CONSOLIDATED REPORT OF PHILRICE AND IRRI'S GR2E RICE APPLICATION FOR DIRECT USE AS FOOD AND FEED, OR FOR PROCESSING*

EXECUTIVE SUMMARY

On February 28, 2017, Philippine Rice Research Institute (PhilRice) and International Rice Research Institute (IRRI) submitted GR2E Rice application for direct use as food and feed, or for processing to the Bureau of Plant Industry (BPI) under the DOST-DA-DENR-DOH-DILG Joint Department Circular (JDC) No. 1 Series of 2016. After reviewing the accomplished biosafety forms and socio-economic, ethical and cultural questionnaire, and supporting documents submitted by the applicant, the assessors namely: Scientific and Technical Review Panel (STRP), BPI- Plant Products Safety Services Division (BPI-PPSSD) and Bureau of Animal Industry (BAI), concurred that GR2E Rice is as safe for human food and animal feed as its conventional counterpart.

The Department of Environment and Natural Resources – Biosafety Committee (DENR-BC), after a thorough scientific review and evaluation of the documents related to Environmental Risk along with the submitted sworn statement and accountability of the proponent, recommended the issuance of a biosafety permit for this regulated event provided that the conditions set by them are complied.

The Department of Health – Biosafety Committee (DOH-BC), after a thorough scientific review and evaluation of documents related to Environmental Health Impact, concluded that GR2E Rice will not pose any significant risk to health and environment and that hazards, if any, could be managed by the measures set by the department. DOH-BC also recommended for the issuance of biosafety permit for GR2E Rice.

Furthermore, the Socio-economic, Ethical and Cultural (SEC) expert also recommended for the issuance of biosafety permit for this regulated article after assessing the socio-economic, social and ethical indicators for the adoption of Genetically Modified Organisms.

BACKGROUND

In accordance with Article VII. Section 20 of the JDC, no regulated article, whether imported or developed domestically, shall be permitted for direct use as food and feed, or for processing, unless: (1) the Biosafety Permit for Direct Use has been issued by the BPI; (2) in the case of imported regulated article, the regulated article has been authorized for commercial distribution as food and feed in the country of origin; and (3) regardless of the intended use, the regulated article does not pose greater risks to biodiversity, human and animal health than its conventional counterpart.

The BPI Biotech Office provided the assessors the complete dossier submitted by Philippine Rice Research Institute (PhilRice) and International Rice Research Institute (IRRI).

Upon receipt of the individual reports from the assessors, the BPI Biotech Office prepared this consolidated risk assessment report for the information of the public.

*This document is subject to DA-BC review

SCIENTIFIC AND TECHNICAL REVIEW PANEL (STRP) ASSESSMENT AND RECOMMENDATION

HOST ORGANISM

Rice has a long history of use as processed food for at least 4000 years. In OECD 2015, it was reported that rice is cultivated in more than 100 countries in the world and is a staple food of about half of the world's population. The worldwide production area of rice is nearly 150 million hectares and the annual production of rice (paddy rice) is 590 million tonnes (FAO, 2004). Asia is the main producer of rice with 92% of total world production. The country with highest production of rice is People's Republic of China with 189 million tonnes, or 31% of total production. The second is India with 133 million tonnes or 22%. India has the largest production area with 43 million hectares. Rice is mostly consumed in each producing country. The trade amount of rice is about 25 million tonnes which is less than 5% of total production. Rice accounts for over 20% of total global caloric intake (FAO, 2001).

Rice is seldom used for animal feed, although the by-products coming from rice milling like bran and husks are utilized for feed purposes.

According to OECD, 2015, most of the available carbohydrates such as starch are found in the endosperm of rice grain. Starch, the principal component of rice, consists mainly of amylose and amylopectin. The key protein in rice is glutelin and the most limiting amino acid is lysine. Most of the rice lipids are neutral. The fat content of rice grain is concentrated primarily in the germ, aleurone layer, and sub-aleurone layer, mostly as triglycerides in which glycerol is esterified with three fatty acids, oleic, linoleic and palmitic acid. It was also reported that rice is not a significant source of micronutrient minerals.

For the anti-nutrients of rice, it was reported by assessors that rice, as consumed, is not considered a source of anti-nutrients, however, the bran has phytic acid, trypsin inhibitors, lectins, oryzacystatin and subtilizin inhibitors. Except for phytic acid, the rest are proteinaceous and are denatured by heat.

Furthermore, two STRPS reported that rice is not a source of toxicants. One assessor reported that according to IRRI, arsenic, cadmium, mercury, and lead are four ubiquitous trace elements known to have a harmful effect on human health. These elements are naturally present at very low concentrations in the environment, and human bodies are able to detoxify them in limited amounts. Rice plants can take up these toxic elements from polluted soil or irrigation water. However, IRRI also reported, and the assessors agreed that consumers need not change their rice-eating habits based on any known risks from toxic elements. Scientists can now detect very low amounts of these elements in rice grains. Some studies are being done on how these elements move within soil and rice plants.

As source of allergens, the assessors agreed with the applicant that rice is not considered to be a common allergenic food although there are two putative rice food allergens: *Oryza* trypsin alpha-amylase inhibitors and *Oryza* glyoxalase I.

TRANSGENIC PLANT

For the list of countries that have approved GR2E rice as food, the assessors have reported that FSANZ has already finished their biosafety assessment and has drafted a report already and is available at http://www.foodstandards.gov.au/code/applications/Pages/A1138GM riceGR2E.aspx. In addition, GR2E rice has not yet been approved in any country for use in feed.

Furthermore, the assessors agreed with the applicant that there will be no change in the consumption pattern of rice as a consequence of the introduction of GR2E rice.

DONOR ORGANISM

The assessors have reported that the protein-encoding sequences (for the proteins phytoene synthase, carotene desaturase and phosphomannose isomerase, and for the transit peptide signal sequence) were properly described by the applicant with respect to source, and potential pathogenic/ allergenic properties. In addition, the documents provided adequately described the potentially inserted regulatory sequences.

The introduced expressible sequences are: *crtI* gene from *Pantoea ananatis* (Misawa et al., 1990) that is fused in-frame at the 5' terminus with the pea (*Pisum sativum)* RUBISCO SSU transit peptide encoding sequence (Coruzzi et al., 1984); *psy1* gene, isolated from Zea Mays (Buckner et al., 1996) under the control of the rice *GluA-2* promoter with termination sequences derived from the 3' untranslated region of the *A. tumifaciens* NOS. Expression of phytoene synthase (*Zm*PSY1) in the rice endosperm catalyzes the conversion of geranylgeranyl diphosphate to phytoene; *pmi (*phosphomannose isomerase) gene from *Escherichia coli* (Miles and Guest, 1984). Expression of the pmi gene is controlled by the maize polyubiquitin promoter (Christensen et al., 1992), providing constitutive expression of the PMI protein in rice.

Pantoea ananatis was the source of the *crtI* gene (Misawa et al., 1990). According to De Maayer et al., 2014, although strains of *P. ananatis* have been found to be pathogenic on a broad range of plant hosts as well as humans, a recent analysis of the *P. ananatis* genome to identify potential molecular determinants of its underlying pathogenicity revealed the absence of many of the factors that are central to the pathogenicity and virulence arsenal of related plant and animal pathogens, including animal toxins, hemolysins, phytotoxins, and their associated effectors.

Zea mays (maize) was the source of the *psy 1* gene (Buckner et al., 1996) and the polyubiquitin promoter (Christensen et al., 1992). No significant endogenous toxins are reported to be associated with the genus *Zea* (IFBC, 1990). Food allergy to maize is relatively rare, and the only significant reported food allergen is a non-specific lipid transfer protein (Pastorello et al., 2000).

Pisum sativum (pea) is the source of the ribulose-1,5-bisphosphate carboxylase (RUBISCO) small sub-unit (SSU) transist peptide signal sequence (Coruzzi et al., 1984) that is fused to the N- terminus of the CRTI protein to direct transport to the chloroplasts. There are few reports of allergy to garden peas, with clear IgE binding to a few major proteins (Sanchez-Monge et al., 2004; Sell et al., 2005), but no reports of IgE binding to the chloroplast transit peptide sequence or the full- length or mature RUBISCO.

Escherichia coli strain K12, a non-pathogenic strain, was the source of the *pmi* gene (miles and Guest, 1984). Certain serotypes are enteropathogenic and are known to cause diarrhea in infants. Some strains also cause diarrhea in adults. *E. coli* is a normal inhabitant of the intestinal flora of humans and animals, where it generally does not cause disease.

Other donor organisms, including *Oryza sativa* and *Agrobacterium tumifaciens* were used as sources of gene regulatory sequences that are not expressed in the transformed plant. These sequences include the *glutelin promoter* (*GluA-2)* from rice and the nopaline synthase (NOS) 3' untranslated regions from *A. tumifaciens*. Since none of these sequences encode expressed products in GR2E rice, their donor organisms are of little relevance to assessing potential toxicity or allergenicity.

All assessors reported that all proteins encoded by the expressible sequences such as ZmPSY1, CRTI, and PMI were not known to be toxic or allergenic.

