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Full Length Research Paper

Compositional assessment of carotenoid-biofortified rice using substantial equivalence

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One important aspect in assessing the safety of genetically modified (GM) crops for human consumption is characterizing their nutrient composition. A β-carotene-biofortified rice was generated by inserting phytoene synthase (*Psy*) and carotene desaturase (*Crtl*) genes isolated from *Capsicum* and *Pantoea* into the genome of a conventional variety of rice (Nakdongbyeo). Nutrients (proximates, amino acids, fatty acids, minerals, and vitamins), anti-nutritive components (trypsin inhibitors and phytic acid), and ferulic acid in GM rice were compared with those in the parent line Nakdongbyeo. Statistical comparisons to test for equivalence showed that all of the analyzed components in the GM plants were equivalent to those in its non-transgenic counterpart, and most nutritional components fell within the range of values reported for other commercial lines, indicating the safety of the GM plant.

Key words: Genetically modified crop, β-Carotene, Transgenic rice, Nutrient, Substantial equivalence.

INTRODUCTION

Carotenoids are very important for human health as a source of provitamin A and components to reduce the incidence of several diseases such as cancers, cardio-vascular diseases, age-related macular degeneration, diseases related to low immune function, and other degenerative diseases (Landrum and Bone, 2001; Perera and Yen, 2007). We have developed transgenic β-carotene biofortified rice using a bicistronic system to coordinately express two carotenoid biosynthetic genes, phytoene synthase (*Psy*) from *Capsicum* and carotene desaturase (*Crtl*) from *Pantoea* (Ha et al., 2010). A synthetic 2A sequence optimized for the rice codon was used to generate the *PAC* (*Psy-2A-Crtl*) construct under the control of a single rice globulin promoter. The PAC rice grain accumulated an average of 0.13 mg/100 g total carotenoids. However, the commercialization of a genetically modified (GM) plant requires an extensive safety assessment for use in food. Commercialization of GM rice has lagged behind that of other cereals such as maize. The reason could be that rice is cultivated in more than 100 countries around the world and is a staple for about a half of the world's population; thus its safety must be strictly evaluated prior to market availability (Jiao et al., 2010). Safety assessment of GM food is based on the concept of substantial equivalence, developed by the Organization for Economic Co-operation and Development (OECD) and further elaborated by the Food and Agriculture Organization/World Health Organization (FAO/WHO). This concept embraces a comparative approach to identify possible differences between GM food and its traditional counterpart. Therefore, substantial equivalence is not a safety assessment per se but is an important starting point for a safety assessment. Composition analysis is a major factor assessed when determining substantial equivalence.

To assess substantial equivalence, the OECD consensus document on rice has identified the key food and feed nutrients and anti-nutrients under consideration for assessment in new rice varieties (OECD, 2004). Comparative compositional analyses have been reported for glufosinate-tolerant rice in the USA (Oberdoerfer et al.,

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2005) and insect-resistant rice developed in China (Li et al., 2007). The statistical analyses used in those studies demonstrated the safety approach. Equivalence boundaries were set to 20% of the means, as recommended by the Nordic Council of Ministers (Nordic Council, 2000). Here, we conducted a series of chemical analyses following the guidelines of the OECD consensus document to assess the effects of the new gene insertion on the nutritional composition and anti-nutrient content of novel biotechnology-derived rice samples.

MATERIALS AND METHODS

Rice samples

GM rice (cv. Nakdongbyeo) was developed to increase carotenoids content using *Agrobacterium tumefaciens*-mediated transformation. A comprehensive description of the transformation process has been provided by Ha et al. (2010). Non-transgenic cultivar, Nakdongbyeo, was used as the counterpart for equivalence comparisons. GM rice and its parent cultivar were grown in adjoining fields under the same environmental conditions and field management strategies. After harvest, the whole grain (rough rice) samples were dried to a final moisture content of 10 to 14%. Rice samples were manually hulled and ground to obtain a fine powder using a cyclone mixer mill (HMF-590, Hanil, Seoul, South Korea), a mortar, and a pestle. The powder was stored at -80°C prior to analysis.

Compositional analysis

The extraction method used for carotenoid analysis was similar to that described elsewhere (Howe and Tanumihardjo, 2006). Briefely, carotenoids were released from the rice samples (0.3 g) by adding 3 ml of ethanol containing 0.1% ascorbic acid (w/v), vortex mixing for 20 s and placing in a water bath at 85°C for 5 min. The carotenoid extract was saponified with potassium hydroxide (120 µl, 80% w/v) at the 85°C water bath for 10 min. After saponification, samples were placed immediately on ice, and cold deionised water (1.5 ml) was added. β-Apo-8'-carotenal (0.05 ml, 25 µg/ ml) was added as an internal standard. Carotenoids were extracted twice with hexane (1.5 ml) by centrifugation at 1,200 × g to separate the layers. Aliquots of the extracts were dried under a stream of nitrogen and re-dissolved in 50:50 (v/v) dichloromethane/methanol before analysis by HPLC.

