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Rice (*Oryza sativa* L.) Containing the *bar* Gene Is Compositionally Equivalent to the Nontransgenic Counterpart

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This publication presents an approach to assessing compositional equivalence between grain derived from glufosinate-tolerant rice grain, genetic event LLRICE62, and its nontransgenic counterpart. Rice was grown in the same manner as is common for commercial production, using either conventional weed control practices or glufosinate−ammonium herbicide. A two-season multisite trial design provided a robust data set to evaluate environmental effects between the sites. Statistical comparisons to test for equivalence were made between glufosinate-tolerant rice and a conventional counterpart variety. The key nutrients, carbohydrates, protein, iron, calcium, thiamin, riboflavin, and niacin, for which rice can be the principal dietary source, were investigated. The data demonstrate that rice containing the genetic locus LLRICE62 has the same nutritional value as its nontransgenic counterpart, and most results for nutritional components fall within the range of values reported for rice commodities in commerce.

KEYWORDS: Nutrition; transgenic rice; glufosinate-tolerant rice (*Oryza sativa* L.)

INTRODUCTION

The *bar* gene has been introduced into numerous plant species due to its utility as a selectable marker in the transformation process and its ability to provide tolerance to glufosinate−ammonium, the active ingredient of Liberty herbicide (*1*). It is a bialaphos resistance gene isolated from the soil microorganism *Streptomyces hygroscopicus* strain HP632 (*2*) that expresses the enzyme phosphinothricin-*N*-acetyltransferase (PAT), which detoxifies the herbicide by acetylation. Using recombinant DNA, the *bar* gene was cloned from *S. hygroscopicus* and fused with the 35S promoter from cauliflower mosaic virus. The rice event LLRICE62 was produced by direct gene transfer, using a purified plasmid fragment containing the 35S/*bar* expression cassette. Southern blot, PCR, and sequence analysis have demonstrated that the glufosinate-tolerant rice event LLRICE62 contains one copy of the *bar* gene cassette and no antibiotic resistance marker genes and that the inserted *bar* gene is inherited as a stable and simple dominant trait. The original transformant LLRICE62 was obtained from the variety Bengal, a tropical japonica, medium-grain rice released by the Rice Research Station of the Louisiana Agricultural Experiment Station, Louisiana State University (*3*). LibertyLink (trademark of Bayer CropScience) rice is distinguishable from other rice only by its tolerance to glufosinate−ammonium herbicide, the genetic locus defined as LLRICE62, and the presence of the PAT protein. For purposes of international trade, LLRICE62 is designated by the OECD unique identifier code of ACS-OS002-5.

Before transgenic crops are commercialized, they are the subject of an extensive safety assessment for use in food and feed. In 1993, the OECD formulated the concept of substantial equivalence as a starting point for the safety assessment of crops derived from modern biotechnology. A joint FAO/WHO consultation in 1996 and the Codex Alimentarius Commission of FAO/WHO in 2000 and 2002 endorsed this concept as a key starting point for the safety assessment of transgenic crops, and this important concept has been reviewed by a number of workers, including Chesson (*4*), Kuiper et al. (*5*), Aumaitre et al. (*6*), and Cockburn (*7*). The process of establishing substantial equivalence alone is not a safety assessment per se, but provides a basis to identify similarities and differences between the new variety and a suitable comparator variety. Composition analysis is the major factor assessed in the determination of substantial equivalence.

Comparative compositional analyses have been reported for nutritionally enhanced rice in Japan (*8*), mineral-enhanced rice for The Philippines (*9*), and flour from a Bt rice line developed for China (*10*). These reports presented the mean and standard error from single-site data and found good correspondence with the comparators. In a similar manner, the nutritional impact data package for LLRICE62 provided by Bayer CropScience (BCS) to the U.S. Food and Drug Administration (FDA) early in 1999 presented data for a single season, which was used to complete the premarket review, and this was followed by the acceptance

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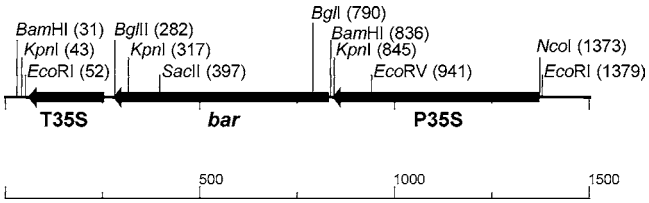

Figure 1. Linear map of the 1501 bp *Hin*dIII–*Pvu*I fragment of pB5/35S*bar*.

of this rice by the FDA in 2000 (www.cfsan.fda.gov/~rdb/bfnm063.html; *11*).

Guidelines published by the European Union have requested that the size of the data package be expanded to demonstrate equivalence to multiple sites over two seasons (minimum six sites per season—U.K. authorities; *12*) and, in the case of herbicide tolerance, to include treatments with and without the recommended herbicide applications. The data set for LLRICE62 was expanded in the 1999 season to collect data from more field locations.

Statistical analysis of the expanded data set is reported here. The data comprise up to 51 components tested for each grain sample and 105 grain samples, representing 2 growing seasons, 14 sites, 3 treatments from almost every site, and a 3-fold replication per treatment. The three treatments consist of (1) nontransgenic rice grown using conventional herbicide weed control, (2) transgenic rice grown using conventional herbicide weed control, and (3) transgenic rice grown with Liberty herbicide weed control.

