BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Modified Cry3A Protein and the Genetic Material Necessary for its Production (Via Elements of pZM26) in Event MIR604 Corn SYN-IR604-8

U.S. Environmental Protection Agency
Office of Pesticide Programs
Biopesticides and Pollution Prevention Division

Table of Contents

Modified Cry3A protein and the Genetic Material Necessary for its Production (Via Elements of pZM26) in Event MIR604 corn SYN-IR604-8 Corn Regulatory Action Team

Product Characterization and Human Health

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I. Overview

A. Background

On October 3, 2006, EPA conditionally registered (with an expiration date of September 30, 2010), Syngenta Seeds Inc.'s active ingredient, modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8. Additionally, on January 24, 2007, the Agency conditionally registered (with an expiration date of October 15, 2008, subsequently extended to September 30, 2010) Syngenta Seed Inc.'s stacked product MIR 604 and Bt11 with mCry3A and Cry1Ab. The Agency determined that the use of these pesticides was in the public interest and that they would not cause any unreasonable adverse effects on the environment during the time of conditional registration.

Results of efficacy trials conducted in 2002, 2003, and 2004 indicated that MIR604 corn provides effective control of key rootworm pests of field corn. MIR604 corn has unique biochemical properties which may benefit insect resistance management for this and other CRW-protected corn products. MIR604 and Bt 11 with mCry3A and Cry1Ab effectively controls key rootworm pests and European corn borers. The availability of multiple CRW-protected corn products and CRW/CB-protected corn products will increase grower choice and price competition, resulting in lower seed prices for consumers and higher adoption rates. Registration of these products is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for CRW-control are highly toxic to humans and the environment, while mCry3A-expressing corn poses no foreseeable human health or environmental risks.

In order to reduce the possibility of corn rootworm or European corn borer developing resistance to Bt, EPA required Syngenta Seeds, Inc. to ensure that 20 percent of the planted acreage of this product be set aside where non-CRW-protected Bt corn will be grown to serve as a "refuge." These refuge areas will support populations of corn rootworm not exposed to the Bt corn. The insect populations in the refuges will help prevent resistance development when they cross-breed with insects in the Bt fields. This resistance management strategy was developed as a condition of the registration, and EPA will require routine monitoring and documentation that these measures are followed. The submitted insect resistance management data support a registration until 2010.

A tolerance exemption (40 CFR Part 174.505) was established for *Bacillus thuringiensis* modified Cry3A protein and the genetic material necessary for its production in corn.

B. Executive Summary

On October 1, 2009, EPA announced a policy to provide a more meaningful opportunity for the public to participate on major registration decisions before they occur. According to this policy, EPA intends to provide a public comment period prior to making a registration decision for, at minimum, the following types of applications: new active ingredients; first food uses; first outdoor uses; first

residential uses; and other actions for which the Agency anticipates that there will be significant public interest.

Consistent with the policy of making registration actions more transparent, the amendments to the expiring mCry3A corn products were subject to a 30-day comment period because the Agency believed, given past experiences with PIPs in general, these actions would be of significant interest to the public. During this comment period, several comments were received from the following stakeholders: Mycogen Seeds c/o Dow AgroSciences LLC; Pioneer Hi-Bred International, Incorporated; Monsanto Company; National Corn Growers Association; Agricultural Biotechnology Stewardship Technical Committee; Center for Science in the Public Interest; and Association of American Seed Control Officials. After reviewing and considering all of the public comments received, the Agency still maintains that, based on all data submitted in support of the mCry3A corn registrations (both for initial registrations and as responses to conditions of registration), it is in the best interest of the public and the environment to amend the currently existing mCry3A registrations by extending their expiration dates in accordance with the scheme explained in section III(C) of this Biopesticides Registration Action Document (BRAD). The basis for this decision can be found in both the risk assessment for the mCry3A corn products, which is characterized throughout this BRAD, and the Agency's response to comments document.

All data and findings for the mCry3A corn products are presented within the standard BRAD configuration for PIPs (i.e., information is placed into separate and distinct chapters according to scientific discipline or regulatory focus); this should be the most familiar format to outside stakeholders interested in reading further about these actions. In addition to the mCry3A corn products, there are other *Bt* corn PIPs, expressing different proteins effective in controlling corn borers or corn rootworm, that were due to expire in 2010, and for which the associated registrants formally requested an extension to expiration dates. Therefore, within the same docket (EPA-HQ-OPP-2010-0607) as this document, the following information[a] is also available for public examination:

