Novel Food Information – Provitamin A Biofortified Rice Event GR2E (Golden Rice)

Health Canada has notified the International Rice Research Institute that it has no objection to the food use of Provitamin A Biofortified Rice Event GR2E (Golden Rice). The Department conducted a comprehensive assessment of this rice event according to its Guidelines for the Safety Assessment of Novel Foods. These Guidelines are based upon internationally accepted principles for establishing the safety of foods with novel traits.

Background:

The following provides a summary of the notification from the International Rice Research Institute and the evaluation by Heath Canada and contains no confidential business information.

1. Introduction

The International Rice Research Institute (IRRI) has developed a genetically modified rice event (GR2E) using recombinant-DNA techniques that is biofortified with provitamin A. GR2E rice will be grown commercially in major rice-producing regions, primarily in Asia. The main intended market for this product is in countries such as Bangladesh and the Philippines where diets are typically low in vitamin A. Intrinsic food supplementation could be a useful tool for alleviating vitamin A deficiency (VAD) in children, a known and preventable cause of blindness. The IRRI has indicated that this product is not intended to be sold in Canada.

The safety assessment performed by Food Directorate evaluators was conducted according to Health Canada's Guidelines for the Safety Assessment of Novel Foods. These Guidelines are based on harmonization efforts with other regulatory authorities and reflect international guidance documents in this area (e.g., Codex Alimentarius). The assessment considered: how this rice event was developed; how the composition and nutritional quality of event GR2E compared to non-modified rice; and the potential for the GR2E rice event to be toxic or cause allergic reactions. The IRRI has provided data that demonstrate that Provitamin A Biofortified Rice Event GR2E is as safe as traditional rice varieties used as food in Canada.

The Food Directorate has a legislated responsibility for pre-market assessment of novel foods and novel food ingredients as detailed in the Food and Drug Regulations (Division 28). Provitamin A Biofortified Rice Event GR2E is considered a novel food under the following part of the definition of novel foods: "c) a food that is derived from a plant, animal or microorganism that has been genetically modified such that

i. the plant, animal or microorganism exhibits characteristics that were not previously observed in that plant, animal or microorganism."

2. Development of the Modified Plant

The petitioner provided information describing the methods used to develop GR2E rice and molecular biology data that characterized the genetic change that results in the accumulation of provitamin A carotenoids. GR2E rice was produced using Agrobacterium tumefaciens (A. tumefaciens) mediated transformation of the japonica rice cultivar Kaybonnet with the transformation vector pSYN12424. This transformation vector was constructed to contain three gene cassettes, one for the crtI gene, one for the Zmpsy1 gene and one for the phosphomannose isomerase (pmi) selectable marker gene.

The crtI gene in the first expression unit derives from the bacteria Pantoea ananatis and is fused in frame at the 5' end with the RUBISCO SSU of Pisum sativum. P. ananatis is a Gram-negative species and is found widely in the environment, both in water and soil and as part of the flora associated with plant and animal hosts. While P. ananatis is an uncommon opportunistic human pathogen, the derived DNA encoding for CRTI lacks similarity to potential pathogenicity determinants. The RUBISCO SSU encodes a transit peptide in order to localize the protein to the chloroplast. The expressed phytoene desaturase enzyme, CRTI, catalyzes the conversion of 15-cis-phytoene to all-trans-lycopene.

The psy1 gene in the second expression unit derives from Zea mays (maize). The expressed enzyme, ZmPSY1, is a phytoene synthase which converts geranylgeranyl diphosphate into phytoene, and acts upstream of CRTI in the carotenoid biosynthesis pathway.

Together the expression of these proteins results in the production of lycopene in rice, which is used by naturally occurring mechanisms in the plant to produce provitamin A carotenoids. The expression of both of these gene cassettes is driven by the rice glutelin promoter (GluA-2), which targets expression to the rice endosperm.

The gene expressed in the third cassette, pmi, encodes a phosphomannose isomerase enzyme derived from Escherichia coli. This enzyme catalyzes the reversible isomerization of mannose-6-phosphate to fructose-6-phosphate. This serves as a selection marker for the ability of transformed calli and plantlets to grow on medium that contains mannose. The expression of pmi is controlled by the maize ubiquitin (ZmUbi1) promoter, associated intron, and 5'-UTR resulting in constitutive expression. The PMI protein has previously been assessed by Health Canada in several authorized maize events.

