulic acid standard, we discovered that the peak at 45.37 min was isoferulic acid but not cis-ferulic acid. This is because the *p*-OH group of ferulic acid makes the benzoic ring more polar than *m*-OH group of isoferulic acid. As can be seen in Fig. 2A, the fragments of both ferulic acid and isoferulic acid in MS/MS spectrum had ions of 134 (*m/z*, $[M\text{-}CH3\text{-}COO]^-$), 178 (*m/z*, $[M\text{-}CH3]^-$), and 149 (*m/z*, $[M\text{-}COO]^-$) (Fig 2A and B). For isoferulic acids, however, its relative abundances of *m/z* 178 and 149 were lower than those recorded for ferulic acid. This is likely due to the *p*-OH group (ferulic acid) which makes the ring more nucleophilic than the *m*-OH group (isoferulic acid). In other words, medium ions *m/z* 178 and 149 of isoferulic acid were so unstable to further divide to *m/z* 134 which was a more stable ion. The structural differences between *trans*- and *cis*-ferulic acid have little effect on nucleophilicity of the ring. Therefore, the different abundances of ions of 178 (*m/z*) and 149 (*m/z*) and the retention time of the two peaks resulted from group positional isomers, but not trans/cis isomers. Isoferulic acid has rarely been reported to occur in cereal grains, besides wheat, rye and triticale (Weidner, Amarowicz, Karamać, & Dabrowski, 1999), we identified and quantified it in white, black and light-purple rice grains.

Besides the above four monomeric phenolic acids, four phenolic acid dehydrodimers were also detected. By plotting the typical molecular ions at *m/z* 385, four peaks were observed with retention times at 46.81, 52.14, 55.54 and 60.37 min, respectively, on the reversed-phase HPLC in the insoluble bound fractions. The four peaks were recognised as ferulate dehydrodimers which are also referred to as diferulic acids and reported to be the most common dimeric phenolic acids observed in cereal grains. These dimers are abundant in the cell wall and occur only in the insoluble fraction (Doberstein & Bunzel, 2010; Qiu et al., 2010). However, ferulic acid and isoferulic acid share the same molecular weight, and both occurred in the rice samples. It seems reasonable to assume that the four dehydrodimers at *m/z* 385 could be constituted by two molecules of either ferulic acid, isoferulic acid or ferulic/isoferulic combination. Moreover, the four dehydrodimers showed different MS/MS spectra (Fig. 2C–E). Fragment ion peaks of 385, 341, 297 and 282 (*m/z*) for the dimer at retention time of 46.81 min, was tentatively identified as 8-8′ coupled diferulic acids (DFA) (Fig. 2C). Among all samples, contents of 8-8′ DFA ranged from 6.68 to 26.86 μg/g rice (Table 3). Fragment ion peaks of 385, 341, 326, 282, and 267 (*m/z*) for the dimer at retention time of 52.14 min was tentatively identified as 8-5′-DFA benzofuran form (Fig. 2D). Among all samples, contents of 8-5′ DFA benzofuran form ranged from 0 to 20.84 μg/g rice (Table 3). Fragment ion peaks of 385, 313, 241 and 193 (*m/z*) for the dimer at retention time of 55.54 min was tentatively identified as 5-5′-DFA (Fig. 2E). Among all samples, contents of 5-5′-DFA ranged from 3.34 to 16.01 μg/g rice (Table 3). Fragment ion peaks of 385, 339, 325 and 192 (*m/z*) for the dimer at retention time of 60.35 min was not identified as its MS/MS spectrum information was not enough (Fig. 2F). It needs further extraction and purification, and then analyse by nuclear magnetic resonance (NMR) technique or other chemical analysis methods. As seen in Fig. 2 and Table 3, the four dehydrodimers at *m/z* 385 could be differently linked together and the contents of 8-8′ DFA and 8-5′- DFA benzofuran form were significantly higher in black samples than those of the light-purple and white rice ($p < 0.05$).

