Identification and quantification of flavonoids in yellow grain mutant of rice (Oryza sativa L.)

Backki Kim, Sunmin Woo, Mi-Jung Kim, Soon-Wook Kwon, Joohyun Lee, Sang Hyun Sung, Hee-Jong Koh

Abstract

Flavonoids are naturally occurring phenolic compounds with potential health-promoting activities. Although anthocyanins and phenolic acids in coloured rice have been investigated, few studies have focused on flavonoids. Herein, we analysed flavonoids in a yellow grain rice mutant using UHPLC-DAD-ESI-Q-TOF-MS, and identified 19 flavonoids by comparing retention times and accurate mass measurements. Among them, six flavonoids,isorientin, isoorientin 2′-O-glucoside, vitexin 2′-O-glucoside, isovitexin, isocaparin 2′-O-glucoside and iso-scopolin, were isolated and fully identified from the yellow grain rice mutant, and the levels were significantly higher than wild-type, with isoorientin particularly abundant in mutant embryo. Significant differences in total phenolic compounds and antioxidant activity were observed in mutant rice by DPPH, FRAP and TEAC assays. The results suggest that the representative six flavonoids may play an important role in colouration and antioxidant activity of embryo and endosperm tissue. The findings provide insight into flavonoid biosynthesis and the possibility of improving functionality in rice.

Keywords:
Yellow rice mutant
UHPLC-DAD-ESI-Q-TOF-MS
Flavonoid
Isoorientin
Antioxidant activity

1. Introduction

Rice (Oryza sativa L.) is one of the most produced and consumed staple food crops worldwide, and provides approximately 19% of the daily supply of calories (545 kcal) for the world population (IRRI, 2011). For this reason, rice directly affects the health of the people, particularly those living in Asia, since it is the major foodstuff in this region. Polished white rice is still the most commonly consumed; however, pigmented rice is becoming increasingly popular in Europe and the USA, as well as Asian countries (Kushwaha, 2016). Furthermore, although rice consumption is decreasing in some Asian countries including Korea and Japan due to changes in eating habits and lifestyle, the demand for coloured/pigmented or functionally enhanced rice is increasing due to its bioactive and health-promoting components such as vitamins, minerals and phytochemicals, including phenolic compounds (Cha, Han, & Chung, 2012). Therefore, understanding the compositional characteristics and functional properties of pigments could provide sight to the utilisation of rice pigments as a health supplement contained within a staple food.

Yellow, brown, purple, red and black coloured rice contains an abundance of naturally occurring phytochemicals such as tocopherols, tocotrienols, oryzanols, flavonoids and phenolic compounds in the bran layer (Friedman, 2013). Several components with antioxidant properties have been identified in rice (Goufo & Trindade, 2014). Researches have shown that furulic acid and p-coumaric acid are the main phenolic acids in light brown rice (Tian, Nakamura, & Kayahara, 2004), whereas cyanidin-3-glucoside and peonidin-3-glucoside are the two predominant anthocyanins, and cyanidin-3-rutinoside, cyanidin-3,5-di-glucoside and malvidin-3-glucoside are present in black and coloured rice grains (Hou, Qin, Zhang, Cui, & Ren, 2013; Pereira-Caro et al., 2013; Yawadio, Tanimori, & Morita, 2007). Unlike phenolic acids and anthocyanins, the identification of other flavonoids such as flavonols, flavones, flavonoids and flavonones in rice has been scarcely reported on. Several flavonoids were recently identified and characterised in black rice (Mohanlal, Parvathy, Shalini, Helen, & Jayalekshmy, 2011; Pereira-Caro et al., 2013; Sriseadka, Wongpornchai, & Rayanakorn, 2012) and
transgenic rice (Cho et al., 2013). However, the identification and quantification of flavonoids in rice has received relatively little attention due to limitations in analytical methods and the low abundance of constituents in rice grains (Sriseadka et al., 2012).

Flavonoids are one of the major classes of plant secondary metabolites found in various crops, including fruits and vegetables. In plants, flavonoids generally display a wide range of colour, from pale-yellow to blue, and are produced in different parts (Winkel-Shirley, 2001). They are involved in various biological and physiological functions, such as attracting pollinators, auxin transportation, fertility/sterility and protecting against UV-B radiation and phytopathogens (Koes, Quattrocchio, & Mol, 1994; Kumar & Pandey, 2013). In addition, flavonoids play an important role in human health, due to their pharmacological properties as nutraceuticals and radical scavengers (Tapas, Sakarkar, & Kakde, 2008). They are valuable as a source of nutrients, essential vitamins and antioxidants. Thus, flavonoids are receiving increasing interest for their use in obesity prevention and suppression of cholesterol levels, as well as anti-inflammatory, antiviral, anti-atherosclerotic disease, anticancer, antitumor and antioxidant activities (Kumar & Pandey, 2013; Pietta, 2000).