The original transformant T0, was self-crossed to create the T1 generation, which was then crossed into three Indica rice cultivars, PSB Rc82, BRRI dhan 29, and IR64. Each of these crosses was then backcrossed and self-crossed multiple times until they reached the BC5F4, BC5F3 and BC5F3 generations respectively. The data presented in the molecular characterisation of GR2E rice is from generations throughout the breeding trees of each of the T1 x Indica rice variety crosses.

3. Characterization of the Modified Plant

The number of insert sites for the pSYN12424 plasmid T-DNA and the integrity of the genetic elements were investigated using Southern blot analyses using three probes and a combination of restriction enzymes. The Southern blot data supports the assertion that there is a single intact copy of the T-DNA insert contained in the GR2E rice, containing three expression cassettes for Zmpsy1, crtI, and pmi expression. No partial insertions or rearrangements of the insert were detected. The results were the same between the original genetic background, as well as three hybrid crosses with other lines of Indica rice, which suggests that inheritance of the entire cassette is stable in a breeding program.

Genomic samples from the controls and GR2E rice in the Kaybonnet background were also tested by Southern blot analysis using a set of five probes, which together covered the entire pSYN12424 plasmid backbone. No hybridizing fragments were detected in the GR2E rice samples demonstrating that no part of the plasmid backbone was inserted into the GR2E rice genome.

In order to assess the overall integrity of the insert and all its component elements, as well as to detect any single base-pair changes that may have occurred, the petitioner sequenced the entire insert in GR2E rice. Based on the known sequence of the insert and preliminary sequence information for the 5’ and 3’ flanking regions, seven PCR primer sets were designed to amplify the entire region of interest in overlapping fragments. Two deletions were found in the border regions, 23 bp at the right border and 11 bp at the left border. This type of deletion is common in Agrobacterium-mediated transformation. Aside from the border deletions, the remaining sequence was intact and identical to the T-DNA region of pSYN12424.

The sequenced genomic DNA was subject to ORF analysis in order to assess the possibility that the insertion could have created new unintended proteins that could be homologous to toxins or allergens. The deduced amino acid sequences of the identified ORFs were used to query toxin and allergen databases. No significant hits were returned for either database and it was concluded that the potential spurious ORFs are unlikely to be potential toxins or allergens.

The stability of the inserted DNA was assessed for four generations of GR2E rice using Southern blots. The consistent and expected banding patterns in the Southern blots observed across breeding generations supports the conclusion that the T-DNA is stably integrated and inherited in a typical breeding program.

Trait stability was also investigated by phenotypic analysis, specifically elevated production of β-carotene in the rice grain of the same four generations used in the Southern blots. The presented data demonstrated carotenoid expression was correlated with the presence of the T-DNA, although there was variation in expression levels. However, the observation that carotenoid accumulated in GR2E rice seed contained in different germplasm backgrounds and across several generations supports the conclusion that the carotenoid expression trait is stably inherited.

The petitioner also conducted a study of the inheritance of the inserted T-DNA using a PCR-based method to detect the presence or absence of the insert in progeny from three segregating generations. Statistical analysis of this data demonstrated a segregation pattern consistent with the expected Mendelian pattern for a single insertion site.

Enzyme-linked immunosorbent assays (ELISAs) were used to approximately measure the amount of protein in the different tissues, namely grain and straw (stems) from plants grown in four locations during either the rainy season (2015) or the dry season (2016). The range and mean protein levels were reported for each tissue type as nanograms per gram fresh tissue weight, uncorrected for extraction efficiency. The highest levels were measured in the dough stage grain, with levels ranging from 308-359 ng/g and 54-68 ng/g for ZmPSY1 and CRTI, respectively, across both growing seasons. In mature grain, the highest protein levels detected were 245 ng/g for ZmPSY1 and 30 ng/g for CRTI. The concentration of PMI protein was higher than for the other two expressed proteins, with a mean concentration in mature rice grain of 1282 ng/g across both seasons. PMI was also detected in straw tissue at a mean concentration of 482 ng/g.

Expression of ZmPSY1 and CRTI proteins in GR2E rice is low and thus could not be purified in sufficient quantity for use in subsequent testing. Therefore, these proteins were expressed in a bacterial (i.e., Escherichia coli) expression system and were characterised using SDS-PAGE, reverse phase HPLC, amino acid analysis, MALDI MS/MS peptide mapping, N-terminal amino acid sequencing, and enzymatic activity. Taken together, the data presented by the petitioner supports the equivalence between the E. coli expressed reference proteins and their plant produced counterparts.

The amino acid sequence identity the of PMI protein expressed in GR2E rice is identical to that of PMI expressed in maize events MIR162, 3272 and 5307. Based on this information no additional characterization was required to demonstrate the equivalence to the proteins used in previously submitted studies.