For the contents of dehydrodimers, it was reported that the dehydroferulic and dehydrosinapic acid are the common ones in commercial wild rice (Qiu et al., 2009), and the structures of diferulic acid included 8-8′, 5-5′, 8-O-4′ and 8-5′ (benzofuran form) coupled dimers with 8-O-4′ as the most abundant which had a content up to 34 μg/g (Qiu et al., 2010). In rice grains, diferulic acid mainly existed in the structures of 8-8′, 8-5′ (benzofuran form) and 5-5′ coupled dimers, all of which had almost the same content except some breeding lines which had higher 8-8′ or 8-5′ (benzofuran form) coupled dimers with the contents up to 26.86 or 20.84 μg/g, respectively. However, the composition of the dimers needs to be further clarified because of the existence of ferulic isomers (isoferulic acid).

3.4. Total anthocyanin content (TAC) and anthocyanin composition

In plants, anthocyanins are being recognised as important antioxidants, which have physiological functions of reducing risks of chronic diseases including hypercholesterolemia, hyperglycemia, and cancer (Abdel-Aal, Young, & Rabalski, 2006). Anthocyanins are the largest group of water soluble pigments in cereal grains and are found in glycosylated forms to be linked with sugars including glucose, galactose, arabinose (Choi, Jeong, & Lee, 2007; Hosseinian et al., 2008). The total anthocyanin content (TAC) of samples, expressed as cyanidin 3-glucoside equivalent, is shown in Table 1. TAC of breeding lines ranged from 0.02 to 2.07 mg/g. Although TAC in white rice was very low from 0.02–0.09 mg/g, anthocyanins were not detected by HPLC–DAD–MS in these samples. This indicated that TAC of white rice could result from non-anthocyanin flavonoids. Determination of TAC is not useful for white rice. However, TAC of the black-coloured grains showed significant differences, ranging from 1.47 to 2.07 mg/g, and was about 8 times higher than those of the light-purple rice. Other coloured grains such as black rice, purple corn, and blue wheat, had remarkable levels of anthocyanins (Abdel-Aal et al., 2006; Min, Gu, McClung, Bergman, & Chen, 2012).

In terms of the anthocyanin composition of the experimental samples, two anthocyanin and one anthocyanidin compounds were detected in the samples of black and light-purple rice. The fragment ion peaks of HPLC–MS corresponding to two anthocyanins were 301 and 287 (*m/z*), respectively, with the former as peonidin and the latter as cyanidin. The difference of *m/z* 14 between both ions attests the presence of a methoxyl group in peonidin in spite of a hydroxyl group in cyanidin. The neutral loss of 162 (*m/z*) from their respective molecular ions indicates the existence of a glucose moiety in the structures of both identified anthocyanins (data not shown). Through a comparison with anthocyanin standards, cyanidin 3-glucoside and peonidin 3-glucoside were detected in these samples. For black rice samples, contents of cyanidin 3-glucoside and peonidin 3-glucoside ranged from 0.564 to 0.792 and from 0.107 to 0.161 mg/g, respectively (Table 1). For light-purple rice samples, three samples contained anthocyanins, and other two samples were not detected to contain anthocyanins. The absence of these compounds in the two light-purple samples may be due to genotypic differences. It is possible that some regulator genes that control the anthocyanins biosynthesis were not passed to these lines. In light-purple lines, contents of cyanidin 3-glucoside and peonidin 3-glucoside ranged from 0.256 to 0.273 and 0.06 to 0.15 mg/g, respectively. Significant differences were observed between contents of cyanidin 3-glucoside and peonidin 3-glucoside ($p < 0.05$). Cyanidin 3-glucoside was reported to be the most abundant anthocyanins in coloured cereal grains (Bellido & Beta, 2009; Laokuldilok, Shoemaker, Jongkaewwattana, & Tulyathan, 2011; Min et al., 2012). Cyanidin 3-glucoside and peonidin 3-glucoside were identified in black rice (Hu et al., 2003; Laokuldilok et al., 2011; Min et al., 2012; Yawadio, Tanimori, & Morita, 2007) besides other four anthocyanins such as cyanidin-3-galactoside, cyanidin-3-rutinoside, ayanidin and peonidin reported in the study by Min et al. (2012).