In plants, flavonoids are usually found as glycoside forms, conjugated through O- or C-glycosidic bonds. O/C-glycosylation affects their solubility, stability, antioxidant activity, biological activities and drug-related properties by changing their structures and properties, and is also important for transportation, compartmentalisation and storage of many specialised metabolites (Bowles & Lim, 2010; Ogo, Mori, Nakabayashi, Saito, & Takaiwa, 2016). In addition, differences in the position, structure, type and total number of sugars attached to flavonoids have an effect on their biological functions (Kumar & Pandey, 2013). In general, flavonoids accumulate in vacuoles as O-glycosides; however, microbes, gymnosperms and angiosperms also accumulate C-glycosyl flavonoids (Harborne, 1993). C-Glycosyl flavonoids are the predominant class and comprise a major portion of the polyphenolic compounds in some cereals and medicinal and herbal plants (Brazier-Hicks et al., 2009; Li et al., 2014). However, the limited production of flavonoid glycosides in nature restricts their identification and exploration of their diverse biological activities.

Several previous studies have attempted to identify and determine the constituents in white and pigmented rice. However, only a few flavonoids have been fully quantified due to the limited availability of rice germplasm including pigmented rice. In the present study, a yellow grain mutant, which is a rare and unique phenotype in rice, was subjected to investigations aimed at determining the specific compounds responsible for the yellow and black colour in endosperm and embryo tissue using UHPLC-DAD-ESI-Q-TOF-MS method. In addition, we compared the differences in phenolic content and antioxidant properties with wild-type rice. The results provide fundamental information on flavonoid biosynthesis in rice that will benefit breeding programs to develop pigmented rice with high levels of bioactive compounds for pharmacological and industrial uses.

2. Materials and methods

2.1. Samples

The yellow grain rice mutant was obtained using the chemical mutagen N-methyl-N-nitrosourea (MNU) and was derived from the Hwacheong (Oryza sativa L. ssp. japonica) parent cultivar (Fig. 1A). This mutant has been fixed over 20 generations in the Crop Molecular Breeding Lab, Department of Plant Science, Seoul National University. The yellow grain mutant and its parent variety were grown by conventional cultural practices at the Experimental Farm of Seoul National University, Suwon, in 2014. Harvested rice grains were air-dried, and the moisture content was reduced to approximately 13%. Samples were stored in a controlled room at 11 °C for 2 months, and then de-husked and hand-selected to eliminate cracked or discoloured seeds. The whole grain was dissected into embryo and endosperm tissue, and the boundary between embryo and endosperm was removed. Each sample was ground using a mill (JKA A118, Staufen, Germany) and sieved by passing through a 300 µm filter prior to further experiments.

2.2. Chemicals and reagents

HPLC grade water and acetonitrile (ACN) were purchased from J.T. Baker (Avantor, Phillipsburg, NJ, USA), and formic acid, leucine-enkephalin, sodium hydroxide and ferulic acid were bought from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was triple deionised (Millipore, Bedford, MA, USA). Dichloromethane (DCM) and MeOH were purchased from Daegu Chemicals Co., Ltd. (Siheung, Korea). Distilled water and DCM were used for extractions. Standards of iso-orientin, orientin, isovitexin, vitexin and acacetin were purchased from Sigma-Aldrich, and other flavonoid reference compounds were obtained from our laboratory. The purity was higher than 95% in all cases as demonstrated by HPLC-DAD analysis. Dimethylsulfoxide-d$_4$ or methanol-d$_4$ were purchased from Cambridge Isotope Laboratories (Cambridge, MA, USA) and used as solvent for NMR analysis. 2.2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethyleclarchroman-2-carboxylic acid (Trolox), 2,2’-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), iron (III) chloride hexa-hydrate, iron (II) sulphate-hepta-hydrate, potassium persulphate, sodium acetate trihydrate, sodium carbonate, 2,4,6-tri-(2-pyridyl)-striaizine (TPTZ), benzene, 2,2-dimethoxypropan, Folín-Gioacalteu reagent, heptane, n-hexane, hydrochloric acid, methanol and sulphuric acid were also purchased from Sigma-Aldrich (MO, USA).

2.3. General experimental procedure

A Branson 8510 ultrasonic bath (Branson Ultrasonics Corporation, Danbury, CT, USA) was used for extraction. Centrifugation was performed using a HANIL micro centrifuge (Micro17TR, Hanil Scientific Industrial, Seoul, Korea). Diaion HP-20 resin was purchased from Mitsubishi Chemical Industry (Tokyo, Japan). Medium-pressure liquid chromatography (MPLC) separation was carried out on a Revelers C$_{18}$ reverse phase cartridge (120 g, Grace, Columbia, MD, USA) using a MPLC-Reveleris system from Grace. HPLC separations were performed using a Gilson 321 HPLC system equipped with a Gilson UV/Vis-151 detector (Gilson, Middleton, WI, USA). Sample solutions were filtrated with a PVDF filter (0.2 µm pore size, HYUNDAI Micro, Korea), 1D and 2D NMR spectra were recorded on Varian 300 (300 MHz for 1H NMR and 75 MHz for 13C NMR) and Bruker AMX 400 (400 MHz for 1H NMR and 100 MHz for 13C NMR) spectrometers.