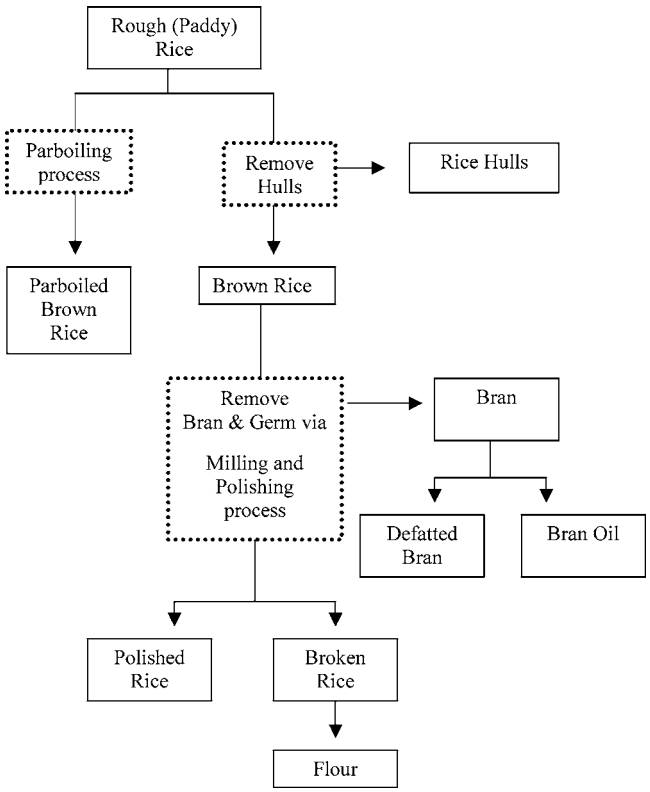

Figure 2. Flowchart of industrial rice processing.

MATERIALS AND METHODS

Transformation and Molecular Characterization. A single linear DNA fragment containing one copy of the *bar* gene expression cassette (**Figure 1** showing a linear map of the expression cassette) was introduced into the rice variety Bengal using particle acceleration. The *bar* gene was isolated from genomic DNA of *S. hygroscopicus*, a common soil microbe. The *bar* gene encodes the enzyme phosphinothricin acetyltransferase (PAT). Expression of PAT allows the rice plant to be sprayed with the biodegradable nonselective, herbicide active ingredient phosphinothricin, commonly known as glufosinate−ammonium, without crop injuries.

The insertion of the *bar* gene has been verified through a characterization of the insert by Southern blot and PCR analysis. Both the inserted DNA and the plant genome flanking DNA have been sequenced. The inserted DNA consists of one copy of the gene cassette, carrying the 35S promoter sequence, the *bar* ORF, and the 35S terminator sequence. The absence of the antibiotic resistant marker (ARM) and origin of replication, present in the pB5/35S*bar* vector that was the source for the transforming fragment, was demonstrated by Southern blot analysis.

The inserted gene is inherited as a simple dominant trait. Stability of the gene insertion has been demonstrated by Southern blot analyses and Mendelian crosses. Furthermore, current molecular techniques were used to make a description of the insertion site on rice chromosome 6.

The 35S promoter and terminator sequences control expression of the *bar* gene in LLRICE62. The 35S promoter directs high-level constitutive expression. Specificity of expression in LLRICE62 is consistent with the published reports for 35S-driven expression in rice (highest expression in leaves). The amount of PAT protein in the leaves of LLRICE62 during the vegetative life cycle of the plant has an upper limit of ~150 μg/g of fresh weight. Roots and grain contain ~12 μg/g of fresh weight PAT protein. No PAT protein was detected in rice pollen.

Plant Materials. The rice varieties were identical in genetic background, except for the presence of the *bar* gene in the glufosinate−ammonium-tolerant rice. Rice is a self-pollinated crop, and the purity of rice varieties is maintained by a limited generation certification system wherein breeder seed from a small population of hand-selected plants is the foundation for each certified seed increase. Certified planting seed from the Bengal variety was used as the nontransgenic comparator for equivalence comparisons. The source of the transgenic seeds was breeder seed from line LL401 derived from panicle rows evaluated by the plant breeder for uniform plant type and tested by PCR analysis to be homozygous for the LLRICE62 genetic insertion.

Grain Production. To produce grain for the studies, glufosinate−ammonium-tolerant rice (transformation event LLRICE62) was grown in the same manner as for commercial rice production, using either conventional weed control practices [336 g of active ingredient (ai)/ha propanil + 84 g of ai/ha bentazon applied at two-leaf stage, followed by 336 g of ai/ha molinate at four−five-leaf stage) or glufosinate-ammonium herbicide (400 g of ai/ha glufosinate applied twice, at the two-leaf and at the four−five-leaf plant growth stage). The use of glufosinate−ammonium-tolerant rice varieties with tolerance to the nonselective glufosinate−ammonium herbicide has proven to provide new control options for weeds difficult to control such as rice mimic (*Echinochloa phyllopogon*) and red rice (*Oryza sativa*) that have life cycles similar to that of cultivated rice. Glufosinate−ammonium has been shown to control biotypes of early watergrass (*Echinochloa oryzoides*) and barnyardgrass (*Echinochloa crus-galli*), which were identified to be resistant to currently registered rice herbicides.

Rough Rice Samples. The geographic range included the southern U.S. rice-growing regions of Louisiana, Arkansas, and Mississippi. Grain samples were collected from 2 growing seasons (1998 and 1999), 14 locations, 3 treatments from almost every location, and a 3-fold replication per treatment (nontransgenic rice treated with conventional herbicides, transgenic rice treated with conventional herbicides, and transgenic rice treated with Liberty herbicide).

A randomized complete plot design was established at 10 of the locations. At the 4 other trial sites, replicates consisted of harvesting several samples from one plot of a treatment.

