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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

FEB 15 2006

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: Review of Product Characterization and Human Health Data for Modified Cry3A (mCry3A) *Bacillus thuringiensis* insect control protein and maize (Corn) Plants Derived from Event MIR604 (EPA Reg. No. 67979-L) in support for a Permanent Exemption from Tolerances and Section 3 Registration, submitted by Syngenta Seeds, Inc. – Field Crops- NAFTA.

TO: Mike Mendelsohn, Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

FROM: Annabel Fellman, Environmental Protection Specialist [signed]
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

THROUGH: John L. Kough, Ph.D., Biologist [signed]
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

ACTION REQUESTED: To review additional product characterization, human health and analytical methods data submitted by Syngenta Seeds, Inc.-Field Crops - NAFTA in support for section 3 registration and a permanent exemption from tolerances for Modified Cry3A insect control protein and maize (Corn) Plants Derived from Event MIR604.

CONCLUSION: The product characterization, protein expression, toxicological and allergenicity data support the finding that there is a reasonable certainty of no harm to humans from the aggregate exposure to the residues of the mCry3A protein, including all anticipated dietary exposures and all other exposures for which there is reliable information.

REVIEW DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

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DATA REVIEW RECORD:

Active Ingredient:	Modified Cry3A (mCry3A) *Bacillus thuringiensis* insecticidal protein and the genetic material necessary for their production (via pZM26) in transgenic maize (corn) plants derived from Syngenta Seeds' transformation Event MIR604.
Product Name:	Event MIR604 Maize Plants Expressing Modified Cry3A *Bacillus thuringiensis* Protein
Company Name:	Syngenta Seeds, Inc. – Field Corps- NAFTA
ID No:	67979
Chemical Number:	006509
Decision Number:	343070
DP Barcode:	319746

MRID No:

465974-01 **Independent Laboratory Validation of ELISA method for the Detection of mCry3A in Event MIR604 corn grain**

BACKGROUND:

Syngenta synthetically modified a *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionis* to optimize the gene for expression in maize (corn) and to enhance its activity against the western corn rootworm (WCRM; *Diabrotica virgifera vigifera*) and northern corn rootworm (NCRM; *D. longicornis barberi*) (Sekar *et al.,* 1987). Syngenta's maize Event MIR604 corn plants were a result of a corn plant transformation with the synthetic modified *cry3A* gene, which provides resistance to these pests (Chen, E. and Stacy, C., 2003). Transformation was conducted using immature maize embryos derived from a proprietary *Zea mays* line, *via Agrobacterium*-mediated transformation. By this method, genetic elements within the left and right border regions of the transformation vector are efficiently transferred and integrated into the genome of the plant cell.

Event MIR604 maize also contains the *pmi* gene, which was introduced along with the mCry3A protein via the same pZM26 transformation vector. The gene represents the *manA* gene from *Escherichia coli* and encodes the enzyme phosphomannose isomerase (PMI), which was employed as a selectable marker during the process of regenerating plant material following transformation (Negrotto, *et al.,* 2000). The PMI protein is a common enzyme involved in carbohydrate metabolism to allow for selection of transformants in cell culture, by only allowing transformed corn cells to utilize mannose as a sole carbon source, while corn cells lacking the *pmi* gene fail to grow. An existing permanent exemption from the requirement of a tolerance has been established for PMI in all crops when used as a plant-incorporated protectant inert ingredient (see 40 CFR 180.1252, effective May 14, 2004). This regulation eliminates the need to establish a maximum permissible level for residues in or on all plant commodities of phosphomannose isomerase and the genetic material necessary for its production in all plants when part of a plant-incorporated protectant.

In the Federal Register of April 6, 2005 (70 FR 17323), the Agency established a temporary exemption from the requirement of a tolerance for Modified Cry3A and the genetic material

necessary for their production in corn which will expire October 15, 2006. In addition, EPA issued an experimental permit for the use *Bt* mCry3A protein and the genetic material necessary for their production (via plasmid pZM26) in Event MIR604 corn and associated activities (such as collection of field data; harvesting & processing of seed after last planting) on 575 acres of field corn. To support their requests for EUP and a temporary tolerance, Syngenta Seeds, Inc. submitted product characterization and protein expression analyses, toxicological and allergenicity data [see memoranda from A. Fellman through J. Kough to M. Mendelsohn, dated 02/11/2005, 02/23/2005, 03/03/2005, and 05/31/2005]. The submitted study titles, conclusions, and their MRID numbers are provided (see Table 1) in this report. The final study to complete data requirements for Section 3 registration for Modified Cry3A protein and the genetic material necessary for their production (via plasmid pZM26) in Event MIR604 corn is reviewed in this report as well.