2.4. Isolation of C-glycosidic flavonoids

Powdered endosperm tissue from yellow grain mutant seeds (288.4 g) was extracted with 70% MeOH (1.5 L × 3) by maceration at room temperature for 24 h. The extract was filtered and concentrated in a rotary vacuum evaporator to yield a yellow residue (7.0 g). The residue was suspended in 500 mL of water and partitioned with DCM (500 mL × 4), yielding concentrated extracts in DCM (2.0 g) and H$_2$O (4.6 g). The flavonoid-rich aqueous residue was subjected to Diaion HP-20 column chromatography (4.0 × 30 cm) with a stepwise gradient of aqueous MeOH (10% MeOH → 100% MeOH → 100% acetone). The 100% MeOH fraction was separated by MPLC and eluted with aqueous MeOH (10% MeOH → 100% MeOH) to yield 15 fractions (OSAM-1 to OSAM-15). Among them, the OSAM-6, OSAM-7, OSAM-8, OSAM-9 and OSAM-10 fractions were further separated by semi-preparative HPLC using a YM Hydrosphere C$_{18}$ column (250 × 10.0 mm, 5 µm) eluted with ACN/H$_2$O (containing 0.1% v/v formic acid) using 83:17, 84:16, 77:23, 78:22, 81:19 and 77:23 (v/v) solvent ratios, respectively, to yield the six flavonoid compounds. The isolated compounds were identified using one-dimensional (1D) $^1$H NMR, 1D $^{13}$C NMR and two-dimensional
(2D) NMR (1H-1H COSY, HSQC, HMBC) spectroscopy (Table S1 and Table S2, Supplementary Material), as well as negative mode electron spray ionisation (ESI) mass spectrometry. The molecular structures of the six isolated and identified flavonoids, isoorientin 2″-O-glucoside (25.1 mg; from OSAM-6), isoorientin (16.7 mg; from OSAM-7), vitexin 2″-O-glucoside (43.8 mg; from OSAM-8), isovitexin (0.8 mg; from OSAM-9), isoscoparin 2″-O-glucoside (19.3 mg; from OSAM-8) and isoscoparin (11.1 mg; from OSAM-10) are shown in Fig. 2.

2.5. Sample preparations for analysis

For UHPLC-DAD-ESI-Q-TOF-MS analysis, 500 mg of yellow mutant and wild-type rice samples were extracted with 1.0 mL of MeOH/H2O (7:3, v/v), sonicated at room temperature for 20 min and centrifuged at 15,000g for 15 min, and 0.5 mL of supernatant was collected. The above steps were repeated once, and collected supernatants were pooled together into a 5 mL glass vial and concentrated by evaporation under nitrogen gas at 40 °C. Extracts were dissolved in MeOH/H2O (1:1, v/v) containing 10 μg/mL of the internal standard acacetin and filtered through a PVDF filter prior to UHPLC-DAD-ESI-Q-TOF-MS analysis.

For quantitative analysis of the six representative flavonoid compounds, whole grain, embryo and endosperm tissue from yellow grain mutant and wild-type rice were powdered and three technical replicates were prepared. Each sample (350 mg) was extracted with 1.0 mL of 70% MeOH twice using ultrasonication for 20 min at room temperature. The 500 mL supernatant from each extraction step was collected and concentrated by evaporation under nitrogen gas at 40 °C. Extracts were dissolved in MeOH/H2O (1:1, v/v) at a final concentration of 2 mg/mL containing 10 μg/mL of the internal standard acacetin and filtered through a PVDF filter prior to UHPLC-DAD-ESI-Q-TOF-MS analysis.

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yellow grain mutant), 16 mg/mL (endosperm of yellow grain mutant) and 32 mg/mL (whole grain, endosperm and embryo of wild-type), and filtered through a PVDF filter for quantitative analysis. A mixed standard solution containing six isolated compounds was prepared. Each standard compound was dissolved in 50% MeOH.

2.6. UHPLC-DAD-ESI-Q-TOF-MS system and conditions

HR-ESI-MS spectra were measured on a Waters XEVO G2 Q-TOF MS apparatus. UHPLC analysis was performed on a BEH C18 column (100 × 2.1 mm, 1.7 μm; Waters, Milford, MA, USA) using an Ultra Performance Liquid Chromatography apparatus equipped with a binary pump, a degasser, an autosampler and a PDA detector. UV–vis absorption spectra were recorded on-line from 200 nm to 500 nm during UHPLC analysis. The mobile phase consisted of A (0.1% v/v formic acid aqueous solution) and B (0.1% v/v formic acid in ACN). Gradient elution was performed as follows: 10% B at 0 min, 11% B at 5 min, 12% B at 8 min, 16% B at 12 min, 40% B at 13 min, 50% B at 15 min, 90% B at 18 min and 100% B at 21 min. The column and autosampler temperatures were set at 40 °C and 15 °C, respectively. The injection volume of the sample was 1 μL.

For the MS² detection, the ESI source was operated in negative ion mode and optimal parameters were as follows: capillary voltage of 3.2 kV, sampling cone voltage of 30 V, extraction cone voltage of 4 V, source temperature of 120 °C, desolvation temperature of 350 °C, cone gas flow of 50 L/h, desolvation gas flow of 800 L/h. The mass range was from m/z 50 to 2000. Argon was used as the collision gas, and nitrogen was used as the nebuliser and desolvation gas. For the low-energy function, a collision energy of 6 eV was applied, and, for the high-energy scan function, a collision energy ramp of 20–40 V was used. Data were calibrated using a Lockspray (leucine-enkephalin at m/z 554.2615 in negative ion detection mode) to ensure mass accuracy. All operations, acquisition and processing of data were performed with Masslynx 4.1 software (Waters).