TRANSFORMATION SYSTEM

The transformation method used, as confirmed by the assessors, was *Agrobacterium*-mediated transformation. Nuclear DNA was the target of the modification. The transformation was performed using plasmid pSYN12424 which contains three gene expression cassettes within the T-DNA:

The first cassette contains a copy of the *crtI* gene from *Pantoea ananatis* (Misawa et al., 1990. Transcription of the *crtI* gene is controlled by the rice *GluA-2* promoter (Takaiwa et al., 1987) for targeted expression in the rice endosperm. Transcription termination of the *crtI* gene is provided by the polyadenylation signal and 3' untranslated region from the NOS gene of *Agrobacterium tumifaciens* Ti-plasmid pTiT37 (Depicker et al., 1982). The CRTI enzyme catalyzes the conversion of 15-*cis*-phytoene to all-*trans*-lycopene (Schaub et al., 2012).

The second cassette contains the *psy1* gene, isolated from *Zea mays* (Buckner et al., 1996).

The third and final cassette contains a copy of the phosphomannose isomerase (*pmi)* gene from *Escherichia coli* (Miles and Guest, 1984). This region also includes the 5' untranslated region (UTR) and intron associated with the native polyubiquitin promoter.. Transcription termination is via the NOS 3' untranslated region from *A. tumifaciens*. Expression of PMI catalyzes the reversible isomerization of mannose-6-phosphate to fructose-6-phosphate, allowing positive selection of transformed calli and plantlets on mannose-containing medium (Negrotto et al., 2000).

INSERTED DNA

The STRP reported that Southern blot analysis showed that there was one insertion site for GR2E. Integrity and order were demonstrated by the results of the Southern blot analysis wherein the restriction enzyme digests of GR2E were shown to hybridize with probes for *Zmpsy, pSSU-crt1 and pmi.*

In addition, the STRP confirmed that based on nucleotide sequencing of the inserted DNA and the flanking regions in the DNA of GR2E rice, it was shown that there were deletions of 15

base pairs (bp) in the rice DNA, in addition to truncations in the left and right borders of the insert of 11 bp and 23 bp, respectively.

It was reported that bioinformatics analyses were conducted to evaluate any open reading frames (ORFs) created as a consequence of the T-DNA insertion to assess their potential to encode amino acid sequences with significant similarity to known toxins or allergens. It was found out that no new novel ORFs were created as a consequence of the DNA insertion that would have the potential to encode proteins with any significant amino acid sequence similarity to known or suspected toxins or allergens.

Moreover, the assessors confirmed that the main transgenes for phytoene synthase and carotene desaturase have not been expressed in other crops, but the marker PMI has been used in GM corn MIR602, MIR 162, 3272, and 5307, that have been approved in several countries. When said corn events were reviewed, the safety of PMI as selectable marker was also reviewed and found safe. Plasmid backbone sequences were not present in GR2E, as determined by Southern blot analysis. The analysis was sufficient for detecting presence of backbone sequences.

GENETIC STABILITY

Assessors confirmed that the stability of the inserted DNA across multiple generations of GR2E was assessed by Southern blot analyses of genomic DNA samples prepared from T_n generation (Kaybonnet) and the BC_3F_5, BC_4F_3 and BC_5F_3 generations in GR2E in BRRI *dhan* 29 ,IR64, and PSB R*c*82 germplasm. Restriction enzyme digestions with *Hin*dIII, *Sph*I, and *Asc*I+*Xma*I were separated by agarose gel electrophoresis and blots were probed with DNA probes specific for the *Zmpsy1, pssU-crtl,* or *pmi* genes, respectively. Single hybridizing fragments of ~7900 bp, ~ 6900 bp, or 8747 bp were detected using the *Zmpsy1, pssU-crtl,* or *pmi* probes, respectively, in corresponding Southern blots of *Hin*dIII, *Sph*I, and *Asc*I+*Xma*I digests of genomic DNA from each generation of GR2E rice. The observation of consistent hybridization patterns for each tested probe across each of the plant generations confirmed stable integration and inheritance of the inserted DNA in GR2E rice (Cueto et al., 2016).

The demonstration of carotenoid accumulation in the endosperm of GR2E rice samples collected from different germplasm backgrounds across four breeding generations (T_n, BC_3F_5, BC_4F_3 and BC_5F_3) provided further evidence that the introduced phenotypic trait was stable and inherited across multiple generations (Swamy and Samia, 2016).

Inheritance pattern was investigated using a PCR-based zygosity test to determine segregation of the inserted DNA. Three different segregating backcross generations in each of three different genetic backgrounds were tested. The results from these analyses were consistent with Southern blot hybridization data indicating the stable integration of the pSYN12424 T-DNA at a single site within the GR2E rice genome and segregation of the introduced DNA as a single genetic locus according to Mendelian rules of inheritance.

EXPRESSED MATERIAL

The level of expression of novel protein was determined and presented for all the 3 expressed proteins using ELISA and Western blot analysis. Using ELISA, the highest expression was observed in mature grains, and was not detected in the roots, stem (except for PMI), foliage and pollen. Results of Western blot analysis were positive only for PMI, which was detected

in roots, stem, foliage and grain. This is expected since PMI is controlled by the constitutive promoter polyubiquitin.

The assessors agreed with the applicant that the expression of the ZmPSY1 and CRTI enzymes in the rice endosperm complete the carotenoid biosynthetic pathway leading to the accumulation of ß-carotene in the endosperm.

TOXICOLOGICAL ASSESSMENT

ZmPSY1 Protein

Pepsin was used in a standardized digestion model. It was reported that the in vitro pepsin digestibility of ZmPSY1 protein was investigated by Oliva (2016) by incubating purified ZmPSY1 protein for 0, 0.5, 1, 2, 5, 10, 20, 10 and 60 minutes at 37^0C in the presence of simulated gastric fluid (SGF) pH 1.2 containing pepsin. Following exposure to SGF containing pepsin for 30 seconds, the earliest time point sampled during digestion, no intact ZmPSY1 protein (ca. 42 kDa) was evident as assessed by either SDS-PAGE or western immunoblot analysis. Low molecular weight degradation products were visible in samples removed up to two minutes of digestion, but not at later time points, and these were not detected in the western blot.

On the other hand, thermal stability was measured by measuring enzymatic activity. Data showed that after heat treatment at 42 degrees C, activity of ZMPSY1 was at 50%, while pre-incubation at 50 degrees C for 15 minutes completely inactivated phytoene synthase. The methods used to measure the effect of temperature on enzyme activity were sufficient to show that temperatures lower than those used for cooking rice can inactivate phytoene synthase.

For amino acid comparison, the ZmPSY1 protein (with and without the transit peptide) did not show a match at an E (0) lower than $1x10^{-5}$, and as such does not show similarity to known toxins.

Furthermore, the acute oral gavage study was not performed for ZmPSY1 protein. According to the STRP, reason presented by applicant is acceptable in consideration of the evidence presented showing safety of ZmPSY1; that there is no toxin/allergen homology, it had high digestibility and underwent heat inactivation.

CRTI protein

The STRP reported that the *in vitro* pepsin digestibility of CRTI was investigated by Oliva and Cueto (2016) by incubating purified CRTI protein for 0, 0.5, 1, 2, 5, 10, 20, 10 and 60 minutes at 37^0C in the presence of simulated gastric fluid (SGF) pH 1.2 containing pepsin. CRTI protein was rapidly and completely digested when incubated in SGF containing pepsin. Following 30 seconds of incubation, the earliest time point sampled during digestion, no intact CRTI protein was evident as assessed by either SDS-PAGE or western immunoblot analysis. These data support the conclusion that CRTI protein will be readily digested as conventional dietary protein in a typical mammalian gastric environment, and there would not be a concern of increased potential allergenicity or toxicity due to stability of the CRTI protein in pepsin.

CRTI activity was lost by 50% following 15 minutes incubation at 51^0C. Activity was totally lost when CRTI was incubated at 55 degrees for 15 minutes. Enzyme activity was measured using spectrophotometric assay.

It was reported that the CRTI protein was shown to share homology with 3 known toxins from snake venom. However, there was limited sequence homology with these snake venom proteins. The similarities were at the N-terminal motifs, which is not considered as structural alert for sequence similarity.

Acute oral gavage was performed for CRTI in mice. The study done by Mukerji (2016) evaluated the acute toxicity of the test substance, CRTI protein, in Crl:CD1 (ICR) mice following oral exposure at 100 mg/kg (administered in a split dose over a period of approximately 4 hours). All animals survived to scheduled euthanasia. There were no clinical abnormalities or overall (test day 1-15) losses in body weight among any of the animals tested. Gross findings were limited to a discolored tooth in one animal, which was considered non-specific. Under the conditions of this study, oral administration of CRTI protein to male and female mice at 100 mg/kg did not result in mortality or other evidence of acute oral toxicity, based on evaluation of body weight, clinical signs, and gross pathology. Therefore, an LD_{50} was not determined.