Permanent Exemption for the Requirement of a Tolerance (4F6838)

EPA has established an exemption from tolerance requirements pursuant to FFDCA section 408(j)(3) for *Bacillus thuringiensis* Cry3A delta-endotoxin and the genetic material necessary for its production in potatoes, and this tolerance exemption has been reassessed and meets the 408(c)(2) standard (see 40 CFR 180.1147). An exemption from the requirement of a tolerance has been established for PMI in all crops when used as a plant-incorporated protectant inert ingredient (see 40 CFR 180.1252, effective May 14, 2004).

In the Federal Register of April 6, 2005 (70 FR 17323), the Agency established a temporary exemption from the requirement of a tolerance for Modified Cry3A and the genetic material necessary for their production in corn which will expire October 15, 2006. In addition, EPA issued an experimental permit for the use *Bt* mCry3A protein and the genetic material necessary for their production (via plasmid pZM26) in Event MIR604 corn and associated activities (such as collection of field data; harvesting & processing of seed after last planting) on 575 acres of field corn.

Syngenta Seeds, Inc. – Field Crops- NAFTA has submitted a petition for a permanent exemption from the requirement of a tolerance pursuant to section 408(d)(1) of the Federal Food, Drug, and Cosmetic Act with respect to the plant-incorporated protectant modified Cry3A *Bacillus thuringiensis* insect control protein and the genetic material necessary for its production in all corn.

Previously submitted studies demonstrated the lack of toxicity of the mCry3A protein following acute oral high-dose exposure to mice, rapid degradation of mCry3A upon exposure to simulated mammalian gastric fluid and the lack of significant amino acid sequence homology of the mCry3A protein to proteins known to be mammalian toxins or human allergens. Therefore, dietary exposure to mCry3A in corn is not anticipated to pose any harm for the U.S. population as stated in the memorandum from A. Fellman, through J. Kough, Ph.D., to M. Mendelsohn, dated May 31, 2005.

The only outstanding data gap identified in the May 31, 2005 memorandum was the submission of a validated analytical method for detection of mCry3A in corn. A new submission for the analytical method employing a monoclonal antibody is reviewed (MRID No. 465974-01) in this report.

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Preliminary Safety Assessment

Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is Asafe.@ Section 408(c)(2)(A)(ii) of the FFDCA defines Asafe@ to mean that Athere is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information.@ This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Section 408(b)(2)(C) of the FFDCA requires EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to Aensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue. . . @ Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider Aavailable information concerning the cumulative effects of a particular pesticide's residues and other substances that have a common mechanism of toxicity.@ EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

Product Characterization Profile

A mCry3A *Bacillus thuringiensis* (*Bt*) insect control protein is produced in transgenic corn plants derived from transformation Event MIR604. A *cry3A* gene from *Bt* subsp. *tenebrionis* was recreated synthetically to optimize for expression in corn. Additional changes in this corn-optimized gene were made, such that the encoded mCry3A protein has enhanced activity against larvae of the western corn rootworm (WCRM; *Diabrotica virgifera vigifera*) and northern corn rootworm (NCRM; *D. longicornis barberi*). Introduced *via* transformation vector pZM26, a *mcry3A* specific probe, consisting of 1797 base pairs (bp), was incorporated between an MTL promoter (2556 bp) from the *Zea mays* metallothionein-like gene and a terminator sequence from the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens* used to provide a polyadenylation site. An *Escherichia coli manA* gene encoding a phosphomannose isomerase *pmi* gene (1176 bp) was incorporated between a promoter region from the *Zea mays* polyubiquitin gene (ZmUbInt (1993 bp)) and the same NOS terminator sequence described above. This *pmi* gene, which was introduced along with the mCry3A protein via the same pZM26 transformation vector, encodes the enzyme phosphomannose isomerase (PMI), which is employed as a selectable marker during the process of regenerating plant material following transformation. The PMI protein is a common enzyme involved in carbohydrate metabolism to allow for selection of transformants in cell culture, by only allowing transformed corn cells to utilize mannose as a sole carbon source, while corn cells lacking the *pmi* gene fail to grow.

