Biotechnology Notification File No. 000158
Note to the File

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From: Carrie McMahon, Ph.D., Consumer Safety Officer, DBGNR/OFAS/CFSAN

To: BNF 000158 Administrative Record

Subject: GR2E rice with altered composition (provitamin A carotenoids (mainly beta (β)-carotene))

Keywords:

Rice; *Oryza sativa* (L.); Gr2E rice; Golden Rice; provitamin A carotenoids; beta-carotene; β-carotene; *Zmpsy1* gene from *Zea mays* (corn); phytoene synthase; *Zm*PSY1; *crtI* gene from *Pantoea ananatis*; carotene desaturase I; CRTI; *pmi* gene from *Escherichia coli*; phosphomannose isomerase; PMI; selectable marker for plant transformation; New Protein Consultation No. (NPC) 000002; OECD unique identifier IR-ØØGR2E-5; International Rice Research Institute; IRRI.

Purpose

This document summarizes the Food and Drug Administration's (FDA's, our) evaluation of biotechnology notification file (BNF) No. 000158. International Rice Research Institute (IRRI) submitted a safety and nutritional assessment of genetically engineered (GE) rice, transformation event GR2E, which we received on November 14, 2016. In its submission, IRRI informed FDA that although GR2E rice is not currently intended for cultivation or marketing in the United States,[1] it anticipates that GR2E rice, or human and animal food products derived from GR2E rice, may enter the U.S. food supply via imports from countries of production. We evaluated the information in IRRI's submission to ensure that regulatory and safety issues regarding human or animal food derived from GR2E rice have been resolved.

In our evaluation, we considered all information provided by IRRI as well as publicly available information and information in the agency's files. Here we discuss the outcome of the consultation, but do not intend to restate the information provided in the final consultation in its entirety.

Intended Effect

The intended effect of the modification in GR2E rice is the production of provitamin A carotenoids (mainly β-carotene) in the rice endosperm. To confer this trait, IRRI introduced two genes that encode components of the carotenoid biosynthetic pathway: the *Zmpsy1* gene from *Zea mays* encodes phytoene synthase (PSY1) and the *crtI* gene from *Pantoea ananatis* encodes carotene desaturase I (CRTI). These enzymes catalyze the conversion of geranylgeranyl diphosphate to phytoene and the

[1] According to IRRI, GR2E rice is intended for cultivation and use in human food as a source of dietary provitamin A carotenoids (mainly β-carotene) in certain south and southeast Asian countries where vitamin A deficiency is common.

conversion of phytoene to lycopene, respectively. Lycopene is the precursor to provitamin A carotenoids, including β-carotene.

IRRI also introduced the *pmi* gene from *Escherichia coli*. This gene encodes phosphomannose isomerase (PMI) and was used by IRRI as a selectable marker for plant transformation.[2]

Regulatory Considerations

The purposes of this evaluation are (1) to assess whether the developer has introduced into human or animal food a substance requiring premarket approval as a food additive and (2) to determine whether use of the new plant variety in human or animal food raises other regulatory issues under the Federal Food, Drug and Cosmetic Act (FD&C Act).

Genetic Modification and Characterization

Transformation Plasmid and Method

IRRI transformed embryogenic calli of the japonica rice cultivar Kaybonnet with plasmid pSYN12424 using Agrobacterium-mediated transformation. The T-DNA region of pSYN12424 contains three expression cassettes between right and left border sequences:

- Cassette I: *crtI*[3] gene from *P. ananatis*, preceded by the N-terminal RUBISCO SSU transit peptide coding sequence from *Pisum sativum*, with regulatory elements, including the glutelin *GluA-2* promoter from *Oryza sativa* and nopaline synthase (NOS) 3′-untranslated region (3′-UTR) from *A. tumefaciens* Ti plasmid (pTiT37)
- Cassette II: *Zmpsy1* gene from *Z. mays* with regulatory elements, including the *GluA-2* promoter from *O. sativa* and NOS 3′-UTR from *A. tumefaciens* pTiT37
- Cassette III: *pmi* gene from *E. coli* K-12 with regulatory elements, including the polyubiquitin *ZmUBI* promoter region and first intron from *Z. mays* and NOS 3′-UTR from *A. tumefaciens* pTiT37

Following transformation, IRRI selected for transformants using mannose selection media to inhibit the growth of untransformed cells lacking expression the selectable marker, PMI. The proliferating transformed colonies were transferred to plant regeneration media. IRRI selected a transformed plant (T0) containing the GR2E event for propagation. The GR2E event was subsequently crossed into indica rice cultivars PSB Rc82, BRRI dhan 29, and IR64 for molecular characterization and compositional analyses.