4. Product Information

Provitamin A Biofortified Rice Event GR2E differs from traditional counterparts through the presence of provitamin A in milled rice. All photosynthetic tissues of higher plants produce and accumulate β-carotene. Rice is usually consumed in milled form, which does not contain provitamin A carotenoids. Milling removes the carotenoid-containing embryo and aleurone layer from rice grains to leave only the endosperm. Germplasm screening has not succeeded in identifying any rice cultivars that accumulate provitamin A in the endosperm, and so conventional breeding was not a viable avenue for introducing the desired trait.

Immature rice endosperm produces a precursor for carotenoid biosynthesis, geranylgeranyl diphosphate (GGDP). The incorporation of the genes that produce the enzymes, phytoene synthase (PSY) and multifunctional CRTI, permits GR2E rice to convert GGDP to lycopene. Bridging this gap in the carotenoid biosynthetic pathway allows the endogenous lycopene cyclase enzymes expressed in the rice endosperm to convert lycopene into a mixture of α- and β-carotene, the provitamin A compounds. Rice event GR2E can accumulate up to 30 µg/g total carotenoids in the endosperm, of which about 80% are mixed isomers of β-carotene.

5. Dietary Exposure

GR2E is intended for cultivation and use in a number of South and Southeast Asian countries. However, it may be possible that raw commodity or food products derived from GR2E rice may unintentionally enter Canada via imports from countries of production. The introduction of GR2E rice in the target markets is not expected to alter the use or consumption patterns of rice in Canada.

For the purposes of estimating daily dietary exposure, the petitioner used historic data from the highest rice-consuming countries in Asia. Based on this data, the upper limit of mean daily dietary intake of rice was set at 12.5 g/kg body weight, which accounts for consumption by all subpopulations, including children who are the highest consumers.

Although the petitioner indicated that Canada is not the intended market for this product, Health Canada analyzed baseline intake data for rice and estimated β-carotene intake if all the rice consumed by Canadians was replaced with GR2E rice. Replacement of all rice and rice products in Canada with GR2E rice would result in a very small 0.8-8% (34 µg-239 µg per day) increase in β-carotene intake. It should be noted that this estimate is conservative as it is unlikely that all rice consumed would be GR2E rice containing β-carotene at the highest level measured. A

6. Nutrition

The data provided by the petitioner on the compositional studies conducted on GR2E rice were reviewed by Health Canada to determine whether the use of GR2E rice is safe relative to its conventional counterparts. The efficacy of the GR2E rice in helping vitamin A deficiency in affected populations was not evaluated. B

Compositional data for GR2E rice and its near-isogenic, non-transgenic conventional counterpart were collected from four field trials over 2 years in the rice growing regions of the Philippines. In each study three blocks (replicates) of each entry (event GR2E and control) were planted at each test site in a randomized complete block design. The petitioner states that at each site, planting and cultivation were done according to local agronomic practices. Normal pest control and maintenance practices consistent for the locale were used to produce the rice..

The grain samples were collected from mature rice plants, which represented the state where typical grain harvest occurs. The nutritional components measured in grain, straw, and bran were chosen based on recommendations of the OECD for comparative assessment of the composition of new varieties of rice (2016). The analyses for each component were conducted on all samples by a single laboratory using internationally approved and validated analytical methods and following consistent and appropriate sample storage and preparation procedures.

Specifically, test and control samples were analyzed for nutrients, anti-nutrients, and secondary metabolites, in paddy rice, as follows: Proximate(s) and fiber: moisture, crude protein, crude fat, ash, carbohydrates; fiber (crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF)); Sugars: amylose Minerals: calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc; Vitamins: B1 (thiamine), B2 (riboflavin), B3 (Niacin), B6 (pyridoxine), B9 (folic acid), E (α-tocopherol); Fatty Acid(s): Caprylic (C8:0), Capric (C10:0), Lauric (C12:0), Myristic (C14:0), Pentadecanoic (C15:0), Palmitic (16:0), Palmitoleic (C16:1), Heptadecanoic (C17:0), Stearic (18:0), Oleic (C18:1), Linoleic (C18:2), Alpha-linolenic

(C18:3), Arachidic (20:0), Eicosenoic (C20:1), Eicosadienoic (C20:2), Eicosatrienoic (C20:3), Arachidonic (C20:4), Behenic (C22:0), Erucic (C22:1), Lignoceric (C24:0), Nervonic (C24:1); Amino Acid(s): Alanine, Arginine, Aspartic acid, Cysteine, Glutamic acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Trytophan, Tyrosine, Valine; Anti-nutrients: Phytic acid, and Trypsin inhibitor.

