academicJournals

Vol. 14(23), pp. 1936-1946, 10 June, 2015
DOI: 10.5897/AJB2015.14632
Article Number: 3B9454853542
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Toxicity assessment of modified Cry1Ac1 proteins and genetically modified insect-resistant Agb0101 rice

Si Myung Lee[1]*, Yun Soo Yeo[1], Myung Ho Lim[1], Hyun Suk Cho[1], Sung Duk Oh[1], Seon Woo Oh[1], Soo Yun Park[1], Soon Jong Kweon[2], Seok Cheol Suh[3] and Soon Ki Park[4]

[1]Division of Biosafety, National Academy of Agricultural Science, Rural Development Administration, Jeonju, Republic of Korea.
[2]Division of Central Area Breeding, National Institute of Crop Science, Rural Development Administration, Jeonju, Republic of Korea.
[3]Division of Molecular Breeding, National Academy of Agricultural Science, Rural Development Administration, Jeonju, Republic of Korea.
[4]School of Applied Biosciences, Kyungpook National University, Daegu, 702-701, Korea.

Received 10 April, 2015; Accepted 18 May, 2015

Insect-resistant Agb0101 rice was recently developed by modifying the *cry1ac1* gene by changing codon usage changes relative to the native truncated *cry1ac1* gene. To assess the toxicity of genetically modified Agb0101 rice, we conducted bioinfomational comparisons of the amino acid sequences that are not similar to known toxic proteins. Sufficient quantities of mCry1Ac1 protein were produced in *Escherichia coli* for *in vitro* evaluation and animal study. We compared the amino acid sequences and molecular mass. There have the same amino acid sequences and molecular masses after purifying the modified Cry1Ac1 (mCry1Ac1) protein from highly expressed bacteria and genetically modified rice were identical. We also investigated the acute and 90-days oral toxicities. No adverse effects were observed in mice following acute oral exposure to 2,000 mg/ kg body weight mCry1Ac1 protein of body weight and 90 days oral exposure to Agb0101. These results indicate that mCry1Ac1 proteins and Agb0101 rice demonstrate no adverse effects in these tests when applied via gavage and feed, respectively.

Key words: Modified Cry1Ac1, food safety assessment, toxicity, insect- resistant rice Agb0101.

INTRODUCTION

Genetically modified (GM) crops are becoming an increasingly important feature of the agricultural land-scapes. In 2013, approximately 175 million hectares of GM crops were planted by 18 million farmers in 27 countries. Due to the unprecedented 100-fold increase between 1996 and 2013, biotech crops are now the

*Corresponding author. E-mail: tataby@korea.kr. Tel: +82-63-238-4709. Fax: +82-63-238-4704.

Abbreviations: **ALP,** alkaline phosphatase; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **BUN,** urea nitrogen; **TG,** triglyceride; **A/G Ratio,** albumin/globulin ratio; **IP,** inorganic phosphorus.

fastest-adopted crop technology in the history of modern agriculture. Products with insect-resistant traits were the fastest growing group between 2009 and 2013 (James, 2013). Rice (*Oryza sativa*) is an important crop and staple food that used to main energy source in Korea. Not only the major food but also economical impact, Rice production affects all around of Korea economy. In Korea, 1 million people are cultivating rice in about 1 million ha of rice field. Various factors limit rice productivity, including pest which annually destroy 20 to 30% of rice crops (Estruch et al., 1996). Pest managements are very important factor to consider when attempting increasing rice production. In Korea, C*nophalocrocis medinalis guenee* is the one of major problem in rice cultivation especially. Generally, this insect can be managed using several kinds of pesticides (Warren et al., 1997). However, many farmers and consumers want to use less pesticide due to environmental and economic concerns. Insect-resistant rice was developed to prevent pest explosions and seek eco-friendly purposes. Many GM Crops for pest managements are developed and commercialized. Almost 32% of commercial GM crops are insect-resistant due to prevent pest damage. Almost all GM Crops use the gene as commonly known as Bt gene from the bacterium *Bacillus thuringiensis* that code for insecticidal crystal proteins were engineered into plants in the mid-1980s in order to develop the insect-resistant genetically modified plant. Bt corn was the first genetically modified by introducing the *Bt* gene since 1987 to combat the crop damage. Bt products have been used as insecticides for more than 40 years without documented evidence of adverse effects. Additionally, a number of toxicological studies on insecticidal Cry proteins have not identified any safety concerns for using Bt proteins (McClintock et al., 1995).

Genetic modification using insect-resistant genes is one of the most common modifications used to generate transgenic crops, including rice (Bajaj and Mohanty, 2005; Maqbool et al., 2001; Ramesh et al., 2004; Tu et al., 2000; Ye et al., 2003). Commercializing GM rice lags behind other cereals such as maize. One reason is that rice is cultivated more than 100 countries around the world and is a staple for about a half of the world's population; thus its safety must be strictly evaluated prior to its release to the market (Jiao et al., 2010). The *cry1Ac* gene can be isolated from *B. thuringiensis* and encodes the Cry protein, which exhibits toxic effects by forming pores in the cell membrane, thereby injuring epithelial cells in the midgut of insect. The effect is highly specific to target insects such as Lepidoptera and Coleoptera species, but is harmless to plants and mammals including humans (Bravo et al., 2007). We recently developed insect-resistant Agb0101 rice that contains the *mcry1Ac1* gene, a modified synthetic and truncated version of the *cry1Ac1* gene that expresses the same toxic protein with Cry1Ac1. Agb0101 rice contains a single copy of the truncated *cry1Ac* gene, and the toxic protein expressed by this transgene targets the chloroplast (Lee et al., 2009). Furthermore, research has revealed that Agb0101 demonstrates high resistance to rice leaf folder, rice green caterpillar, and rice skipper under laboratory conditions and to rice leaf folder under field conditions (Kim et al., 2009).

This study was performed to assess the toxicity of insect-resistant rice, develop scientific methodologies assess the safety of GM crops. These results could be used to further commercialization of insect-resistant Agb0101 rice. Although, this study described toxicity only, our results could help elucidate the food safety of Agb0101 rice.