Plots were harvested by mechanical grain combine designed for yield evaluation of small plots. Plot size varied by location and was either 8 or 16 m^2. Grain samples were collected from the combine grain bin. The plot combine cuts the panicles and removes the grain from the panicles using mechanical rollers, the grain passes over a screen to remove the chaff, and finally the grain is mixed by the mechanical auger when it passes into the grain bin. The whole grain (rough rice) samples were dried to a final moisture content of 11−14%. Three

Table 1. Rice Products and Components Analyzed

component of the compositional analysis	whole grain/ rough rice	polished grain/ white milled rice	brown rice	parboiled brown rice	rice flour	rice bran	crude rice bran oil
proximates[a]	X[h]	X	X	X	X	X	
total dietary fiber[b]	X						
acid detergent fiber	X					x	
neutral detergent fiber	X					x	
crude fiber	X						
total amino acids[c]	X	X	X	X	X	X	
total fatty acids[d]	X	X				X	X
phosphorus	X					X	
potassium	X						
calcium	X	X	X	X	X		
iron	X	X	X	X	X		
niacin	X	X	X	X	X	X	
pantothenic acid	X	X	X	X			
vitamin B_1	X	X	X	X	X	X	
vitamin B_2	X	X	X	X	X	X	
vitamin E	X	X	X	X			
antinutrients[e]	X	X				X	
four Osborne protein fractions[f]			X				
bioactives[g]							X

[a] Proximates include moisture, crude protein, crude fat, ash, and total carbohydrate (calculated). [b] Total dietary fiber includes soluble and insoluble forms. [c] Full spectrum of amino acids after protein hydrolysis. [d] Major fatty acids are 16:0, 18:0, 18:1, 18:2, and 18:3; longer chain fatty acids <1% relative. [e] Antinutrients of rice include trypsin inhibitors, lectins, and phytic acid. [f] Four protein fractions are albumin, globulin, prolamin (oryzin), and glutelin (oryzenin). [g] Bioactives of rice are oryzanol, tocopherols, and tocotrienol (found in crude oil). [h] X = analysis was completed for the indicated component.

replicate 500 g samples (~18750 seeds) were taken from each treatment in this manner. Because the study design consists of small adjacent plots planted with nontransgenic and transgenic rice plants and the harvester transverses the field in plot order, some comingling of samples could be expected. Adventitious presence of <10% was the threshold used for compositional studies. This level was approached in samples from only two of the sites. In the other control samples, PAT protein was not detectable in the nontransgenic samples or was below the limit of quantification for ELISA testing (the limit of detection is 2.82 ng/g in grain matrix or ~1 seed in 4000 seeds).

Processing of Rough Rice. The nontransgenic rice variety Bengal and the transgenic Bengal line based upon LLRICE62 were produced at the Louisiana State University Agricultural Center, Rice Research Station (Crowley, LA) in 1998, using conventional weed control and the recommended glufosinate−ammonium herbicide practice, respectively. To prepare the processed rice products, 40 kg samples of rough rice were frozen immediately after harvest and kept frozen until processed. The rough rice was dried to a final moisture content of 11−14% and processed after cleaning by the Food Protein Research and Development Center, Texas A&M University (Riverside Campus, Bryan, TX). Two kilograms of rough rice was parboiled, dried, and dehulled to produce samples of parboiled brown rice. The remaining rough rice was dehulled to produce 25 kg of brown rice. Most of the brown rice was milled to produce bran and white, milled rice (polished rice). A sample of the bran was solvent-extracted to produce rice bran oil. A sample of the white milled rice was used to produce rice flour. After processing, the fractions were kept frozen until analyzed. A flowchart of the industrial rice processing is shown in **Figure 2**.

Analysis of Chemical Composition. Rice is primarily an energy source in human nutrition, and carbohydrates comprise ~80 wt % of the whole grain. However, in regions of the world where it is considered a staple, rice can be the principal dietary source not only of energy but also of protein, iron, calcium, thiamin, riboflavin, and niacin.

The components selected for compositional and nutritional analyses shown in **Table 1** include the important nutrients of rice and were identified in consultation with the FDA (*11*). These are proximates, fiber compounds, total amino acids, total fatty acids, micronutrients, such as minerals and vitamins, and antinutrients, such as phytic acid, trypsin inhibitors, and lectins. In this publication, we provide data from selected components and rice products to demonstrate our approach. A more recent effort sponsored by the OECD has produced a consensus document for the nutritional evaluation of rice. There is good correspondence between our list and that published by the OECD (*13*).

Most of the nutritional components could be measured in whole rice grain (rough rice), whereas others required further processing for assessment. The analysis was carried out using validated methods by accredited laboratories. A summary of the components that were measured in each commodity is given in **Table 1**. A complete list of the analytical methods applied is provided in **Table 2**.

Compilation of the Reference Ranges in Commerce. Published literature was consulted to get an estimate of the range of values to be expected for each component. However, directly comparable information is not available for every component. Thus, a range of values was established following compilation of values from a number of reference volumes (*15*−*31*). It should be noted that the primary source of the data in these publications is often not given, the analytical and statistical methods used are not described, the variety and the number of rice samples tested are not known, and much of the data is >20 years old.

Statistical Analysis. The statistical approach is a modification of the *t*-test procedure. Instead of using the *t*-test table for the comparison of means to determine the difference, the equivalence analysis tests whether the treatment differences exceed the range of normal variation of the comparator (nontransgenic plants).

It must be noted that performing an ordinary two-sided *t* test and inferring equivalence from the absence of a significant difference entails an uncontrolled increase in risk of false positive conclusions, that is, the assumption of "equivalence". In other words, "nonsignificant difference" is different from "significant equality" (*32*). Statistical equivalence for a component is assumed if the mean values of two treatments do not differ "too much", that is, the difference of the mean values is within a certain interval. Another power of this approach is that natural variation caused by the environment is evident in the site interaction term of the control data.

In the first step of the statistical assessment of the data, the means of the nontransgenic (reference) group were calculated for each nutritional component, using the data of the single site and of all the sites. Then equivalence boundaries were set to ±20% of the means.