Characteristics, Inheritance, and Stability of the Introduced DNA

IRRI characterized the insertion event in GR2E rice using Southern blot analyses and direct sequencing of PCR products amplified from the insertion site. IRRI concluded that GR2E rice contains a single, intact DNA insertion that is identical to the T-DNA region of plasmid pSYN12424, with the exception of small deletions at the ends of both the right and left borders. The two deletions do not affect the integrity of the three expression cassettes. IRRI used five probes corresponding to the entire plasmid backbone region to demonstrate by Southern blot analysis that GR2E rice does not contain plasmid backbone sequences, including antibiotic resistance marker genes.

IRRI used a combination of methods to demonstrate the inheritance as a single locus according to Mendelian principles and the stability of the inserted DNA, as well as the stability of the β-carotene

[2] PMI is the subject of New Protein Consultation No. (NPC) 000002. FDA responded to NPC 000002 on February 10, 2009.

[3] The *crtI* gene sequence in pSYN12424 contains an A3992 to G change compared to GenBank accession no. D90087 sequence. This nucleotide difference does not alter the amino acid sequence of the protein.

phenotype. IRRI confirmed the inheritance and stability of the inserted DNA using Southern blot analyses to analyze genomic DNA samples from four different germplasm backgrounds carrying the GR2E event (Kaybonnet, PSB Rc82, BRRI dhan 29, and IR64) and from multiple breeding generations.

IRRI also analyzed nucleotide sequences of the T-DNA insert and flanking rice genomic sequences to determine whether the integration event created new start-to-stop open reading frames (ORFs) that could potentially encode sequences homologous to known toxins or allergens. The analysis identified two potential new ORFs. IRRI then compared the amino acid sequences of the potential ORFs to the sequences of known and putative toxins[4] and allergens,[5] and reported that it did not identify significant sequence similarities. Based on these results, IRRI concluded that there were no new novel ORFs created as a consequence of the DNA insertion that would have the potential to encode a protein with any significant amino acid sequence similarity to known and putative toxins or allergens.

Protein Characterization

Identity and Function

GR2E rice was genetically engineered to express PSY1 from *Z. mays* and CRTI from *P. ananatis*. In immature rice grains, the carotenoid biosynthetic pathway functions up to the synthesis of geranylgeranyl diphosphate (GGPP). The PSY1 and CRTI enzymes bridge the gap between GGPP and lycopene, which is a precursor for α- and β-carotenoids. PSY1 catalyzes the condensation of two molecules of GGPP to form 15-*cis*-phytoene. CRTI then catalyzes the *cis* to *trans* conversion of 15-*cis*-phytoene to all-*trans*-lycopene. Lycopene is subsequently converted to α- and β-carotene by endogenous rice cyclases.

GR2E rice was also genetically engineered to express PMI from *E. coli*. PMI catalyzes the interconversion of mannose-6-phosphate and fructose-6-phosphate. PMI is used as a selectable marker by genetic engineers because plants expressing PMI are able to utilize mannose as their only or primary carbon source and thus grow on media lacking other carbon sources.

Protein Expression Levels

IRRI measured the levels of PSY1, CRTI and PMI in tissue samples from plants grown in the Philippines at four sites in 2015 (rainy season) and in 2016 (dry season). Composite samples were collected from at least five representative plants of each entry (GR2E introgressed into PSB Rc82 background (PSB Rc82+GRE2 rice) and from the near-isogenic, non-GE PSB Rc82 (the control)) within each of three replicated blocks. Grain samples were collected at the milk, dough, and mature stages of development; straw samples were collected at harvest. IRRI analyzed the samples using quantitative enzyme-linked immunosorbent assay and reported the results of its analyses. PSY1, CRTI, and PMI protein levels were below the limit of quantification (LOQ) in all tissue samples collected from the control rice. In tissue samples from the PSB Rc82+GR2E rice:

- Under the endosperm specific rice *GluA*-2 promoter, the PSY1 protein was present in grain but not straw samples. The highest levels in grain were measured in dough-stage samples and ranged from 308 – 359 ng/g fresh weight (FW) across both growing seasons. The highest level of PSY1 measured in mature rice grain samples was 245 ng/g FW.

[4] IRRI evaluated potential similarities to known and putative toxins using FASTA bioinformatic alignment (*E*-score of 1 x 10^{-5} or less) against a toxin database created from a subset of sequences from the UniProt Knowledgebase.