- Cry1F and Cry1Ab BRAD (Draft - August 2010; Final - September 2010)
- Cry3Bb1 BRAD (Draft - July 2010; Final - September 2010)
- Cry34/35Ab1 BRAD (Draft - July 2010; Final - September 2010)
- Cry1A.105 and Cry2Ab2 BRAD (Draft - August 2010; Final - September 2010)
- Optimum® AcreMax™ *B.t.* Seed Blends BRAD (Draft - August 2010; Final - September 2010)
- Current Registration Terms and Conditions for *Bt* Corn Registrations Set to Expire in 2010
- Proposed Registration Terms and Conditions for *Bt* Corn Registrations Set to Expire in 2010
- Registration Terms and Conditions Established with the Finalized Amendments

[a] Each of the Biopesticides Registration Action Documents in this action are modified from previous versions to account for data/information submitted to fulfill terms and conditions of registration (see draft and final versions) and to respond, in part, to comments received on the information presented in Docket Number EPA-HQ-OPP-2010-0607 (see final versions only). All documents presented in the list can be retrieved from the following website: *http://www.regulations.gov*.

- BPPD mCry3A, Cry3Bb1, and Cry34/35Ab1 Corn Rootworm Monitoring Reviews (June 2010)
- Public Comments on EPA Docket Number EPA-HQ-OPP-2010-0607
- EPA's Response to Comments

EPA made the decision to amend the registrations of eighteen (18) expiring *Bt* corn PIP registrations to extend the expiration dates. We conducted comprehensive assessments of each of these registrations, considering all toxicity and environmental effects data, data from insect resistance monitoring, and insect resistance refuge compliance reports, received and obtained since the last comprehensive evaluation of these products in 2001. Based upon our comprehensive assessment, we reached significant conclusions regarding the positive environmental impact of *Bt* corn PIPs, and we took several actions to strengthen the insect resistance management requirements to ensure continued success in the prevention of the evolution of resistance in target pests.

Since the commercialization of *Bt* crops, there have been a significant number of published field studies that, combined with the post-registration field studies required to be submitted to the Agency, have demonstrated that non-target invertebrates are generally more abundant in *Bt* cotton and *Bt* corn fields than in non-transgenic fields managed with chemical insecticides. Thus, these published and registrant-produced studies demonstrate that, not only are the *Bt* crops not causing any unreasonable adverse effects in the environment, but, arthropod prevalence and diversity is greater in *Bt* crop fields.

To strengthen insect resistance management of these corn PIPs and to address reports that compliance with the mandated refuge requirements has been decreasing, EPA is requiring enhanced compliance assurance programs (CAPs), and a phased requirement for seed bag labeling that clearly shows the refuge requirements. Also, given the increasing variety of PIP products and combinations, and the differing risk of resistance evolution that the various products represent, we are granting registrations for the corn PIP products for different time frames, based on assessments of their likelihood of forestalling the evolution of insect resistance. We are registering differing categories of products for differing time periods to reflect the assessed level of risk of resistance posed by the various corn PIP products. The scheme that we are following includes registration periods generally of five, eight, and twelve years; with the possibility of a fifteen-year registration period for products that are demonstrated to meet specified criteria. We retain, however, the discretion to register products for time periods differing from these defaults where circumstances warrant.

Product Characterization

The modified Cry3A (mCry3A) *Bacillus thuringiensis* (*Bt*) insect control protein is produced in transgenic corn plants derived from transformation Event MIR604 and has activity against certain beetles. 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 and northern corn rootworm.

Protein characterization data demonstrate that the plant-produced protein is of sufficiently similar biological activity to that of the two modified Cry3A protein variants produced in the recombinant E. coli system (designated as test material MCRY3A-0102) for the purposes of human health and ecological effect risk assessments. Although the MCRY3A-0102 test material was not as active towards target pests as the plant-produced modified Cry3A protein, the doses in submitted studies were much higher than would occur via the modified corn.

Bt corn Events MIR 604 and Bt11 with modified Cry3A and Cry1Ab is a stacked product that has activity against the rootworm varieties previously mentioned and European corn borer. The product characterization and protein expression analyses of Bt Cry1Ab and mCry3A insect control proteins and the genetic material necessary for its production in maize (corn) plants derived from Event Bt11 x MIR604 hybrid cross via traditional plant breeding were found to be similar and functionally equivalent to the Cry1Ab protein expressed in Bt11 and to mCry3A protein expressed in MIR604.

Human Health Assessment

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, or MIR 604 and Bt11 with mCry3A and Cry1Ab.

An acute oral toxicity study was submitted for the mCry3A protein and MIR 604 and Bt11 with mCry3A and Cry1Ab. Both studies support the prediction that the products would be non-toxic to humans.

Amino acid sequence comparisons showed no similarity between the mCry3A protein or MIR 604 and Bt11 with mCry3A and Cry1Ab to known toxic proteins available in public protein databases.