2.7. Determination of the flavonoid content using HPLC analysis

For quantitative analysis, HPLC was performed on a XBridge C18 column (250 × 4.6 mm, 5 μm; Waters, Milford, MA, USA) using an Ultimate 3000 UHPLC system (Thermo Fisher Scientific Inc.) equipped with an autosampler, a gradient system and a diode array detector (DAD). Chromatograms were acquired at 365 nm, and photodiode array spectra were recorded from 190 to 800 nm during HPLC analysis. Eluent A was 0.1% formic acid aqueous solution, and eluent B was 0.1% formic acid in ACN. A gradient elution protocol was used as follows: 10.0% B at 0 min, 15.5% B at 10 min, 15.9% B at 22 min, 60.0% B at 23 min, 80.0% B at 30 min and 100.0% B at 32 min. The flow rate was 1.0 mL/min, and the column and autosampler temperatures were maintained at 30 °C and 15 °C, respectively. The injection volume of each sample was 5 μL.

2.8. Determination of the total phenol content

The total phenol content was determined as described by Kim et al. (2004). Rice powder (1.0 g) was extracted with 10 mL of 80% methanol for 24 h in a shaking incubator with stirring at 25 °C. Extracts were filtered through a Whatman No. 42 filter paper, and quantitative analysis of the total phenol content was measured by the Folin-Denis method (AOAC, 1980). Extract (0.5 mL) was mixed with 5 mL of distilled water and 5 mL of Folin-Ciocalteu phenol reagent in a screw-top flask. After 3 min, 2 mL of 10% sodium carbonate (Na2CO3) was added and mixed. After 1 h, the absorbance was measured at 760 nm using a spectrophotometer (U-2900, Hitachi Ltd., Tokyo, Japan). A standard curve was constructed using 1, 50 and 100 ppm of ferulic acid. The total phenolic content was expressed as milligrams of ferulic acid equivalents per gram of dry weight (DW) for each sample.

2.9. Determination of antioxidant activity by DPPH, FRAP and TEAC assays

The DPPH assay was performed as previously described (Kim et al., 2014). DPPH solution was prepared by dissolving 138 mg of DPPH in 100 mL of 50% ethanol and storing at −20 °C until use. Reaction mixtures were prepared by mixing 2.5 mL of DPPH and 0.2 mL of methanolic sample extract. Reactions were incubated for 10 min at room temperature, and the change in absorbance at 517 nm was measured with a spectrophotometer. The DPPH radical scavenging activity was calculated in terms of the percentage inhibition caused by the hydrogen donor activity.

The ferric reducing antioxidant power (FRAP) assay was performed as previously described (Kim et al., 2014; Hu & Xu, 2011). The FRAP reagent consisted of 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM FeCl3. Methanolic rice extracts (0.1 mL) were mixed with 1.8 mL of FRAP reagent and 3.1 mL of HPLC grade water. The reaction mixture was incubated at 37 °C for 0.5 h, and the absorbance at 593 nm was measured with a spectrophotometer. FRAP values were calculated based on the FeSO4 standard curve (100–1000 μM) and expressed as μmole of Fe (II) per gram of DW.

The Trolox equivalent antioxidant capacity (TEAC) assay using ABTS was performed as previously described (Kim et al., 2014) with slight modifications. The stock solution consisted of 7 mM/L ABTS⁺ and 2.45 mM/L potassium persulfate. The stock solution was incubated in the dark at room temperature for 16–24 h before use and diluted with methanol to an absorbance of 0.700 ± 0.050 at 734 nm. All samples were diluted appropriately to provide 20–80% inhibition of the blank absorbance. Diluted extract (50 μL) was mixed with 1.9 mL of diluted ABTS⁺ solution and incubated for 6 min at room temperature, and the absorbance was immediately measured at 734 nm. The results were expressed as μM Trolox equivalents (TE) per gram of DW. All experiments were carried out in triplicate.

2.10. Statistical analysis

The results are reported as mean ± standard deviation. The t-test at p < 0.001 was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) to assess significant differences in total phenolic compounds and antioxidant activities among samples.

3. Results

3.1. Comparative analysis of yellow grain mutant and wild-type rice grains

Artificial mutations in rice can be induced by chemicals and radiation, and the resultant mutants can display enhanced rice grain yield and quality. The yellow grain mutant investigated in the present study was selected from the MNU-induced progeny of japonica cultivar Hwacheong. The mutant grain as a yellow pericarp and black embryo (Fig. 1A) has not been reported previously, and represents a unique phenotype.