The carotenoids were separated on a C_{30} YMC column (250 × 4.6 mm, 3 µm; Waters Corporation, Milford, MA, USA) by Agilent 1100 HPLC (Massy, France) equipped with a photodiode array (PDA) detector. Chromatograms were generated at 450 nm. Solvent A consisted of methanol/water (92:8 v/v) with 10 mM ammonium acetate. Solvent B consisted of 100% methyl *tert*-butyl ether. Gradient elution was performed at 1 ml/min under the following conditions: 0 min, 83% A/17% B; 23 min, 70% A/ 30% B; 29 min, 59% A/41% B; 35 min, 30% A/70% B; 40 min, 30% A/70% B; 44 min, 83% A/17% B; and 55 min, 83% A/17% B. Lutein, α-carotene, β-carotene and zeaxanthin were obtained from CaroteNature (Lupsingen, Switzerland). β-Apo-8'-carotenal were purchased from Sigma Chemical Co. (St. Louis, MO).

Moisture content was determined by gravimetrically measuring weight loss after drying the samples to constant weight in a 105°C hot-air oven. Crude protein content was estimated by determining the total nitrogen content using the Kjeldahl method and crude fat was analyzed by the Soxhlet extraction method (Association of Official Agricultural Chemists, 2005). Ash content was determined by gravimetrically measuring sample residue after ignition in an oven at 600°C to constant weight (Association of Official Agricultural Chemists, 2005). Crude fiber was quantified as the loss on ignition of dried residue remaining after sample digestion with 1.25% sulfuric acid and 1.25% sodium hydroxide solutions (Association of Official Agricultural Chemists, 2005).

For the amino acids analysis, the sulfur-containing amino acids cysteine and methionine were oxidized by performic acid before hydrolysis with hydrochloric acid, and the remaining 15 kinds of amino acids were analyzed with an automatic amino acid analyzer (L-8500-A, Hitachi, Japan) directly after protein hydrolysis with hydrochloric acid (Association of Official Agricultural Chemists, 2005).

Fatty acid content was determined by lipid extraction and saponification with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride/methanol, and the resulting methyl esters were extracted with pentane. The methyl esters of the fatty acids were analyzed by gas chromatography (Hewlett Packard 5890A, Avondale, PA, USA) (American Oil Chemists' Society, 1997). Pentadecanoic acid was used as the internal standard.

Levels of calcium, potassium, sodium, magnesium, zinc, iron, and copper were determined by inductively coupled plasma optical emission spectrometry (Integra XL inductively coupled plasma optical emission spectrometer, GBC Co., Melbourne, Australia), according to Association of Official Agricultural Chemists (2005).

Vitamins B_1 and B_2 were extracted according to a slight modification of the methods of Sims and Shoemaker (1993) and Esteve et al. (2001), respectively. Vitamins were determined using a HPLC method with fluorometric detection (Shimadzu, Kyoto, Japan). Vitamin E was detected as described by our group (Kim et al., 2011).

Phytic acid was analyzed by ion exchange chromatography following Association of Official Agricultural Chemists (2005). Trypsin inhibitor activity was determined in alkali solvent-extracted rice samples using American Oil Chemists' Society (1997). Ferulic acid was extracted according to a slight modification of the method of Ridley et al. (2002).

Statistical analysis

Equivalence tests were used to determine whether treatment differences exceeded the range of normal variation of the comparator. We used the two one-sided test (TOST) procedure for equivalence testing. The statistical analysis was conducted with the SAS 9.2 software package (SAS Institute, Cary, NC, USA). In the TOST procedure, the null hypothesis tested is "treatment 1 is not equivalent to treatment 2" versus the alternative hypothesis "treatment 1 is equivalent to treatment 2." To test for equivalence, the 90% confidence intervals for the difference between the two treatments were constructed. The confidence intervals, 100 $(1 - 2 \times \alpha)$% (= 90%), where α = 0.05, were calculated in pairs for the treatment differences. Thus, the 90% confidence interval method was equivalent to conducting TOST at the 5% significance level. Equivalence boundaries were set to ±20% of the means.