Amino acid sequence comparisons showed no similarity between the mCry3A protein or MIR 604 and Bt11 with mCry3A and Cry1Ab to known allergens available in public allergen databases.

Environmental Assessment

The Agency is aware of no identified significant adverse effects of mCry3A proteins or MIR 604 and Bt11 with mCry3A and Cry1Ab on the abundance of non-target beneficial organisms in any population in the field environment, whether they are pest parasites, pest predators, or pollinators. Further, the EPA believes that cultivation of these products may have fewer adverse impacts on non-target organisms than use of chemical pesticides for corn production, because under normal circumstances, these products require substantially fewer applications of chemical pesticides, compared to production of non-Bt corn. And fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers, in corn fields.

In addition, no adverse effect on endangered and threatened species listed by the US Fish and Wildlife Service is expected from the proposed MIR604 CRW resistant corn registration. Further, the EPA has determined that there is no significant risk of gene capture and expression of mCry3A protein or MIR 604 and Bt11 with mCry3A and Cry1Ab by wild or weedy relatives of corn in the U.S., its possessions, or territories, available data do not indicate that Cry proteins have any measurable adverse effect on microbial populations in the soil, nor has horizontal transfer of genes from transgenic plants to soil bacteria been demonstrated.

Insect Resistance Management

The proposed IRM strategy and data to support it are "acceptable" except that the in-field strip refuge must be at least 4 rows wide based on recent larval movement data. If resistance is recessive, then the proposed IRM plan using a 20% structured refuge will be adequate to delay resistance for at least 15 years given the assumptions of Syngenta's model. If MIR604 maize is planted in areas with observable rotation-resistance in WCRW, then planting transgenic corn only in rotated maize fields is a good IRM strategy that will delay the evolution of resistance by at least 15 years regardless of gene expression.

Benefits

Registration of modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8 is in the public interest because:

1. Results of efficacy trials conducted in 2002, 2003, and 2004 indicate that MIR604 corn provides effective control of key rootworm pests of field corn.

2. MIR604 corn has unique biochemical properties which may benefit insect resistance management for this and other CRW-protected corn products.

3. If MIR604 corn is registered, it will be the third CRW-protected *Bt* corn product on the market. The availability of multiple CRW-protected corn products will increase grower choice and price competition, resulting in lower seed prices for consumers and higher adoption rates.

4. Registration of MIR604 corn is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for CRW-control are highly toxic to humans and the environment, while mCry3A-expressing corn poses no foreseeable human health or environmental risks.

C. Use Profile

1. **Pesticide Name**: Modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8

Trade and Other Names: Agrisure RW Rootworm-Protected Corn; MIR 604 corn

EPA Registration Number: 67979-5

OPP Chemical Code: 006509

Basic Manufacturer: Syngenta Seeds, Inc,

Type of Pesticide: Plant-Incorporated Protectant

Uses: Field Corn

Target Pest(s): Corn Rootworm.

2. **Pesticide Names:** Modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8
and
Bacillus thuringiensis Cry1Ab delta-endotoxin and the genetic material necessary for its production (Plasmid Vector pZ01502) in corn

Trade and Other Names: Bt11 x MIR 604 Corn Seed; Agrisure CB/LL/RW, Bt11 x SYN-IR6Ø4-5 corn seed Bt11 x SYN-IR6Ø4-8 corn seed

EPA Registration Number: 67979-8

OPP Chemical Code: 006509 and 006505

Basic Manufacturer: Syngenta Seeds, Inc,

Type of Pesticide: Plant-Incorporated Protectant

Uses: Field Corn

Target Pest(s): European Corn Borer and Corn Rootworm

II. Science Assessment

The classifications that are found for each data submission are assigned by the EPA science reviewer and are an indication of the usefulness of the information contained in the documents and if the data meet the intent of the test guidelines. A rating of "ACCEPTABLE" indicates the study is scientifically valid and has been satisfactorily performed according to accepted EPA guidelines or other justified criteria. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. However, the studies may either have certain aspects not determined to be scientifically acceptable (SUPPLEMENTAL, but UPGRADABLE) or that the studies have not been done to fulfill a specific EPA guideline requirement. If a study is rated as "SUPPLEMENTAL, but UPGRADABLE," EPA always provides an indication of what is lacking or what can be provided to change the rating to "ACCEPTABLE." If there is simply a "SUPPLEMENTAL" rating, the reviewer will often state that the study is not required by current EPA guidelines or does not need to be reclassified as "ACCEPTABLE." Both ACCEPTABLE and SUPPLEMENTAL studies may be used in the risk assessment process as appropriate.

II.A. Product Characterization

1. 2007 Position.