The next step is the analysis of equivalence for each component, first for each site separately—as recommended by the EU national competent authorities advising the European Commission (*12*)—and then over all sites. For the overall analysis, an analysis of variance (ANOVA) was calculated with the factors treatment, location, and their interaction term (fixed effects). A p value of <0.05 for the treatment × location interaction is considered to be indicator not only to look at the overall analysis but also to examine the site-by-site results (**Figures 3** and **4**).

Table 2. Methods of Analysis

component	method[a]
moisture	AOAC 934.01 (1995)
crude protein	AOCS Ba 4e-93 (1995)
crude fat	AOAC 920.39 (1990)
ash	AOAC 942.05 (1990)
total carbohydrate	difference between 100 and the sum of moisture, crude protein, crude fat, and ash
total dietary fiber	AOAC 991.43 (1991)
acid detergent fiber	ANCOM 200/220 fiber analyzer
neutral detergent fiber	ANCOM 200/220 fiber analyzer
crude fiber	AOCS Ba 6-84 (1989)
total amino acids	AOAC 982.30 (1990)
total fatty acids	AOCS Ce 1e-91 (1990)
phosphorus	AOAC 965.17 (1995 modified)
potassium	AOAC 968.08 (1990 modified)
calcium	AOAC 968.08 (1990 modified)
iron	AOAC 968.08 (1990 modified)
niacin	AOAC 944.13 (1995)
pantothenic acid	AOAC 945.74, microbiological
vitamin B_1	AOAC 942.23 (1995)
vitamin B_2	AOAC 970.65 (1995)
vitamin E	AOAC 971.30 with HPLC quantitation
trypsin inhibitors	modified from AACC 71-10 (1995)
lectins	method based on the hemagglutination activity of lectins Klurfeld, Kritchevsky. Isolation and quantitation of lectins from vegetable oils. *Lipids* **1987**, *22*, 667–668 Liener. Photometric determination of the hemagglutinating activity of soyin and crude soybean extracts. *Arch. Biochem. Biophys.* **1955**, *54*, 223–231
phytic acid	by ion exchange, modified from AOAC 986.11 (1995)
four Osborne protein fractions	Osborne method, quantification of protein by Kjeldahl method
bioactives (tocopherols, tocotrienols + oryzanol)	HPLC with spectrometric detection (*14*)

[a] AOAC, *Official Methods of Analysis of AOAC*, 17th ed.; Association of Official Analytical Chemists: Gaithersburg, MD. AOCS, *Official Methods and Recommended Practices of the AOCS*, 5th ed.; American Oil Chemists' Society: Champaign, IL. AACC, *Approved Methods*, 9th ed.; American Association of Cereal Chemists: St. Paul, MN.

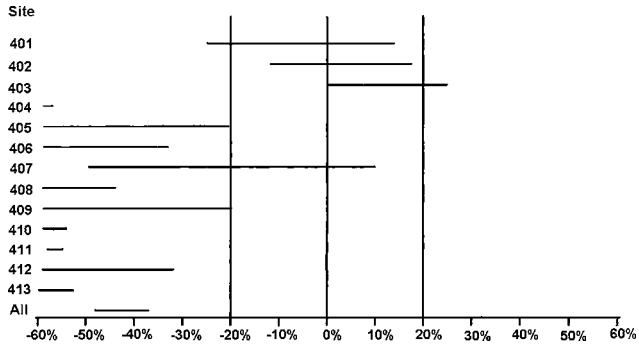

Figure 3. Iron content of rough rice—13 sites and combined data (all). Confidence intervals for differences in means are shown in percent of mean of reference. Nontransgenic reference and LLRICE62 were produced using a conventional herbicide system.

On the basis of the ANOVA, two-sided confidence intervals 100 $(1-2 \times \alpha)$ % (= 90%), where $\alpha = 0.05$, were calculated in pairs for the treatment differences (LSMEANS statement in the SAS procedure PROC MIXED; this procedure was used due to the convenient output facilities in SAS 6.12). In the absence of any other guidance, the authors followed the advice of the FDA (*33*) and the Nordic Council (*34*) for accepted limits for equivalence. Two treatments were considered to be equivalent in these studies if the 90% confidence interval of the difference was within ±20% of the mean value of the respective reference treatment (nontransgenic plants).

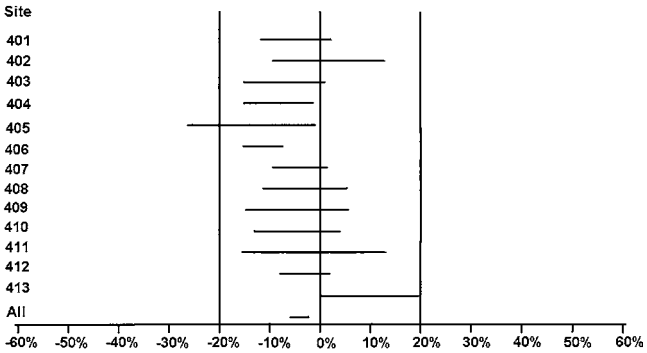

Figure 4. Phytic acid content of rough rice—13 sites and combined data (all). Confidence intervals for differences in means are shown in percent of mean of reference. Nontransgenic reference and LLRICE62 were produced using a conventional herbicide system.

The results of the equivalence analysis can also be graphically displayed as shown in **Figures 3** and **4**.

RESULTS AND DISCUSSION

Composition Analysis of Rough Rice. For the nutritional components that could be measured in rough rice a robust data set from the two-season multisite design can evaluate potential environmental effects between the sites and allow a sound statistical comparison between the nontransgenic and transgenic samples. Means are reported for all locations, comparing the nontransgenic comparator with the transgenic rice grain produced either with conventional herbicides or by the application of glufosinate−ammonium herbicide (**Tables 3**−**5**). Following the guidance published by the European Commission (*35*), part of the substantial equivalence evaluation uses a comparison with reference ranges to look for consistent differences as an indication of unintended effects. The reference ranges or reference values from the literature for rice in commerce are included to provide an expected range for each of the nutrients. Most measured levels were in good compliance with the literature ranges. Deviation from literature data occurred in cases where only a single literature data set was available for comparison.