MATERIALS AND METHODS

Test materials

The modified c*ry1Ac1* gene (*mcry1Ac1*: GenBank accession no. AY126450) used in this study was derived from the truncated *cry1Ac1* gene (GenBank accession no. AAA22551). This *mcry1Ac1* gene encodes the same amino acid sequences with the truncated Cry1Ac1 protein. For transformation into rice, some nucleotides were changed for optimal use in plants. Because the expression level of the mCry1Ac1 protein is extremely low in transgenic Agb0101 rice (typically less than 100 mg/kg), it is impractical for use in animal studies. Therefore, we conducted safety assessments using mCry1Ac1 protein that was produced in *Escherichia coli*. Proper characterization of the equivalence between recombinant and rice expressed protein is a necessary pre-requisite for use in safety evaluations of specific transgenic events. Agb0101 and control rice (Nak-dong) were grown in the experimental field of the National Academy of Agricultural Science (Suwon, Korea). To purify the mCry1Ac1 protein from rice, transgenic rice leaves containing the *mcry1ac1* gene were used as the plant material. Samples were collected, and the leaves were stored at -80°C until use.

Expression and purification of mCry1Ac1 protein from *E. coli*

The mCry1Ac1 proteins were produced and characterized. The pMAL-p2g/mcry1ac1 vector (BioLabs Inc., USA) was used to express the mCry1Ac1 protein. *mCry1Ac1* referred to the modified *cry1ac1* gene in order to be expressed in *E. coli* strain BL21 CodonPlus (DE3) RIPL (Stratagene, La Jolla, CA) as a fusion protein containing a maltose binding protein(MBP) tag and was purified using immobilized amylase resin chromatography. The MBP tag was cleaved from the affinity purified protein with cysteine protease from Tobacco Etch virus (TEV). And the fusion tag and TEV were removed by dialysis. For the acute toxicity study, the protein was lyophilized, mixed and stored at −80°C. The purity of the total protein was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and scanning densitometry. The concentration of the total protein was determined using the Bradford method. The identities of both lots of proteins were characterized using amino acid analysis and western blot analysis using a proprietary mCry1Ac1- specific antibody.

Using bioinformatic analysis to determine similarities between toxic proteins and the mCry1Ac1 protein

Sequence similarity searches were conducted on the amino acid sequence of the mCry1Ac1 protein using the BLASTP algorithm of the NCBI protein database. Sequence similarities were manually

Table 1. Study design of feed composition for the sub-chronic study.

Ingredient (%)		G1	G2	G3	G4
No. of animals	Male	10	10	10	10
	Female	10	10	10	10
Agb0101 rice		0	0	5	20
Nak-dong rice		0	20	0	0
Feed		100	80	95	80
Total		100	100	100	100

inspected manually to identify known toxic proteins. Generally, a st atistically significant sequence similarity requires a match with an E score of <0.01; however, a threshold E score 1.0 provides greater assurance that proteins with even limited similarities will not be overlooked. The BLOSUM62 scoring matrix was used, low complexity filtering was turned off, and the number of alignments returned was set to a maximum value of 1,000 proteins.

Protein sequencing and In-gel trypsin digestion and protein identification by MS/MS

The mCry1Ac1 protein was electroporated and transferred to PVDF membranes. Targeted band were cut and amino acid sequencing was carried out by Edman Sequencer ABI494. MALDI-TOF mass experiments were carried out according to Sun et al. (2007), with minor modifications. Protein bands with mCry1Ac1 were carefully cut out from CBB R250-stained gels and subjected to in-gel trypsin digestion according to Sun et al. (2007), with minor modifications. MALDI-TOF/TOF-GPS Explorer™ software version 3.6 (Applied Biosystems) was used to create and search files with the MASCOT search program for peptide and protein identification.

Acute toxicity assessments of the mCry1Ac1 protein in ICR mice

Acute toxicity studies were conducted in accordance with OECD guidelines (OECD, 2001) and the Laboratory animal Act by Korea Food and Drug Administration (KFDA). 60 ICR mice (3 weeks old, 18 to 22 g) were obtained from Hanlim Animal Experiment Institute, (Hwasung, Korea). After 5 day acclimatization period, the mice were randomly divided into 3 groups. Each group was related to mCry1Ac, Bovine serum albumin (BSA) and water as control. Each group contained 20 mice / group (10 males and 10 females per treatment). All mice were kept in stainless steel wire cages (2/cage) at 21 to 23°C, relative humidity 40 to 60%, 15 air change times per hour, and electric lights were turned on from 9 AM to 9 PM. Mice were allowed free access to both food and water. The mCry1Ac1 protein was dissolved in distilled water to 95% purity, and the mCry1Ac1 protein concentration was adjusted to 250 mg/mL. Each mouse received about 0.2 mL/kg protein (2,000 mg/kg mCry1Ac1 protein for each mouse) by gavage on the first day. BSA was used as the negative control and water was used as the blank control. Mice were given basal diet and tap water and observed for 14 days for any signs of morbidity or mortality. During the experimental period all animals were inspected twice daily (cage side). At study completion day 15, all animals were anaesthetized by carbon dioxide inhalation and sacrificed by exsanguinations for the subsequent gross and histopathological examinations.

Subchronic toxicity of Agb0101 rice in SPF rats

Subchronic toxicity study was conducted in accordance with OECD guidelines (OECD, 1998) and the Laboratory animal Act by KFDA. 80 SPF rats (40 male and 40 female) were obtained from Orient Co. (Kapyung, Korea). All rats were 5 weeks old at study initiation. Following a 7 day acclimatization period, they were randomly divided into 4 groups, 20 mice/ group (10 males and 10 females per treatment). All diets were administered to rats for 90 consecutive days. Animals were housed pair wise in stainless steel wire cages at 23 ± 2°C, relative humidity 55 ± 5%, 10 to 15 air change per hour and electric light with 200 to 300 lux from 9 AM to 9 PM. Rats were allowed free access to both food and water. Insect resistant Agb0101 rice was mixed with the basal diet in order to take into account nutritional balance. We made 4 kinds of feed for this experiment. The composition of the feed administrated to each group is listed in Table 1. Diets were identically adjusted to assure an adequate supply of macronutrients and vitamins after substitution with 60% rice, but no adjustments were made to balance differences in the constitution of the rice (as observed by the compositional/chemical analyses). Rats were allowed free access to both food and water (KFDA, 2007).

Serum biochemistry and haematology

All animals were fasted overnight to minimize fluctuations in the measured parameters. Blood samples were taken from the eyeball veniplex and stabilized using heparin. The following biochemical parameters were measured: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (BUN), triglyceride (TG), albumin/globulin ratio (A/G ratio), inorganic phosphorus (IP), sodium, potassium, cholesterol, protein, albumin, creatinine and glucose. All analyses on blood plasma were performed on a Express plus (Bayer Diagnostics Inc., USA). Blood samples used to assess the hematology characteristics were stabilized using EDTA. The following characteristics were assessed using the Baker system 9118 Hematology Analyzer (Biochem Immunosystems Inc., Allentown, PA). White blood cells, Red blood cells, hemoglobin concentration, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration and platelet count. The differential count distinguishes between basophils, eosinophils, lymphocytes, monocytes and neutrophils.