PMI protein

Pepsin was used in the digestibility study of PMI protein. The susceptibility of PMI to proteolytic degradation in simulated mammalian gastric fluid was evaluated using SDS PAGE and Western blot analysis. The results showed that PMI was readily degraded with no intact protein or degradation fragments detected following digestion for one minute.

The heat stability of PMI protein was also evaluated by measuring enzymatic activity following pre-incubation for 30 min at 25-95 C. PMI enzymatic activity was below limits of quantification following pre-incubation at 65°Celsius and above.

For amino acid sequence comparison, the STRP reported that no homologies were found with known/putative toxins using BLASTP.

Moreover, Korgaonkar in 2009 performed single-dose oral (gavage) toxicity study using test substance PMI-0105, containing the active ingredient phosphomannose isomerase protein (89.5% purity w/w), which was administered as a single oral gavage dose to groups of five male and five female Crl:CD-1(ICR) mice at 0 or 2000 mg active ingredient/ kg body weight. All animals survived the 14-day observation period following dosing, up until the scheduled necropsy. There were no test substance-related clinical observations. There were no test substance-related effects on body weight or weight gain, food consumption or hematology parameters. There were no macroscopic or microscopic findings that were attributable to the test substance (Korgaonkar, 2009).

Higher urea nitrogen levels (males only), slightly higher alkaline phosphatase levels (males only) and slightly higher alanine aminotransferase levels (females only) were noted in the 2000 mg/kg group compared with the control group, and were considered test substance-related. However, these changes in serum chemistry parameters were considered non-

adverse as there were no histopathological correlates and the group mean values were within WIL Historical control ranges (Version 2.5), except for one male mouse which had urea nitrogen levels exceeding the historical control range (Korgaonkar, 2009).

Statistically significantly lower testicular and epididymal weights in males and slightly higher adrenal weights in females were noted in the 2000 mg/kg group compared with the control group, and were considered test substance-related. However, there were no distinct microscopic changes in these organs, and the organ weights were within the WIL Historical control ranges (Version 2.6), suggesting that organ weight alterations probably represented physiologic responses of a non-adverse nature (Kargaonkar, 2009).

The assessors agreed with the applicant that the ZmPSY1, CRTI, and PMI proteins are independently expressed. Functional activity of both the ZmPSY1 and CRTI proteins is evidenced by the accumulation of b-carotene in GR2E rice endosperm, which would not occur if either of these proteins was inactive. Functional expression of the PMI protein was evidenced by the ability to select transformed plantlets in tissue culture on media containing only mannose as a carbon source.

In addition, ZmPSY1 and CRTI are expressed in plastids while PMI is expressed in the cytoplasm. Only the ZmPSY1 and CRT1 enzymes interact in a metabolic pathway. Their sequential activities complete the carotenoid biosynthetic pathway in the GR2E rice endosperm. The condensation of two molecules of geranylgeranyl diphosphate to yield the first carotenoid C40 15-cis-phytoene, is catalyzed by ZmPSY1. CRT1 catalyzes consecutive modifications of phytoene, including desaturation and isomerization to form all-trans-lycopene. The endogenous rice B-cyclase enzyme is responsible for production of B-carotene from all-trans-lycopene.

ALLERGENICITY ASSESSMENT

ZmPSY1 Protein

Pepsin in SGF was used and phytoene synthase was found to be digested with T_{50} of less than 30 seconds. In addition, data presented shows that phytoene synthase was inactivated by heat, with T50 of 51°C. No amino acid homology with known allergens were found in allergen database.

It was reported that the expression of the *Zm*PSY1 proteins in event GR2E is driven by the endosperm-specific rice *GluA-2* promoter and measurable concentrations of this protein were found in all grain developmental stages but not in stem tissue (straw). The highest concentration was measured in samples of dough-stage grain (BBCH 85) ranging between *ca.* 308-359 ng/g. Across the four locations and two growing seasons, the highest concentration for *Zm*PSY1 measured in samples of mature grain was 245 ng/g FWT. Total protein intake in grain was 8.1% DB; moisture content was 12.26%. Therefore, *Zm*PSY1 was 0.00034% of total protein.

Serum screening was not performed since the introduced gene product was found to be not a major allergen.

CRTI Protein

Digestibility study used pepsin in simulated gastric fluid. Results showed that T_{50} was less than 30 secs, and no fragments were observed after exposure to pepsin in SGF.

The thermal stability of the phytoene desaturase (CRTI) protein was evaluated by measuring enzymatic activity using a spectrophotometric assay to monitor the conversion of liposome-incorporated 15-*cis*-phytoene to all-*trans*-lycopene. Samples of microbial-expressed CRTI protein were subjected to heat treatment over a temperature incubation range of *ca* 30-60^{0}C for 15 minutes, following which enzyme activity was measured at 37^{0}C in the presence of 7µM phytoene, 150 µM Flavin adenine dinucleotide (FAD), 50 mM Tris-HCl pH8.0, and 200 mM NaCl. Under conditions of this study, the CRTI enzyme lost half of its activity when pre-incubated at *ca*. 51^{0}C for 15 minutes and was completely inactivated following pre-incubation at 55^{0}C for 15 minutes. The temperatures required to completely inactivate the CRTI enzyme were significantly lower than temperatures normally employed during cooking or processing, and it is therefore expected that dietary exposure to functional CRTI will be negligible (Schaub and Beyer, 2016).

The 492 amino acid sequence encoded by the CRTI gene was compared to a peer reviewed database of 1956 known and putative allergen and celiac protein sequences found in FARRP16 dataset at University of Nebraska. No identity matches of >35 % over 80 residues were observed and no matches of eight contiguous identical amino acids.

It was reported that CRTI was 0.00004% of total protein. Computed from 0.03 ug/gFWT of CRTI in mature grain, Total Protein content in grain was 8.1% DB, moisture content was 12.26%.

PMI Protein

The assessors reported that the digestibility study used pepsin in simulated gastric fluid. Results showed that T_{50} was less than 30 seconds, and no fragments of PMI were observed after exposure to pepsin in SGF.

Furthermore, no significant sequence similarity between any 80 amino acid peptide of the PMI amino acid sequence and any entry in the FARRP AllergenOnline database (2010).

Further investigation was done by using sensitive serum screening methodology. No cross reactivity between PMI and the serum from the single individual known to have demonstrated IgE-mediated allergy to this specific a-paravalbumin was found. The patient's serum did not recognize any portion of the PMI protein as an allergenic epitope. Thus, the sequence identity between PMI an a-paravalbumin from Rana species CH2001 is not biologically meaningful and has no implications for the potential allergenicity of PMI.

NUTRITIONAL DATA

For the proximate and fibre analysis of straw, the assessors concluded that there were no statistically significant differences in proximates and fibre between samples of straw obtained from GR2E and control PSB Rc82 rice.

Furthermore, the mean values for all proximates and fibre in rice straw were similar to the range reported in literature with the exception of moisture content, which is dependent on the extent of drying straw following harvest.

On the other hand, comparison of proximates and fibre in grain (paddy) samples derived from GR2E and control PSB Rc82 rice grown during the rainy season resulted in no statistically significant differences in ash, crude fat, crude protein, carbohydrate, amylose, moisture, acid detergent fiber (ADF), neutral detergent fiber (NDF), and total dietary fiber (TDF). Although there was a statistically significant difference in the mean concentration of crude fibre between samples of GR2E and PSB Rc82 rice grain, the difference was relatively small (10.5 percent) and unlikely to be biologically meaningful.

Some of the parameters like crude fat, acid detergent fiber, neutral detergent fiber and starch were not within the literature values; however, for these values, there were no statistically significant differences between GR2E rice and control PSB Rc82 rice.

For the proximate and mineral analysis of bran, no statistically significant differences were noted for any of the measured parameters between bran samples derived from GR2E and control PSB Rc82 rice grain. The measured values for the analytes were within the respective ranges reported in the literature except for crude fat and phosphorus which were slightly higher in both GR2E and control PSB Rc82rice.

For the analysis of minerals in straw, the assessors confirmed that there were no statistically significant differences in concentrations of calcium and phosphorus measured in samples of GR2E and control PSB Rc82 rice straw across locations and growing seasons. Calcium and phosphorus levels in straw samples of GR2E and PSB Rc82 were within range reported in the literature.

Moreover, comparison of the mineral composition in samples of GR2E and control PSB Rc82 rice grain did not reveal any statistically significant differences in the concentrations of any measured analytes. The mean concentrations of each of the minerals measured in samples from GR2E and control PSB Rc82 rice grain were within the ranges reported in the literature.

A comparison of amino acid composition of event GR2E and control PSB Rc82 rice (grown during the rainy and dry season) grain showed no statistical differences in the concentrations of any amino acids between samples of Gr2E and PSB Rc82. The mean concentrations of each of the amino acids except tryptophan (lower but not statistically different) in samples from GR2E and PSB Rc82 rice were within the ranges of literature values.

In addition, samples of event GR2E and control PSB Rc82 rice grain were analyzed for concentrations of the water-soluble B vitamins (thiamine, riboflavin, niacin, pantothenic acid, pyridoxine, and folic acid), β-carotene, and α-tocopherol (Vitamin E). Except for B-carotene which was intended to be elevated in GR2E rice, there were no statistically significant

differences noted in the concentrations of any measured vitamins between GR2E and control PSB Rc82 rice.