[5] IRRI evaluated potential similarities to known and putative allergens using FASTA bioinformatic alignment (>35% match over an 80 amino acid sliding window) against the Food Allergy Research and Resource Program (FARRP) allergen protein database. IRRI also screened for any 8 contiguous, identical amino acid matches to allergens in the FARRP database.

- Under the endosperm specific rice *GluA*-2 promoter, the CRTI protein was present in grain but not straw samples. The highest levels in grain were measured in dough-stage samples and ranged from 54 – 68 ng/g FW across both growing seasons. The highest level of CRTI measured in mature rice grain samples was 30 ng/g FW.
- Under the constitutive control of the *Z. mays* polyubiquitin promoter, the PMI protein was present in both grain and straw samples. The highest levels in grain were measured in dough-stage samples, which averaged about 2015 ng/g FW across the four locations over both growing seasons. PMI levels were on average about 1282 ng/g FW in mature rice grain across both growing seasons and ranged from 320 – 796 ng/g FW in straw depending on location and growing season.

Potential for Toxicity and Allergenicity

IRRI used a "weight of the evidence" approach to assess the potential toxicity and allergenicity of PSY1, CRTI, and PMI. In its safety assessment, IRRI discussed information about the history of dietary exposure to *Z. mays* PSY1 and to *E. coli* PMI protein homologs present in common food sources, as well as the likely incidental dietary exposure to *P. ananatis* CRTI. IRRI noted the lack of evidence of toxicity and allergenicity associated with these genetic sources. IRRI summarized unpublished acute oral toxicity studies of CRTI and PMI that demonstrated a lack of any test substance-related adverse effects. IRRI discussed information about the enzymatic functions of the three proteins and explained that these functions are not similar to the activities of known protein toxins. IRRI provided the results of standard bioinformatics analyses, which showed no significant amino acid sequence similarities to known and putative toxins[4] or allergens.[5,6] IRRI concluded that the PSY1, CRTI, and PMI proteins are unlikely to be toxic or allergenic to humans or animals.

IRRI discussed digestibility and heat stability test results for PSY1, CRTI, and PMI.[7] Digestibility studies using standard methods did not identify significant resistance to pepsin proteolysis. Heat stability studies using standard methods showed rapid and irreversible enzymatic inactivation of the proteins at temperatures below those normally used during processing or cooking of rice. IRRI concluded from these results that dietary exposure to intact and functional PSY1, CRTI, and PMI proteins will be negligible.

Based on the weight of this combined evidence, IRRI concluded that the PSY1, CRTI, and PMI proteins are unlikely to be toxic or allergenic to humans or animals through oral exposure.

Human and Animal Food Use

Oryza sativa L., commonly known as rice, is the dominant species of rice cultivated around the world for a variety of human and animal food uses. There are three types of *O. sativa* L.: japonica, indica, and javanica. After threshing and winnowing to remove chaff and other material, rice grain is known as paddy rice. Beginning with dehulling, paddy rice is further processed into a variety of food products for human consumption. Commonly consumed rice products are brown rice, milled rice, and parboiled rice. Processed rice is also consumed in prepared products such as rice noodles, cakes, crackers, sweets, and alcoholic beverages.

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[6] IRRI identified one 8-amino acid identity match between PMI and a known allergen: α-parvalbumin from *Rana* species CH2001. However, IRRI concluded the match is not biologically meaningful because no cross-reactivity was observed between PMI and the serum of an individual with a documented IgE-mediated allergy to *Rana* species CH2001 α-parvalbumin.

[7] Because the concentrations of PSY1 and CRTI in GR2E rice are low, IRRI conducted the digestibility and heat stability studies using microbially-expressed PSY1 and CRTI. Prior to conducting the studies, IRRI confirmed the equivalence of the plant- and microbe-expressed proteins using mass spectral analysis of trypsin-digested protein samples as well as immunochemical cross-reactivity. For assessment of digestibility and heat stability of PMI, IRRI relied on published reports.

The by-products of food processing, as well as low grade paddy rice and whole crop silage, are used in animal food to varying degrees in different regions of the world. In the United States, rice grain, bran, hulls and polishings (material that is removed during the polishing process) are used in a wide range of livestock diets while rice may be used in pet foods.

Composition

Scope of Analyses

IRRI analyzed the composition of paddy rice, milled rice (hull removed), and straw, as well as bran samples, collected from event GR2E introgressed into PSB Rc82 background (PSB Rc82+GRE2 rice) and from the near-isogenic, non-GE PSB Rc82 (the control).