Table 4
DPPH radical scavenging activity and ORAC of soluble and insoluble fractions of hybrid rice samples.[A]

	DPPH scavenging activity (μmol trolox/g rice)		ORAC (μmol trolox/g rice)	
	Soluble free fraction	Insoluble bound fraction	Soluble free fraction	Insoluble bound fraction
White				
YF43 (parent)	1.43 ± 0.02^{i}	1.70 ± 0.03^{ef}	16.71 ± 2.10^{ij}	15.96 ± 0.55^{e}
YF45	1.39 ± 0.04^{i}	2.09 ± 0.06^{b}	23.66 ± 3.52^{fg}	25.31 ± 2.83^{a}
YF50	1.50 ± 0.06^{i}	0.90 ± 0.01^{k}	21.54 ± 3.85^{fgh}	17.72 ± 2.07^{de}
YF47	1.46 ± 0.02^{i}	1.65 ± 0.01^{fg}	17.29 ± 2.14^{hi}	16.02 ± 1.55^{e}
YF55	1.47 ± 0.03^{i}	1.33 ± 0.01^{i}	12.00 ± 2.49^{jk}	17.77 ± 1.70^{de}
YF56	1.45 ± 0.04^{i}	1.71 ± 0.06^{ef}	9.36 ± 2.37^{k}	17.36 ± 1.06^{de}
Light-purple				
YF44	1.55 ± 0.03^{i}	1.49 ± 0.04^{h}	19.66 ± 2.29^{ghi}	11.75 ± 0.88^{fg}
YF49	2.39 ± 0.14^{gh}	2.01 ± 0.05^{c}	25.92 ± 1.11^{ef}	18.91 ± 1.07^{cd}
YF63	2.24 ± 0.07^{h}	1.69 ± 0.05^{fg}	15.07 ± 0.87^{ij}	17.05 ± 2.17^{de}
YF62	2.50 ± 0.07^{g}	1.93 ± 0.10^{d}	28.80 ± 1.48^{e}	17.75 ± 0.47^{de}
YF67	1.52 ± 0.09^{i}	1.88 ± 0.06^{d}	24.65 ± 2.32^{ef}	27.10 ± 2.32^{a}
Black				
YF46	7.69 ± 0.04^{d}	1.52 ± 0.04^{h}	49.53 ± 5.15^{bc}	12.22 ± 1.22^{f}
YF53	9.11 ± 0.43^{a}	1.25 ± 0.03^{j}	60.18 ± 3.43^{a}	22.45 ± 1.90^{b}
YF54	8.36 ± 0.29^{b}	1.76 ± 0.06^{e}	52.96 ± 2.15^{b}	9.65 ± 0.96^{g}
YF57	7.49 ± 0.17^{e}	1.63 ± 0.06^{g}	45.15 ± 6.48^{c}	9.56 ± 0.56^{g}
YF64	6.73 ± 0.16^{f}	2.14 ± 0.03^{b}	37.77 ± 6.54^{d}	20.23 ± 1.39^{c}
YF68 (parent)	8.07 ± 0.32^{c}	2.25 ± 0.07^{a}	52.81 ± 5.18^{b}	22.38 ± 1.20^{b}

[A] Values in each column with the same superscript are not different ($p > 0.05$).

In the present study, one type of anthocyanidin, cyanidin, was observed to occur in black and light-purple rice samples. Contents of cyanidin in black and light-purple rice samples ranged from 0.095 to 0.181 and 0.038 to 80.065 mg/g, respectively. Among samples, the black rice YF53 had the highest content of cyanidin 3-glucoside, peonidin 3-glucoside and cyanidin, which indicated that the anthocyanin content of black rice could be improved by hybridisation.

3.5. Antioxidant properties

To distinguish the antioxidant properties of different coloured rice, two assays were applied. Free radical scavenging activity against DPPH radical assay is a method widely used to evaluate the antioxidant activity of plant extracts, although it is focused on hydrogen-donating antioxidants against nitrogen radicals. As seen in Table 4, the DPPH radical scavenging activity ranged from 1.39 to 9.11 μM TE/g in soluble fractions of the rice samples. Mean values of the black showed significantly higher than the light-purple and white rice, and those of the light-purple were significantly higher than the white ($p < 0.05$). Mean values of DPPH radical scavenging activity did not show significantly different in comparison of insoluble bound fractions of three colour rice samples. It is probably because the phenolic compounds are mainly present as insoluble bound form.