RESULTS AND DISCUSSION

Safety assessment of GM crops is based on an assessment of statistical equivalence between the food

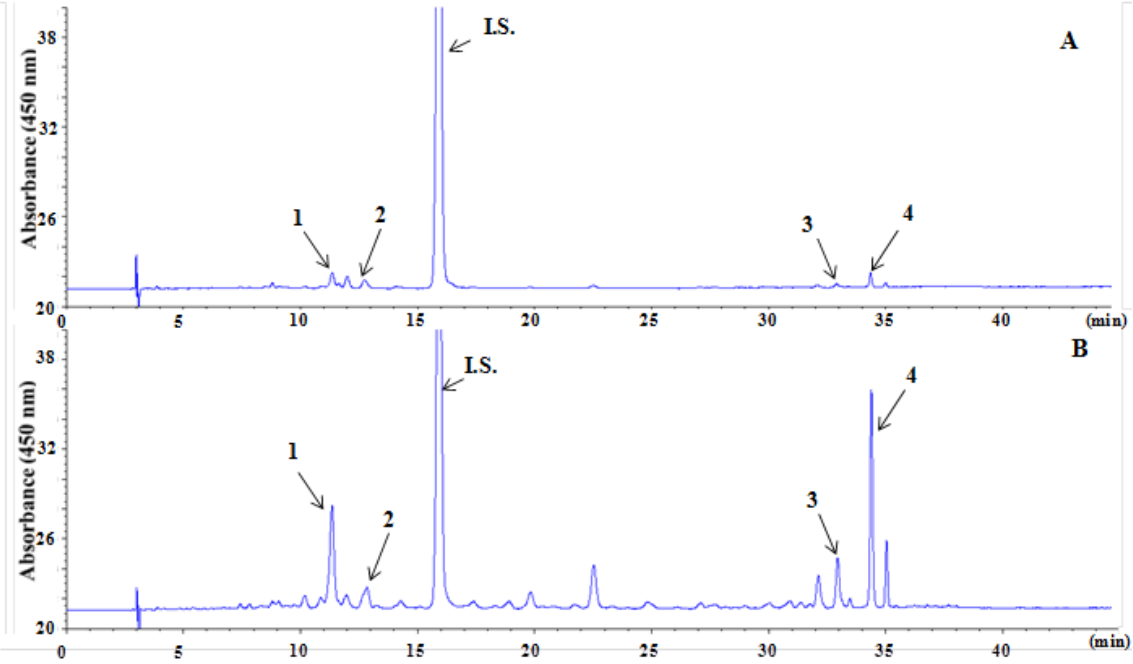

Figure. 1. The metanolic extracts from rice seeds were subjected to quantitative high performance liquid chromatography analysis. (**A**) Non-transgenic seeds (Nakdongbyeo). (**B**) Transgenic seeds (PAC rice). The peaks correspond to the following: 1, lutein; 2, zeaxanthin; 3, α-carotene; 4, β-carotene; and IS, internal standard (β-apo-8'-carotenal).

derived from the GM crop and its conventional counterpart. In this study, the nutritional composition of the PAC rice was compared with that of a conventional counterpart (cv. Nakdongbyeo), which was grown in the same field trials in Korea. Total carotenoid level was 0.04 µg/g in nontrangenic rice and 1.14 µg/g in PAC rice, respectively (Figure 1). The PAC rice accumulated lutein (0.3 µg/g), zeaxanthin (0.1 µg/g), α-carotene (0.1 µg/g), and β-carotene (0.6 µg/g) in its grain. In addition, the composition values of the PAC rice were compared with values obtained from previously published study (Ha et al., 2010).

The comparative analysis of the rice grain was completed using a statistical procedure to assess equivalence. Performing an ordinary *t*-test and inferring equivalence from the absence of a significant difference entails an uncontrolled increase in the risk of false-positive conclusions; that is, the assumption of "equivalence." In other words, a "nonsignificant difference" is different from "significant equality." Statistical equivalence for a component was assumed if the mean values of the two treatments did not differ "too much"; that is, the difference in the mean values was within a certain interval. Thus, we followed the advice of the FDA and the Nordic Council for accepting equivalence limits.

The moisture levels in all samples were maintained consistently, and no significant differences were found for the mean values of protein, lipid, ash, fiber, and carbohydrate between the transgenic rice grain and its nontransgenic counterpart (Table 1).