In this study, components from extracts of de-hulled wild-type and yellow grain mutant rice were separated and analysed by UHPLC-DAD-ESI-Q-TOF-MS in negative ion mode to obtain accurate m/z and MS² information. Total ion chromatograms (TICs) of wild-type and mutant rice obtained from UHPLC-DAD-ESI-Q-TOF-MS analysis is shown in Fig. 1B. The chromatograms reveal dramatic differences in the flavonoid profiles between wild-type and yellow grain mutant. A total of 19 components were detected in the extract of the yellow grain mutant, indicating variation in the flavonoid content, and these components were investigated further to identify those responsible for the colouration of the yellow grain mutant.
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**Table 1**

List of flavonoids tentatively identified in the yellow grain mutant rice.
3.2. Analysis of C-glycosidic flavonoid compounds in the yellow grain mutant by UHPLC-DAD-ESI-Q-TOF-MS

The 19 different C-glycosidic flavonoids included isoorientin, iso-orientin 2′-O-glucoside and vitexin 2′-O-glucoside, were identified or tentatively characterised based on UHPLC-DAD-ESI-Q-TOF-MS analysis (Table 1). Identification was confirmed by comparing m/z values, retention times \( t_r \), UV spectra and fragmentation patterns with those of reference substances. Table 1 lists the \( t_r \) values, calculated and observed m/z \([M−H]^{−}\) values, calculated errors (ppm), proposed molecular formula and most characteristic fragment ions of the 19 peaks in the TICs. Peaks for which standards are not commercially available in the TICs of whole grain samples from yellow grain mutant extracts were compared with tandem mass spectral data taken from the scientific literature, or Massbank, KNaPSSaCk, MoTo DB, METLIN, HMDB and ReSpect databases. Based on relative quantification of the yellow grain mutant in the whole grain, six most abundant compounds (except for chrysoeriol 6, 8-di-C-hexoside) were further isolated (see Materials and Methods) and fully identified as isoorientin 2′-O-glucoside, iso-orientin, vitexin 2′-O-glucoside, isovitexin, isoscoparin 2′-O-glucoside and isoscopyrin (Fig. 2).

MS\(^2\) product ions generated from \([M−H]^{−}\) precursor ions were observed to determine the flavone components, and all 19 compounds derived from three flavone aglycone moieties, namely, apigenin, luteolin or chrysoeriol. Based on the number of conjugated sugars and the type of linkage between the sugar and flavone aglycone moieties, the 19 C-glycosidic flavonoids were divided into three groups: mono C-glycosidic flavonoids, and diglycosidic \( \text{O, C-glycosidic or C, C-glycosidic flavonoids.} \)

In this study, three pairs of mono C-glycosidic flavonoid isomers were completely separated in the UHPLC chromatograms. Matching the MS\(^2\) spectra with those of reference compounds identifies characteristic fragment ions (m/z) and intensities, and provides insight into the structures of the individual compounds. For product ions, we used the nomenclature system described previously (Domon & Costello, 1988), and previously reported diagnostic product ions were used to identify the 6-C and 8-C isomers (Guo et al., 2013). The \([M−H−\text{H}_2\text{O}]^{−}\) product ion was only found among those from 6-C-glycosidic flavonones in the negative ion mode. In the MS\(^3\) spectra, the relative intensity of product ions \([0.63\text{X}_{6−}−\text{H}]^{−}\) and \([0.63\text{X}_{6−}−\text{H}_2\text{O}]^{−}\) derived from 6-C-glycosidic flavonones was obviously higher than that of 8-C-glycosidic flavonones. Specifically, the 6-C-glycosidic isoform was the predominant mono C-glycosidic flavonoid isomer in the yellow grain mutant. By comparing m/z values, \( t_r \) values and the fragmentation patterns with standards, five mono C-glycosidic flavonoids were identified as isoorientin, orientin, vitexin, isovitexin and isoscoparin. Furthermore, peak 19 in Table 1, corresponding to the mono-C-glycosyl isomer of isoscoparin, was identified as scoparin by comparing the product ions.

In the O, C-glycosidic flavonoids of the yellow grain mutant, O-glycosylation on the sugar moiety of the C-glycosyl flavone was revealed by product ions \([\text{(M−H)}−180\ \text{(hexose)}]^{−}\) and \([\text{(M−H)}−150\ \text{(pentose)}]^{−}\). Specially, the AGly + 71, AGly + 71-18, AGly + 41 and AGly + 41-18 ions indicated O-glycosylation at the 2′-position of the sugar in the C-glycosyl flavone (Ferreres, Andrade, Valentao, & Gil-Izquierdo, 2008). It was difficult to determine the position of C-glycosylation in these compounds using MS spectra because there was only one isomeric form for each molecular weight. For unambiguous identification, NMR spectral data from previously isolated compounds were compared with the literature, and the three O,C-glycosidic flavonoids were assigned as isoorientin 2′-O-glycoside (Fig. 1C), vitexin 2′-O-glycoside and isoscoparin 2′-O-glycoside (Table S1 and Table S2, Supplementary Material). However, for the compound with O-glycosylation at the 6′ position of the sugar of the C-glycosyl flavone, the relative abundance of AGly + 41 ions was obviously higher than for those with O-glycosylation at the 2′ position (Ferreres, Gil-Izquierdo, Andrade, Valentao, & Tomas-Barberan, 2007).