Organ weights, gross necropsy and histopathology

Necropsy was performed and the following organs were sequentially excised: brain, thymus, heart, lungs, liver, spleen, stomach, duodenum, adrenals, kidneys, ovaries, and testes. The brain, heart, liver, spleen, lungs, kidneys, thymus, adrenals, ovaries and testes

were immediately weighed, and the stomach, duodenum, kidneys and liver were immediately fixed in 4% buffered formaldehyde for histological processing. Tissue samples were embedded in paraffin, and sectioned to 3 to 5 µm thick, and then stained with standard hematoxylin–eosin for light microscopy.

Statistical analysis

Statistical comparisons were designed to determine if differences in the mentioned response variables between groups could be attributed to the mCry1Ac1 protein and Agb0101 rice. Data obtained from the mCry1Ac1 protein groups were compared with the values from the vehicle and BSA control groups. Similarly, each Agb0101-treated group was compared with the values from the control groups. Homogeneity variance was analyzed using one-way analysis of variance (ANOVA) using Statistical Product and Service Solutions (SPSS) v12.0 (SPSS Inc., Chicago, IL). Differences were considered significant when $p<0.05$ and then a step-down analysis was conducted using least squares differences (LSD).

RESULTS

BlastP comparison of the mCry1Ac1 proteins to other toxic proteins

BLASTP similarity searches were conducted using the amino acid sequence of mCry1Ac1 in order to identify the 1000 closest matches. The highest E score returned was 0.85, confirming that sequences with limited similarity were not overlooked by the search. 95 of the accessions returned by the searches demonstrated complete significance (E = 0) and represent very closely related Cry proteins from various bacterial species. A total of 827 other sequences were identified as Cry proteins from various bacterial species. The remaining 173 hits represented a variety of proteins that are all functionally related by the possession of one or more well characterized conserved thiamine pyrophosphate binding domains. No information is available on the toxicity of these proteins returned by the BLASTP search identified similarities to proteins known toxic. Assessing the safety of a novel protein requires determining its amino acid sequence similarity with known toxic proteins that have potential safety concerns. Accordingly, amino acid sequences were compared as part of the current food safety decision tree strategy as recommended by FAO/WHO (2001) and the CODEX (2003). Amino acid sequence similarities between the mCry1Ac1 protein and known toxic proteins were determined according to published guidelines (CODEX, 2003). The results of the *in silico* analysis revealed no evidence for similarities between mCry1Ac1 and any known toxic protein.

Isolation and characterization of heterologously produced mCry1Ac1 protein

The recombinant mCry1Ac1 protein was expressed in *E. coli* and purified as a soluble protein. The mCry1Ac1 protein migrated as a major band and demonstrated a molecular weight of approximately 66 kDa according to our SDS–PAGE analysis. The purity of the mCry1Ac1 protein was greater than 95% according to densitometry analysis which was performed the SDS–PAGE analysis (Figure 1). After blotting to PVDF membrane, we performed N-terminal sequencing by Edman degradation. The N-terminal sequence of the analyzed mCry1Ac1 protein was identical to deduced amino acid sequence of mCry1Ac1. MALDI-TOF MS analysis confirmed 71% sequence coverage for the mCry1Ac1 protein (data not shown).

Acute toxicity of mCry1Ac1 protein

The acute toxicity of the mCry1Ac1 protein was assessed in mice following the oral administration of the purified heterologously expressed protein at 2,000 mg/kg body weight of the test substance (corresponding to approximately 2,000 mg/kg of body weight of mCry1Ac1 protein) via oral gavage. Control groups were administered either vehicle (that is, water) alone or BSA at 2,000 mg/kg body weight. All mice survived the study period and no clinical signs of systemic toxicity were observed in any treatment groups (data not shown). All mice in all treatment groups gained weight relative to day 0 of dosing (Table 2), and no gross lesions were present in any of the mice at necropsy, thereby indicating that the mCry1Ac1 protein was not acutely toxic. The mCryAC1 protein demonstrates no special toxicity according to our acute toxicity tests. We did not observe animal death and special symptoms at 2,000 mg/Kg body weight.

Subchronic toxicity of genetically modified rice Agb0101

Mortality and clinical signs

There was no instance of treatment-related mortality in the animals treated with Agb0101 rice during this study. No significant clinical signs were observed in any other group.

Body weight and feed consumption

No reliable differences in body weight (Figure 2), feed consumption, weight gains, or food efficiency were observed between rats in the Agb0101-treated groups and those in the related control group (that is, rat that received Nak-dong rice). Also, no reliable changes in consumption between treated groups were observed during the test periods (Figure 3).