Hybridization patterns indicate that one full length copy of each of the *mcry3A* and *pmi* genes were integrated into the maize genome. Moreover, DNA sequencing revealed that there was a 44 and 43 bp truncation at the right and left break points of the T-DNA insert, respectively, during the transformation process that resulted in MIR604. Therefore, the overall integrity of the insert and the contiguousness of the functional elements were confirmed.

The native Cry3A protein of *Bt* subsp. *tenebrionis* is a *ca.* 73 kDa polypeptide of 644 amino acids. By comparison, the mCry3A protein expressed in Event MIR64 corn is a *ca.* 67 kDa polypeptide of 598 amino acids. The amino acid sequence of the mCry3A protein corresponds to that of the native Cry3A protein, except: 1) Its N-terminus corresponds to methionine-48 of the native protein; and 2) A cathepsin-G protease recognition site has been introduced, beginning at amino acid residue 155 of the native protein. This cathepsin-G recognition site has the sequence alanine-alanine-proline-phenylalanie, and has replaced the amino acids valine-155, serine- 156, and serine-157 in the native protein.

Toxicological Profile

Consistent with section 408(b) (2) (D) of the FFDCA, EPA has reviewed the available scientific data and other relevant information in support of this action and considered its validity, completeness and reliability and the relationship of this information to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children.

Data have been submitted demonstrating the lack of mammalian toxicity at high levels of exposure to the pure mCry3A protein. These data demonstrate the safety of the products at levels well above maximum possible exposure levels that are reasonably anticipated in the crops. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.740(b)(2)(i)). For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study, to verify the observed effects and clarify the source of these effects (Tiers II and III).

An acute oral toxicity study was submitted for the mCry3A protein. The acute oral toxicity data submitted support the prediction that the mCry3A protein would be non-toxic to humans. Male and female mice (5 of each) were dosed with 2,377 milligrams/kilograms bodyweight (mg/kg bwt) of mCry3A protein. With the exception of one female in the test group that was euthanized on day 2 (due to adverse clinical signs consistent with a dosing injury), all other mice survived the study, gained weight, had no test material-related clinical signs, and had no test material-related findings at necropsy.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. 1992). Therefore, since no effects were shown to be caused by the plant- incorporated protectants, even at relatively high dose levels, the mCry3A protein is not considered toxic. Amino acid sequence comparisons showed no similarity between the mCry3A protein to known toxic proteins available in public protein data bases. According to the Codex Alimintarius guidelines, the assessment of potential toxicity also includes stability to heat (FAO/WHO Standards Programme, 2001). Further data demonstrate that mCry3A is inactivated against WCRM, when heated to 95 °C for 30 minutes.

Since mCry3A is a protein, allergenic sensitivities were considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a

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weight of the evidence approach where the following factors are considered: source of the trait; amino acid sequence similarity with known allergens; prevalence in food; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF), and glycosylation. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation acid and proteases; may be glycosylated; and present at high concentrations in the food.

Data have been submitted that demonstrate that the mCry3A protein is rapidly degraded by gastric fluid *in vitro.* In a solution of simulated gastric fluid 1 mg/mE mCry3A test protein mixed with simulated gastric fluid (pH 1.2, containing 2 mg/mL NaCl, 14 *pL* 6 N HC1, and 2.7 mg/mL pepsin) resulting in 10 pepsin activity units/ jag protein (complies with 2000 US Pharmacopoeia recommendations), complete degradation of detectable mCry3A protein occurred within 2 minutes. A comparison of amino acid sequences of known allergens uncovered no evidence of any homology with mCry3A, even at the level of 8 contiguous amino acids residues. Further data demonstrate that mCry3A is not glycoslylated, is inactivated when heated to 95 °C for 30 minutes, and is present in low levels in corn tissue.

Therefore, the potential for the mCry3A protein to be a food allergen is minimal. As noted above, toxic proteins typically act as acute toxins with low dose levels. Therefore, since no effects were shown to be caused by the plant-incorporated protectant, even at relatively high dose levels, the mCry3A protein is not considered toxic.

Aggregate Exposures

In examining aggregate exposure, section 408 of the FFDCA directs EPA to consider available information concerning exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectant chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Exposure via residential or lawn use to infants and children is also not expected because the use sites for the mCry3A protein are all agricultural for control of insects. Oral exposure, at very low levels may occur from ingestion of processed corn products and, potentially, drinking water.