Comparison with a range of values from the literature showed that GR2E had detectable and high values for β- carotene. All other vitamins were within the published range of values.

For the analysis of fatty acids in grain, it was reported that the only statistically significant different observed between GR2E and control PSB Rc82 rice samples was in the concentration of stearic (C18:0) acid which was ~ 6.5% higher for GR2E rice. The data for the grain fatty acids from GR2E were within the range reported in the literature.

Analysis of anti-nutrients present in grain were also conducted. The assessors have confirmed that there were no statistically significant differences in the concentrations of phytic acid or in levels of trypsin inhibitor between samples of GR2E and PSB Rc82 control rice. On the other hand, data on levels of phytic acid and trypsin inhibitor in conventional rice grain are limited or non-existent. Mean concentrations of phytic acid in grain samples from GR2E and control PSB Rc82 rice were both slightly outside the range reported from the ILSI Crop Composition Database, but were not significantly different.

The assessors concurred with the applicant stating that the trypsin inhibitor is affected by heat and activity is expected to be significantly reduced following cooking. On the other hand, concentrations of phytic acid in rice may be reduced up to 82 percent when rice is cooked after steeping in excess water and then the excess water discarded, or up to 31 percent when rice is cooked without excess water removal. Reductions in phytic acid content of approximately 54 percent following boiling have also been reported.

Moreover, the assessors reported that rice event GR2E was developed through the use of recombinant-DNA techniques to express elevated levels of provitamin A (mainly β-carotene in the rice endosperm, which is converted in the body to vitamin A). Vitamin A is required for normal functioning of the visual system, maintenance of cell function for growth, epithelial integrity, production of red blood cells, immunity and reproduction. Vitamin is an essential nutrient in humans that cannot be synthesized *de novo* in the body, so it must be obtained through the diet. It is a natural component of certain food crops with a history of long use for its beta -carotene nutrient content, such as those found in raw carrots that contain 82.9 ug beta-carotene/ g carrot. The applicant presented data on results of studies wherein different amounts of β-carotene were taken for several years. The conclusion was that there was no toxic effect of the prolonged uptake of β-carotene.

In addition, data shows that the amount of β-carotene in GR2E is 7.31 ug/g. It was reported that there are no adverse effects when β-carotene is taken in excess of average consumption and below 15 mg per day.

STRP RECOMMENDATION

The assessors found scientific evidence that the regulated article applied for human food and/or animal feed use is as safe as its conventional counterpart and shall not pose any significant risk to human and animal health.

BPI-PLANT PRODUCTS AND SAFETY SERVICES DIVISION (PPSSD) ASSESSMENT AND RECOMMENDATION

HOST ORGANISM

Rice *(Oryza sativa* L.) has been a staple food for Filipinos and several countries around the world (OECD, 2016). It is a basic food for about half of the world population and is being widely cultivated, as one of the three major staple crops, in more than 100 countries around the world. It is being consumed as food upon cooking, parboiling and steaming (USDA, 2017). Rice by-products are often processed through milling, polishing and pulverization. In 2013, about 11.8 million tons of rice is potentially available for consumption of Filipinos (FAOSTAT, 2017). In 2006, ordinary rice was consumed by approximately 15 million Filipino families at an average of 463 kg per family per year (PSA, 2010). The daily per capita consumption of rice in the Philippines in 2014 is 312.93 g/ day (PSA, 2015). The consumption pattern of rice for average Filipino adult is 278.6 g/day (FNRI, undated document). The mean daily rice consumption by children and adult in the Philippines is approximately 112 g/day (5.72 g/kg body weight) and 279 g/day, respectively (PhilRice and IRRI, 2017).

Rice grain is seldom used as feed, except for damaged grains and sweepings from mills and warehouses. By-products of processing are used as feed in the form of bran, hulls, bran oil, germ and defatted bran (OECD, 2016)

Rice is a known source of key nutrients such as carbohydrates (starch), proteins, fiber, B vitamins, and fatty acids (PhilRice and IRRI, 2017). It is not a significant source of micronutrient minerals iron or zinc, and of fat soluble vitamins A, D, and K (OECD, 2016). It also contains few anti-nutrients such as phytic acid, trypsin inhibitors and hemagglutinin-lectins, oryzacystatin and alpha-amylase/subtilisin inhibitor (RASI) which are present at low levels in rice. Except for phytic acid, all other anti-nutrients are proteins readily denatured and inactivated by heat (PhilRice and IRRI, 2017). Most of the anti-nutrients are concentrated in the bran (OECD, 2016).

Rice is not known to be associated with any known toxicants and is generally considered as a safe source of food (PhilRice and IRRI, 2017; OECD, 2016). It is not considered a common allergenic food although there were some reports of rice allergy in Asian and European countries (e. g., Japan, Malaysia, and Indonesia, Finland, France, Spain, etc.). Two commonly accepted rice food allergens, *Oryza* glyoxalase I and *Oryza* trypsin alpha-amylase inhibitors are included in Food Allergy Research and Resource Program (FAARRP, 2014) (OECD, 2016). No data for the levels of the two putative allergens in GR2E rice were provided by the proponent. However, since no consensus or guidance on threshold levels for these allergens are available, there would be no standard to which such data may be compared. It is expected that people who are allergic to these antigens will also show allergenic responses to GR2E rice

History of safe use is attributed to rice since it is not known to cause toxicity and is being consumed as staple food worldwide (OECD, 2016).

TRANSGENIC PLANT

GR2E rice has not yet been approved in any country for use as food (PhilRice and IRRI, 2017). The transgenic plant was not found in the approval list in ISAAA GM Crop Database as of July 10, 2017. Dossiers have been submitted to US Food and Drugs Administration, Health Canada and Food Standards Australia New Zealand.

DONOR ORGANISMS

Based on the documents provided by the proponent, the following donor organisms were used in the transformation of GR2E rice:

a. *Zea mays L. (*maize; corn) – is the donor organism for *Zmpsy1* gene and polyubiquitin promoter. History of safe use is being attributed with *Z. mays* which is being widely consumed as staple food for several countries worldwide and is not associated with any known toxin. The only known allergen associated with maize is a non-specific lipid transfer protein (Pastorello et al., 2000).
b. *Pantoea ananatis* – is the donor organism for *crtI* gene. It belongs to the family of *Enterobacteriaceae* and is found in a wide range of natural environments. Strains of *P. ananatis* have been reported to cause pathogenicity to plants and humans. However, a recent analysis showed the absence of many of the factors that are central to pathogenicity and virulence arsenal of related plant and animal pathogens (De Maayer, et al., 2014). History of safe use of *P. ananatis* in association with food uses has not yet been established. However, the *crtI* gene isolated from this organism is not associated with pathogenicity or virulence of the donor organism.
c. *Escherichia coli* strain K12– is the donor organism for *pmi* gene. *E. coli strain K12* is a non-pathogenic strain and a normal inhabitant of the intestinal flora of humans and animals. It is also widely used as donor organisms for *pmi* and other genes in several approved genetically modified products.
d. *Pisum sativum* L. – is the donor organism for ribulose-1,5-bisphosphate carboxylase (RUBISCO) small sub-unit (SSU) transit peptide signal sequence. History of safe use is attributed to *P. sativum* which is widely consumed worldwide

No food safety concern with regards to the other donor organisms used in the transformation including *Oryza sativa* and *Agrobacterium tumefaciens* since the regulatory sequences obtained from these organisms are not being expressed in GR2E rice.

For this entire section, in accordance with the characterization provided by the proponent and obtained from other literatures, there is no food safety concern with regards to the donor organisms due to their history of safe use except for *P. ananatis*. However, literature indicates that the *crt*I gene is not involved in pathogenicity and virulence.

TRANSFORMATION SYSTEM

The transformation method for GR2E rice is through ***Agrobacterium tumefaciens* – mediated transformation** with plasmid vector **pSYN12424** (PhilRice and IRRI, 2017). The plasmid vector contains the *crtI*, *Zmpsy1* and *pmi* gene expression cassettes. The *crtI* gene expression cassette is composed of non-protein coding sequences such as: glutelin GluA-2

promoter from *Oryza sativa*, RUBISCO SSU transit peptide coding sequence from *Pisum sativum*, termination sequence of nopaline synthase gene (NOS 3') from *Agrobacterium tumefaciens* and intervening sequences from the cloning vector. No matured protein products are produced from these sequences except for the peptide product that has cleaved out from the phytoene desaturase protein during post translation. The *Zmpsy1* gene expression cassette is composed of glutelin GluA-2 promoter from *Oryza sativa*, termination sequence of nopaline synthase gene (NOS 3') from *Agrobacterium tumefaciens* and intervening sequences. No matured protein products are produced from these sequences except for the peptide product that was cleaved from the phytoene synthase protein post-translationally. The *pmi* gene expression cassette is composed of polyubiquitin promoter gene (*ZmUBI*) from *Zea mays*, termination sequence of nopaline synthase gene (NOS 3') and intervening sequences.