Analysis of Equivalence. Summary results compiling the data from all 14 sites of the analysis for equivalence for the proximates, fiber compounds, total amino acids, micronutrients (minerals, vitamins), and phytic acid measured in the whole rice grain (rough rice) are provided in **Tables 3**−**5**.

Equivalence in rough rice was demonstrated for all proximates, fiber compounds, total amino acids, and phytic acid. Statistical analysis of the data resulted in no equivalence for calcium, pantothenic acid, and vitamin E, as well as for iron and vitamin B_1 in one of the comparisons with the transgenic treatment groups. However, when equivalence for these micronutrients was not statistically proven, the mean values calculated for the transgenic samples were in fact higher than those of the nontransgenic reference and are within the reference range reported in the literature for rice in commerce, except for vitamin B_1 (**Table 5**). Vitamin B_1 levels were higher than those reported in the literature for all treatments, including the nontransgenic control, indicating that this is not an effect caused by the genetic modification.

Table 3. Comparison and Analysis of Equivalence of the Proximates and Fiber Compounds Measured in Rough Rice

component	*p* value[a]	nontransgenic	transgenic (conventional herbicide system)	analysis of equivalence[b]	transgenic (Liberty herbicide system)	analysis of equivalence[c]	value from lit. (ref range)
proximates							
moisture, % fw	<0.05	10.99 ± 4.72[d]	10.42 ± 3.28	yes[s]	12.93 ± 4.86	yes	11.0–13.7 (*15*, *18*, *20*, *27*, *30*)
crude protein, % dm	<0.05	8.10 ± 0.61	8.41 ± 0.42	yes	8.31 ± 0.51	yes	6.7–8.9 (*15*, *17*, *18*, *20*, *27*, *30*)
crude fat, % dm	<0.05	2.57 ± 0.18	2.61 ± 0.18	yes	2.62 ± 0.20	yes	1.8–2.7 (*15*, *17*, *18*, *20*, *27*, *30*)
ash, % dm	<0.05	4.55 ± 0.54	4.47 ± 0.53	yes	4.69 ± 0.74	yes	3.4–6.0 (*15*, *17*, *18*, *20*, *27*, *30*)
total carbohydrates, % dm	>0.05	84.78 ± 0.82	84.51 ± 0.64	yes	84.38 ± 0.92	yes	83.0–87.8 (*15*, *17*, *18*, *20*, *27*, *30*)
fiber compounds							
total dietary fiber, % dm	<0.05	18.84 ± 1.41	19.41 ± 1.54	yes	18.42 ± 1.40	yes	19.1 (*17*)
acid detergent fiber, % dm	<0.05	14.68 ± 1.45	14.31 ± 1.15	yes	14.13 ± 1.58	yes	
neutral detergent fiber, % dm	<0.05	18.10 ± 1.95	19.44 ± 1.64	yes	17.93 ± 2.09	yes	16.4 (*18*)
crude fiber, % dm	<0.05	10.36 ± 0.59	10.61 ± 0.91	yes	10.45 ± 1.02	yes	8.4–12.1 (*15*, *17*, *18*, *20*, *27*, *30*)

[a] Analysis for interaction between the field site and component, comparison of all three treatments, across all sites. A significant interaction is indicated if the *p* value is <0.05. [b] Analysis of equivalence over all sites reflects the comparison between the pooled data of the respective component in the transgenic, conventionally treated samples and the pooled data for the same component in the nontransgenic samples. [c] Analysis of equivalence over all sites reflects the comparison between the pooled data of the respective component in the transgenic, Liberty-treated samples and the pooled data for the same component in the nontransgenic samples. [d] Means ± standard deviations across all locations for the transgenic, LLRICE62 rough rice grain (produced using a conventional herbicide and a Liberty herbicide program) and for the nontransgenic reference (cv. Bengal), together with the reference ranges/values for rice in commerce. [e] The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (nontransgenic).

Table 4. Comparison and Analysis of Equivalence of the Total Amino Acids Measured in Rough Rice