The Modified Cry3A (mCry3A) *Bacillus thuringiensis* (*Bt*) insect control protein is produced in transgenic corn plants derived from transformation Event MIR604. The Agency's detailed assessment of the product characterization for *Bt* mCry3A is found in Fellman (2005a, b, e and 2006a and c). 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 virgifera*) and northern corn rootworm (NCRW; *D. longicornis barberi*). Introduced *via* transformation vector pZM26, the *mcry3A* gene, 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 (1176 bp) encoding a phosphomannose isomerase (PMI) 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 gene* 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 and allows for selection of transformants in cell culture by enabling only transformed corn cells to utilize mannose as a sole carbon source, while corn cells lacking the *pmi* gene fail to grow.

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 (MRID No. 461556-01, reviewed in Fellman, 2005a).

The molecular properties of the *mcry3A* gene and the *pmi* gene, both present in Event MIR604 corn, were evaluated by utilizing Southern blotting analyses, DNA sequencing of the T-DNA insert, and by studying the Mendelian inheritance of the transgene insert (MRID No. 461556-02, reviewed in Fellman, 2005a). Maize genomic DNA was digested with *Kpn*I restriction enzyme and hybridized to probes for *mcry3A* (1797 bp)*, pmi* (1176 bp) and plasmid backbone (5309 bp; not inserted in the host genome). The T-DNA insert (via the pZM26 plasmid) in MIR604 was analyzed *via* Southern blot analyses and single copies of *mcry3A* and the *pmi* genes were present and closely linked in MIR604. The results also verified that the backbone DNA sequence from the transformation plasmid pZM26 in MIR604 was not inserted into MIR604.

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. In addition, T_5 generation plants generated from T_4 generation plants known to be heterozygous for the *mcry3A* and *pmi* genes were analyzed by an enzyme-linked immunosorbent assay (ELISA) of mCry3A and Polymerase Chain Reaction (PCR) analysis for both the *mcry3A* and *pmi* genes. Results showed that the two genes were closely linked with the expected Mendelian inheritance ratio.

Expression levels of the mCry3A protein in transgenic corn tissues were also provided for both inbred and hybrid varieties (MRID 461556-04, reviewed in Fellman, 2005a) and were found comparable. The data on expression levels in two hybrids lines are summarized in Table 1.

TABLE 1. mCry3A Levels on a Dry Weight Basis in Event MIR604-Derived Corn Plants					
Tissue	**Genotype**	**Whorl**	**Anthesis**	**Seed Maturity**	**Senescence**
		mean µg mCry3A/g dry weight ± S. D. (range)			
Leaves	MIR604-B Hybrid	18.64 ± 2.08 (16.27 - 20.90)	15.93 ± 3.65 (11.40-20.87)	13.29 ± 2.37 (9.61 - 15.40)	5.05 ± 2.37 (1.69 - 8.24)
	MIR604-C Hybrid	25.76 ± 4.11 (22.23 - 31.86)	16.83 ± 2.88 (13.16-21.18)	24.49 ± 3.34 (19.58-28.87)	6.57 ± 2.97 (3.65 - 10.33)
Roots	MIR604-B Hybrid	25.50 ± 1.36 (23.55 - 27.07)	15.57 ± 4.35 [b] (10.58 - 18.57)	7.29 ± 1.96 (4.99 - 9.03)	10.31± 6.34 (1.68 - 19.36)
	MIR604-C Hybrid	19.41 ± 2.17 (16.29 - 21.18)	14.46 ± 2.57 (11.56 - 18.30)	12.96 ± 5.36 (7.10 - 19.46)	9.87 ± 5.43 (5.45 - 17.60)
Kernels	MIR604-B Hybrid	N/A	N/A	1.09 ± 0.45 (0.74 - 1.83)	0.77 ± 0.20 (0.59 - 0.99)
	MIR604-C Hybrid	N/A	N/A	1.95 ± 0.74 (1.26 - 3.13)	0.94 ± 0.47 (0.43 - 1.59)
Silk	MIR604-B Hybrid	N/A	<0.34 (ND-DNQ)	0.99 [d]	N/A
	MIR604-C Hybrid	N/A	<0.56 (DNQ)	3.04 ± 0.78 [a] (2.31 - 3.92)	N/A
Pollen	MIR604-B Hybrid	N/A	ND [e]	N/A	N/A
	MIR604-C Hybrid	N/A	ND [e]	N/A	N/A
Whole Plant	MIR604-B Hybrid	7.31 ± 3.40 (1.33 - 9.51)	11.16 ± 5.48 (4.23- 16.99)	16.97 ± 8.24 (5.88 - 28.88)	8.96 ± 3.96 (5.03 - 15.40)
	MIR604-C Hybrid	9.83 ± 1.83 (8.56 - 13.01)	11.22 ± 2.87 (9.05 - 14.41)	23.77 ± 4.91 [a] (16.82 - 28.35)	11.85 ± 3.51 (7.44 - 16.78)