INSERTED DNA

Southern blot analyses confirmed that only a single copy of the cassette was integrated in a single insertion site in GR2E rice (PhilRice and IRRI, 2017). The integrity and order of genetic elements were demonstrated through Southern Blot Analyses using different probes (*Zmpsy1*, *pSSU-crtI* and *pmi*) and restriction enzymes (*Asc*I+*Xma*I, *Sph*I and *Hind*III). Integrity and order of genetic elements was confirmed upon detection of the predicted fragments for each hybridization probes tested. Host sequences flanking the 5' and 3' insertion points were also determined.

Small deletions of 23 bp and 11 bp in the right and left border sequences, respectively, of the T-DNA was detected (PhilRice and IRRI, 2017). Bioinformatics analyses of putative transcripts and translated amino acid sequence from each ORF were performed using FASTA/BLOSUM 50. The analysis did not indicate significant homology with any known toxin or allergens. The truncation and deletions observed in the inserted DNA are outside the promoter sequences and stop codons, and as such would not be expected to affect transcription initiation and termination of translation.

Southern blots of genomic DNA digests with plasmid as probe confirmed the absence of plasmid backbone.

GENETIC STABILITY

The multigenerational stability of the inserted DNA was assessed through Southern Blot Analyses of genomic DNA samples from the T_n generation (Kaybonnet) and three generations of backcrosses (BC_3F_5, BC_4F_3 and BC_5F_3) of GR2E in BRRI dhan 29, IR64 and PSB Rc82 germplasm (PhilRice and IRRI, 2017). Southern blot profiles of the inserts were consistent across the generations tested.

Stability of functional expression of the introduced genes was assessed using consistency of carotenoid levels in Kaybonnet (T_n germplasm) and BC_3F_5 generations in PSB, Rc82, and IR64 backgrounds and BC_4F_3 and BC_5F_3 in PSB Rc82, IR64 and BRRI dhan 29 germplasm backgrounds. (2 generations of backcrosses) (PhilRice and IRRI, 2017). The inserted DNA in GR2E rice is being stably integrated and inherited from one generation to the other as illustrated by the consistent hybridization patterns for each tested probe across each of the plant generations.

The stability of the introduced trait, elevated β-carotene trait, in GR2E rice was assessed through determining the concentrations of total carotenoids in GR2E rice grain samples in Kaybonnet (T_n germplasm and BC_3F_5 generations in PSB Rc82 and IR64 backgrounds and BC_4F_3 and BC_5F_3 in PSB Rc82, IR64 and BRRI dhan 29 germplasm backgrounds (2 generations of backcrosses) (PhilRice and IRRI, 2017). Data showed stable integration of the genes and consistent levels of functional expression of the cassette, as indicated by the levels of carotenoids in the grain.

The segregation analysis within three generations was assessed using PCR-based zygosity test (PhilRice and IRRI, 2017). Based on the results of Chi-square analysis, there are no significant differences between the observed and expected segregation ratios for the three generations of GR2E rice in PSB Rc82, BRRI dhan 29 and IR 64 genetic backgrounds. This indicates that the introduced DNA segregates following Mendelian Principle of Inheritance.

EXPRESSED MATERIAL

ZmPSY1 and *CRTI* is involved in the carotenoid biosynthetic pathway leading to accumulation of β-carotene in rice endosperm (PhilRice and IRRI, 2017). *PMI* is involved in the catalysis of mannose-6-phosphate to fructose-6-phosphate, and this trait served as a selectable marker for transformants.

*Zm*PSY1, CRTI and PMI levels were measured using quantitative Enzyme-linked Immunosorbent Assay (ELISA) (PhilRice and IRRI, 2017). Using the level of expression of ZmPSYI (2016) and the Philippine Daily Rice Intake, the daily intake of ZmPSY1 protein was calculated as approximately 1425 ng/kg body weight. Using the level of expression of CRTI and PMI proteins (2016), the Philippine Daily Rice Intake and the No Observed Effect Level (NOEL) of CRTI and PMI proteins, the Margin of Exposure (MOE) of CRTI and PMI proteins are approximately 678,000 and 259,000, respectively.

TOXICOLOGICAL AND ALLERGENICITY ASSESSMENT

The novel proteins, CRTI, ZmPSY1 and PMI, were subjected to digestibility, heat inactivation, oral toxicity and amino acid sequence comparison studies to determine its potential to cause toxicity or allergenicity to humans (PhilRice and IRRI, 2017). The test proteins used in these analyses were CRTI, ZmPSY1 and PMI from *Escherichia coli.* SDS-PAGE analysis, reverse HPLC, amino acid analysis, MALDI MS/MS peptide mapping, and enzyme functionality tests demonstrated the equivalence of *E. coli*-produced ZmPSY1 to plant-produced ZmPSY1. SDS-PAGE, western blot analysis, protein sequence alignment, and immunocross-reactivity in the lateral flow test strip assay demonstrated the equivalence of the *E. coli*- and plant-produced PMI. SDS-PAGE analysis, HPLC, N-terminal sequencing (via Edman degradation), functional enzymatic equivalence, and immunochemical cross-reactivity of western blotted proteins demonstrated the equivalence of *E. coli*- and plant-produced CRTI.

Digestibility study using Simulated Gastric Fluid (SGF) with pepsin demonstrated that ZmPSY1 is readily degraded within 2 minutes of incubation with SGF, in presence of pepsin at pH 1.2, a characteristic of most non-toxic proteins (PhilRice and IRRI, 2017). The same were observed in CRTI and PMI proteins upon incubation for 30 and 60 seconds, respectively.

Heat inactivation of ZmPSY1 was evaluated by monitoring the production of 15-cis-phytoene from *in situ* generated precursor geranylgeranyl diphosphate (PhiRice and IRRI, 2017).

Functional activity of CRTI was determined by monitoring the conversion of liposome-incorporated phytoene to all-trans-lycopene Results both analyses showed complete loss of activity for ZmPSY1 and CRTI was observed after incubation at 50°C for 15 minutes and 55°C for 15 minutes, respectively.

The heat stability of PMI was determined through monitoring its immunoreactivity using Enzyme Linked Immunosorbent Assay (ELISA) (Mims, 2009). Results of analysis provided by the developer showed that the enzymatic activity of PMI was below the limit of quantitation upon incubation at 65°C and above. This indicates that upon cooking or subjection to high temperatures, *PMI* is readily denatured. PMI is a transgenic protein in several maize events approved for food and feed under Department of Agriculture Administrative Order No. 8.

Amino acid sequence comparisons of ZmPSY1, CRTI and PMI protein were provided by the proponent and were confirmed using the similar Bioinformatics tools, BLASTp and FASTA (PhilRice and IRRI, 2017, AIS-FRA-17-08-BIA). ZmPSY1 protein were confirmed to have no significant homology to any known toxin or allergen (PhilRice and IRRI, 2017, AIS-FRA-17-08-BIA).

PMI protein was confirmed to have one eight-amino acid identity match to α-parvalbumin, a known allergen from unidentified edible frog, *Rana* species. Sensitive serum screening methodology indicated no-crossreactivity between *PMI* and the serum from the single individual known to have demonstrated IgE-mediated allergy to this specific α-parvalbumin. This indicates that the sequence identity between *PMI* and α-parvalbumin is not biologically relevant and has no implications for the potential allergenicity of PMI.

CRTI protein were confirmed to have 32-37% identity to sequence alignments from three species of venomous snakes, *Bungarus multicinctus, B. fasciatus* and *Daboia russelii* in 81 amino acid overlap. However, limited sequence similarity between *CRTI* protein from *P. ananatis* and the three LAAOs was due to homology between N-terminal motifs involved in FAD binding and was not considered to be a structural concern for potential toxicity. N-terminal sequence similarity was also observed between the native rice phytoene desaturase and a range of L-amino acid oxidases. Also, literatures had stated that snake venom LAAOs are non-toxic via oral route. Weight of evidence approach indicating that oral ingestion of *CRTI* protein will not pose hazard to human or animal health and that the enzyme activity of CRTI protein is irreversibly destroyed upon heat treatment at temperatures lower than cooking or processing.

Acute oral toxicity study of CRTI and PMI indicated no clinical signs of toxicity nor mortality, no gross lesions found in mice at necropsy, no treatment-related effects on body weight, food consumption or haematology parameters and no macroscopic or microscopic findings were observed. The No Observed Effect Level for CRTI and PMI proteins were 100 and 2000 mg/kg body weight, respectively. The developer did not provide an acute oral gavage study for *ZmPSY1* protein. However, weight of evidence approach indicated that *ZmPSY1* is not likely to act as a toxin.

In terms of the prevalence in food, *Zm*PSY1, CRTI and PMI constitute 0.00034%, 0.00004% and 0.0027% of the total protein of the grain.