The data collected from the multi-year combined site studies were analyzed using statistically appropriate methodology. Where a statistically significant difference ($P < 0.05$) was identified in the multi-year combined site analysis, further context for interpreting the possible biological significance of the difference was gathered through comparisons with the range of values for each analyte reported in the published literature or available from the ILSI Crop Composition Database. Analyte ranges for GR2E rice that fell within the combined literature range for that analyte were considered to be within the range of normal variability of conventional rice.

In addition to the compositional components listed above, milled rice samples of GR2E and control were also analysed for all-trans-β-carotene and other carotenoids (β-cryptoxanthin, all-trans-α-carotene, 9′-cis-β-carotene, and total carotenoids). The concentrations of all-trans-β-carotene ranged from 1.96–7.31 µg/g dry matter across locations and years and on average comprised of approximately 59% of the total carotenoids as determined by HPLC. This was followed by all-trans-α-carotene (12%) and β-cryptoxanthin (5%).

With the exception of provitamin A carotenoids, which were intended to be elevated in the GR2E rice, there were no significant differences observed between the non-genetically modified conventional counterpart and the GR2E rice for any of the proximates, minerals, amino acids, vitamins, fatty acids (except for stearic acid), and secondary metabolites. The mean value for stearic acid was within the range reported values found in literature and the ILSI database.

The absorption of β-carotene from foods ranges from ~2% to 65%. Several factors affect the bioavailability of carotenoids including the food matrix, extent of cooking, and amount of co-ingested fat, e.g. in a typical mixed North American diet, β-carotene absorption ranges from 12-16%. There is negative feedback regulation of β-carotene intestinal absorption and of conversion due to increasing cellular levels of all-trans-retinoic acid. The vitamin A equivalency for β-carotene from foods ranges from 3.8:1 (Golden Rice) to 28:1(leafy green vegetables) but even for pure β-carotene in oil, extremes of 55:1 or more have been reported based on vitamin A status and genetic polymorphisms, e.g., in the genes coding for enzymes involved in the conversion of β-carotene to vitamin A (Haskell 2012). The Institute of Medicine currently estimates a vitamin A equivalency ratio for plant sources of β-carotene is 12:1 by weight (i.e. 12 µg β-carotene is equal to 1 µg retinol), whereas for β-cryproxanthin and α-carotene is 24:1. The provitamin A activity of 9-cis and 13-cis isomers of β-carotene is less than 10% of all-trans-β-carotene.

If adequate retinol is provided by the diet, there are no known clinical effects of consuming diets high in carotenoids over the short term. Harmless skin discolouration in the form of carotenodermia (yellow discolouration) or lycopenodermia (orange discolouration) is the only observed adverse effect associated with the excess consumption of carotenoids from food and supplements. The condition is reversible when carotene ingestion is discontinued. The Institute of Medicine has not set any DRIs specifically for carotenoids.

7. Chemistry/Toxicology

The petitioner used data bridged from previously approved GM corn submissions to support the toxicological safety of PMI protein. The data included an acute oral toxicity study performed with mice (6 to 7 mice/sex/group) given microbially produced PMI and a bioinformatics assay using the predicted amino acid sequence of PMI. The results of these studies showed that PMI does not share amino acid sequence homology with known toxins and no adverse effects were reported in treated-mice.

Similarly, the petitioner used data bridged from previously approved GM corn submissions to support the allergenic safety of PMI protein. The data included simulated gastric fluid, simulated intestinal fluid, thermostability and bioinformatics assays. The results of these studies showed that PMI does not share significant amino acid sequence homology with putative allergens (e.g. 35% identity over 80 amino acids). PMI did share homology with an 8 amino acid sequence with frog α-paravalbumin; however, this epitope was not found to be clinically relevant as determined by an IgE screening assay. Further, PMI readily degraded and digested under conditions normally found during food preparation and in the gastrointestinal tract. As such, intact and functional PMI protein is not expected to be systemically available or pose an allergenic health concern to consumers.