Except where noted otherwise, five samples were used to determine the means and standard deviations.
[a] Four samples were used to determine the mean and standard deviation.
[b] Three samples were used to determine the mean and standard deviation.
[c] Two samples were used to determine the mean and standard deviation.
[d] One sample analyzed.
[e] Result of one pooled sample.
N/A Not analyzed at this stage.
ND Not detectable.
DNQ. Detectable but not quantifiable

Production of bacterial-derived mCry3A protein was chosen in order to obtain sufficient material for testing, therefore, its equivalence to the plant-derived protein was determined. The mCry3A protein was produced in recombinant *E. coli* by over-expressing the same modified *cry3A* gene that was introduced into Event MIR604 corn plants.

The similarity of mCry3A protein expressed in corn event MIR604 and the recombinant *E. coli* test system (designated as test material MCRY3A-0102) were evaluated (MRID 461556-03, reviewed in Fellman, 2005a). The mCry3A proteins from both sources had the same approximate molecular weight (ca. 67,700 Da.) based on mass spectral analysis. Moreover, Western blot analysis of the test material showed a single immunoreactive band corresponding to the predicted molecular weight of ca. 67,700 Da. SDS-PAGE showed the purity of mCry3A in the test material to be ca. 90.3% by weight. 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 also no evidence of post-translational glycosylation of mCry3A protein from either source. Therefore, it was concluded that the mCry3A proteins from corn event MIR604 and from recombinant *E. coli* were equivalent.

However, further testing revealed that the test material, MCRY3A-0102, contained two closely related components in a ratio of *ca.* 2:3 *via* SDS-PAGE and MALDI TOF mass spectrometry (MRID 461556-06, reviewed in Fellman, 2005a). 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. To satisfy Agency' concerns with the long form component of mCry3A, Syngenta conducted an in-depth examination of the nucleotide sequence of the cry1Ac promoter in the cassette utilized for expression of the mCry3A protein in the MCRY3A-0102 test substance. The registrant reported that an "ATG" initiation codon sequence was identified within the cry1Ac promoter indicating the presence of an additional putative open reading frame (ORF). This demonstrated that the resultant putative ORF would be in-frame with the *mcry3A* gene and would result in an additional 16 amino acids on the N-terminus of the intended mCry3A protein (MRID 464667-01, reviewed in Fellman, 2005b).

Visual confirmation of the molecular weights for the long and short forms of mCry3A protein was also provided *via* SDS-PAGE and western blot analysis. These gels indicated single intense bands consistent with the predicted molecular weights of *ca.* 69,500 Da and *ca.* 67,700 Da for the mCry3A-LF (long form) and mCry3A-SF (short form) samples, respectively. Therefore, 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 (MRID 461556-07). The molecular weight data showed the two proteins to have masses of 67,519 and 69,138 Da.

Because both forms of mCry3A were insecticidally active against WCRW 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 equivalent. Moreover, the test material was re-analyzed ca.

9 months after its initial characterization and found to be substantially stable when stored at -20 °C (MRID 461556-05, reviewed in Fellman, 2005a).

On March 14-15, 2006, EPA held a FIFRA Scientific Advisory Panel (SAP) meeting, http://www.epa.gov/scipoly/sap/meetings/2006/index.htm#march to address the scientific issues that arose during the risk assessment of mCry3A. The SAP Report (SAP, 2006) is reviewed in Fellman (2006c). EPA asked the SAP to comment on the equivalence of the mCry3A proteins from corn event MIR604 and from recombinant *E. coli* - specifically the presence of two forms in the bacterially produced mCry3A protein and the differences in bioactivity in the WCRW bioassay. The majority of the Panel concluded that the two forms of the mCry3A are of relatively comparable biological activity for the purposes of the human health assessments based on the amino acid sequence identity, lack of glycosylation, and general stability.

2. Product Characterization Data. The product characterization data is summarized in Table 2.

Table 2. Product characterization studies (2010 Update)

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**

MRID	Title	Summary
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 associated with 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 *E. 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**
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 varieties 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**

MRID	Title	Summary
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**

MRID	Title	Summary
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 2.12 µg mCry3A/g. **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	A monoclonal antibody 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 (EnviroLogix, 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. Therefore, the monoclonal 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. EPA's Analytical Method Laboratory located in Fort Meade (Maryland) will have to independently validate Syngenta's ELISA protocol for accuracy, precision, and sensitivity. **Classification: Acceptable**