Serum screening was performed in *Rana* species CH2001(unidentified edible frog). Results indicated that the patient's serum did not recognize any portion of the PMI protein as an allergenic epitope

ZmPSY1, CRTI and PMI proteins are not likely to interact due to the differences in mode of action. The phytoene synthase and Phytoene desaturate act sequentially in the carotenoid metabolic pathway. They act on substrates far different from mannose, the substrate of PMI. Phytoene synthase catalyzes the conversion of geranylgeranyl diphosphate to phytoene in the endosperm. In turn the *CRTI* desaturase catalyzes the conversion of phytoene to all trans lycopene via four desaturation steps and one cis-trans isomerization step. The all-trans lycopene is the precursor for the B carotene. Both proteins are regulated by the same promoter and therefore are expressed together and directed to the plastids. PMI remains in the cytoplasm to catalyze the interconversion of mannose 6 phosphate and fructose 6 phosphate.

NUTRITIONAL DATA

Compositional analysis was provided by the developer indicating the nutritional data of GR2E in comparison with the non-transgenic rice (PSBRc82), and range of literature values (PhilRice and IRRI, 2017). The trials were conducted in four (4) locations in the Philippines during one dry and one wet season. Results of the analysis indicated that there is no differences in the proximate, fiber, mineral, amino acid, fatty acid, vitamins and anti-nutrient of GR2E rice and the non-transgenic rice that can be considered biologically relevant except for the fortification with β-carotene which is the induced trait in GR2E rice.

GR2E rice grain contains β-carotene and other carotenoids such as β-cyptoxanthin, all trans-α-carotene, and 9'-cis- β-carotene. The purpose of having the regulated produce this new substance is to enhance nutritional quality of rice through introduction of β-carotene, a precursor of vitamin A to complement Vitamin A Deficiency (VAD) in the Philippines.

Carotenoids are one of the most widespread groups of pigments in nature. They are essential in human diet as antioxidants and protective agents against various diseases (Namitha and Negi, 2010). B-carotene is a precursor of vitamin A which is an essential nutrient in humans required for normal functioning of visual system, maintenance of cell function, epithelial integrity, production of red blood cells, immunity and reproduction (PhilRice and IRRI, 2017; NCBIe). Other carotenoids found in GR2E are not converted into Vitamin A in significant amounts (Khoo, *et al.,* 2011) Animals convert β-carotene to Vitamin A enzymatically through either β-carotene 15,15'-oxygenase 1 or β-carotene 9',10'-oxygenase 2. The other carotenoids can also serve as precursors to Vitamin A although the conversion is reportedly less efficient than that observed for β-carotene.

β-carotene is a safe source of vitamin A in human body commonly found in cellular tissues of fruits and vegetables such as carrots, spinach, mango, lettuce, spinach, sweet potato, onion, eggplant and other food crops that have been part of human diet (FNRI, 2016; USDA; Linus pauling Institute). β-carotene and the other carotenoids are also found in the grain. These substances are also naturally present in the leaves of the rice plant. B-carotene and other carotenoid structures in GR2E rice are identical to those that are synthetically produced and also those naturally found in other food crops (PhilRice and IRRI, 2017; Linus Pauling Institute).

The β-carotene content of carrot is 78.6 μg/g; onion is 11.2 μg/g; mango is 7.8 μg/g; and, eggplant is 0.8 μg/g (FNRI, 2016; Food and Nutrition Board, USA, 2000). The amount of β-carotene in GR2E is 1.96-7.31 μg/g dry weight. Total carotenoid content ranges from 3.5-10.9 μg/g dry weight.

The Linus Pauling Institute provides the carotenoid content of various foods in terms of cups. Conversion tables assume that 1 cup is equivalent to 340 g. Based on data presented, the amount of α-carotene, β-cryptoxanthin, and 9-cis-β-carotene that can be consumed by an average adult is much less than that obtainable from raw or cooked carrots.

The potential β-carotene intake of average Filipino adults is 2.04 mg/day (PhilRice and IRRI, 2017). The average Filipino commonly consume at least one medium-sized mango per day (approximately 100 g edible portion) which contains 1.17 mg β-carotene. This indicates that the daily intake of β-carotene from daily consumption of rice (278.6 g/ day) is equivalent to consuming two medium sized mango.

No adverse effects upon prolonged consumption of β-carotene in food have been reported. The proponent indicated in the dossier that standard toxicological tests have been performed on β-carotene, as recently reviewed by EFSA (2012). Results of the analyses showed no evidence of permanent harmful effects. Excess ingestion of carotenoids can lead to hypercarotemia in light-skinned individuals, but this condition is reversible once ingestion rates are reduced. Diplock (1995) also reported that supplementary intakes of 15-50 mg β-carotene /day has no side effects except for discolouration of the skin related to hypercarotenemia in some subjects at high intakes which are reversible upon reduction of b-carotene intake.

β-carotene is not known to be toxic or have adverse effects when ingested in excess of the average consumption. Diplock (1995) reported no evidence that the conversion of β-carotene to vitamin A contributes to vitamin A toxicity even when ingested in large amounts.

Heat, digestion, processing, interaction with atmospheric oxygen, light and storage may lead to degradation of β-carotene. Stability of β-carotene in GR2E rice during storage had been assessed. Results of analysis identified trace amounts of β-cyclocitral, ionene, 5,6-epoxy-β-ionone, β-ionone, dihydroactinidiolide and 4-oxo-β-ionone (FASEB Journal). Effect of storage temperature on the stability of β-carotene in rice varieties showed a decrease in the carotenoids levels in rice upon storage within two weeks at room temperature and at four weeks at 4^0C (Minatel et al, 2013).

OTHER CONCERNS

Concerns had been included in the assessment such as the other carotenoids found in GR2E rice, safety of β-carotene degradation products, conversion of β-carotene to vitamin A, effect of β-carotene supplementation to smokers and the effect of β-carotene to alcoholics. Upon review of the documents provided by the proponent and other available literatures, the following assessment were made:

a) History of safe use was attributed to the other carotenoids present in GR2E rice such as β-cryptoxanthin, α-carotene, all-trans-β-carotene and 9-cis-β-carotene.

b) Some β-carotene degradation products that may be found in GR2E rice may pose certain toxicological effect to human but with considerably high concentrations. β-carotene degradation products may be present in GR2E but not at that level which may alter human health. It has to be considered that the daily intake of β-carotene in GR2E rice is equivalent to the daily intake of β-carotene in other food crops that has a long history of safe use as presented in the Risk Assessment Report.
c) There is a wide variation in conversion factors for vitamin A reported not only between different studies but also between individuals in a particular study. These findings show that the vitamin A value of individual plant foods rich in provitamin A carotenoids may vary significantly.
d) The increase in incidence of neoplasia in male smokers having a high risk of lung cancer cannot be directly attributed as a possible effect of supplementation of β-carotene through GR2E rice. The rapid non-enzymatic oxidative cleavage of β-carotene could be a result of heavy oxidative stress such as smoking, asbestos and photo irradiation in the skin and eyes. Such activities are known to have carcinogenic effects on human body. It was also indicated in the Risk Assessment Report that the structure β-carotene in GR2E rice is identical to those found in other food crops. This indicates that any effects of the β-carotene cleavage products in GR2E rice on the respiratory burst and human neutrophils can also be found in carrots, mango, cabbage, lettuce and other commonly consumed food crops that have a long history of safe use.
e) Intake of high amount of β-carotene is considered safe but can become toxic to liver when taken in combination with alcohol. However, it has to be considered that the daily intake of β-carotene in GR2E rice is equivalent to the daily intake of β-carotene in other food crops that has a long history of safe use as presented in the Risk Assessment Report. Hence, normal consumption of GR2E rice does not indicate high intake of β-carotene which could lead to toxicity upon combination with alcohol.

RECOMMENDATION

For the Provitamin A Biofortified Rice Event GR2E, enough evidence was considered to support the substantial equivalence of the genetically modified crop in terms of nutritional composition and food safety, with the conventional rice other than the biofortification with β-carotene. After reviewing the provided material of PhilRice and IRRI, and other literatures taking into considerations the other concerns pertaining to the genetic modification of GR2E rice, it is therefore concluded that GR2E rice is as safe as its conventional counterpart.

BUREAU OF ANIMAL INDUSTRY ASSESSMENT AND RECOMMENDATION

HOST ORGANISM

The main nutrients rice provides are carbohydrates and protein. Milled rice is composed of carbohydrate in the form of starch and dietary fiber is contained in the hull and rice bran and germ lost during hulling, milling and polishing. Proteins are present in the outer layer and the inside of milled rice in the form of glutelin and prolamin. Other proteins are found in the outer layer of brown rice. There are also vitamins found in the bran of the rice grains which significantly decreased during milling.

The BAI reported that rice contains anti-nutrient factors but not at a significant level to raise an alarm. It is not considered a common allergenic food, although there are reported cases of allergy in

a number of countries in Asia and Europe. The two-known putative rice food allergens are *Oryza* trypsin alpha-amylase inhibitors and *Oryza* glyoxalase.

Rice is used for food and feed. As food, it is used in various forms including whole and milled grain, flour and bran. Rice used for food is milled rice. The pulverized form from milled rice is rice flour. An oil used for cooking is made from rice bran. The by-products from rice milling, such as rice bran, rice polishing and rice mill sweepings are considered important sources of feed especially in the Philippines. Rice grain at its normal state is not used as animal feed because of its high cost. Rice straw is good source of energy but it contains high silica, has poor digestibility and a low protein content. It is fed when there is no available or scarcity of green forage.