Hematology

As shown in Table 3, no significant changes were observed

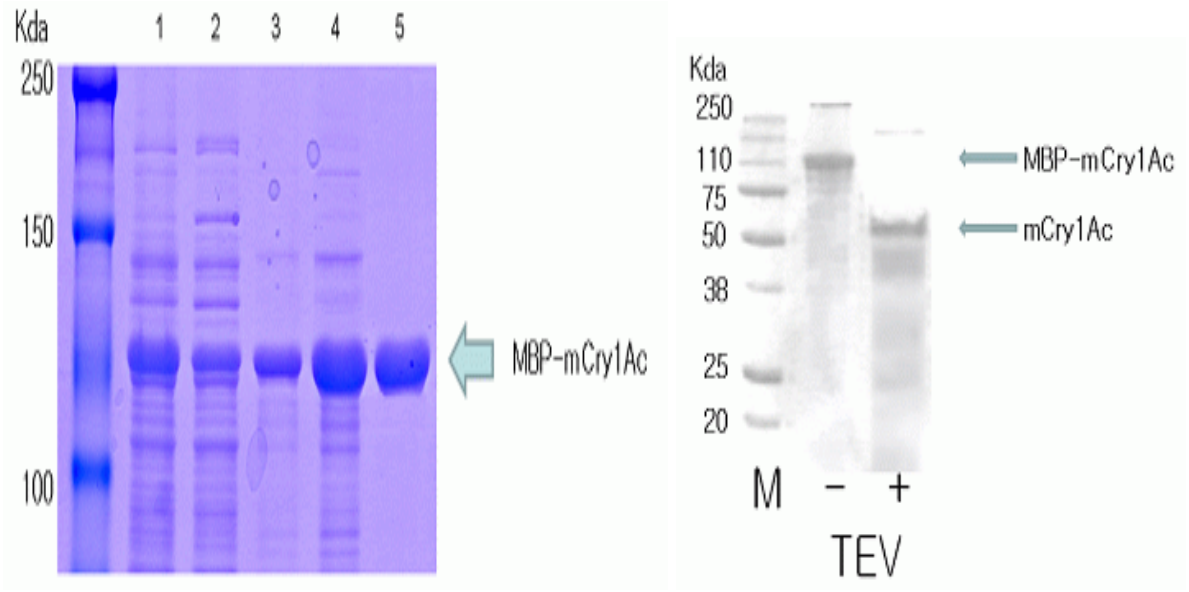

Figure 1. Purification of microbial mCry1Ac1 protein. (A). SDS-PAGE analysis of microbial mCry1Ac1 protein. Lane 1 contains total cell extract; Lane 2 contains soluble fraction of cell extract; Lane 3 contains amylase resin eluted fraction; Lane 4 contains concentrate of amylase resin eluted fraction; Lane 5 contains anion-exchange chromatography eluted fraction. (B). The Cleavage of MBP-mCry1Ac1 protein. Lane M contains molecular weight marker; Lane – contains MBP-mCry1Ac1 protein without TEV protease; Lane + contains mCry1Ac1 protein with TEV protease.

Table 2. Body weights changes of mice from acute toxicity study with mCry1Ac1 protein.

Materials	Female			Male		
	0	7	14	0	7	14
m Cry1Ac1	22.1±0.79	24.6±1.36	26.8±1.41	31.5±1.34	34.3±1.77	37.6±1.96
BSA	22.7±1.66	25.5±1.93	26.6±1.95	31.6±1.83	35.0±3.23	38.4±3.35
Water	22.5±1.51	24.5±1.73	27.1±1.88	30.6±1.53	34.2±2.32	37.4±2.83

in most of the hematology response variables between groups that consumed the different diets. However, certain hematology variables, such as reticulo-cytes in the male rats in the G4 group demonstrated significant decrease in comparison with G1 whereas female in the G3 and G4 groups demonstrated significant increases in comparison with G1, G2 groups. Reticulocytes are a known indicator of anemia, but related variables like red blood cells, hemoglobin, and hematocrit did not significantly changed in either males or females. These results indicate that the reticulocyte changes in males and females were not cause by Agb0101 rice.

Serum biochemistry

No differences were observed in most of the values of the serum response variables between the groups consumed different diets (Table 4). However, some differences were observed in total protein and some ion parameters. Total protein demonstrated a significant decrease among female in the G4 and G1 groups. However, this value difference was not observed in the G2 group females. Calcium ion also statistically and significantly decreases in G3 and G4 females, but not in males, though this finding was not significantly between the G1 and G4 groups. Chloride ion decreased in G3 and G4 males in comparison with G1 males, but increased in G4 female comparison with G1 females. These changes were not consistently demonstrated in males and females, and the increases/decreases were inconsistent. Therefore, the observed differences in these parameters are attributed to background variability and sporadic deviation. All differences are within the normal ranges for rats. Therefore, they are not considered related to Agb0101 rice.

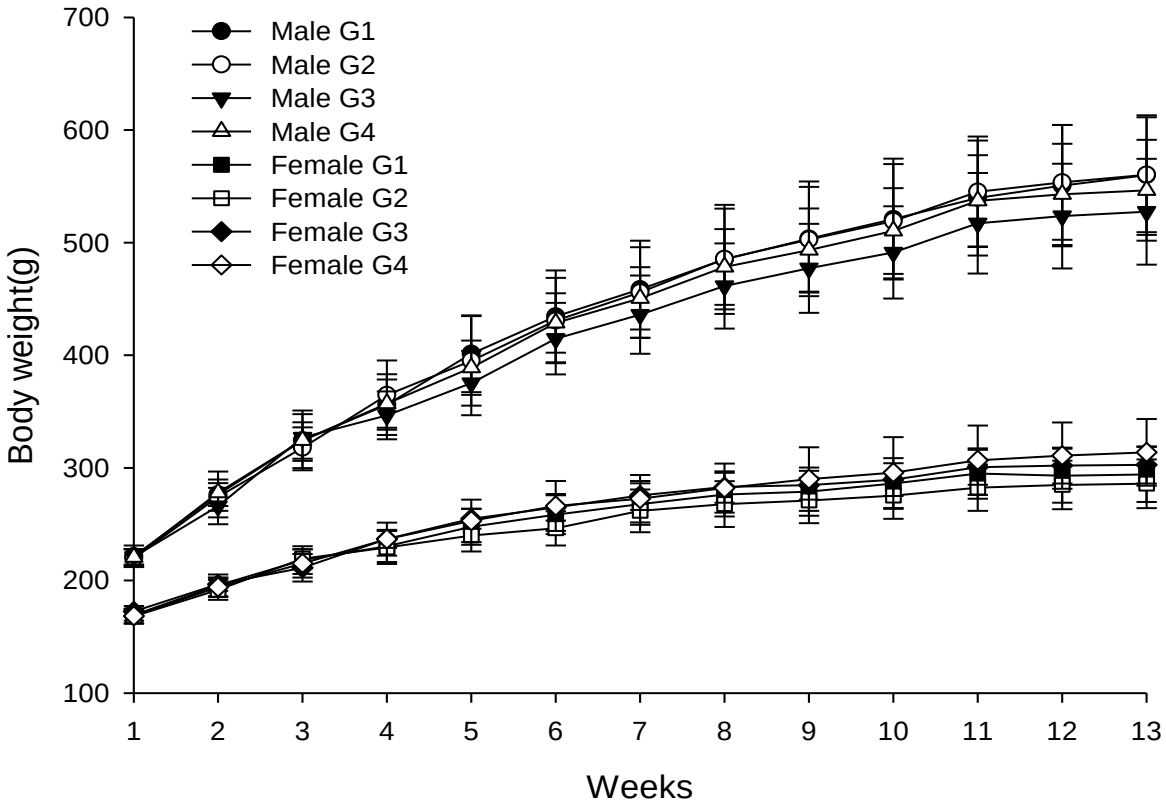

Figure 2. Body weight changes of rats orally treated with Agb0101 rice for 90 days.