However, oral toxicity testing done at a dose in excess of 2 gm/kg showed no adverse effects. Furthermore, the expression of the modified Cry3A protein in corn kernels has been shown to be in the parts per million range,which makes the expected dietary exposure several orders of magnitude lower than the amounts of mCry3A protein shown *to* have no toxicity. Therefore,

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even if negligible aggregate exposure should occur, the Agency concludes that such exposure would present no harm due to the lack of mammalian toxicity and the rapid digestibility demonstrated for the mCry3A protein.

Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity, resulting from the plant-incorporated protectant, we conclude that there are no cumulative effects for the mCry3A protein.

Determination of Safety for U.S. Population, Infants and Children

A. Toxicity and Allergenicity Conclusions
The data submitted and cited regarding potential health effects for the mCry3A protein include the characterization of the expressed mCry3A protein in corn, as well as the acute oral toxicity, heat stability, and *in vitro* digestibility of the proteins. The results of these studies were determined applicable to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were considered.

Adequate information was submitted to show that the mCry3A protein test material derived from microbial cultures was biochemically and functionally similar to the protein produced by the plant-incorporated protectant ingredients in corn. Microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the prediction that the mCry3A protein would be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. 1992). Since no effects were shown to be caused by mCry3A protein, even at relatively high dose levels (2,377 mg mCry3A/kg bwt), the mCry3A protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. (See 40 CFR 158.740(b)(2)(i)). Moreover, mCry3A showed no sequence similarity to any known toxinand was inactivated by heat against WCRM.

Protein residue chemistry data for mCry3A were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. However, data submitted demonstrated low levels of mCry3A in corn tissues with less than 2 micrograms mCry3A protein/gram dry weight in kernels and less than 30 micrograms mCry3A protein/gram dry weight of whole corn plant.

Since modified Cry3A is a protein, its potential allergenicity is also considered as part of the toxicity assessment. Data considered as part of the allergenicity assessment include that the modified Cry3A protein came from *Bacillus thuringiensis* which is not a known allergenic

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source, showed no sequence similarity to known allergens, was readily degraded by pepsin, and was not glycosylated when expressed in the plant. Therefore, there is a reasonable certainty that modified Cry3A protein will not be an allergen.

Neither available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children); nor safety factors that are generally recognized as appropriate for the use of animal experimentation data were evaluated. The lack of mammalian toxicity at high levels of exposure to the mCry3A protein, as well as the minimal potential to be a food allergen demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated in the crop.

The genetic material necessary for the production of the plant-incorporated protectant active ingredients are the nucleic acids (DNA, RNA) which comprise genetic material encoding these proteins and their regulatory regions. The genetic material (DNA, RNA) necessary for the production of mCry3A protein has been exempted under the blanket exemption for all nucleic acids (40 CFR 174.475).

B. Infants and Children Risk Conclusions
FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity.

In addition, FFDCA section 408(bfl2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the data base unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for the mCry3A protein and the genetic material necessary for their production. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

C. Overall Safety Conclusion
There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the mCry3A protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as previously discussed, no toxicity to mammals has been observed, nor any indication of allergenicity potential for the plant-incorporated protectant.

Other Considerations

A. Endocrine Disruptors
The pesticidal active ingredient is a protein, derived from sources that are not known to exert

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an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of the plant-incorporated protectant at this time.

B. Analytical Method(s)
A method for extraction and ELISA analysis of mCry3A protein in corn has been submitted and found acceptable by the Agency.

C. Codex Maximum Residue Level
No Codex maximum residue levels exist for the plant-incorporated protectant *Bacillus thuringiensis* mCry3A protein and the genetic material necessary for its production in corn.

RECOMMENDATION: As previously noted, there is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the mCry3A protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, the product characterization and human health data submitted are sufficient to support Section 3 registration and a permanent exemption from the requirement of a tolerance.

The data submitted for the ELISA method for determining mCry3A protein in Event MIR604 grain are classified as **ACCEPTABLE** and satisfies the EPA Residue Chemistry Guidelines OPPTS 860.1340(c)(6) Residue Analytical Methods and PR Notice 96-1. However, the EPA's Analytical Method Laboratory located in Fort Meade (Maryland) will have to independently validate Syngenta's ELISA protocol for accuracy, precision, and sensitivity.