MRID	Title	Summary
67956-01	Comparative Southern Analysis of a Bt11 x MIR604 Maize Hybrid with the Parental Event Bt11 and Event MIR604 Maize Inbreds	The purpose of this study was to use Southern blot analysis to confirm the presence of the *cry1Ab* and *pat* genes from the parental Event Bt11 and *mcry3A* and *pmi* genes from parental Event MIR604 in the hybrid Bt11 x MIR604 in a predictable manner. For the *cry1Ab*-specific probe, the *Nde*I digest resulted in a single hybridization band of ~4.6 kb in both Event Bt11 and Bt11 x MIR604. For the *pat*-specific probe, the *Nde*I digest resulted in a single hybridization band of ~1.9 kb in both Event Bt11 and Bt11 x MIR604. For the *mcry3A*-specific probe, the *Kpn*I digest resulted in a single hybridization band of ~5.6 kb in both Event MIR604 and Bt11 x MIR604. Likewise, for the *pmi*-specific probe, the *Kpn*I digest resulted in a single hybridization band of ~5.2 kb in both Event MIR604 and Bt11 x MIR604**.** The predicted DNA hybridization patterns were retained and stability of the transgenic locus from parent to progeny was demonstrated. However, a more detailed explanation with documentation and/or literature reference for the unexpected cross hybridization of the Bt11 transformation plasmid control lane (observed on Figure 7, lane 8) and the commercial DNA ladder is needed to verify the results of the Bt11 x MIR604 Southern blot, hybridized with the *mcry3A*-specific probe. **CLASSIFICATION: SUPPLEMENTAL but UPGRADEABLE-** pending submission of a more detailed explanation for the unexpected hybridization band detected in the Bt11 transformation plasmid control lane on the Bt11 x MIR604 Southern blot, hybridized with the *mcry3A*-specific probe.

MRID	Title	Summary
469165-01	Comparison of Transgenic Protein Expression in Event Bt11, Event MIR604 and Stacked Bt11 x MIR604 Maize (corn) Hybrids	The purpose of this study was to compare expression of the four transgenic proteins (Cry1Ab, mCry3A, PAT and PMI) in a Bt11 x MIR604 maize (field corn) hybrid with expression in corresponding near-isogenic hybrids derived from the individual transformation events to determine whether any unexpected differences in protein expression had occurred as a result of combining the traits by breeding via ELISA. Mean Cry1Ab concentrations in leaves and roots of the Bt11 x MIR604 hybrid ranged from *ca*. 19.7 to 27.7 µg/gdw and *ca.* 5.6 to 10.0 µg/gdw, respectively. Mean Cry1Ab concentrations in kernels were *ca*. 1.7 µg/gdw. The Cry1Ab concentrations in pollen from all three locations ranged from below the LOQ to 0.06 µg/gdw. Mean mCry3A concentrations in leaves and roots of Bt11 x Event MIR604 hybrid ranged from *ca.* 33.4 to 46.3 µg/gdw and *ca.* 18.9 to 23.9 µg/gdw, respectively. Mean mCry3A concentrations in kernels were *ca*. 0.7 µg/gdw. The mCry3A levels in the pollen collected at anthesis from all three locations range from below the LOQ to *ca.* <0.03 µg/gdw. Mean PAT concentrations in leaves and roots of the Bt11 x MIR604 hybrid ranged from *ca*. <0.05 to 0.17 µg/gdw and *ca*. 0.08 to 0.19 µg/gdw, respectively. Mean PAT concentrations in the kernels were both <LOQ to <0.04 µg/gdw. The PAT levels in the pollen collected at anthesis from all three locations range from below the LOQ to *ca.* <0.034 µg/gdw. Mean MIR604 PMI concentrations in leaves and roots of the Bt11 x MIR604 hybrid ranged from *ca*. 5.7 to 10.4 µg/gdw and *ca*. 2.3 to 6.0 µg/gdw, respectively. Mean MIR604 PMI concentrations in kernels were *ca*. 1.9 µg/gdw. The PMI levels in the pollen collected at anthesis from all three locations range from 39.3 to 56.9 µg/gdw. Overall, concentrations of Cry1Ab, mCry3A, PAT, and PMI protein levels were found comparable and all control tissues were negative for the expression of Cry1Ab, PAT, mCry3A and PMI. Therefore, transgenic protein expression in the Bt11 x MIR604 hybrid are not substantially different from that of the hybrids derived from the individual Bt11 and MIR604 transformation events. **Classification: Acceptable**