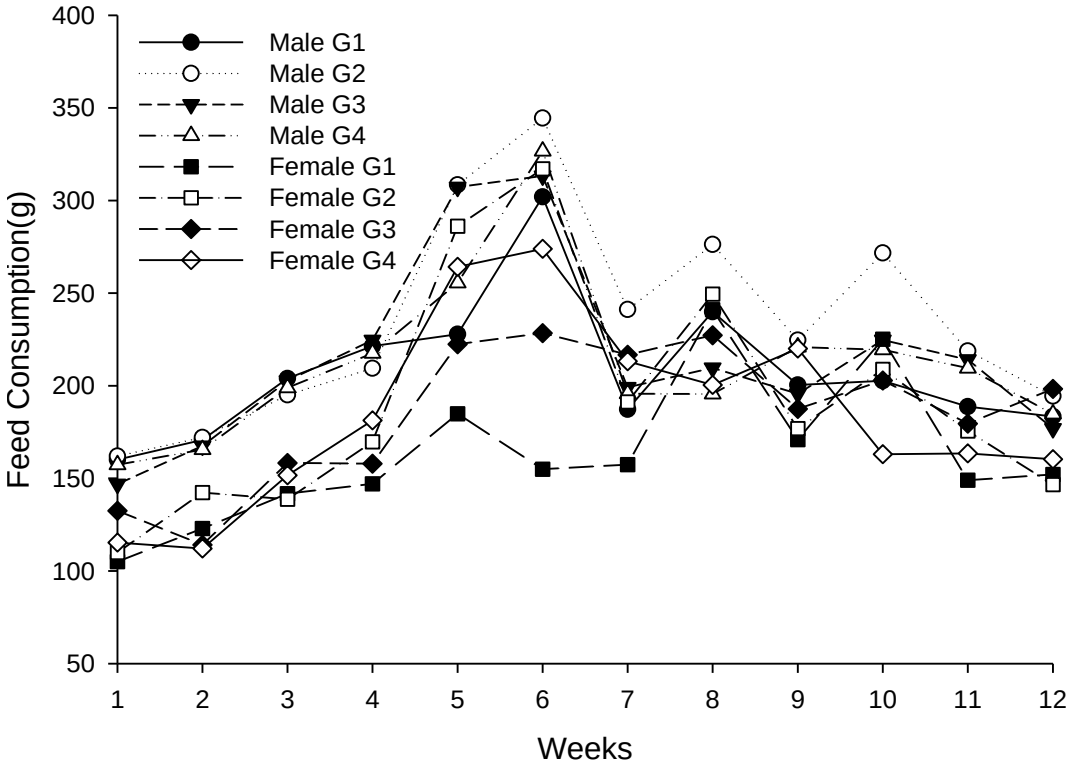

Figure 3. Feed consumption of rats orally treated with Agb0101 rice for 90 days.

Table 3. Analysis of Blood components contents treated with Agb0101 rice.

Sex	Blood components Group (n=10)	G 1	G 2	G 3	G 4
	Basophils(%)	0.06±0.03	0.03±0.02	0.04±0.02	0.03±0.02
	Eosinophils (%)	0.42±0.27	0.35±0.23	0.44±0.21	0.53±0.34
	Hematocrit (%)	42.54±3.61	42.53±2.62	42.08±3.90	44.55±3.94
	Hemoglobin (g/dl)	16.42±0.74	16.57±1.04	16.48±0.92	17.06±0.61
	Lymphocytes (%)	47.35±3.72	48.45±5.83	46.44±8.04	43.54±6.41
	Monocytes (%)	9.92±1.71	11.87±3.05	11.32±4.64	14.35±4.21
	Neutrophils (%)	37.28±3.49	38.83±4.61	41.75±6.01	41.51±6.14
Male	Red Blood Cell (10^6/mm^3)	7.82±1.06	7.82±0.53	8.01±0.59	8.37±0.64
	Reticulocyte (%)	0.93±0.21	0.91±0.22	0.82±0.18	0.69^{a}±0.10
	White Blood Cell (10^3/mm^3)	7.67±2.29	8.17±2.09	7.88±2.46	5.92±1.89
	Activated Partial Thromboplastin Time (sec)	23.74±2.87	22.65±2.10	22.76±1.75	23.63±3.41
	Prothrombin Time (sec)	20.58±3.26	19.82±1.74	18.98±1.45	19.64±3.24
	Mean Corpuscular Hemoglobin (pg)	21.13±1.37	21.61±0.91	20.84±2.82	21.49±2.36
	Mean Corpuscular Hemoglobin Concentration (g/dl)	38.39±2.14	39.07±1.84	39.37±2.45	38.44±2.38
	Mean Corpuscular Volume (fL)	53.37±3.45	54.56±2.82	52.63±2.43	52.81±2.84
	Basophils (%)	0.03±0.02	0.03±0.02	0.05±0.03	0.04±0.02
	Eosinophils (%)	0.76±0.44	0.61±0.25	0.63±0.23	0.71±0.47
	Hematocrit (%)	42.58±4.32	42.85±5.11	39.83±6.87	44.35±5.14
	Hemoglobin (g/dl)	15.28±0.72	15.83±1.21	15.65±0.79	15.32±1.59
	Lymphocytes (%)	53.82±7.91	48.94±7.89	55.64±9.85	50.97±9.52
	Monocytes (%)	10.03±2.66	11.48±1.77	10.69±2.82	11.31±2.26
	Neutrophils (%)	35.44±7.31	39.53±7.92	34.53±9.25	37.08±8. 43
Female	Red Blood Cell (10^6/mm^3)	7.51±1.05	7.65±0.88	7.39±1.32	7.87±0.78
	Reticulocyte (%)	0.74±0.21	0.81±0.22	$1.28^{a,b}$±0.29	$1.31^{a,b}$±0.25
	White Blood Cell (10^3/mm^3)	3.68±1.38	3.53±1.51	3.25±0.75	4.18±1.29
	Activated Partial Thromboplastin Time (sec)	21.57±1.46	21.13±1.30	22.03±1.85	21.28±1.18
	Prothrombin Time(sec)	15.69±1.23	15.84±0.67	16.34±0.61	16.37±0.60
	Mean Corpuscular Hemoglobin (pg)	21.11±1.75	21.35±1.94	22.47±5.63	19.85±1.55
	Mean Corpuscular Hemoglobin Concentration (g/dl)	36.78±2.29	38.89±3.69	40.16±9.71	35.39±3.75
	Mean Corpuscular Volume (fL)	55.91±2.05	55.78±2.26	54.86±1.88	55.33±2.96

Significant differences as compared with G1: $^{a}P < 0.05$. Significant differences as compared with G2 : $^{b}P < 0.05$.