component	*p* value[a]	nontransgenic	transgenic (conventional herbicide system)	analysis of equivalence[b]	transgenic (Liberty herbicide system)	analysis of equivalence[c]	value from lit. (ref range)
alanine, % dm	<0.05	0.41 ± 0.03[d]	0.42 ± 0.02	yes[e]	0.42 ± 0.02	yes	0.47 (*24*)
arginine, % dm	<0.05	0.55 ± 0.03	0.55 ± 0.04	yes	0.55 ± 0.04	yes	0.52–0.80 (*18*, *24*, *29*, *30*)
aspartic acid, % dm	<0.05	0.73 ± 0.05	0.74 ± 0.04	yes	0.73 ± 0.04	yes	0.81 (*24*)
cystine, % dm	<0.05	0.18 ± 0.01	0.19 ± 0.01	yes	0.18 ± 0.01	yes	0.09–0.14 (*18*, *24*, *29*, *30*)
glutamic acid, % dm	<0.05	1.25 ± 0.07	1.30 ± 0.07	yes	1.26 ± 0.09	yes	1.59 (*24*)
glycine, % dm	<0.05	0.36 ± 0.02	0.36 ± 0.02	yes	0.36 ± 0.01	yes	0.39–0.69 (*18*, *24*, *30*)
histidine, % dm	>0.05	0.19 ± 0.02	0.20 ± 0.02	yes	0.21 ± 0.03	yes	0.10–0.20 (*18*, *24*, *29*, *30*)
isoleucine, % dm	<0.05	0.28 ± 0.02	0.29 ± 0.02	yes	0.29 ± 0.02	yes	0.30–0.43 (*18*, *24*, *29*, *30*)
leucine, % dm	<0.05	0.58 ± 0.05	0.59 ± 0.04	yes	0.59 ± 0.04	yes	0.60–0.68 (*18*, *24*, *29*, *30*)
lysine, %	<0.05	0.29 ± 0.01[d]	0.29 ± 0.01	yes	0.29 ± 0.01	yes	0.28–0.34 (*18*, *24*, *29*, *30*)
methionine, %	<0.05	0.19 ± 0.01	0.20 ± 0.01	yes	0.19 ± 0.01	yes	0.15–0.20 (*18*, *24*, *29*, *30*)
phenylalanine, %	<0.05	0.37 ± 0.03	0.38 ± 0.03	yes	0.37 ± 0.02	yes	0.34–0.42 (*18*, *24*, *29*, *30*)
proline, %	<0.05	0.34 ± 0.03	0.35 ± 0.03	yes	0.35 ± 0.03	yes	0.37 (*24*)
serine, %	<0.05	0.38 ± 0.03	0.38 ± 0.02	yes	0.38 ± 0.03	yes	0.41–0.56 (*18*, *24*, *30*)
threonine, %	<0.05	0.28 ± 0.02	0.28 ± 0.02	yes	0.28 ± 0.02	yes	0.26–0.35 (*18*, *24*, *29*, *30*)
tryptophan, %	<0.05	0.10 ± 0.01	0.10 ± 0.01	yes	0.10 ± 0.01	yes	0.10–0.14 (*18*, *24*, *29*, *30*)
tyrosine, %	<0.05	0.13 ± 0.01	0.12 ± 0.02	yes	0.13 ± 0.02	yes	0.26–0.71 (*18*, *24*, *30*)
valine, %	<0.05	0.41 ± 0.03	0.43 ± 0.03	yes	0.42 ± 0.03	yes	0.44–0.58 (*18*, *24*, *29*, *30*)

[a] Analysis for interaction between the field site and component, comparison of all three treatments, across all sites. A significant interaction is indicated if the *p* value is <0.05. [b] Analysis of equivalence over all sites reflects the comparison between the pooled data of the respective component in the transgenic, conventionally treated samples and the pooled data for the same component in the nontransgenic samples. [c] Analysis of equivalence over all sites reflects the comparison between the pooled data of the respective component in the transgenic, Liberty-treated samples and the pooled data for the same component in the nontransgenic samples. [d] Means ± standard deviations across all locations for the transgenic, LLRICE62 rough rice grain (produced using a conventional herbicide and a Liberty herbicide program) and for the nontransgenic reference (cv. Bengal), together with the reference ranges/values for rice in commerce. [e] The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (nontransgenic).

In the following section, the graphical representation of the equivalence analyses of iron and phytic acid are presented and explained (**Figures 3** and **4**). The 0% line represents the mean value of the respective component in the reference treatment (nontransgenic rough rice). The −20% and +20% lines are the upper and lower borders of the variation in the nontransgenic reference treatment. The horizontal bar in the figure represents the 90% confidence interval of the treatment differences for each site. If the horizontal bar is between the upper and lower borders of the reference range, equivalence between the treatments is confirmed.

For iron, equivalence between the treatments could be confirmed at only 1 of the 13 single sites (**Figure 3**). At 12 sites, the treatment differences exceeded the 20% range of the reference mean, and at these sites equivalence between the treatments was not demonstrated. The 90% confidence interval of the mean built over all sites is also clearly outside the equivalence limits. The mean data provided in **Table 5** show that the LLRICE62 grain has more iron than the nontransgenic grain. The *p* value for the site by treatment interaction is statistically significant ($p < 0.05$), and the analysis of equivalence is recorded as "no (+)", indicating the mean of all sites is outside the ±20% range and the difference is positive. However, most of the single-site mean values and the mean values built over all sites fall within the literature range.

For phytic acid, equivalence between the treatments could be confirmed at 12 of the 13 single sites and for the comparison over all sites (**Figure 4**). With 90% probability, the real

Table 5. Comparison and Analysis of Equivalence of the Minerals, Vitamins, and Antinutrient Measured in Rough Rice

component	*p* value[a]	nontrans-genic	transgenic (conventional herbicide system)	analysis of equivalence[b]	transgenic (Liberty herbicide system)	analysis of equivalence[c]	value from lit. (ref range)
minerals							
phosphorus, %	>0.05	0.268 ± 0.025[d]	0.278 ± 0.025	yes[e]	0.286 ± 0.030	yes	0.24–0.36 (*15*, *18*, *20*, *24*, *27*, *30*)
potassium, %	<0.05	0.286 ± 0.026	0.297 ± 0.021	yes	0.294 ± 0.022	yes	0.18–0.53 (*15*, *18*, *20*, *24*, *27*, *30*)
calcium, %	<0.05	0.022 ± 0.008	0.027 ± 0.008	no (+)	0.028 ± 0.005	no (+)	0.02–0.07 (*15*, *18*, *20*, *24*, *27*, *30*)
iron, mg/kg	<0.05	35.85 ± 14.75	50.52 ± 8.59	no (+)	41.44 ± 10.16	yes	16.2–57.0 (*15*, *20*, *24*, *27*, *30*)
vitamins							
niacin, mg/kg	>0.05	48.76 ± 5.18	49.86 ± 5.84	yes	54.73 ± 5.31	yes	14.6–65.0 (*15*, *17*, *18*, *20*, *22*, *30*)
pantothenic acid, mg/kg	<0.05	9.10 ± 1.50	10.52 ± 1.79	no (+)	11.10 ± 1.59	no (+)	4.0–12.4 (*15*, *18*, *20*, *22*, *30*)
vitamin B_1, mg/kg	<0.05	5.28 ± 1.04	5.89 ± 0.67	yes	5.96 ± 0.67	no (+)	1.4–3.8 (*15*, *17*, *20*, *22*, *30*)
vitamin B_2, mg/kg	>0.05	1.11 ± 0.28	1.10 ± 0.14	yes	1.12 ± 0.31	yes	0.4–1.3 (*15*, *17*, *18*, *20*, *22*, *30*)
vitamin E, IU/kg	>0.05	17.30 ± 6.50	20.76 ± 6.59	no (+)	19.70 ± 5.54	no (+)	6.7–34.7 (*18*, *20*, *22*, *30*)
antinutrient							
phytic acid, %	<0.05	0.83 ± 0.05	0.86 ± 0.06	yes	0.81 ± 0.09	yes	0.72–1.20 (*18*, *21*)

