Comparative analysis of nutritional compositions of transgenic high iron rice with its non-transgenic counterpart

Dipak Gayen [a], Sailendra Nath Sarkar [a], Swapan K. Datta [a,b], Karabi Datta [a,*]

[a] Plant Molecular Biology and Biotechnology Laboratory, Department of Botany, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, WB, India
[b] Division of Crop Science, Indian Council of Agricultural Research (ICAR), Krishi Bhavan, Dr. Rajendra Prasad Road, New Delhi 110001, India

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ABSTRACT

Iron is an essential micronutrient for human nutrition and polished rice contains very low amount of iron. Rice with high iron content in seed endosperm has been developed by insertion of soybean *ferritin* gene under the control of the endosperm specific glutelin promoter into the genome of *indica* rice line IR68144. The nutritional composition of the brown and milled rice grain has been compared with that of the non-transgenic rice of the same variety. In this study, the nutritional components, as well as the anti-nutrient levels, were measured. Our studies established that apart from the increased level of iron and zinc in transgenic seeds, the nutritional quality of both the brown and milled rice grains from the transgenic line was substantially equivalent to that of the non-transgenic rice plants. The result clearly shows that the measured amounts of the nutritional components are well within the range of values reported for other commercial lines.

1. Introduction

Rice is one of the most important cereal crop in the world, supplying a staple source of energy, protein and other nutrients to half of the world population. About 8.20 billion people in developing countries are suffering from micronutrient deficiencies, of which about 2 billion people are suffering from iron deficiency (Stein et al., 2008). Iron is an essential nutrient for humans, and must be available in the diet for proper growth and development. Mostly women and children of the world population suffer from iron deficiency anaemia, especially where vegetable based diet is the primary food source (Goto, Yoshihara, Shigemoto, Toki, & Takaiwa, 1999). The removal of the outer layers of rice seeds by commercial milling dramatically reduces the level of iron in the grains because most of the iron is accumulated in the aleurone layer.

Tremendous progress has been made in the development of transgenic crops for nutritional improvement. The improvement of the iron content of rice has been reported by the expression of the soybean *ferritin* gene driven by the endosperm-specific glutelin promoter. It has been shown that the iron and zinc content remains high in milled and brown rice as compared to that of non-transgenic rice (IR68144) after different degrees of commercial milling (Vasconcelos et al., 2003). The nutritional quality improvement of rice by insertion of the desired gene is very essential, but at

the same time, the nutritional assessment of transgenic crops is an important aspect of biosafety evaluation as recommended by food safety regulatory agencies (Chassy, 2010).

To facilitate rapid approval and risk assessment for genetically modified foods, the "substantial equivalence" concept was introduced by the Food and Agriculture Organisation of the united nation (FAO) and World Health Organisation (WHO) in the early 1990s. In 1993 the organization for Economic Co-operation and Development (OECD) developed the concept of substantial equivalence. Targeted analyses of key compounds have been considered extensively in substantial equivalence studies of genetically modified (GM) crops and provide detailed information of macro and micronutrients as well as anti-nutrients (Barros et al., 2010). Substantial equivalent analysis has been studied for different events of transgenic crops, such as corn (George et al., 2004), soybean (Zhu et al., 2008), potato (El-Khishin, Hamid, El-Moghazy, & Metry, 2009), wheat (Baker et al., 2006) and rice (Li et al., 2007; Momma et al., 1999; Oberdoerfer, Shillito, de Beuckeleer, & Mitten, 2005). In the present study, the compositional analysis of the transgenic event of rice seeds expressing *ferritin* gene in the endosperm (ferritin rice) and its counterpart having the same genetic background (non-transgenic) were evaluated through a series of chemical analyses following the guidelines of the OECD consensus document to assess the effect of the new gene insertion on the nutritional composition as well as anti-nutrients content of brown and milled rice. The results revealed that the nutritional components of ferritin rice are substantially equivalent with that of the non-transgenic counterpart and the measured amounts of nutritional components are

* Corresponding author. Tel.: +91 33 24614688; fax: +91 33 24614849.
E-mail address: krbdatta@yahoo.com (K. Datta).

well within the range of values reported for other commercial rice lines.

2. Materials and methods

2.1. Rice sample for analysis

Homozygous transgenic IR68144 rice line expressing *ferritin* gene (developed at IRRI, Vasconcelos et al., 2003) and respective non-transgenic control IR68144 rice samples were used in this study for nutritional compositional analysis. For nutritional quality analysis, rice seeds were dehulled by a Satake THU-35C dehuller (Tokyo, Japan). About 500 g of dehulled brown rice seeds were milled for 30 s by Satake TM-05C (Tokyo, Japan) grain miller. The resultant brown and milled rice were used for substantial equivalence analysis.

2.2. Molecular analysis

2.2.1. PCR analysis

Genomic DNA was extracted from fresh leaves (2–3 cm) of transgenic and non-transgenic rice plants following rapid DNA isolation protocol (Huang et al., 1997) and 50–100 ng of template DNA were used for PCR analysis (780 bp) with gene specific primer. The primer sequences used were as follows:

fer F: 5′-ATGGCTCTTGCTCCATCCAAAGTT-3′.
fer R: 5′-TTG ATCAAAGTGCCAAACACCGTG-3′.