Necropsy findings and organ weight

There were no gross indications of adverse effect in any rat organs on necropsy. No statistically significant differences were observed in the mean relative organ weights between the different treatment groups and the control group (Table 5). Additionally, no adverse effects were noted on histopathological observation (Table 6). Insect-resistant Agb0101 rice was mixed with normal feed at two different concentrations, 5 and 20%. The amounts of protein incorporated into the diet of each groups were 92 and 132 mg/Kg, respectively. As control groups, normal Nak-dong rice also mixed with normal feed into the diet at 20%. Over the course of the in-life phase of this study, no statistically significant differences in body weights (Figure 2) or feed consumption were observed between the control groups and the groups that consumed Agb0101 rice-containing diets (Figure 3). The consumed doses of mCry1Ac1 protein averaged 0.42 and 1.62 mg/kg body weight/day in males and 0.3 and 1.3 mg/kg body weight/day in females that calculate from the results of Kim et al. (2013).

DISCUSSION

This study describes our toxicity assessment that was

Table 4. Biochemical values of SD rats orally treated with Agb0101 for 90 days.

Sex	Biochemical values Group (n=10)	G 1	G 2	G 3	G 4
	T-Protein (g/dl)	6.01±0.17	6.09±0.25	6.16±0.22	6.09±0.20
	Albumin (g/dl)	2.29±0.16	2.34±0.08	2.28±0.12	2.35±0.11
	A/G ratio	0.62±0.26	0.62±0.13	0.59±0.07	0.63±0.09
	ALP (IU/L)	203.2±44.38	208.1±38.57	202.6±38.32	186.2±39.15
	AST (IU/L)	101.7±29.47	98.9±18.38	97.8±17.89	103.5±14.88
	ALT (IU/L)	38.9±5.65	39.5±6.23	39.7±8.91	38.5±6.83
	BUN (mg/dl)	14.76±2.38	15.06±1.79	16.94±2.41	17.16±2.38
	Cholesterol (mg/dl)	68.6±15.44	71.4±16.06	72.5±10.84	70.5±7.79
Male	Creatinine (mg/dl)	0.54±0.06	0.55±0.06	0.56±0.06	0.56±0.08
	Glucose (mg/dl)	164.1±36.28	170.0±17.68	165.2±34.92	165.9±19.93
	TG (mg/dl)	83.83±39.65	84.10±36.92	82.30±26.81	73.40±19.76
	Calcium (mg/dl)	10.19±0.62	9.96±0.61	10.84±0.55	9.98±0.43
	Cl-(mmol/L)	124.6±5.56	117.3±3.02	114.3[a]±3.07	109.9[a,b]±1.17
	IP (mg/dl)	6.13±0.85	6.24±0.67	5.99±0.49	6.13±0.54
	K+(mmol/L)	4.32±0.83	4.33±0.56	4.46±0.45	4.53±0.47
	Na+(mmol/L)	145.1±1.45	143.8±1.94	146.3±1.62	145.7±1.46
	T-Protein (g/dl)	7.27±0.23	6.92±0.29	6.95±0.32	6.78[a]±0.28
	Albumin (g/dl)	3.19±0.21	3.15±0.33	3.19±0.17	2.96±0.33
	A/G ratio	0.78±0.09	0.84±0.11	0.85±0.08	0.77±0.13
	ALP (IU/L)	103.1±45.43	98.7±20.21	93.8±24.55	96.5±20.19
	AST (IU/L)	123.9±31.14	99.1±24.88	93.6±26.31	101.5±27.65
	ALT (IU/L)	41.8±19.23	36.7±22.44	39.5±10.86	38.9±20.42
	BUN (mg/dl)	16.45±2.32	17.14±2.43	18.34±3.97	17.18±1.81
	Cholesterol (mg/dl)	85.1±12.77	84.4±17.30	89.2±19.31	87.1±13.11
Female	Creatinine (mg/dl)	0.63±0.11	0.67±0.06	0.65±0.13	0.64±0.09
	Glucose (mg/dl)	139.4±16.22	140.5±15.37	142.3±11.19	139.3±11.27
	TG (mg/dl)	11.6±6.86	10.3±3.54	9.7±3.85	12.5±11.06
	Calcium (mg/dl)	11.90[b]±0.23	10.87[a]±0.16	10.11[a]±0.17	10.24[a]±0.16
	Cl-(mmol/L)	109.1±2.03	108.5±1.47	108.1±3.08	124.2 [a,b]±4.93
	IP (mg/dl)	5.81±1.48	5.55±1.13	5.72±0.52	5.65±0.83
	K+(mmol/L)	3.97±0.45	3.93±0.33	4.03±0.29	3.99±0.23
	Na+(mmol/L)	145.9±1.25	145.9±1.61	146.1±1.78	146.3±1.32

Significant differences as compared with G 1 : [a]$P < 0.05$. Significant differences as compared with G 2 : [b]$P < 0.05$.

conducted on the mCry1Ac1 protein and Agb0101 rice. This assessment evaluates the potential toxicity of transgenic proteins using weight-of-evidence and tiered approaches, respectively (CODEX, 2003; EC 2003). No evidence for potential toxicity was identified for the *mcry1Ac1* gene according to the components of a first tier. The bt gene was obtained from *Bacillus sp.,* which has a long history of safe use in agricultural pesticides. Rice also has a long safe history as a common component of the human diet. Human exposure to the mCry1Ac1 protein is most likely extremely low because it is present at low concentrations in the entire genetically modified Agb0101 rice plant. The effects of the mCry1Ac1 protein determined by the 90-day toxicity study should be considered in comparison to the concentration determined in rice seeds obtained from genetically modified insect-resistant Agb0101 rice as determined using mCry1Ac1-specific ELISA (0.2 mg/Kg mCry1Ac1 protein in dry tissue). The average human (average body weight 60 kg) would need to consume about 60 kg/day of grain expressing the mCry1Ac1 protein to approximate the same daily dose consumed by the high dose group enrolled

Table 5. Organ weight in treated rat.