DONOR ORGANISM

The BAI reported that the description of all protein encoding sequences were provided. All three genes were derived from sources which are not known to possess pathogenic or allergenic properties.

The assessor listed and describe the toxicity and allergenicity of all donor organisms:

a) Zea mays: Has long history of safe use. No significant endogenous toxins are reported to be associated with it. Food allergy is relatively rare and the only significant reported food allergen is a nonspecific lipid transfer protein.
b) *Pantoea ananatis*: Some strains have been found to be pathogenic on a broad range of plant and human hosts, a recent analysis of its genome revealed the absence of many of the factors that are central to the pathogenicity and virulence arsenal of related plants and animal pathogens including animal toxins, hemolysins, phytotoxins and their associated effectors.
c) *Escherichia coli* strain K12 is a non-pathogenic strain.
d) Other donor organisms:
    i. *Pisum sativum* L. (pea) – Source of ribulose-1,5-bisphosphate carboxylase (RUBISCO) small sub-unit (SSU) transit peptide signal sequence. There are few reports of allergy with clear IgE binding to a few major proteins, but no reports of IgE binding to the chloroplast transit peptide sequence or the full-length or mature RUBISCO.
    ii. *Oryza sativa* – Source of glutelin promoter (GluA-2) which is not expressed.
    iii. *Agrobacterium tumefaciens* – Source of the nopaline synthase (NOS) 3' UTR, which is non-expressible

TRANSFORMATION SYSTEM

They confirmed that the transformation method used was *Agrobacterium*-mediated transformation and the target of modification is the nuclear DNA. The BAI reported that the description of the experimental protocol and the table of genetic elements with descriptions were sufficiently provided by the applicant.

THE INSERTED DNA

The BAI concurred that Southern blot analyses sufficiently confirmed that there is a single insertion site. The analysis of genomic DNA from GR2E demonstrated the integrity and order of genetic elements within the insertion site.

A bioinformatics analysis identified two ORFs but these had no significant alignments or amino acid sequence similarities to any known toxins or allergens. Furthermore, the BAI confirmed that both the *Zmpsy1* and *crtI* genes have not been expressed in other approved GM crops while the *pmi* gene has been incorporated in MIR604, MIR162, 3272 AND 5307.

GENETIC STABILITY

The BAI agreed that Southern blot analysis demonstrated the stable heritability of the inserted T-DNA and the demonstration of carotenoid accumulations in the endosperm of GR2E rice samples using spectrophotometric quantification affirms the inheritance of the introduced trait across four breeding generations.

They confirmed that segregation was assessed by multiplex PCR zygosity testing followed by gel electrophoresis. A Chi-square analysis of the results found no statistically significant differences between the observed and expected segregation ratios for the three generations of GR2E rice. This confirms that the T-DNA insert segregated as a single locus according to Mendelian rules of inheritance.

EXPRESSED MATERIAL

Western blot analysis was used to confirm the tissue specificity of ZmPSY1, CRTI and PMI expression in GR2E rice. Expression of ZmPSY1 and CRTI was detected only in milk, dough and mature stage grain but not in samples of bran, hulls, leaf, stem or root tissue. PMI was expressed in all rice tissue types tested. Level of expression was determined by specific quantitative ELISAs and results were provided.

It was confirmed that the Phytoene synthase (ZmPSY1) and CRTI enzyme complete the carotenoid biosynthetic pathway leading to accumulation of β-carotene in the endosperm, which is not possible in conventional rice.

TOXICOLOGICAL ASSESSMENT

ZmPSY1 Protein

The BAI reported that the SDS-PAGE and Western Immunoblot analysis revealed no intact fragments of the protein remaining after digestion at 30 seconds.

Furthermore, samples of ZmPSY1 were subjected to heat treatment over a temperature incubation range of 30-65°C for 15 minutes after which the enzymatic activity was measured by monitoring the production of 15-cis-phytoene using the HPLC method. It was also confirmed that a FASTA36 bioinformatic alignment search was performed against a toxin database (UniProt Knowledgebase) and there were no sequence homology structural alerts for potential toxicity.

For this protein, the applicant stated that no animal toxicity studies were performed since sufficient data has been derived to rule out potential for toxicity.

CRTI protein

Pepsin was used in the digestibility study. The estimated T_{50} is <30 seconds. There was no fragment remaining after digestion for 30 seconds as demonstrated by SDS-PAGE and Western immunoblot analyses.

It was reported that the thermal stability was determined by measuring enzymatic activity using spectrophotometric assay to monitor conversion of 15-*cis*-phytoene to all-*trans*-lycopene. T_{50} is 51°C

On the other hand, a search of the UniProt Knowledgebase returned sequence similarities with 3 L-amino acid oxidase (LAAO) accessions form the database due to homology between N-terminal motifs involved in FAD binding and was not considered to be a structural alert for potential toxicity. The three sequence alignments were from three species of venomous snakes.

PMI protein

The BAI reported that pepsin was used in the digestibility study. T_{50} is less than 1 minute. SDS-PAGE and Western blot analysis demonstrated that PMI was completely degraded. Heat stability of PMI was also evaluated. This was determined by measuring enzymatic activity.T_{50} is 65^0C. The BLASTP search of the NCBI Entrez Protein Database identified 1384 protein sequences as having significant sequence similarity to PMI amino acid sequence but none were known or putative toxins.

The BAI concluded that all of the three proteins are expressed independently of each other and the functional activities of each are maintained. Both ZmPSY1 and CRTI are expressed in the plastid while PMI is in the cytoplasm. No interactions were found.

ZmPSY1 and CRTI participate in the carotenoid biosynthetic pathway. ZmPSY1 catalyzes the condensation of GGPP to yield 15-cis-phytoene and CRTI catalyzes the next consecutive stages to yield all-trans-lycopene. Endogenous rice lycopene cyclases act to produce β-carotene. On the other hand, PMI functioned as a selective marker for transformed plantlets in tissue culture.

ALLERGENICITY ASSESSMENT

ZmPSY1 Protein

The BAI confirmed that pepsin was used in the digestibility study of the protein. Results of the SDS-PAGE and Western Immunoblot analysis revealed no intact fragments of the protein remaining after digestion at 30 seconds.

Samples of ZmPSY1 were subjected to heat treatment over a temperature incubation range of 30-65^0C for 15 minutes after which the enzymatic activity was measured by monitoring the production of 15-*cis*-phytoene using the HPLC method. T_{50} is 42^0C.

The BAI found that no identity matches of >35% over 80 residues were observed.

CRTI protein

Pepsin was used in the digestibility study. No intact CRTI protein was detected by SDS-PAGE or Western Immunoblot analysis after digestion. T_{50} is <30 seconds.

Furthermore, enzymatic activity was measured using spectrophotometric assay by monitoring the conversion of 15-*cis*-phytoene to all-*trans*-lycopene. T_{50} =51^{0}C. In addition, no identity matches of >35% over 80 residues were observed using the FARRP16 allergen dataset. There were also no eight contiguous amino acid matches observed.

PMI protein

It was confirmed by BAI that pepsin was used in the digestibility study. SDS-PAGE and western blot analysis did not show any intact protein or degradation products. T_{50} is less than 1 minute.

Enzymatic activity was measured following pre-incubation for 30 minutes at temperatures ranging from 25^0-95^{0}C. It was found out that the T_{50} = 65^{0}C.

Furthermore, using the FARRP AllergenOnline database, no identity matches of >35% over 80 residues were observed. A sequence similarity search for any eight contiguous identical amino acid matches yielded a match to a α-parvalbumin from Rana species CH2001 (unidentified edible frog). Sequence identity is not biologically meaningful and has no implication for the potential allergenicity of PMI.

No cross-reactivity was demonstrated between PMI and serum from the single individual known to have demonstrated IgE-mediated allergy to α-paravalbumin. Patient's serum did not recognize any portion of the PMI protein as an allergenic epitope.

NUTRITIONAL DATA

For the proximate and fibre analysis of straw, no statistically significant differences in proximate and fibre between samples of straw obtained from GR2E and control PSB rice. With the exception of moisture content, which is dependent on the extent of drying of straw following harvest. The mean values for all proximate and fibre in rice straw were similar to the ranges reported. Furthermore, for the proximate and fibre analysis of grain, there is also no statistically significant differences identified in grain samples derived from GR2E and control PSB Rc82 rice. The data derived from the transgenic line are within the reported range. For those values slightly outside the range, there were no statistically significant differences between GR2E rice and control PSB Rc82 rice.

The BAI reported that no statistically significant differences were identified between samples of GR2E and control PSB Rc82 rice as a result of the proximate and mineral analysis of bran. In addition, values measured from the analytes were within respective ranges reported in the literature except for crude fat and phosphorus which were slightly higher in both GR2E and control PSB Rc82.