MRID	Title	Summary
470062-01	Response to EPA questions regarding *mcry3A* Southern blot in the Bt11 x MIR604 Comparative Southern Analysis Report (MRID No. 467956-01, Figure 7)	On November 22, 2006, EPA and representatives from Syngenta Seeds Inc., held a conference call to discuss the deficiencies noted in MRID 467965-01. EPA required clarification for the Southern blot (Figure 7) where an unexpected hybridization band (as 6.2 kb) was observed in the Bt11 transformation plasmid (the *Not*I-digested pZO1502) control lane. In response, Syngenta submitted a report that explained that the unexpected hybridization band was due to a small amount of pUC plasmid present in the KB ladder (used as the molecular weight marker). Syngenta ascertained this after discussion with a tech service representative of Stratagene (the KB ladder supplier), who indicated trace amounts of pUC plasmid may remain as contaminants in the KB ladder stock (as a result of the manufacturing process). Due to the pUC plasmid presence in the KB ladder, Syngenta subsequently hybridized the membrane with KB ladder probe, *mcry3A*-specific probe, and pUC plasmid probe. Syngenta provided an additional rationale based on the generated Southern data for another project (Bt11 x MIR604 x GA21) in which they utilized a different ladder (Analytical Marker DNA, Wide Range, Promega Cat. No. DB1931). There was no unexpected hybridization band in the pZO1502 lane on the blot probed with the *mcry3A*-specific probe. This further confirmed that the unexpected hybridization band (Figure 7, lane 8) was due to the radiolabeled pUC plasmid, present in the KB ladder, hybridizing to COLE1in pZO1502. EPA also questioned Syngenta's method of radiolabeling the ladder DNA. Syngenta stated a preference to hybridize blots with the ladder and element-specific probes simultaneously so that the ladder can be visualized directly on the blot. The registrant utilizes this technique to eliminate the potential for introducing errors when manually transferring the position of stained ladder bands from the gel to the membrane. Syngenta also stated that they are using a different molecular weight marker preparation (consisting of a mixture of restriction enzyme digests of lambda DNA and Phi X174 DNA) to avoid any future potential non-specific sequence binding of the labeled ladder probe. Since this ladder is made from phage DNA, no unexpected hybridization bands in the plasmid control lanes is either expected nor observed in other Southern analyses (when probing with a combination of the radiolabeled Analytical Marker DNA, Wide Range ladder and a radiolabeled element-specific probe). Syngenta also noted that choosing different enzymes would not eliminate the presence of any of the plasmid backbone sequences in the plasmid control lanes, because the plasmid controls on the Southern blots contain all the fragments of the digested plasmids. **EPA Reviewer's Comment:** The registrant has submitted a sufficient explanation for the non-specific binding in the Southern blot (Figure 7, lane 8). However, visual verification is needed to confirm the lack of any unexpected hybridization band in the pZO1502 lane on the blot probed with the mcry3A-specific probe. Therefore, Syngenta should submit a Southern blot containing genomic DNA from Bt11, MIR604, Bt11 x MIR604 and a negative control as well as the plasmid controls pZO1502 and pZM26 hybridized with a combination of the mcry3A-specific and the newly employed Analytical Marker DNA, Wide Range ladder, which does not contain the COLE1 origin of replication vector sequence. **Classification: Supplemental, But Upgradeable-** pending submission of an additional Bt11 x MIR604 Southern blot probed with the *mcry3A*-specific probe to confirm the lack of non-specific sequence binding in the Bt11 transformation pZO1502 plasmid control lane as confirmatory data to complete the Bt11 x MIR604 database.

MRID	Title	Summary
472648-01	Supplement to Report Titled: Comparative Southern Analysis of a Bt11 x MIR604 Maize Hybrid with the Parental Event Bt11 and Event MIR604 Maize Inbreds	The original submission of Southern blot data for the registration of this combination PIP product expressing both Cry1Ab and mCry3A proteins did not have clear results for the reactivity of the molecular weight standards with labeled plasmid components. Syngenta was required as a condition of the registration to submit new Southern blot data to clarify the cross reactivity and other issues. The study presents Southern blot data with appropriate plasmid maps to explain the expected fragment banding patterns. The combination of several endonuclease digestions and probes gives the necessary background to judge the results from the single parental lines as well as the hybrid cross Bt11 x MIR604. There was no aberrant probe binding to the molecular weight markers. The control samples in each gel were free from probe reactivity except in the case of positive controls. These data demonstrate stability and presence of both the Cry1Ab and mCry3A proteins and PAT and PMI marker genes in the parental line and the hybrid cross. **Classification: Acceptable**
none	Provide to the EPA Laboratory (Ft. Meade, MD) methodology and/or reagents necessary for validation of mCry3A analytical method.	More recently, the Agency decided to allow this requirement to be satisfied when the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) has verified the performance of a qualitative rapid test kit for detecting the presence of the biotechnology event in grains and oilseeds. In the case of mCry3A, the Agency has confirmed that a test kit has been verified by GIPSA and, therefore, the aforementioned requirement has been satisfied. **Classification: Acceptable**

3. 2010 Update: Terms and Conditions of the Event MIR 604 expressing modified Cry3A

1. Validation of analytical method for mCry3A. When Event MIR 604 expressing modified Cry3A Corn (EPA Reg. No. 67979-5) was initially registered on October 03, 2006, the Agency issued registration notices to Syngenta Seeds, Inc. that contained the following requirement for further product characterization information:

> "Provide to the EPA laboratory (Ft. Meade, MD) methodology and/or reagents necessary for validation of a mCry3A analytical method within 6 months from the date that the Agency requests them."