[a] Analysis for interaction between the field site and component, comparison of all three treatments, across all sites. A significant interaction is indicated if the *p* value is <0.05. [b] Analysis of equivalence over all sites reflects the comparison between the pooled data of the respective component in the transgenic, conventionally treated samples and the pooled data for the same component in the nontransgenic samples. [c] Analysis of equivalence over all sites reflects the comparison between the pooled data of the respective component in the transgenic, Liberty-treated samples and the pooled data for the same component in the nontransgenic samples. [d] Means ± standard deviations across all locations for the transgenic, LLRICE62 rough rice grain (produced using a conventional herbicide and a Liberty herbicide program) and for the nontransgenic reference (cv. Bengal), together with the reference ranges/values for rice in commerce. [e] The criterion for equivalence (yes) is met when the 90% confidence interval of the difference does not exceed the 20% range of the reference (nontransgenic). If the 90% confidence interval of the difference exceeds the 20% range of the reference (nontransgenic), this is indicated by "no (+)".

Table 6. Minerals and Vitamins in Brown Rice

	on dry matter basis		
component	nontransgenic	transgenic Liberty herbicide	value from lit. (ref range)
Ca, %	<0.01	0.02	0.02–0.04 (*15*, *16*, *18*, *19*, *24*, *31*)
Fe, mg/kg	17.2	17.1	14.7–40 (*15*, *16*, *18*, *19*, *24*, *31*)
niacin, mg/kg	56.31	62.14	41–58 (*15–19*, *24*, *25*, *31*)
pantothenic acid, mg/kg	12.60	11.33	10–17 (*16–19*, *25*, *31*)
vitamin B_1, mg/kg	6.21	7.44	3.0–4.71 (*15–19*, *24*, *25*, *31*)
vitamin B_2, mg/kg	1.29	1.19	0.5–1.6 (*15–19*, *24*, *25*, *31*)
vitamin E, IU/kg	29.29	24.56	13–29 (*17–19*, *24*, *25*, *31*)

Table 7. Minerals and Vitamins in Rice Bran

	on dry matter basis		
component	nontransgenic	transgenic Liberty herbicide	value from lit. (ref range)
phosphorus, %	1.61	1.35	1.59–2.02 (*15*, *18*, *20*, *23*, *26–28*, *30*, *31*)
niacin, mg/kg	318	280	330–580 (*15*, *17*, *18*, *20*, *25*, *30*, *31*)
vitamin B_1, mg/kg	30.26	32.49	11.0–29.3 (*15*, *17*, *18*, *20*, *25*, *30*, *31*)
vitamin B_2, mg/kg	4.58	3.7	1.2–4.0 (*15*, *17*, *18*, *20*, *25*, *30*, *31*)

difference of the two treatments is completely contained within the defined limits of equivalence (±20% of the reference mean − mean value from nontransgenic samples). In addition, most of the single-site mean values and the mean values built over all sites are within the reference range reported from the literature (**Table 5**).

Analysis of Postprocessing Nutritional Composition. Rice grain from one location was processed to provide samples of processed products for analysis.

Rice bran gives brown rice its color and nutty flavor. Important nutritional factors were measured in the transgenic LLRICE62 brown rice samples and the respective conventional brown rice. Vitamin determinations showed an excellent correspondence between LLRICE62 and the comparator for brown rice samples (**Table 6**).

Bran is generally high in minerals and vitamins, especially the B-complex group, and is used as an ingredient in cereals and mixes as well as in vitamin concentrates. The minerals and vitamins of the transgenic rice bran samples (**Table 7**) have values that are comparable to the values for the respective conventional rice bran sample and the reference ranges reported from the literature.

Rice bran oil is a high-quality cooking oil, and we found that the desired lipid profile is preserved in LLRICE62. The fatty acids and unsaponifiable lipids were measured in the transgenic LLRICE62 crude rice bran oil samples and the respective conventional crude rice bran oil. The fatty acid values for the nontransgenic and transgenic samples agree well (**Table 8**). The unsaponifiable lipid constituents in unbleached rice bran oil (tocopherols, tocotrienols, and oryzanol) showed very little difference in content between the nontransgenic and transgenic rice samples. It was difficult to find reference values in the literature for unsaponifiable lipid constituents in crude rice bran oil. Comparison with the only literature source (*22*) available

Table 8. Fatty Acid Composition of Crude Rice Bran Oil

	relative %		
fatty acid	nontransgenic	transgenic Liberty herbicide	value from lit. (ref *31*)[a]
saturated			
C14:0	0.39	0.38	0.41–0.70
C16:0	15.1	14.6	16.9–18.55
C18:0	1.82	1.86	1.60–1.94
C20:0	0.71	0.73	
C22:0	0.30	0.28	
C24:0	0.59	0.54	
total	18.9	18.4	19.70–20.90
monounsaturated			
C16:1	0.26	0.26	0.20–0.39
C18:1	40.3	40.0	38.97–39.10
C20:1	0.61	0.67	0
total	40.9	40.7	39.30–39.36
polyunsaturated			
C18:2	38.7	39.0	33.40–37.24
C18:3	1.12	1.12	1.60–1.65
total	39.8	40.1	35.00–38.89
grand total	99.6	99.2	93.50

[a] Ranges built from values of rice bran and refined rice bran oil.