The percent fractions of particular amino acids and fatty acids in the total protein and total fatty acid were respectively calculated for the equivalence analysis of total amino acids and fatty acids (Tables 2 and 3). The statistical analysis revealed that all amino acids and fatty acids between the PAC transgenic rice and nontransgenic counterpart were equivalent. The amino acid values did not deviate considerably from ranges for commercial rice provided by the OECD (2004). Although the consensus document published by the OECD (2004) was not available, the measured fatty acid values fell within the ranges of values observed in brown rice for the six typical Korean varieties reported by Choe et al. (2002). Equivalence was demonstrated in the brown rice for all minerals, vitamins, anti-nutrients, and ferulic acid (Table 4). All mean values calculated in the GM rice samples, except for vitamin B_2, were within the ranges reported by the OECD (2004). Vitamin B_2 levels were lower than those provided by the OECD, including in the nontransgenic control, indicating that this was not caused by GM. The inhibition activity by trypsin inhibitors was very low in both the PAC brown rice and the non-transgenic counterpart compared with 100 to 184 trypsin inhibitor units (TIU)/mg detected in soybean (Li et al.,

Table 1. Comparison and analysis of equivalence of the proximates measured in brown rice.

Component	*p*-value[a]	Non-transgenic	PAC	Analysis of equivalence[b]	OECD[c] (2004)
Moisture, % fw[d]	<0.05	10.95 ± 0.61[e]	10.56 ± 0.27	yes	14
Protein, % dw[f]	<0.05	7.04 ± 0.40	7.49 ± 0.17	yes	7.1–8.3
Lipid, % dw	<0.05	2.36 ± 0.10	2.07 ± 0.07	yes	1.6–2.8
Ash, % dw	<0.05	1.26 ± 0.10	1.33 ± 0.09	yes	1.0–1.5
Fiber, % dw	<0.05	1.19 ± 0.26	0.98 ± 0.08	yes	0.6–1.0
Carbohydrates[g], % dw	<0.05	89.56 ± 0.73	89.12 ± 0.20	yes	87.4–90.3

[a]Based on the two one-sided test, confidence intervals, 100 (1 – 2 × α)%, where α = 0.05, were calculated to assess equivalence. The null hypothesis tested was "treatment 1 is not equivalent to treatment 2" versus the alternative hypothesis "treatment 1 is equivalent to treatment 2." [b]The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (cv. Nakdongbyeo). If the 90% confidence interval of the difference exceeds the 20% range of the reference (cv. Nakdongbyeo), this is indicated by "no." [c]Source: OECD data. [d]fw, fresh weight. [e]Each value is the mean ± standard deviation (n = 6). [f]dw, dry weight. [g]Carbohydrate levels were estimated by the formula: % carbohydrates = 100 – (% protein + % lipid + % ash).

Table 2. Comparison and analysis of equivalence of the total amino acids measured in brown rice[a].

Component	*p* value[b]	Non-transgenic	PAC	Analysis of equivalence[c]	OECD[d] (2004)
Alanine	<0.05	5.45 ± 0.29[e]	5.45 ± 0.07	yes	5.8
Arginine	<0.05	7.63±0.35	7.52 ± 0.22	yes	8.5–10.5
Aspartic acid	<0.05	8.90 ± 0.44	8.96 ± 0.24	yes	9.0; 9.5
Cystine	<0.05	2.29 ± 0.22	2.14 ± 0.08	yes	2.2–2.4
Glutamic acid	<0.05	16.90 ± 0.76	17.18 ± 0.33	yes	16.9; 17.6
Glycine	<0.05	4.64 ± 0.22	4.63 ± 0.07	yes	4.7; 4.8
Histidine	<0.05	2.52 ± 0.24	2.52 ± 0.17	yes	2.4; 2.6
Isoleucine	<0.05	3.10 ± 0.13	3.11 ± 0.09	yes	3.6–4.6
Leucine	<0.05	7.41 ± 0.30	7.46 ± 0.15	yes	8.3–8.9
Lysine	<0.05	3.83 ± 0.33	3.75 ± 0.20	yes	3.9; 4.3
Methionine	<0.05	2.22 ± 0.31	2.05 ± 0.08	yes	2.3; 2.5
Phenylalanine	<0.05	4.48 ± 0.22	4.56 ± 0.09	yes	5.0; 5.3
Proline	<0.05	3.97 ± 0.52	4.47 ± 0.28	yes	4.8; 5.1
Serine	<0.05	4.88 ± 0.23	4.88 ± 0.13	yes	4.8–5.8
Threonine	<0.05	3.31 ± 0.18	3.58 ± 0.09	yes	3.9–4.0
Tyrosine	<0.05	3.86 ± 0.14	3.95 ± 0.17	yes	3.8–4.6
Valine	<0.05	4.65 ± 0.22	4.59 ± 0.07	yes	5.0-6.6

[a]The values of amino acids were calculated as a percentage of total protein. [b]Based on the two one-sided test, confidence intervals, 100 (1 – 2 × α)%, where α = 0.05, were calculated for to assess equivalence. The null hypothesis tested was "treatment 1 is not equivalent to treatment 2" versus the alternative hypothesis "treatment 1 is equivalent to treatment 2." [c]The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (cv. Nakdongbyeo). If the 90% confidence interval of the difference exceeds the 20% range of the reference (cv. Nakdongbyeo), this is indicated by "no." [d]Source: OECD data. [e]Each value is the mean ± SD (n = 6).