Study Design - Compositional Analyses

IRRI grew PSB Rc82+GR2E rice and the control over two growing seasons in 2015 (rainy) and 2016 (dry) in the Philippines at four sites, which IRRI states represent typical rice growing conditions. At each site, the rice varieties were planted using a randomized complete block design with three replicates, within which each entry was planted in a 10m^2 plot with 250 plants. Grain (paddy rice) and straw were collected from mature plants and pooled into a single sample per plot.[8] Composite samples of grain were processed to obtain bran samples.

IRRI analyzed the composition of paddy rice, straw, and bran.

- In paddy rice, IRRI reported the results for moisture, crude protein, crude fat, ash, fiber (acid detergent fiber, neutral detergent fiber, total dietary fiber, and crude fiber), carbohydrates by calculation, starch, amylose, 11 fatty acids, 18 amino acids, 9 minerals, 7 vitamins (including β-carotene), and antinutrients phytic acid and trypsin inhibitor.
- In straw and bran, IRRI reported the results for moisture, crude protein, crude fat, ash, ADF, NDF, crude fiber, carbohydrates by calculation, calcium, and phosphorus.

IRRI explained that compositional data were analyzed using mixed linear model for multi-year combined-sites analysis. The parameters included in the model were entry, site, and growing season and the appropriate interaction terms. For each component, the least squares (LS)-mean value across years and sites was estimated for PSB Rc82+GR2E rice and the control. IRRI used the LS-means to test for least significant differences between the components of PSB Rc82+GR2E rice and the control rice. Statistically significant differences were established at p-value < 0.05.

When a statistically significant difference was identified in the multi-year combined-sites analysis, IRRI evaluated the biological significance of this difference by comparing the individual PSB Rc82+GR2E rice component mean value with the range of values available in ILSI Crop Composition Database (2014) or that are reported in published literature.[9] IRRI concluded that ranges observed for PSB Rc82+GR2E rice that fell within the combined literature range for a given component are within the ranges of normal variability for conventional rice.

Results of analyses

Paddy rice, straw, and bran

IRRI analyzed 69 components in paddy rice and 10 components each in straw and bran. While no statistically significant differences were observed in either straw or bran, statistically significant

[8] Grain was collected from at least 150 plants per plot; straw was collected from at least 8 plants per plot.
[9] Published literature included OECD (2016), NARO (2011), Heuzé and Tran (2015), and Juliano and Bechtel (1985).

differences were observed in paddy rice for stearic acid and for β-carotene (the intended compositional change). IRRI concluded that, with the exception of β-carotene, the components measured in samples of PSB Rc82+GR2E rice, including stearic acid, were within or similar to the range of values observed for conventional rice varieties with a history of safe consumption.

Intended Compositional Change – provitamin A carotenoids (mainly β-carotene)

IRRI reported the results of analysis of β-carotene levels[10] in paddy rice samples from PSB Rc82+GR2E rice and the control. Whereas the LS-mean value for β-carotene in the control is below the LOQ (range = below LOQ to 0.07 mg/kg dry weight (DW)), the LS-mean value for β-carotene in PSB Rc82+GR2E rice is 1.26 mg/kg DW (range = 0.504 to 2.35 mg/kg DW).

IRRI also summarized unpublished results of a compositional analysis for β-carotene and other provitamin A carotenoids[11] in milled rice prepared from grain samples. As expected, the mean values of β-carotene and other carotenoids in the control rice were below the LOQ while the mean values in PSB Rc82+GR2E rice were quantifiable, with the highest mean reported for β-carotene (3.57 μg/g DW) and a total carotenoid mean of 5.88 μg/g DW[12]. IRRI attributes the differences in mean β-carotene levels (grain versus milled rice) to differences in the sample matrix and in the carotenoid extraction efficiencies of the two analytical methods employed.

In considering the safety of increased levels of β-carotene in human food, IRRI discussed the current dietary sources of β-carotene and other provitamin A carotenoids; dietary intakes by consumers in the United States from different sources, and the potential dietary exposure from consumption of GR2E rice. Assuming 100 percent of rice consumed in the United States is replaced with GR2E rice, IRRI used current dietary rice intake (11.8 kg per capita annually) and the highest value of β-carotene measured in samples of milled GR2E rice (7.31 μg/g DW) to estimate the potential increase in dietary exposure to β-carotene. IRRI estimated the potential dietary exposure to β-carotene from GR2E rice to be approximately 0.24 mg/day; this translates to approximately one tenth the current daily β-carotene consumption in the adult population, from all other food sources. IRRI acknowledged that it expects the actual dietary intakes to be lower given (1) that it is unlikely that all rice in the diet would be substituted with GR2E rice and (2) that β-carotene levels in food containing GR2E rice would decline over time due to storage, processing, and cooking.