Oxygen radical absorbance capacity (ORAC) assay, due to its biological relevance to *in vivo* antioxidant efficacy, is one of the most widely used methods to investigate the antioxidant effects of various foods, including cereals. It measures the antioxidant capacity against peroxyl radicals. ORAC values of soluble fractions ranged from 9.36 to 60.18 μM TE/g as shown in Table 4. Consistent with DPPH radical scavenging activity, ORAC values of soluble fractions of the black showed significantly higher than the light-purple and white, and those of the light-purple were significantly higher than the white ($p < 0.05$). ORAC values of insoluble bound fractions ranged from 9.56 to 27.1 μMTE/g, which showed the same trend in comparison of three colour rice samples as DPPH radical scavenging activity. The similar results have been shown that pigment cereal grains had higher antioxidant activity than the nonpigment rice (Liu, Qiu, & Beta, 2010; Min et al., 2012; Qiu et al., 2009; Shen et al., 2009; Vichapong, Sookserm, Srijesdaruk, Swatsitang, & Srijaranai, 2010). DPPH radical scavenging activity and ORAC of soluble fractions of YF53 was the highest among all fractions.

To assess the contribution of TAC, TPC and phenolic acids to the total antioxidant activity of the rice accessions, the relationship among TAC, TPC, phenolic acids and DPPH free radical scavenging activity and ORAC was investigated (Supplementary Table 1). High correlations were found between TAC, TPC in soluble fraction, and DPPH and ORAC in soluble fraction ($r > 0.95^{**}$). TPC in insoluble fraction was highly correlated with ORAC in insoluble fraction ($r = 0.90^{**}$). The isoferulic acid and vanillic acid were correlated with DPPH in insoluble fraction ($p < 0.05$), 8-8′ DFA, 8-5′ DFA and 5-5′ DFA were highly correlated with ORAC in insoluble fraction ($p < 0.01$).

The genotypic diversity of some phytochemicals in rice grain or bran layers has been widely characterised (Dykes & Rooney, 2007; Min et al., 2012; Nam et al., 2006; Qiu et al., 2009; Shen et al., 2009). This study found wide diversity in the phenolics contents and antioxidant capacity in the dehulled grains of rice breeding lines. TPC, TAC, contents of four phenolic acids, DPPH radical scavenging activities and ORAC vary from colour shades. Generally, the black had the highest values in comparison with the light-purple and white. Moreover, the breeding line YF53 displayed the highest in TPC, TAC, DPPH radical scavenging activities and ORAC among all of the breeding lines and the parents. It indicated that cross breeding technique increase not only the yield but also nutritional quality of rice.

4. Conclusions

Phenolic compounds and antioxidant abilities were investigated among the 15 breeding line samples and their parents. In soluble fraction, no free phenolic acid was found because of the lower content and the detection limit. After alkaline treatment, *p*-coumaric acid, ferulic acid, isoferulic acid and vanillic acid were identified in insoluble bound fractions with the most quantities of ferulic acid. Isoferulic acid which was rarely reported to occur in cereal grains was identified and quantified in this study. Phenolic dehydrodimers were only observed in the insoluble bound fractions. They mainly consisted of 8-5′-coupled diferulic acids and 5-5′-coupled diferulic acids. Cyanidin 3-glucoside, peonidin

3-glucoside and cyanidin occurred in black and light-purple rice samples. The TPC, TAC, DPPH radical scavenging activities and ORAC of a sample YF53 displayed the highest among all of breeding lines and were higher than those of the parent samples. It indicated that cross breeding technique could increase not only the yield but also nutritional quality of rice. The study will be helpful for rice breeders to screen the breeding lines with high nutraceutical properties and health benefits. It also provides important clues for the rice breeders to discover new genes that are associated with the biosynthesis of polyphenols.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.118.

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