More recently, the Agency decided to allow this requirement to be satisfied when the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) has verified the performance of a qualitative rapid test kit for detecting the presence of the biotechnology event in grains and oilseeds. In the case of mCry3A, the Agency has confirmed that a test kit has been verified by GIPSA and, therefore, the aforementioned requirement has been satisfied.

2. Product Characterization for Events MIR 604 x Bt11, expressing the proteins mCry3A and Cry1Ab

The product Agrisure CB/LL/RW contains Bt corn events MIR 604 x Bt11 expressing the proteins mCry3A and Cry1Ab. This stacked product targets both lepidopteran corn pests that are susceptible to Cry1Ab as well as corn rootworm that is susceptible to Cry3A. Both Bt11 and MIR 604 have been previously registered as single gene products. Specific product characterization information on this stacked product is presented below.

The purpose of the Transgenic Protein Expression Study was to compare expression of the four transgenic proteins (Cry1Ab, mCry3A, PAT and PMI) in a Bt11 x MIR604 maize (field corn) hybrid with expression in corresponding near-isogenic hybrids derived from the individual transformation events to determine whether any unexpected differences in protein expression had occurred as a result of combining the traits by breeding via ELISA.

Overall, concentrations of Cry1Ab, mCry3A, PAT, and PMI protein levels were found comparable and all control tissues were negative for the expression of Cry1Ab, PAT, mCry3A, and PMI. Therefore, transgenic protein expression in the Bt11 x MIR604 hybrid are not substantially different from that of the hybrids derived from the individual Bt11 and MIR604 transformation events.

The Southern blot analysis is used to confirm the presence of the *cry1Ab* and *pat* genes from the parental Event Bt11 and *mcry3A* and *pmi* genes from parental Event MIR604 in the hybrid Bt11 x MIR604 in a predictable manner. The predicted DNA hybridization patterns were retained and stability of the transgenic locus from parent to progeny was demonstrated. However, a more detailed explanation with documentation and/or literature reference for the unexpected cross hybridization of the Bt11 transformation plasmid control lane and the commercial DNA ladder was needed to verify the results of the Bt11 x MIR604 Southern blot, hybridized with the *mcry3A*-specific probe.

On November 22, 2006, the EPA and representatives from Syngenta Seeds Inc., held a conference call to discuss the deficiencies noted in the Southern Blot data (MRID 467965-01). The Agency wanted further clarification for the Southern blot where an unexpected hybridization band (as 6.2 kb) was observed in the Bt11 transformation plasmid (the *Not*I-digested pZO1502) control lane.

In response, Syngenta submitted a report that explained that the unexpected hybridization band was due to a small amount of pUC plasmid present in the KB ladder (used as the molecular weight marker). Syngenta ascertained this after discussion with a tech service representative of Stratagene (the KB

ladder supplier), who indicated trace amounts of pUC plasmid may remain as contaminants in the KB ladder stock (as a result of the manufacturing process). Due to the pUC plasmid presence in the KB ladder, Syngenta subsequently hybridized the membrane with KB ladder probe, *mcry3A*-specific probe, and pUC plasmid probe.

Syngenta provided an additional rationale based on the generated Southern data for another project (Bt11 x MIR604 x GA21) in which they utilized a different ladder (Analytical Marker DNA, Wide Range, Promega Cat. No. DB1931). There was no unexpected hybridization band in the pZO1502 lane on the blot probed with the *mcry3A*-specific probe. This further confirmed that the unexpected hybridization band was due to the radiolabeled pUC plasmid, present in the KB ladder, hybridizing to COLE1in pZO1502.

EPA also questioned Syngenta's method of radiolabeling the ladder DNA. Syngenta stated a preference to hybridize blots with the ladder and element-specific probes simultaneously so that the ladder can be visualized directly on the blot. The registrant utilizes this technique to eliminate the potential for introducing errors when manually transferring the position of stained ladder bands from the gel to the membrane.

Syngenta also stated that they are using a different molecular weight marker preparation (consisting of a mixture of restriction enzyme digests of lambda DNA and Phi X174 DNA) to avoid any future potential non-specific sequence binding of the labeled ladder probe. Since this ladder is made from phage DNA, no unexpected hybridization bands in the plasmid control lanes is either expected nor observed in other Southern analyses (when probing with a combination of the radiolabeled Analytical Marker DNA, