**RISK ASSESSMENT REPORT OF THE FOOD SAFETY TECHNICAL TEAM (FSTT ) FOR GM MAIZE EVENT MIR604**

**I. Introduction**

MIR604 maize is the product of Syngenta claimed to provide benefit for resistance against corn rootworm, a Coleopteran pest ubiquitous to corn growing areas of the United States. MIR604 maize produces mCry3A protein and phosphomannose isomerase (PMI) protein.

MIR604 maize has been used as a food and or feed in 10 countries namely Australia (2006), Taiwan (2007), Canada (2007), Japan (2007), Korea (2007), Mexico (2007), Philippines (2007), United States (2007), Russian (2007), and China (2008)

Food Safety Technical Team has conducted food safety studies for MIR604 maize based on genetic information and food safety information consisting of substantial equivalence, allergenicity, and toxicity according to the regulation of Indonesia’s National Agency for Drug and Food Control (NADFC/BPOM) Number HK.00.05.23.3541 of 2008 concerning Guidelines of Food Safety Study for Genetically Modified Products. Summary of assessment are specified below.

**II. Genetic Information**

**II.1 Genetic Element**

MIR604 maize contains two novel genes namely *mCry3A* gene and *PMI* gene. *mCry3A* gene produces mCry3A protein responsible for resistance against Coleopteran (*Diabrotica virgifera virgifera* Le Conte, *D. longicornis barberi* Smith *and* Lawrence, and *D. virgifera zeae* Krysan *and* Smith); and *pmi* gene encodes *phosphomannose isomerase* protein as selectable marker. Promotor used in *mCry3A* genes are MTL (*metallothionein-like Zea mays*); it confers root-preferential gene expression in corn while NOS (nopaline synthase) from *Agrobacteria tumefaciens is used as terminator*. Promotor and terminator used for PMI gene are ZmUbilInt and NOS (nopaline synthase).

**II.2 Gene Source**

*mCry3A* gene is isolated from *Bacillus thuringiensis* subsp. *tenebrionis* and *pmi* gene is isolated from *Escherichia coli*

**II.3 Transformation Method**

Event MIR604 maize was produced by *Agrobacterium tumefaciens*-mediated transformation of immature embryos. Plasmid pZM26 is the vector used for the transformation of MIR604.

**II.4 Genetic Stability**

Molecular analysis using Southern blot is conducted to see the stability of inserted genes from generation to generation. Hibridization pattern of backcross four (BC4), backcross five (BC5) and backcross six (BC6) generations of Event MIR604 were identified. The result shows that the TDNA inserted gene of pZM26 is stable up to the generation of back-cross 6 (BC6). Protein level of mCry3A and PMI are also stable over four successive backcross generations. Genetic stability of Mendelian inheritance Law.

The Southern blot hybridization data provided confirmation of evidence supporting the TaqMan® PCR analysis that MIR604 maize contained single copy of the *mcry3A* gene and *pmi* gene. Southern blot analysis using backbone specific probe revealed no hybridization bands detected in the genomic samples, demonstrating that MIR604 maize does not contain any backbone sequences from the transformation plasmid pZM26. Genetic stability of MIR604 maize data was provided by Syngenta study report: Hope Hart and Scott Rabe, 2004. *Molecular Characterization of Event MIR604 Maize (Corn) Expressing a Modified Cry3A Bacillus thuringiensis Protein.*

**II.5.** Based on genetic information study concludes that:

1. MIR604 maize contains single copy of *mCRY3A* genes and PMI (*phosphomannose isomerase*) genes;
2. MIR604 maize does not contain any backbone sequences from the transformation plasmid pZM26;
3. Both genes of interest, *mCry3A* and *pmi*, introduced to MIR604 maize remain stable up to the generation of back-cross 6;
4. Both genes of interest, *mCry3A* and *pmi*, introduced to MIR604 maize are segregated according to Mendelian inheritance Law.

**III. Food Safety Information**

**III.1 Substantial Equivalence**

Complete substantial equivalence study for MIR604 maize is explained under company study report Nr SSB-111-04 A2 “*Compositional Analysis of Grain and Whole Plants from Transgenic Maize (Corn) Event MIR604”* (C.Kramer, 2005).

Composition of kernels and all parts of corn plants (forage) of MIR604 maize and nontransgenic were obtained from corn grown in 2002 in 3 locations in the USA and planted in 2003 at 7 locations in the USA. All 2002 samples were analyzed by Woodson-Tenent Laboratory Inc., Goldston NC. Whereas all 2003 samples were analyzed by Covance Laboratories Inc., Madison, WI.

Composition analysis and chemical components in forage is proximate including Total Dietary Fiber (TBF), mineral (calcium, phosphor, potassium, sodium, iron, copper, and Zinc) vitamin (β-karoten, α-, β-, δ-, γ-tocopherol, folic acid, thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, and vitamin C) amino acid, fatty acid, secondary metabolite such as ferulic acid and p-coumaric acid, furfural, inositol, phytic acid, and inhibitor trypsin.

From the results of the analysis it was found that for the 2002 planting, the composition of MIR604 maize was not different from nontrangenic. For 2003 planting, there was a slight difference, i.e MIR604 maize was higher by around 4-7% compared to nontransgenic, also for water content (3% higher) and calcium (4-10% higher). Generally the average value is still within the range of compositions reported in the literature, as reported by OECD (2002).

The result of substantial equivalence study above concludes that MIR604 maize is substantially equivalent with nontransgenic maize.

**III.2 Allergenicity**

Amino acid sequence analysis has been carried out for mCry3A protein produced from the *mCry3A* gene and PMI enzyme produced from the PMI gene. The results are reported as a company study report, namely:

1. “Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Toxins”, oleh Jennifer Zawodny. Penelitian dilakukan di Syngenta Seeds, Inc. Product Registration Group, Post Office Box 12257, 3054 Cornwallis Road, Research Triangle Park, North Carolina, USA 27709-2257.
2. “Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Toxins”, oleh Jennifer Zawodny. Penelitian dilakukan di Syngenta Seeds, Inc. Product Registration Group, Post Office Box 12257, 3054 Cornwallis Road, Research Triangle Park, North Carolina, USA 27709-2257.

Homology sequence of amino acid was analyzed using a bioinformatics program of SWISSPROT, BLASTP, FASTA, and FARRP Data Base. mCry3A amino acid sequence was screened and compared in each peptide of 80 amino acids and in segments 8 of amino acids with allergens protein to track the presence of binding epitopes to IgE. The result showed no homology among overall proteins, peptide of 80 sequential amino acids and segment of 8 sequential amino acids with known allergen data. PMI sequence also showed no homology result among overall proteins with any known allergenic protein. There was one sequence identity match of eight contiguous identical amino acids between PMI and a known allergen, α-parvalbumin (110 amino acid described as *Rana* species). The specific amino acid sequence in common was “DLSDKETT,” which occurs at positions 327 – 334 of PMI and positions 77 – 84 of the allergen sequence.

Hilger *et al*. (2002) proceeded to identify the causative agent of this anaphylactic response as α-parvalbumin using PMI produced from an *Escherichia coli* overexpression system. The allergic patient’s serum IgE indicate no cross-reactivity to related parvalbumins from *Rana esculenta* and it does not recognize any portion of PMI as an allergenic epitope. This study supports the conclusion that PMI shows no biologically relevant amino acid sequence similarity to any known or putative protein allergens.

Protein characterization was carried out on recombinant proteins derived from Maize plants and recombinant proteins derived from *Escherichia coli*. The study consist of immunoreactivity test, molecular weight, glycosylation and N-terminal amino acid sequence, and was conducted in Syngenta laboratory in compliance with the relevant provisions of Good Laboratory Practice (GLP). Both proteins were extracted and purified using column chromatography

Quantity and quality testing of protein were conducted according to the method described by Bradford, SDS-PAGE, ELISA, Western blot methods, enzymatic activity tests and bioassays, glycosylation analysis were analyzed using the DIG Glycan Detection Kit, Edman degradation and peptide map. In vitro digestibility was done using simulated gastric fluid (SGF) and simulated intestine fluid (SIF).

PMI enzyme produced from *E. coli* is different on 16 non-functional amino acids in N-terminals. However, the biochemical characteristics, immunoreactivity, biological activity and enzymatic reactions of both PMI enzymes (E. coli and GM corn) were the same.

The average mCry3A protein is 0.0001% of total protein (less than 1 μg of mCry3A protein per gram of grain or less than 1 ppm). In all growth stage, the average protein level is 3-23 μg / g (leaves), 2-4 μg / g (roots) and 0.9-11 μg / g (whole plants). The expression of mCry3A protein of inbred is higher than that of the hybrid. PMI enzymes range from 0-0.4 μg / g. In seeds less than 0.14 µg / g and in inbred similar to hybrids.

The stability analysis of the mCry3A protein and PMI enzyme showed that mCry3A protein was stable for up to four generations and the average mCry3A protein level was 2,3 - 3,1 μg / g and the PMI enzyme was also stable for generations.

Rapid degradation was shown for in vitro digestibility of mCry3A protein from MIR604 maize and recombinant *E. coli* using SGF containing pepsin. No intact mCry3A (*ca.* 67KD) were detected following digestion in SGF for 2 minutes. Whereas the PMI enzyme digestibility test from recombinant *E. coli* in SGF did not find PMI molecules intact after 1 minute.

Heat stability at 4°C, 25°C, 37°C, 65°C, 95°C showed that at 95°C the mCry3A protein was inactivated (denaturation or degradation) and at 65°C the loss all bioactivity. The PMI enzyme still detected at 37°C for 30 minutes, and at a temperature of more than 55°C the PMI enzyme was unstable and protein activity decreased to 3% after incubation at a temperature of 65°C.

Based on the results of allergenicity study it is concluded that the mCry3A protein and PMI protein do not show any potential for allergic reactions.

**III.3 Toxicity**

Acute toxicity study of mCry3A protein was conducted on mice and its result was reported as a company study report: “*Acute Oral Toxicity of Modified Cry3A Protein (mCry3A-0102) in the Mouse”* by Ian Johnson. The research was conducted at Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, United Kingdom, SK10 4TJ.

Test substance MCRY3A-0102 was administered to 5 male and 5 female albino mice strain APfCD-1 *via* a gavage dose of 2623 mg/kg body weight. Based on the Syngenta’s analysis that test substance MCRY3A-0102 contained ca. 90.3% modified Cry3A protein (w/w), the dose administered was equivalent to ca. 2377 mg modified Cry3A protein/kg bodyweight. A control group (5 males and 5 females) concurrently received the dosing vehicle alone, a suspension of 1% carboxymethylcellulose, at the same dosing volume as was used for the test material mixture.

Observations for bodyweight, food consumption, clinical pathology, mortality and clinical/behavioral signs of toxicity were made, and at least once daily thereafter for 14 days.

At the end of the scheduled period, the animals were killed and examined *post mortem*. The test was completed on November 11, 2003.

No test substance-related effects were seen in mice following an acute oral dose of 2632 mg MCRY3A-0102/kg bodyweight. The dose administered was equivalent to ca. 2377 mg modified Cry3A protein/kg bodyweight. MCRY3A-0102 is not acutely toxic to mice.

Acute toxicity of PMI protein was conducted to mice and its result was reported as a company study report: “*Phosphomannose Isomerase (Sample PMI-0198): Acute Oral Toxicity Study in Mice*“ by Janice O Kuhn. The research was conducted STILLMEADOW Inc., 12852 Park One Drive, Sugar Land, TX 77478.

Test substance PMI-0198 was administered to 7 male and 6 female albino mice strain HSD:ICR  *via* a gavage dose of 5050 mg/kg body weight. Based on the Syngenta’s analysis that test substance PMI-0198 contained ca. 60% phosphomannose isomerase (w/w). The protein substance was given twice with a giving distance of about 1 hour, with a concentration of 20% (weight / volume), which was dissolved in a 0.5% carboxy-methyl-cellulose solution. A control group (6 males and 5 females) concurrently received the dosing vehicle alone, a suspension of 0.5% carboxymethylcellulose 25.25 ml/w at the same dosing volume as was used for the test material mixture.

Observations for bodyweight, food consumption, clinical pathology, mortality and clinical/behavioral signs of toxicity were made, and at least once daily thereafter for 14 days.

At the end of the scheduled period, the animals were killed and examined *post mortem*. The test was completed on August 11, 1999.

No test substance-related effects were seen in mice following an acute oral dose of 5050 mg PMI-0198/kg bodyweight. PMI-0198 is not acutely toxic to mice

From the results of the toxicity study it can be concluded that the mCry3A protein is considered non-toxic and PMI protein is included in the practically non toxic group of substances.

**IV. Conclusion**

According to the explanations on genetic information of *mCry3A* gene which is isolated from *Bacillus thuringiensis* strain *tenebrionis* and *pmi* gene cloned from *Escherichia coli* that inserted in MIR604 maize; substantial equivalence analysis between the composition of MIR604 maize and nontransgenic maize; as well as allergenicity and toxicity study of mCry3A and PMI protein, it can be concluded that MIR604 maize is safe to be consumed as food.