Regarding di-C-glycosidic flavonoids, the characteristic AGly + 113 and AGly + 83 ions were detected. The 6-C-pentosyl-8-C-hexosyl-flavone \([\text{(M−H)}−120]^{−}\) ion was more abundant than the \([\text{(M−H)}−120]^{−}\) ion. Conversely, for the 6-C-hexosyl-8-C-pentosyl-flavone, the \([\text{(M−H)}−120]^{−}\) ion was more abundant than the \([\text{(M−H)}−90]^{−}\) ion (Ferreres et al., 2008, 2011). Based on the fragmentation patterns, the three di-C-glycosidic flavonoids were tentatively identified as luteolin 6, 8-di-C-hexoside, apigenin 6, 8-di-C-hexoside and chrysoeriol 6, 8-di-C-hexoside. Three isomers of luteolin 6-C-pentosyl-8-C-hexoside, two isomers of apigenin 6-C-pentosyl-8-C-hexoside and apigenin 6-C-hexosyl-8-C-pentoside were tentatively identified by comparing the relative intensity of product ions.

3.3. Quantitative analysis of flavonoids in the yellow grain mutant

The 19 flavonoids identified or tentatively identified in the whole grain of the yellow grain mutant were quantified in relative terms by UHPLC-DAD with an internal acetalin standard (Fig. S1, Supplementary Material). This method is useful for the rough quantification and comparison of similar compounds, but it requires the complete separation of all components in a single UHPLC run. An almost complete separation of the 19 most abundant flavonoids was achieved under the optimised conditions based on monitoring by UV absorbance and MS. Isoorientin was identified as the major flavonoid present in the yellow grain mutant, accounting for 30% of the 19 flavonoids, followed by isoorientin 2′-O-glucoside, vitexin 2′-O-glucoside, isoscoparin 2′-O-glucoside, chrysoeriol 6, 8-di-C-hexoside, isoscopyrin and isovitexin in that order.

For quantitative analysis, we selected the most abundant among the 19 flavonoids based on their relative quantification, which identified chrysoeriol-6, 8-di-C-hexoside as the 5th most abundant flavonoid, but this component could not be isolated. Thus, we estimated the content of isoorientin, isoorientin 2′-O-glucoside, vitexin 2′-O-glucoside, isoscoparin 2′-O-glucoside, isoscopyrin and isovitexin in the whole grain, endosperm and embryo tissue of wild-type and yellow grain mutant grain. The mixed standard solution was diluted to six different concentrations for construction of calibration curves by plotting the peak areas against the corresponding concentrations in the range of 5–600 \( \mu \text{g/mL} \). The linearity, regression equation and linear ranges of the six compounds were determined (Table 2). Calibration curves were checked based on high-correlation coefficient values \( (R^2 > 0.9986) \). The limit of detection (LOD) and limit of quantification (LOQ) of each analyte were defined by the peak intensity of peaks with signal-to-noise values \( (S/N) \) of 3 and 10, respectively. As shown in Table 2, the LODs and LOQs of the six compounds ranged from 9.8821 to 25.2455 \( \mu \text{g/mL} \), and from 32.9405 to 84.1517 \( \mu \text{g/mL} \), respectively, indicating that the analytical method was effective and suitably sensitive. The content of the six compounds in extracts of whole grain, endosperm and embryo tissue was determined using the calibration equation. Representative HPLC chromatograms for yellow grain mutant grain tissue are shown in Fig. S2, Supplementary Material.

As shown in Table 2, isoorientin was the most abundant of the six flavonoids in the whole grain of the yellow grain mutant, followed by isoorientin 2′-O-glucoside, isoscoparin 2′-O-glucoside, vitexin 2′-O-glucoside, isoscopyrin and isovitexin in that order. It is interesting to note that isoorientin accumulated to remarkably high levels in the embryo of the yellow grain mutant, accounting for 59.8% of the total generated from all six flavonoids. The abundance of other flavonoids in embryo tissue from the yellow grain mutant was ordered isoorientin 2′-O-glucoside, isoscoparin 2′-O-glucoside, isoscopyrin, vitexin 2′-O-glucoside and isovitexin (from highest to lowest). In addition, even though the relative content of isoorientin in endosperm was low compared with that in embryo tissue, the most abundant flavonoid of the six was isoorientin, followed by isoscoparin 2′-O-glucoside, vitexin 2′-O-glucoside, isoscopyrin, isovitexin and isoorientin 2′-O-glucoside. The yellow
grain mutant had markedly higher flavonoid content than the wild-type cultivar, and isoorientin was the most abundant flavonoid in the mutant embryo, but it was not detected at all in wild-type embryo tissue. These results suggest that these six flavonoids may play an important role in the colouration of the yellow grain mutant, and isoorientin may make a major contribution to the black colour of the embryo and yellow colour of the pericarp. The order of abundance of the six flavonoids differed between the embryo and endosperm in the yellow grain mutant. In particular, isoorientin-2'-O-glucoside was the second most abundant compound in the embryo of the yellow grain mutant, but the least abundant in the endosperm among the six flavonoids.

