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

Backki Kim[a], Sunmin Woo[b], Mi-Jung Kim[c], Soon-Wook Kwon[d], Joohyun Lee[e], Sang Hyun Sung[b], Hee-Jong Koh[a,*]

[a] Department of Plant Science, Research Institute for Agriculture and Life Sciences, and Plant Genomics and Breeding Institute, Seoul National University, Seoul 08826, Republic of Korea
[b] College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea
[c] National Institute of Crop Science, RDA, Suwon 16429, Republic of Korea
[d] Department of Plant Bioscience, College of Natural Resources and Life Science, Pusan National University, Milyang 60463, Republic of Korea
[e] Department of Crop Science, College of Life Science, Konkuk University, Seoul 05029, Republic of Korea

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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, isoorientin, isoorientin 2″-*O*-glucoside, vitexin 2″-*O*-glucoside, isovitexin, isoscoparin 2″-*O*-glucoside and isoscoparin, 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.

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 ferulic acid and ρ-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-diglucoside 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, flavanols and flavanones 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

* Corresponding author.
E-mail addresses: uptfamily@hanmail.net (B. Kim), woosm@snu.ac.kr (S. Woo), tyche@korea.kr (M.-J. Kim), swkwon@pusan.ac.kr (S.-W. Kwon), edmund@konkuk.ac.kr (J. Lee), shsung@snu.ac.kr (S.H. Sung), heejkoh@snu.ac.kr (H.-J. Koh).

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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, anticardiovascular 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*-glycoside 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 (IKA A11B, 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 Daejung Chemicals Co., Ltd. (Siheung, Korea). Distilled water and DCM were used for extractions. Standards of isoorientin, 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_6 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-tetramethylchroman-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)-striazine (TPTZ), benzene, 2,2-dimethoxypropane, Folin-Ciocalteu 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 Reveleris 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 ^{1}H NMR and 75 MHz for ^{13}C NMR) and Bruker AMX 400 (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C 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_2O (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 (15% 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 YMC Hydrosphere C_{18} column (250 × 10.0 mm, 5 μm) eluted with ACN/H_2O (containing 0.1% v/v formic acid) using 83:17, 84:16, 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