2007; Kakade et al., 1972).

In conclusion, key nutritional components measured in brown rice derived from GM rice (transgenic *β*-carotene biofortified PAC rice) were found to be substantially equivalent to those of the nontransgenic counterpart. Based on the principle of substantial equivalence, as in conclusion, key nutritional components measured in articulated by the WHO and the OECD, these data support the conclusion that PAC transgenic rice is as safe as its traditional counterpart.

Table 3. Comparison and analysis of equivalence of the fatty acids measured in brown rice[a].

Component	*p* value[b]	Non-transgenic	PAC	Analysis of equivalence[c]
C14:0	<0.05	0.24 ± 0.04[d]	0.25 ± 0.02	yes
C16:0	<0.05	16.32 ± 0.27	16.00 ± 0.10	yes
C18:0	<0.05	1.82 ± 0.11	1.96 ± 0.08	yes
C18:1	<0.05	39.44 ± 0.76	40.94 ± 0.45	yes
C18:2	<0.05	38.81 ± 0.73	37.42 ± 0.33	yes
C18:3	<0.05	1.60 ± 0.02	1.54 ± 0.04	yes
C20:0	<0.05	0.64 ± 0.03	0.65 ± 0.01	yes
C20:1	<0.05	0.55 ± 0.07	0.55 ± 0.05	yes

[a]Fatty acid values are indicated as a percentage of total fatty acids. [b]Based on the two one-sided test, confidence intervals, 100(1 – 2 × α)%, where α = 0.05, were calculated to assess equivalence. The null hypothesis tested was "treatment 1 is not equivalent to treatment 2" versus the alternative hypothesis "treatment 1 is equivalent to treatment 2." [c]The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (cv. Nakdongbyeo). If the 90% confidence interval of the difference exceeds the 20% range of the reference (cv. Nakdongbyeo), this is indicated by "no." [d]Each value is the mean ± SD (*n* = 6).

Table 4. Comparison and analysis of equivalence of the minerals, vitamins, anti-nutrients, and ferulic acid measured in brown rice.

Component	*p* value[a]	Non-transgenic	PAC	Analysis of equivalence[b]	OECD[c] (2004)
Copper, µg/g	<0.05	3.35 ± 0.75[d]	3.22 ± 0.64	yes	1–7
Iron, µg/g	<0.05	13.16 ± 2.35	12.40 ± 1.07	yes	2–60
Sodium, µg/g	<0.05	32.34 ± 1.84	34.91 ± 3.33	yes	20–400
Zinc, µg/g	<0.05	26.92 ± 1.20	26.34 ± 3.91	yes	7–33
Calcium, mg/g	<0.05	0.15 ± 0.01	0.14 ± 0.03	yes	0.1–0.6
Potassium, mg/g	<0.05	2.51 ± 0.11	2.77 ± 0.05	yes	0.7–3.2
Magnesium, mg/g	<0.05	1.17 ± 0.08	1.25 ± 0.10	yes	0.2–1.7
Vitamin B_1, µg/g	<0.05	4.29 ± 0.41	4.38 ± 0.32	yes	2.9–6.1
Vitamin B_2, µg/g	<0.05	0.20 ± 0.02	0.22 ± 0.04	yes	0.4–1.4
Vitamin E, µg/g	<0.05	14.0 ± 1.4	14.4 ± 0.6	yes	9–25
Trypsin inhibition, TIU/mg		<0.1	<0.1	yes	Not available
Phytic acid, %	<0.05	0.87 ± 0.12	0.98 ± 0.06	yes	Not available
Ferulic acid, µg/g	<0.05	60.17 ± 1.41	58.05 ± 2.78	yes	Not available

[a]Based on the two one-sided test, confidence intervals, 100 (1 – 2 × α)%, where α = 0.05, were calculated to assess equivalence. The null hypothesis tested was "treatment 1 is not equivalent to treatment 2" versus the alternative hypothesis "treatment 1 is equivalent to treatment 2." [b]The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (cv. Nakdongbyeo). If the 90% confidence interval of the difference exceeds the 20% range of the reference (cv. Nakdongbyeo), this is indicated by "no." [c]Source is OECD data. [d]Each value is the mean ± SD (*n* = 6).

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