Table 9. Unsaponifiable Lipids in Crude Rice Bran Oil

unsaponifiable lipid	nontransgenic	transgenic Liberty herbicide	value from lit. (ref *22*)
oryzanol, %	2.23	2.17	1.5–2.9
tocopherols, mg/kg			
α	239.2	254.1	190–460
γ	15.49	16.52	10–100
δ	14.80	19.30	4–9
tocotrienols, mg/kg			
α	157.3	138.7	140–330
γ	10.40	12.40	90–690
δ	13.06	18.94	

Table 10. Antinutrients in Rough Rice and Rice Products

		on dry matter basis		
component	rice	nontransgenic	transgenic Liberty herbicide	value from lit. (ref range)
lectin, HU/mg	rough	<0.1	<0.1	no activity (*37*)
	white	<0.1	<0.1	<1.3 (*15*)
	bran	<0.1	<0.1	
trypsin inhibition, TIU/mg	rough	<1.0	<1.0	
	white	<1.0	<1.0	
	bran	2.27	1.36	0.011–0.045 (*15*)
phytic acid, %	white	0.29	0.33	0.1–0.3 (*15, 19, 21*)
	bran	5.14	4.49	1.72–8.76 (*15*)

showed that the results for oryzanol, α- and γ-tocopherol, and α-tocotrienol are in good correspondence with the reported ranges (**Table 9**).

Special Nutritional Considerations of Rice. There are three antinutrients that are important in rice and rice products: phytic acid, trypsin inhibitors, and lectins. These antinutritional factors were measured in rough rice, white, polished rice, and rice bran (**Table 10**). The biochemical analysis did not detect lectins in any of the rice products (limit of quantitation = 0.1 HU/mg). Trypsin inhibition was found in only rice bran, but not in any rough rice or white rice samples. Although the results for trypsin inhibition of rice bran extracts exceed the reference value, the nutritional relevance of the determined levels is very low compared to the trypsin inhibition activity in soybeans of 100–184 TIU/mg (*36*). For phytic acid, equivalence between the transgenic and nontransgenic rough rice samples was demonstrated. Phytic acid was also measured in white rice and rice bran samples. There was no difference in the phytic acid contents of the transgenic and nontransgenic samples, and the measured values were in agreement with literature reference ranges.

Table 11. Osborne Fractionation of Rice Proteins

	% total protein		
Osborne protein class	nontransgenic	transgenic Liberty herbicide	value from lit. (ref *22*)
albumin	3.1	2.8	5–17
globulin	10.3	9.9	4–15
glutelin	85.4	86.2	70–86
prolamin	1.2	1.1	2–5

Prolamins. Rice is an important grain source in diets that require low prolamin protein. For example, rice is used as a substitute for wheat in baby food and special diets for celiac patients, because rice has the lowest prolamin content of all the common food grains (*38*).

Osborne fractionation separates proteins into four fractions of solubility, extracting successively the albumin (water fraction), globulin (saline fraction), and prolamin (ethanol fraction). Glutelin remains in the residue, but is partially soluble in diluted acid and completely soluble after reduction of the disulfide bonds. Brown rice (with hulls removed, but not milled) was analyzed using the Osborne method to confirm that the protein profile remained unchanged (**Table 11**). Classification of the rice proteins, and especially the prolamin content, is the same in nontransgenic and transgenic brown rice. This result is an important indication of the stability of the prolamin fraction in transgenic rice.

Conclusion. Biotechnology applications for rice (*O. sativa* L.) are advancing in all of the rice-growing regions of the world (*39*, *40*). Comparison of nutritional composition is an important consideration in the safety assessment of food and feed products derived from crops for which agricultural productivity has been enhanced using biotechnology and for which no changes to the nutritional composition are intended.

Using a framework of side-by-side analysis, key nutritional components were measured in whole grain (rough rice) derived from transgenic rice (in this case, glufosinate-tolerant rice LLRICE62) and a nontransgenic counterpart. The comparative analysis of the rice grain was completed by using a statistical procedure to assess equivalence. Using the values of the nontransgenic counterpart, a range of equivalence was calculated and criteria for acceptance or rejection were determined.

Literature reference ranges were constructed, defining the variation in composition for the key nutritional factors reported as reference ranges or values in commerce for rice. A comparison was made of the variation found in the grain of LLRICE62 and its nontransgenic counterpart as grown in regions of production resulting in good compliance with most of the reported figures.

Representative grain samples were processed to assess potential differences that may be observed in the normal handling of the grain. Samples of brown rice, parboiled brown rice, white, milled rice, rice bran, rice flour, and rice bran oil were prepared. Nutritional components—key for each product—were analyzed and compared, some components being analyzed in only a processed product.

Equivalence assessed by statistical methods was demonstrated for proximates, fiber compounds, all amino acids, most minerals

and vitamins, and the antinutrients in rough rice samples. For calcium, iron, vitamin B_1, pantothenic acid, and vitamin E, equivalence between the data sets could not be proven statistically, but all mean values calculated for these micronutrients in the transgenic samples, except for vitamin B_1, are within the reference range reported in the literature for rice in commerce and were in fact not lower than those of the nontransgenic reference. Vitamin B_1 levels were higher than those reported in the literature for all treatments, including the nontransgenic control, indicating that this is not an effect caused by genetic modification.

Assessment of the composition data and comparison with the reported ranges lead to the conclusion that LLRICE62 has the same nutritional value and is compositionally equivalent to its nontransgenic counterpart and to other commercial rice varieties.

It should be noted that the conclusions reached in an earlier evaluation presented to the FDA (*11*) to assess the nutritional impact of the new rice were not altered by the expansion of treatments, locations, and seasons.

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