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Summaries of each review supporting the safety findings in the areas of product characterization, human toxicity, and allergenicity for this product are provided below.

Table 1. Previously submitted product characterization, toxicity, and allergenicity studies

MRID	Title	Summary
461556-01	Review of Characterization and Safety of Modified Cry3A protein and maize (corn) plants derived from Event MIR604 with comparison to native Cry3A protein	The mCry3A protein contains 598 amino acids (*ca*. 67 kDa), whereas the native Cry3A protein is *ca*. 73 kDa polypeptide of 644 amino acids. The amino acid sequence of the mCry3A protein corresponds to that of the native Cry3A protein, except: 1) Its N-terminus corresponds to methionine-48 of the native protein; and 2) A cathepsin-G protease recognition site has been introduced, beginning at amino acid residue 155 of the native protein. The susceptibility of insect pest species and insecticidal properties, such as solubilization, proteolytic processing, receptor binding, and membrane pore forming properties, were also determined for mCry3A protein and differentiated with native Cry3A protein. The mCry3A protein has a similar spectrum of activity to the native Cry3A, but with enhanced toxicity to NCRW and WCRW. **Classification: Acceptable**
461556-02	Molecular characterization of event MIR604 maize (corn) expressing a modified Cry3A bacillus thuringiensis protein	Corn Event MIR604 does not contain any of the backbone sequences from the transforming plasmid pZM26. Three nucleotide changes were identified, one in a regulatory region of the *mCry3A* gene and two in the *pmi* coding sequence. The *mCry3A* and *pmi* genes are closely linked. **Classification: Acceptable**
461556-03	Characterization of modified Cry3A protein produced in event MIR604-derived maize (corn) and comparison with modified Cry3A protein expressed in recombinant Escherichia coli	This study evaluated the similarity of modified Cry3A (mCry3A) insecticidal protein expressed in corn event MIR604 and mCry3A protein expressed in a recombinant *E. coli* test system. The mCry3A protein derived from corn event MIR604 and recombinant *E. coli* had the same approximate molecular weight (ca. 67,700 Da.) based on mass spectral analysis (from MRID 461556-06). The mCry3A protein from both sources was immunologically cross-reactive with the same anti-mCry3A antibody. Both proteins produced comparable toxicities toward Western Corn Rootworm larvae, based on LC_{50} values. There was no evidence of post-translational glycosylation of mCry3A protein from either source. It was concluded that the mCry3A proteins from corn event MIR604 and from recombinant *E. coli* were substantially the same. **Classification: Acceptable**

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461556-04	Quantification of modified Cry3A and pmi proteins in transgenic maize (corn) tissues, whole plants, and silage derived from transformation event MIR604	The plant extracts (including leaves, roots, kernels, silk, pollen, silage, and whole plants) from inbred and hybrid corn lines derived from MIR604 field plants were quantitatively analyzed for mCry3A by ELISA. The magnitude of expression for tissue types was as follows in descending order: leaves, roots, silage, and kernels. All control tissues were negative for the expression of mCry3A. The mean extraction efficiency for mCry3A over all tissues (except for pollen or silk tissue, where levels were too low to be determined) was 76.6%. Low, but quantifiable, levels of PMI protein were found in most of the Event MIR604-derived plant tissues analyzed including pollen. The mCry3A and PMI proteins were stably expressed in four backcross generations in leaf tissue analyzed at anthesis stage. Mean levels across all backcross generations were *ca.* 11.8 - 15.5 µg/g dry weight and 1.1 - 1.3 µg/g dry weight for mCry3A and PMI proteins, respectively. **Classification: Acceptable**
461556-05	Characterization of modified Cry3A test substance mCry3A-0102 and certificate of analysis	This study characterized test material MCRY3A-0102, a microbially produced protein preparation containing a modified Cry3A (mCry3A) protein. The purity of mCry3A in the test material was shown to be *ca.* 90.3% by weight using SDS-PAGE analysis. Western blot analysis of the test material showed a single immunoreactive band corresponding to the predicted molecular weight of ca. 67,700 Da. The test material was insecticidally active and had a 144-hour LC_{50} of 1.4 µg/mL diet (95% confidence interval: 0.7 - 2.2 µg/mL) against Western corn rootworm (WCRW) larvae. Two forms of mCry3A were found in the test material, designated mCry3A-SF and mCry3A-LF, respectively, and were both insecticidally active against WCRW. On this basis, and taking into account the high degree of structural homology (97.4% amino acid identity), the two forms of mCry3A in test material MCRY3A-0102 were considered to be equivalent. The test material was re-analyzed ca. 9 months after its initial characterization and found to be substantially stable when stored at -20 °C. **Classification: Acceptable**
461556-06	Further Characterization of Modified Cry3A Test Substance MCRY3A-0102	The test material, MCRY3A-0102, containing mCry3A protein was shown, by SDS-PAGE and MALDI TOF mass spectrometry, to contain two closely related components in a ratio of *ca.* 2:3. The lesser of the two components, with the lower molecular weight, corresponded to the intended mCry3A protein with 598 amino acids. The other component contained the same 598 amino acids as the first component but also contained an additional 16 amino acids at the N–terminal end of the protein. The identity of the two components was conclusively determined by peptide mapping using tandem (MS/MS) mass spectrometry and by MALDI TOF mass spectrometry of the intact proteins. The molecular weight data showed the two proteins to have masses of 67,519 and 69,138 Da. **Classification: Acceptable**

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461556-07	In vitro digestibility of modified Cry3A protein (mCry3A-0102 and IAPMIR604-0103) under simulated mammalian gastric conditions	The susceptibility of mCry3A protein to proteolytic degradation was evaluated in simulated gastric fluid (SGF) containing pepsin. Modified Cry3A protein from transgenic corn and recombinant E. coli (test material MCRY3A-0102) was readily degraded in SGF. The data support a conclusion that mCry3A protein expressed in transgenic plants will be readily digested as conventional dietary protein under typical mammalian gastric conditions. **Classification: Acceptable**
461556-08	Effect of temperature on the stability of modified Cry3A protein (mCry3A-0102)	At 95°C mCry3A protein was completely inactivated against WCRM. At 4°C, 25°C, and 37° C there was little or no effect on mCry3A bioactivity, while at 65°C there was some reduction in the bioactivity. **Classification: Acceptable**
461556-09	Analysis for the presence of modified Cry3A protein in wet and dry milled fractions, corn oil and corn chips from corn (maize) event MIR604	Among the wet-milled fractions, the medium fiber (0.46 µg mCry3A/g), fine fiber (0.26 µg mCry3A/g), and gluten meal (0.24 µg mCry3A/g) fractions yielded quantifiable amounts of mCry3A. Among the dry-milled fractions the highest concentrations were found in the flaking grits (2.12 µg mCry3A/g), the corn hulls (1.42 µg mCry3A/g), and the coarse grit (0.92 µg mCry3A/g) fractions. Levels of mCry3A found in the other dry-milled fractions, including fine grits, corn meal, corn cone and corn flour, were between 0.32 and 0.69 µg mCry3A/g. Although the concentration of mCry3A protein measured in the flour used to prepare the corn chips was 0.32 µg mCry3A /g, no mCry3A protein was detected in the corn chips. Similarly, mCry3A protein was not detectable in oil, whereas the starting material, flaking grits, contained 2.12µg mCry3A/g. **Classification: Acceptable**
461556-10	Acute oral toxicity study of modified Cry3A protein (mCry3A-0102) in the mouse	MCRY3A-0102 was not acutely toxic to mice. There was no evidence of toxicity at 2,632 mg MCry3A-0102/kg body weight, representing ca. 2,377 mg mCry3A protein/kg body weight. The estimated LD_{50} value for pure mCry3A protein in male and female mice was > 2,377 mg/kg body weight, the single dose used. **Classification: Acceptable**
461556-11	Modified Cry3A protein as expressed in transgenic maize event MIR604: assessment of amino acid homology with known toxins	The National Center for Biotechnology Information (NCBI) GenBank Database (NCBI, 2003) containing all publicly available protein sequences was queried for proteins with amino acid sequences having significant homology to mCry3A protein and that were toxins. The query found no significant amino acid homology between any protein toxin and the mCry3A protein. **Classification: Acceptable**