IRRI discussed its consideration of the safety of β-carotene in animal diets. IRRI notes that with the exception of elevated levels of provitamin A carotenoids, there are no biologically meaningful differences in the composition of GR2E rice and conventional rice. IRRI highlights that β-carotene from synthetic or microbial sources are used as an additive in animal nutrition as a source of provitamin A. IRRI concludes that animal food derived from GR2E rice is as safe and nutritious as animal food derived from conventional rice varieties.

Endogenous Allergens

IRRI summarized what is known about rice allergies in humans, noting that while allergic reactions have been documented, rice is not considered to be a commonly allergenic food.

[10] Samples were analyzed using AOAC International Official Method 941.15 and data were reported as all-*trans*-β-carotene. Values obtained using this AOAC method may be influenced by the presence of other carotenoids. IRRI subsequently analyzed milled rice using a method that further differentiates carotenoids.

[11] Samples were analyzed using high performance liquid chromatography and concentrations of carotenoids were reported for β-cryptoxanthin, all-*trans*-α-carotene, β-carotene (as all-*trans*-β-carotene), 9′-*cis*-β-carotene, and total carotenoids.

[12] IRRI also reported mean β-cryptoxanthin (0.312 μg/g DW), all-*trans*-α-carotene (0.713 μg/g DW), and 9′-*cis*-β-carotene (07.62 μg/g DW).

Summary of Compositional Analyses

IRRI reports that, with the exception of provitamin A carotenoids, no consistent patterns emerged to suggest that biologically meaningful changes in composition or nutritive value of the grain or straw had occurred as an unintended consequence of the genetic modifications. Accordingly, IRRI concludes that human and animal food derived from GR2E rice is as safe and nutritious as food derived from conventional rice varieties.

Food Labeling Considerations

IRRI states that GR2E rice is not currently intended for use in human or animal food in the United States. However, in response to a request from FDA, IRRI explained, on October 9, 2017, its view regarding an appropriate common and usual name for rice derived from event GR2E in the ingredient statement of food products containing this rice if such products were to enter the US food supply.

Although GR2E rice is not intended for distribution in the United States, it is a producer's or distributer's responsibility to ensure that labeling of the foods it markets meets applicable legal requirements. Although the levels of β-carotene in GR2E rice are too low to warrant a nutrient content claim, the β-carotene in the endosperm of GR2E rice results in grain that is yellow-golden in color. We note that the name "Golden Rice" has been used by IRRI and others for many years to identify β-carotene-expressing rice varieties under development. CFSAN's Office of Nutrition and Food Labeling, Food Labeling and Standards Staff (ONFL/FLSS) considers that this name would accurately describe GR2E rice if present in human food. If companies market GR2E rice in human food in the United States, we advise them to consult with ONFL/FLSS to discuss any required or voluntary labeling including statements relating to attributes of this rice or any other type of claim.

FDA's Center for Veterinary Medicine, Office of Surveillance and Compliance, Division of Animal Feeds (OSC/DAF) has determined that "rice" is the appropriate name for GR2E rice if present in animal food. OSC/DAF notes that although GR2E rice is altered in color, color is not an important attribute of animal food unless it would be expected to impact food coloration despite the other ingredients that could be present in the food.

On July 19, 2016, the National Bioengineered Food Disclosure Law (Public Law 114-216) charged the United States Department of Agriculture's Agriculture Marketing Service with developing a national mandatory system for disclosing the presence of bioengineered material in food. Producers, distributers, and marketers of GR2E rice are responsible for following requirements issued by USDA relevant to the labeling of their products.

Conclusion

FDA evaluated IRRI's submission to determine whether GR2E rice raises any safety or regulatory issues with respect to its uses in human or animal food. Based on the information provided by the company and other information available to the agency, FDA did not identify any safety or regulatory issues under the FD&C Act that would require further evaluation at this time.

IRRI has concluded that its rice variety, GR2E rice, and the human and animal foods derived from it are as safe as and, with the exception of β-carotene expression, are not materially different in composition or any other relevant parameter from other rice varieties now grown, marketed, and consumed. At this time, based on IRRI's data and information, the agency considers IRRI's consultation on GR2E rice to be complete.

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