Sex	Organ weight group (n=10)	G 1	G 2	G 3	G 4
	Terminal body weight	538.59±50.02	555.67±50.38	522.95±48.67	543.07±46.14
	Adrenal gland	0.029±0.004	0.028±0.005	0.026±0.004	0.029±0.005
	Brain	2.11±0.08	2.12±0.06	2.12±0.09	2.12±0.08
	Epididymis	0.73±0.08	0.74±0.11	0.73±0.06	0.75±0.06
Male	Heart	1.67±0.15	1.68±0.18	1.71±0.13	1.72±0.14
	Kidney	1.53±0.15	1.52±0.12	1.51±0.17	1.55±0.16
	Liver	12.58±1.61	12.77±1.62	12.94±1.94	12.42±1.33
	Pituitary gland	0.014±0.002	0.013±0.002	0.014±0.003	0.016±0.005
	Spleen	0.73±0.12	0.74±0.25	0.73±0.19	0.75±0.16
	Testis	1.73±0.22	1.69±0.24	1.68±0.21	1.72±0.18
	Thymus	0.39±0.11	0.41±0.10	0.38±0.10	0.39±0.13
	Terminal body weight	291.72±25.41	289.71±25.64	299.38±26.40	301.62±28.89
	Adrenal gland	0.034±0.007	0.034±0.006	0.033±0.007	0.034±0.005
	Brain	2.01±0.10	2.04±0.10	2.03±0.09	2.01±0.09
	Heart	1.17±0.21	1.17±0.26	1.09±0.14	1.15±0.21
	Kidney	0.91±0.13	0.89±0.13	0.88±0.14	0.94±0.15
Female	Liver	7.45±0.84	7.25±0.69	7.35±0.87	7.55±0.86
	Pituitary gland	0.020±0.004	0.019±0.004	0.018±0.005	0.017±0.007
	Spleen	0.51±0.08	0.49±0.06	0.48±0.07	0.50±0.06
	Testis	0.060±0.013	0.062±0.012	0.057±0.014	0.062±0.010
	Thymus	0.33±0.08	0.30±0.07	0.31±0.06	0.32±0.04

Table 6. Histopathological findings of rats treated with GM rice for 90 days.

Histopathological finding	Sex	Male		Female	
	Group(n=10)	G1	G4	G 1	G4
Liver	Portal inflammatory cells	9(1)	10(0)	10(0)	10(0)
	Centrilobular apoptosis/vacuolation	10(0)	10(0)	10(0)	10(0)
	Medullary mineralization	10(0)	10(0)	10(0)	10(0)
	Periportal vacuolation	10(0)	10(0)	10(0)	10(0)
	Medullary mineralization	10(0)	10(0)	10(0)	9(1)
	Parenchymal inflammatory cells	10(0)	10(0)	10(0)	9(1)
	Cortical scarring and inflammatory cells	10(0)	10(0)	10(0)	9(1)
Kidney	Papillary mineralization	9(1)	10(0)	10(0)	10(0)
	Inflammatory cells	9(1)	9(1)	10(0)	9(1)
	Cortical vacuolation	10(0)	10(0)	10(0)	10(0)
	Basophilia	10(0)	10(0)	10(0)	10(0)
Adrenal gland	Cortical hypertrophy	9(1)	9(1)	10(0)	10(0)
	Cortical vacuolation	10(0)	6(4)	10(0)	9(1)
Heart	Myocardial inflammation	8(2)	9(1)	9(1)	10(0)
	Endocardial inflammation	10(0)	10(0)	10(0)	10(0)
	Myocardial fibrosis & inflammation	10(0)	10(0)	10(0)	10(0)
	Myocardial necrosis	10(0)	9(1)	10(0)	10(0)

Table 6. Contd.

	Myocardial hemorrhage	10(0)	9(1)	10(0)	9(1)
Lung	Alveolar macrophage	9(1)	9(1)	10(0)	10(0)
	Pneumonitis	9(1)	9(1)	10(0)	10(0)
	Perivascular inflammatory cells	8(2)	10(0)	8(2)	10(0)
	Alveolar osseous metaplasia	9(1)	9(1)	10(0)	10(0)
	Prominent BALT	9(1)	8(2)	8(2)	10(0)
	Alveolar hemorrhage	10(0)	10(0)	10(0)	10(0)
	Pigmented macrophage	10(0)	10(0)	10(0)	10(0)
Spleen	Extramedullary hematopoiesis	8(2)	6(4)	8(2)	6(4)
	Subcapsular vacuolation	9(1)	10(0)	10(0)	10(0)
	Hemosiderosis	10(0)	10(0)	10(0)	10(0)
Epididymis/ Prostate gland	Interstitial infiltration cells	9(1)	10(0)		
	Prostatitis	9(1)	9(1)		
	Interstitial inflammatory cells	9(1)	10(0)		
Uterus	Luminal dilatation			7(3)	8(2)
	Endometrial glandular dilatation			10(0)	10(0)
Thymus	Involution / atrophy	9(1)	10(0)	10(0)	8(2)
	Cortical apoptosis	10(1)	10(0)	10(0)	10(0)
	Cystcs	10(0)	10(0)	10(0)	10(0)
Thyroid gland	Microfollicles	9(1)	10(0)	10(0)	10(0)
	Follicular cell hypertrophy	10(0)	10(0)	10(0)	10(0)
Pancreas	Focal acinar atrophy	9(1)	10(0)	10(0)	10(0)
	Fat replacement	9(1)	10(0)	10(0)	10(0)
	Acinar apoptosis	7(3)	8(2)	9(1)	10(0)
	Inflammatory cells	10(0)	10(0)	10(0)	10(0)
Stomach	Submucosal inflammation	9(1)	10(0)	10(0)	9(1)
	Squamous cyst	9(1)	10(0)	10(0)	10(0)
	Ulceration-non glandular region	10(0)	10(0)	9(1)	10(0)
Urinary bladder	Luminal dilatation	10(0)	9(1)	9(1)	10(0)

*Normal (slight malfunction).

in the 90-day toxicity study. This is an extremely conservative estimate of human exposure to the mCry1Ac1 protein because *in vitro* digestion studies indicate that mCry1Ac1 will not be absorbed intact because it will most likely be degraded within the gut.

Dietary proteins are taken in as nutrients and typically demonstrate no relation to toxic effects. However, some proteins do cause acute toxicity (Metcalfe et al., 1996; Sjoblad et al., 1992). For this reason, guidance documents from CODEX and KFDA described procedures for assessing the potential toxicity of transgenic proteins. Most dietary proteins are non-toxic and absorbed for nutritional purposes. However, because some identified proteins are toxic to mammals and other species, new reliable recommendations were developed to assess transgenic proteins that could cause toxicity using a two-tiered approach (Delaney et al., 2008).

The first tier assesses the history of use of the organism from which the gene is obtained. Generally, similarities between known toxic proteins and transgenic proteins can be determined using bioinformatical analysis, mechanism of action of the protein, *in vitro* stability to digestive enzymes were major research process (Delaney et al., 2008). A second tier analysis may be conducted if there have harmful results. Elements within this tier include acute toxicity studies that use purified transgenic proteins and 90-days oral toxicity (sub-chronic) studies with genetically modified organisms

as requested by regulatory authorities.