### 3.4. Total phenolic content and antioxidant capacity determined by DPPH, FRAP and TEAC assays

Phenolic compounds are major naturally occurring antioxidants in plants, and the total phenolic compound content tends to reflect the overall antioxidant activity. Thus, the total phenolic content of the yellow grain mutant rice was measured. As shown in Table 3, the endosperm and embryo of the yellow grain mutant had a markedly higher total phenolic content compared with these tissues in wild-type rice. Significant differences (p < 0.001) in the total phenolic content were also observed in the whole grain of the yellow grain mutant. The embryo of the yellow grain mutant contained 46.28 mg/g ferulic acid equivalent, which is approximately 17 times higher than the total phenolic content in the wild-type rice embryo. The ferulic acid content of the pericarp. The order of abundance of the six flavonoids identified from whole grain, endosperm and embryo extracts from the yellow grain mutant rice.

### Table 2

<table>
<thead>
<tr>
<th>Peak number</th>
<th>t₀ (min)</th>
<th>Analytes</th>
<th>Regression equation</th>
<th>R²</th>
<th>Linear range (µg/mL)</th>
<th>LOQ (µg/mL)</th>
<th>LOQ (µg/mL)</th>
<th>Content (w/w, mg/g)</th>
<th>Whole grain</th>
<th>Endosperm</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>5.74</td>
<td>Isoorientin 2'-O-glucoside</td>
<td>y = 0.1003 x + 0.4831</td>
<td>0.9996</td>
<td>5.00-600.00</td>
<td>15.2705</td>
<td>50.9015</td>
<td>27.46 ± 1.18</td>
<td>12.55 ± 1.40</td>
<td>125.15 ± 6.87</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>6.65</td>
<td>Luteolin 6-C-glucoside (Isoorientin)</td>
<td>y = 0.0975 x + 0.1291</td>
<td>0.9998</td>
<td>5.00-600.00</td>
<td>9.8821</td>
<td>32.9405</td>
<td>62.46 ± 2.41</td>
<td>23.92 ± 2.51</td>
<td>500.48 ± 17.35</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>9.04</td>
<td>Vitexin 2'-O-glucoside</td>
<td>y = 0.0911 x + 0.2661</td>
<td>0.9998</td>
<td>5.00-600.00</td>
<td>11.0887</td>
<td>36.9623</td>
<td>19.65 ± 1.02</td>
<td>18.22 ± 1.94</td>
<td>41.08 ± 2.05</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>10.30</td>
<td>Apigenin 6-C-glucoside (Isovitexin)</td>
<td>y = 0.0747 x + 0.5332</td>
<td>0.9989</td>
<td>5.00-600.00</td>
<td>25.2455</td>
<td>84.1517</td>
<td>13.20 ± 0.84</td>
<td>13.34 ± 1.35</td>
<td>36.29 ± 1.83</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>10.68</td>
<td>Isoscoparin 2'-O-glucoside</td>
<td>y = 0.1044 x + 0.4697</td>
<td>0.9996</td>
<td>5.00-600.00</td>
<td>15.1384</td>
<td>50.4614</td>
<td>22.40 ± 1.17</td>
<td>18.64 ± 2.05</td>
<td>84.70 ± 3.87</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>11.82</td>
<td>Chrysoeriol 6-C-glucoside (Isoscoparin)</td>
<td>y = 0.1123 x + 0.5826</td>
<td>0.9994</td>
<td>5.00-600.00</td>
<td>18.9419</td>
<td>63.1398</td>
<td>13.66 ± 0.72</td>
<td>16.91 ± 1.79</td>
<td>49.12 ± 2.25</td>
<td></td>
</tr>
</tbody>
</table>

None of the six flavonoids was detected in the whole grain, endosperm or embryo of wild-type rice.

a y = peak area; x = concentration of standard solution.
b LOD refers to the limit of detection (S/N = 3).
c LOQ refers to the limit of quantification (S/N = 10).
d n = 3; Data calculated as an average of three replicates. Values are expressed as mg/g ± standard error.

**Table 3**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenolic content (mg/g, ferulic acid equivalent)</th>
<th>DPPH (%)</th>
<th>FRAP [µmol Fe (II)/g DW]</th>
<th>TEAC [µmol TE/g DW]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>YGM</td>
<td>WT</td>
<td>YGM</td>
</tr>
<tr>
<td>Whole grain</td>
<td>1.25 ± 0.04</td>
<td>3.68 ± 0.07</td>
<td>27.69 ± 0.40</td>
<td>78.66 ± 0.33</td>
</tr>
<tr>
<td>Endosperm</td>
<td>0.97 ± 0.02</td>
<td>1.79 ± 0.00</td>
<td>21.16 ± 0.30</td>
<td>45.93 ± 0.11</td>
</tr>
<tr>
<td>Embryo</td>
<td>2.62 ± 0.05</td>
<td>46.28 ± 0.87</td>
<td>46.69 ± 0.65</td>
<td>87.06 ± 0.19</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 3).

WT, wild-type (Hwacheong); YGM, yellow grain mutant.