For the analysis of straw minerals, the BAI reported that there was no statistically significant differences identified between sample GR2E and control PSB Rc82 as to concentration of calcium and

phosphorus. On the other hand, there was also no statistically significant differences observed in the mineral composition between the samples of GR2E grain and the control.

Moreover, a comparison of amino acid composition of event GR2E and control PSB Rc82 rice grain showed no significant differences. In addition, the data derived from the transgenic line are within the reported range except for tryptophan which was slightly lower and not statistically different from sample and the control.

Among all vitamins tested, no significant differences were observed in vitamins composition between the sample and the control except for Beta carotene which was intended to be elevated and no statistical difference from the sample and the control. In addition, the data derived from the transgenic line are within the reported range of literature except for pyridoxine (B6), folic Acid (B9) and α-tocopherol which were not statistically significantly different between sample and control.

For the analysis of fatty acids in grain, the BAI reported that no statistically significant differences were identified between sample GR2E and control PSB Rc82 rice as to concentration of fatty acids except in the concentration of stearic acid, which was approximately 6.5 % higher for GR2E rice. In addition, the data derived from the transgenic line of fatty acid are within the reported range in literature. Stearic acid comprises approximately two percent of the total fatty acids in rice grain and is not essential fatty acid. The small but statistically significant difference between stearic acid concentrations in samples of GR2E and control PSB Rc82 rice is unlikely to be biologically relevant.

No statistically significant differences were observed in phytic acid composition or in the levels of trypsin inhibitor between the sample and the control. In addition, the data derived from the transgenic line are within the reported range of literature and mean concentrations of phytic acid in grain samples from sample and control rice.

RECOMMENDATION:

The BAI found scientific evidence that the regulated article applied for human food and animal feed use is as safe as its conventional counterpart and shall not pose any significant risk to human and animal health.

DENR ASSESSMENT AND RECOMMENDATION

After thorough and scientific reviews and evaluation of the documents provided by the Bureau of Plant Industry (BPI) on the application of Philippine Rice Research Institute (PhilRice) and International Rice Research Institute (IRRI) for Direct Use as Food and Feed or for Processing of Golden Rice GR2E hereunder are their observations:

1. Evaluation of the application submitted by the proponent, including the scientific evidence from provided references and literature, as well as other related studies, the Committee accepted that the direct use of the regulated article whether for food, feed and/or for processing will not cause any significant adverse effect on the environment (land, air, and water) and non-target organisms, to wit:
    a. The genetic stability in the transgenic crop is ensured both under contained use and in previous multi-location confined testing (conducted year 2015 and 2016) such that no unintended horizontal gene transfer to unrelated species occurs;

b. The protein product produced by the transgenic crop will degrade upon exposure to the natural environment and general conditions (i.e. high temperature (60 degrees and above), varying pH, enzyme digestion, etc.); and
c. The protein product will not increase the weediness potential and will not confer selective advantage to the transgenic crop.

The data evaluated support the conclusion that the regulated article is as safe as its conventional counterpart.

2. The project description report (PDR) discusses the specified environmental management plan indicating the possible risk and harm to the environment and non-target organisms as well as the mitigating measures and contingency plan of the proponent. Upon evaluation of the submitted PDR, the Committee noted that the chances of unintended release or planting of the regulated article is very minimal and will not cause any damaging and lasting effects to the environment. Also, the application clearly states that the use of GR2E for Food, Feed and/or Processing will occur after approval for commercial propagation.

3. The purpose of the application is for the conduct of bioefficacy studies wherein food/feed safety assessment is a necessary prerequisite. The Bureau of Plant Industry to monitor the progress of the applicants in the subsequent studies and experimentation which would follow after approval for food, feed and processing. The use of the transgenic crop only for bioefficacy tests must be strictly followed and under no circumstance should planting be allowed except under approved field trial conditions.

4. The DENR-BC finds scientific evidence that the regulated article applied for Direct Use as food and feed, or for processing is safe as its conventional counterpart ad is not expected to pose any significant risk to the environment and to non-target organisms. Based on the above consideration and with the proponent's sworn statement of accountability, we hereby submit our evaluation relative to the PhilRice and IRRI's Golden Rice GR2E application for biosafety permit for food, feed or for processing.

The DENR-BC found scientific evidence that the regulated article applied for Direct Use as food and feed, or for processing is as safe as its conventional counterpart and is not expected to pose any significant risk to the environment and to non-target organisms.

DOH ASSESSMENT AND RECOMMENDATION

After a thorough review and evaluation of the documents provided by the proponent, PhilRice and IRRI, through the Bureau of Plant Industry (BPI), in support of their application for approval for Direct Use for Food and Feed or for Processing (FFP) of Golden Rice GR2E. The DOH-BC found that the regulated article applied for Direct Use for Food and Feed or for Processing (FFP) is as safe as its conventional counterpart and shall not pose any significant risk to human and animal health, and environment.

The following are their observations and recommendations:

1. Scientific pieces of evidence from toxicity studies and references conclude that the regulated article will not cause significant adverse health effects to human and animal health;
2. Dietary exposure to the regulated article is unlikely to result allergic reaction;

3. The regulated article is as safe and nutritious as food or feed derived from conventional rice varieties;
4. It is suggested that the Bureau of Plant Industry ensure the following: clear labelling of the regulated article from the source down to all levels of marketing stating that it is only for the purpose of direct use for food, feed, or processing and is not to be used as planting materials; and
5. Based on the above considerations and with the submitted sworn statement and accountability of the proponent, this recommendation was submitted to BPI related to the processing and issuance of a biosafety permit for direct use as food and feed, or for processing of Golden Rice GR2E

SEC Assessment and Recommendation

Below is the assessment of the SEC expert for GR2E Rice application:

For Direct Use, the SEC expert stated that the applicant will just have to answer two basic questions:

> Question 1: "How significant is the crop/base crop in terms of production, consumption, and trade? Provide supply and demand data on non-GM data for the past 5 years"?
>
> Question 2: "Will GR2E product change drastically the current patterns of production, consumption/utilization and trade? Provide trend of importation of non-GM rice, as the base commodity for the last five years"?

The questions and PhilRice's responses are shown below.

Question 1: "How significant is the crop/base crop in terms of production, consumption, and trade? Provide supply and demand data on non-GM data for the past 5 years"?

"Rice supply and utilization data from 2009 through 2017, the last year of reporting, are provided in Table 1. Over this period, average annual gross supply was ca. 15.3 million metric tons (MMT), of which 11.4 MMT was derived from domestic production and the balance, approximately 11 percent, from imports.

Table 1: Rice supply utilization accounts, 2009-2015, Philippines

	Supply (in '000 metric tons)					Utilization (in '000 metric tons unless otherwise indicated)			
						Net Food Disposable			
Year	Beginning Stock	Production	Imports	Gross Supply	Exports	Total	Per Capita (kg/year)	Per Capita (gram/day)	Ending Stock
2009	2639	10633	1755	15027	a/	11060	119.92	328.55	2629
2010	2629	10315	2378	15322	a/	10601	114.81	314.55	3424
2011	3424	10911	707	15042	a/	11047	115.30	315.89	2627
2012	2618	11793	1041	15452	a/	11474	118.89	325.73	2509
2013	2509	12059	398	14966	1	11340	115.48	316.38	2126
2014	2126	12405	1074	15605	1	11408	114.22	312.93	2662
2015	2662	11870	1478	16010	a/	11336	112.26	307.56	3199

a/ Less than one (1) thousand metric tons.
Source data from Supply Utilization Accounts (SUA) of Selected Agricultural Commodities published by the Philippines Statistics Authority. Reports for 2009- 2011 (https://www.psa.gov.ph/sites/default/files/sua_09-II.pdf), 2012- 2014 (https://www.psa.gov.ph/sites/default/files/sua_12-14.pdf), and 2013- 2015 (https:/ /www.psa.gov.ph/sites/default/files/sua_2013-201S.pdf) were utilized.

2. "Will GR2E product change drastically the current patterns of production, consumption/utilization and trade? Provide trend of importation of non-GM rice, as the base commodity for the last five years"?

"Pending receipt of a permit for commercial propagation, which is not the subject of the current application, the cultivation of GR2E rice is not anticipated to have any measurable impact on current patterns of rice production, consumption, or trade. Trends in rice imports are provided in Table 1 and depicted graphically in Figure 1.

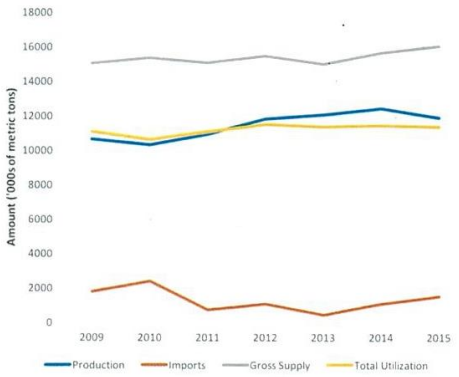

Figure 1. Trends in Philippine rice production, imports, and total utilization between 2009 and 2015

Therefore, given the responses to the SEC requirements above, the SEC expert recommended that the BPI grant approval to PhilRice SEC application for GR2E direct use.

*This document is subject to DA-BC review