The predicted amino acid sequences of the ZmPSY1 and CRTI proteins were compared to sequences of known toxins retrieved from Uniprot Knowledgebase (550 116 sequences), Swiss-Prot (6588 sequences) and TrEMBL Uniprot Consortium databases (17 510 sequences). A single match (37% sequence homology over 81 amino acids within the N-terminal region, 30 amino acids out of 492 amino acids) was found between CRTI and a sequence in a known toxin. However, this known toxin is not an oral toxin and the sequence similarity is in a cofactor binding domain that is common in many metabolic enzymes; this domain is not responsible for the catalytic and toxic activity of the known toxin. Taken together, there is no indication that this small homology would alter the oral safety of CRTI. This argument is further supported as CRTI is readily denatured and digested as demonstrated by the in vitro heat stability and simulated gastric fluid assays, and CRTI showed an absence of toxic effect in the acute oral toxicity study. Thus, no significant matches were found between ZmPSY1 and CRTI proteins and the oral toxins listed in these databases. It was concluded that ZmPSY1 and CRTI do not share sequence similarity with known oral toxins.

GR2E rice expresses a ZmPSY1 protein that was originally derived from corn. ZmPSY1 protein is produced in the endosperm of corn kernels and facilitates the accumulation of carotenoids, which confers a yellowish or orange colour to the kernel. Farmers have selected for yellow and orange corn due its higher levels of lutein, β-carotene and β-cryptoxanthin as a source of vitamin A. By consuming these varieties, humans have had a history of safe food exposure to Z. mays with elevated levels of ZmPSY1 protein.

Due to the absence of a history of safe food exposure to CRTI, the petitioner performed an acute oral toxicity study, that was compliant with Good Laboratory Practices and Organisation for Economic Co-operation and Development (OECD) guidance, to assess the potential toxic effects of CRTI in male and female CRl:CD1 (ICR) mice. Animals (5 mice per sex per group) were administered 100 mg CRTI protein/kg b.w. or 100 mg bovine serum albumin protein/kg b.w.

(control) or vehicle (buffer) administered by gavage as two doses given four hours apart. Animals were monitored for 15 days and then euthanized for necropsy. All animals survived to scheduled sacrifice. No clinical abnormalities or differences in body weight were observed between CRTI-treated animals and controls. A no observed adverse effect level (NOAEL) of 100 mg CRTI protein/kg b.w. was determined.

Children consuming up to 12.5 g rice/kg b.w./day (0.85 µg CRTI/kg b.w./day) will have CRTI exposure levels that are approximately five orders of magnitude less than the NOAEL (100 mg/kg b.w./day) reported in the acute oral toxicity study conducted with mice. This margin of exposure (MOE) for CRTI protein is sufficiently large to be protective of consumer safety.

The petitioner performed a bioinformatics analysis using the predicted amino acid sequence of the ZmPSY1 and CRTI proteins and compared it with sequences of known allergens retrieved from the AllergenOnline database (2016; 1956 sequences). The proteins did not share ≥ 35% amino acid identity with any known allergen or contain potential allergen epitopes. Based on the results of the bioinformatics analysis, it was concluded that ZmPSY1 and CRTI did not match known allergens.

The petitioner demonstrated that microbial ZmPSY1 and CRTI proteins lost enzymatic activity when incubated at temperatures equal to or greater than 50 and 55 ºC, respectively, for 15 minutes. The processing and cooking of GR2E rice products will generally require temperatures that greatly exceed 55°C which will help degrade and/or denature the ZmPSY1 and CRTI proteins in the final food product. These actions will result in a reduced amount of intact or active ZmPSY1 and CRTI in the human diet.

Microbial-derived ZmPSY1 and CRTI were found to be completely digested in simulated gastric fluid (SGF; 10 U pepsin per µg test protein; pH ~ 1.2; incubated at 37 °C) within 5 minutes and 30 seconds, respectively, as visualized by stained SDS-PAGE gel and western blot. As such, ZmPSY1 and CRTI proteins are expected to be digested under the conditions normally found in the stomach such that no intact and functional protein would be absorbed in humans to initiate an allergic response.

Conclusion:

Health Canada’s review of the information presented in support of the food use of Provitamin A Biofortified Rice Event GR2E does not raise concerns related to food safety. Health Canada is of the opinion that food derived from this event is as safe and nutritious as food from current commercial rice varieties.

The petitioner has been informed that, if in the future, there is an interest in selling this rice in Canada, compliance with the Food and Drug Regulations regarding the addition of vitamins to foods would be required. Similarly, they have been informed that, due to the increased levels of provitamin A, the common name of any food products derived from this rice would be required to differentiate this rice from conventional varieties.

Health Canada's opinion deals only with the food use of Provitamin A Biofortified Rice Event GR2E.

This Novel Food Information document has been prepared to summarize the opinion regarding the subject product provided by the Food Directorate, Health Products and Food Branch, Health Canada. This opinion is based upon the comprehensive review of information submitted by the petitioner according to the Guidelines for the Safety Assessment of Novel Foods.

(Également disponible en français)

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