*** Indicates significance at the 0.001 probability level (p < 0.001).
Physicochemicals have been studied using various rice mutants and transformants; however, rice grains with yellow or yellowish-coloured grains are rarely reported. Oryzamaticae acids have been isolated from yellow endosperm mutants of Hatsuyamabuki and Musashino 20 varieties (Nakano et al., 2009, 2011). Recently, Shen et al. revealed that oryzamatic acid A was the main pigment in the W3660 yellow endosperm mutant japonica variety that has a mutation in the OsALDH7 gene that causes oryzamatic acid A to accumulate in the endosperm during late seed development or after a year-long storage (Shen et al., 2012). In this study, we used a rare yellow grain mutant rice which showed a bicoloured seed phenotype which is due to the colour combination a black embryo and the yellow seed pericarp. It was found that the high levels of flavonoids including isoorientin, which was accumulated in the embryo and pericarp of yellow rice grain mutant. Several flavonoids have been identified in rice (Brazier-Hicks et al., 2009; Cho et al., 2013; Iriakl, Samandidou, Biliaderis, & Papadoyannis, 2012; Mohanal et al., 2011) and recently, large-scale methods for detection, identification, and quantification of flavonoids were applied to rice metabolomics study (Chen et al., 2013; Dong et al., 2014) but these are not enough to quantify or confirm the interesting target flavonoids. We identified or tentatively identified 19 flavonoids in rice grain and among them six representative compounds were further isolated, identified and quantified. Four of these flavonoids, isoorientin 2′-O-glucoside, isoscoparin 2′-O-glucoside, vitexin 2′-O-glucoside, iso- scoparin are reported for the first time as constituents in rice grain. Interestingly, isoorientin (luteolin-6-C-glucoside) was the most abundant in the rice grain, and particularly in the embryo. Isoorientin has been identified and quantified in the leaves of bamboo (Wang, Yue, Jiang, & Tang, 2012) and gentians (Gentiana triflora) (Sasaki et al., 2015), but this study is the first report of high-level accumulation in rice. In addition, high concentrations of isoorientin 2′-O-glucoside, vitexin 2′-O-glucoside, isoorientin, isoscoparin 2′-O-glucoside and isoscoparin were also observed in the grain of this rice mutant (Table 2 and Fig. S2, Supplementary Material). In rice grains, phenolic compounds are located mainly in the bran. In this study, the embryo of the yellow grain mutant had much higher levels of flavonoids than the endosperm. In addition, the order of abundance of the six isolated flavonoids was different in embryo and endosperm; while isoorientin was the main compound in both embryo and endosperm, isoorientin 2′-O-glucoside was the second most abundant flavonoid in embryo but the least abundant in the endosperm of the yellow grain mutant (Table 2). Differences in the accumulation of flavonoids, such as the efficiency of flavonoid synthesis, transport and compartmentalisation that are affected by the class of flavonoid and glycosylation, lead to variation in flavonoid accumulation in the endosperm and embryo (Ogo et al., 2016). In particular, glycosylation is important for the transport and storage of various flavonoids. Galland et al. reported that the embryo contains both O- and C-glycosylated flavones, while only C-glycosylated flavones are found in the bran of colourless rice grains, suggesting that the synthesis of O-glycosylated flavones is limited to the embryo (Galland et al., 2014). The amount of secondary metabolites accumulating in the embryo and endosperm differed, even though the accumulation pattern was influenced by the same endogenous mutation, indicating changes in the expression of mutated genes involved in the biosynthesis, modification, transport, accumulation and compartmentalisation of flavonoids in the yellow grain mutant, especially in the embryo and endosperm. The yellow grain mutant showed high levels of flavonoids accumulation in both embryo and endosperm (Table 2 and Fig. S2, Supplementary Material) and the extracts from both of these tissue of the yellow grain mutant showed much higher antioxidant activities in comparison to the wild-type when applied the three different assays. In addition, both flavonoids concentration and antioxidant capacity were higher in embryo tissue than endosperm tissue (Table 3). These results imply that highly accumulated flavonoids including isoorientin in the embryo and endosperm of yellow grain mutant might contribute to high antioxidant activity. These results are consistent with previous studies on passion fruit which demonstrated that isoorientin contributes significantly to the antioxidant activity, and shows directly correlated relationship between DPPH scavenging activity and isoorientin content (Zeraik, Yarivate, Wauters, Tita, & Angenot, 2012). This suggests that the content of flavonoids with respect to isoorientin can be correlated with antioxidant activity in extracts of yellow grain mutant. Therefore, the yellow grain mutant rice could be good source of antioxidants and health-promoting food.

5. Conclusion

In the present study, 19 flavonoids were identified or tentatively identified in a yellow grain rice mutant. Among them, the structure of six isolated compounds, isoorientin, isoorientin 2′-O-glucoside, vitexin 2′-O-glucoside, isoorientin, isoscoparin 2′-O-glucoside and isoscoparin was elucidated. Isoorientin was the most abundant compound in both embryo and endosperm, and levels were particularly elevated in the embryo. Significant differences (p < 0.001) were observed in the total phenolic compound content, and in the antioxidant capacity, as demonstrated using three different antioxidant assay systems. These findings provide new insight into flavonoid biosynthesis in rice grains.

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Notes

The authors declare no competing financial interests.

Appendix A. Supplementary data

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References


214–223.