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461556-12	Modified Cry3A protein as expressed in transgenic maize event MIR604: assessment of amino acid homology with known allergens	No significant similarity was found between any of the mCry3A 80-amino acid peptides and any entries in the SBI Allergen Database. Also, there were no alignments of eight or more contiguous amino acids between the mCry3A protein and any of the proteins in the allergen database. Overall, the mCry3A protein showed no significant amino acid homology to any known or putative allergenic protein. **Classification: Acceptable**
465974-01	Analytical Method for the Detection of the Plant-Incorporated Protectant Modified Cry3A Protein in Event MIR604 Corn Grain and Independent, Third-Party Validation of Said Method	An ELISA method to quantify modified Cry3A (mCry3A) protein in corn tissues was validated by an independent third-party laboratory (EnvironLogix, Inc.) using grain from Event MIR604, which contains mCry3A, and 25 other commercially-available transgenic and non-transgenic corns which do not contain mCry3A. The average mCry3A LOD for three different lots of reagents tested was 5.5 ± 4.6 ppb, which established the sensitivity of the ELISA assay at approximately 25%. In a ground grain assay, the mean mCry3A protein detected was 159.1 ± 70.2 ng/g fresh weight with a coefficient of variation (%CV) of 44.1%. However, the data submitted showed: less than optimal LOD; low sensitivity; high standard deviations; the %CV exceeding the recommended value (20%); anomalous frequency of false positives in the non-MIR604 corn (both transgenic and conventional) as well as false negatives in the MIR604 corn; and unexpected cross reactivity with other Cry3Bb1 samples. **Classification: Supplemental**
461556-13	Phosphomannose Isomerase as expressed in transgenic maize event MIR604: assessment of amino acid homology with known toxins	Two nucleotide changes were discovered in the *pmi* gene sequence inserted in corn Event MIR604. This resulted in two changes in the PMI protein; valine-61 was replaced by alanine, and glutamine-210 was replaced by histidine. These substitutions have not resulted in any apparent functional change in the PMI protein. The NCBI GenBank Database containing all publicly available protein sequences was queried for proteins with amino acid sequences having significant homology to this modified PMI protein that were toxins. The query found no significant amino acid homology between any protein toxin and the PMI protein expressed in corn Event MIR604. **Classification: Acceptable**
464252-01	Phosphomannose Isomerase as expressed in transgenic maize event MIR604: assessment of amino acid homology with known allergens	No significant similarity was found between any of the PMI 80-amino acid peptides and any entries in the SBI Allergen Database. However, in the eight or more contiguous amino acids homology search, there was an alignment between the PMI protein and a recently identified allergen, α-parvalbumin from *Rana species* CH2001 (a frog of Indonesian origin). However, a serum screening concluded that there is no cross-reactivity between PMI and serum IgE (obtained from an allergic individual who displayed food-induce anaphylaxis from α-parvalbumin). Bovine serum albumin was also tested as an internal check. The observed low degree of sequence identity between MIR604 PMI and α-parvalbumin is not biologically relevant. **Classification: Acceptable**

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465974-01 **Independent Laboratory Validation of Monoclonal-based ELISA method for the Detection of mCry3A in Event MIR604 corn grain**

A monoclonal based enzyme-linked immunosorbent assay (ELISA) method was developed by Syngenta to detect modified Cry3A (mCry3A) protein expressed in MIR604 seed and leaf tissues. An independent, third party laboratory (EnvironLogix, Inc.) utilized the assay methodology, Event MIR604 seed, negative isoline seed, and three lots of mCry3A monoclonal antibody for validation of assay protocol, sensitivity and cross-reactivity, according to the USDA GIPSA directive. The assay sensitivity was estimated at 1 positive Event MIR604 kernel in 999 non-MIR604 kernels (0.1%), which was based on a minimum of 120 ground seed samples (with a LOD of 0.33 ppb for seed material). The average quantification was 2.07 ppb (ng/g fresh weight corn). No cross reactivity with other commercial, conventional and transgenic corn was detected with the exception of products expressing the Cry3Bb1 protein. Moreover, extraction efficiency was determined at 59% for ground seed and 75% for leaf tissue.

CLASSIFICATION: ACCEPTABLE- The monoclonal assay antibody-based commercial ELISA detection assay (tested by EnviroLogix, Inc.) satisfies the EPA Residue Chemistry Guidelines OPPTS 860.1340(c)(6) Residue Analytical Methods and PR Notice 96-1. However, the EPA's Analytical Method Laboratory located in Fort Meade (Maryland) will have to independently validate Syngenta's ELISA protocol for accuracy, precision, and sensitivity.

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