Conclusion

Our present data demonstrate the safety of the mCry1Ac1 protein and insect-resistant Agb0101 rice for use in food and feed applications and indicate that the mCry1Ac1 protein and Agb0101 rice presents no risks for adverse health effects when used in the context of agricultural biotechnology. No evidence of toxicity was observed in mice or rats following acute or 90-day oral exposure to heterologously produced mCry1Ac1 protein in Agb0101 rice. Therefore, according to our current study findings, the no-observed-adverse-effect-level (NOAEL) for the mCry1Ac1 protein is more than 10 g/Kg body weight for both male and female mice. Results from these studies further support the use of a tiered approaches that include Tier II studies, such as including hazard characterizations and acute and sub-chronic oral toxicity studies, but do not provide additional information or results that contradict the results of the Tier I studies that do not identify evidence for potential toxicity of the mCry1Ac1 protein.

Conflict of interest

The authors did not declare any conflict of interest.

ACKNOWLEDEGEMENTS

This study was carried out with the support of "Research Program for Agricultural Science and Technology Development (Project No. PJ009654)", National Academy of Agricultural Science, Rural Development Administration, Republic of Korea.

REFERENCES

Bajaj S, Mohanty A (2005). Recent advances in rice biotechnology towards genetically superior transgenic rice. Plant Biotechnol. J. 3: 275-307.

Bravo A, Gill SS, Soberón M (2007). Mode of action of *Bacillus thuringiensis* Cry and Cyt toxin and their potential for insect control. Toxicon. 49:423–435.

CODEX (2003). Codex Alimentarius Commission, Alinorm 03/34: Joint FAO/WHO Food Standard Programme, Codex Alimentarius Commission, Twenty-Fifth Session, Rome, Italy, June 30–July 5, 2003. Appendix III, Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, and Appendix IV, Annex of the assessment of possible allergenicity, pp. 47-60.

Delaney JD, Astwood H, Cunny R, Eichen-Conn C, Herouet-Guicheney S, MacIntosh LS, Meyer L, Privalle Y, Gao J, Mattsson J and Levine M (2008). Evaluation of protein safety in the context of agricultural biotechnology. ILSI International Food Biotechnology Committee Task Force on Protein Safety, Food Chem. Toxicol. 46: S71–S97.

Estruch JJ, Warren GW, Mullins MA, Nye GJ and Craig JA (1996). Vip3A, a novel Bacillus thuringiensis vegetative insecticidal protein with a wide spectrum of activities against Lepidopteran insects. Proc Natl Acad Sci USA, 93:5389-5394.

European Commission (2003). Guidance document for the risk assessment of genetically modified plants and derived food and feed, Prepared for the Scientific Steering Committee by the Joint Working Group on Novel Foods and GMOs.

FAO/WHO (2001). Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology – Evaluation of Allergenicity of Genetically Modified Foods, Rome, Italy. pp. 1–27.

James C (2013). Global status of commercialized biotech/GM crops: 2013, ISAAA brief no. 46. ISAAA, Ithaca, NY, USA.

KFDA (2007). Guidelines for the safety assessment data for genetically modified foods and food additives. KFDA Notification No. 2007-60.

Kim EH, Suh SC, Park BS, Shin KS, Kweon SJ, Han EJ, Park SH, Kim YS, Kim JK (2009). Chloroplast-targeted expression of synthetic cry1Ac in transgenic rice as an alternative strategy for increased pest protection. Planta 23: 397–405.

Kim HY, Lee SM, Kim JK, Shin KS, Yeo YS and Cho HS (2013). Quantification of mCry1Ac1 and PAT proteins during growth of genetically modified rice developed in Korea, 2013, Food Sci. Technol. 22(1): 195-200.

Lee RY, Shin KS, Suh SC, Kim KY, Jeon YH, Park BS, Kim JK, Kweon SJ, Lee YH (2009). Molecular characterization of lepidopteran pest-resistant transgenic rice events expressing synthetic Cry1Ac. Plant Biotechnol. Rep. 3:317–324.

Maqbool SB, Riazuddin S, Loc NT, Gatehouse AMR, Gatehouse JA, Christou P (2001). Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. Mol. Breed. 7:85-93.

McClintock JT, Schaffer CR, Sjoblad RD (1995). A comparative review of the mammalian toxicity of *Bacillus thuringesis*-based pesticides, Pestic. Sci. 45:95-105.

Metcalfe DD, Astwood JD, Townsend R, Sampson HA, Taylor SL and Fuchs RL (1996). Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Critical Rev. Food Sci. Nutr. 36(S): S165-S186.

OECD (1998). Guidelines for the Testing of Chemicals / Section 4: Health Effects-Repeated Dose 90-Day Oral Toxicity Study in Rodents Test No. 408:

OECD (2001). Acute oral toxicity – Acute toxic class method (423). www.oecd.ord .dataoecd/17/50/1948370.pdf

Ramesh S, Nagadhara D, Reddy VD, Rao KV (2004). Production of transgenic indica rice resistant to yellow stem borer and sapsucking insects, using super-binary vectors of *Agrobacterium tumefaciens*. Plant Sci. 166:1077-1085.

Sjoblad RD, McClintock JT and Engler R (1992). Toxicological considerations for protein components of biological pesticide products, Regul. Toxicol. Pharmacol. 15:3-9.

Sun W, Xing BC, Sun Y, Du XJ, Lu M, Hao CY, Lu Z, Mi W, Wu SF, Wei HD, Gao X, Zhu YP, Jiang Y, Qian XH, He FC (2007). Proteome analysis of hepatocellular carcinoma by two-dimensional difference gel electrophoresis, Molecular & Cell Proteomics, 6: 1798–1808.

Tu J, Zhang G, Datta K, Xu C, He Y, Zhang Q, Khush GS, Datta SK (2000). Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* delta-endotoxin. Nat. Biotechnol. 18:1101-1104.

Warren GW (1997). Vegetative insecticidal proteins: novel proteins for control of corn pests. In Advances in Insect Control, the Role of Transgenic Plants ed. Carozzi, N.B. *and* Koziel, M. *pp.* 109–121. London: Taylors & Francis Ltd.

Ye GY, Yao HW, Shu QY, Cheng X, Hu C, Xia YW, Gao MW, Altosaar I (2003). High levels of stable resistance in transgenic rice with a *cry1Ab* gene from *Bacillus thuringiensis* Berliner to rice leaffolder, *Cnaphalocrocis medinalis* (Guenée) under field conditions. Crop Prot. 22:171-178.