2.2.2. RT-PCR analysis

Total RNAs were extracted from the mature dehusked seeds (100 mg) using TRIzol (Invitrogen, USA) based on rapid RNA extraction protocol (Meng & Feldman, 2010). First strand cDNA were synthesized from 4 μg of total RNA using the transcriptor high fidelity cDNA synthesis kit (Roche, USA), following the manufacturer's instruction. 100 ng of cDNA were used to amplify 566 bp of *ferritin* gene through RT-PCR using gene specific primer. Rice *β-tubulin* was selected as housekeeping gene to amplify 64 bp cDNA sequences. The primer sequences used were as follows:

ferrt-F: 5′-CAGTGTTGG GGATGCTCTGAA-3′.
ferrt-R: 5′-GGTCATTGTTGCGATCTGCCA-3′.
BT-F: 5′-GGAGTCACATGCTGCCTAAGGTT-3′.
BT-R: 5′-TCACTGCCAGCTTACGGAGG-3′.

2.3. Nutritional composition estimation

2.3.1. Proximate analysis

The moisture content of rice was determined by gravimetric measurement of weight loss after oven-drying to a constant weight at 105 ± 5 °C (AOAC, 1990).

A total of 100 mg of rice powder sample was used to determine the total nitrogen content using Kjeldahl method described by Sadasivam and Manikam (1991) and the protein content was estimated from nitrogen content by multiplying factor 5.95 (AOAC, 2000).

Crude lipid from rice flour was extracted using Soxhlet apparatus with n-hexane as solvent. Hexane solvent was removed by speed vac system (Eyela, Japan) and stored at 4 °C (AOAC, 1990). The ash content of rice was measured by gravimetric method after ignition in muffle furnace at 550–600 °C to constant weight (AOAC, 1990). The total crude carbohydrate of rice seed was estimated by the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). Gross energy was estimated by the formula (Deepa, Singh, & Naidu,

2008): Gross energy (kJ 100 g^{-1} dry matter) = (crude protein × 16.7) + (crude lipid × 33.7) + (crude carbohydrates × 16.7).

2.3.2. Mineral analysis

Mature dehusked seeds of transgenic and non-transgenic plants (10 g each) were milled in rice miller (Satake, Japan) for 30 s. Sodium, potassium, calcium, magnesium, iron, zinc, manganese and copper were estimated by AAS (AAnalyst200, Perkin Elmer, USA) using modified method of Jiang, Wu, Feng, Yang, and Shi (2007). About 2.0 g unpolished and milled seeds were ignited in muffle furnace at 550–600° for 10 h. The ash sample was dissolved in 0.2 N HCl and filtered through whatman filter paper. The dissolved sample was used for AAS analysis.

2.3.3. Amino acid analysis by AccQ-Tag method

Rice samples (20 mg) were digested with 2 ml 6 N HCl containing 0.1% phenol at 110 °C temperature for 16 h. After neutralisation with equal volume of 6 M NaOH, samples were derivatized with the AccQ-Fluor reagent kit (Waters, USA). A 10 μl of sample was separated by Waters AccQ-Taq Column (150 × 3.9 mm) according to manufacturer's protocol.

2.3.4. Fatty acid analysis

Fatty acids of rice seed were estimated by one step-lipid extraction and methyl ester preparation method (Garces & Mancha, 1993). 100 mg of rice seed power were taken in 2.0 ml micro centrifuge tube. For methylation of fatty acid, 1 ml of methylation reaction mixer containing methanol:*n*-heptane:benzene:DMP:H$_2$SO$_4$ (37:36:20:5:2) was added to the rice sample and incubated at 80 °C for 2 h in water bath (Eyela, Japan). After heating, the tube was cooled to room temperature and shaken again. The two phases were allowed to separate and the upper layer containing fatty acid methyl ester was used for GC–MS analysis. One micro litre of derivative sample was injected by the auto sampler AI/AS 3000 into GC–MS (TRACE GC-ULTRA, Thermo Fisher scientific, USA) having TR-wax column (30 M × 0.25 mm, 0.25 μm film thickness). The injector temperature was 240 °C. The initial temperature of the column was 80 °C followed by a ramp of 20 °C/min to 150 °C (15 min hold), a second ramp of 20 °C/min to 240 °C (8 min hold) and a split ratio 1:20. He gas was used as carrier gas (1 ml/min). All the data were obtained by collecting the full scan mass spectra within scan range of 45–500 amu. Fatty acids were further identified by the NIST mass database and the corresponding fatty acid standard.

2.3.5. Vitamins analysis

The niacin and thiamine content of rice were estimated by a spectrofluorometric method (Sadasivam & Manikam, 1991). Vit-E of rice grains were extracted by ethanol and hexane (Britz et al., 2007). The tocopherols were analysed by an analytical HPLC system (Waters) consisting of a Waters 510 pump, a 717 plus auto sampler equipped with absorbance, and a scanning fluorescence detector (Waters, Milford, MA). Chromatograms were recorded and processed using Waters Empower Chromatography software. Extracts (20 μl) were injected into C18 column (25 cm × 4.6 mm, 5 μm; Supelco, Bellefonte, PA) maintained at room temperature (25 °C). The mobile phase consisted of 25:22:3 (v/v/v) methanol/acetonitrile/methylene chlorides at a flow rate of 1.0 ml/min. The elution of tocopherols was monitored using the fluorescence detector at 290-nm excitation and 330-nm emission wavelengths.

2.3.6. Anti-nutrient factor

Phytic acid was extracted with 2.4% HCl and estimated by spectrophotometer at 500 nm after reaction with 0.03% FeCl$_3$·6H$_2$O solution containing 0.3% sulfosalicylic acid (Rajbhandari & Kawabata, 2006).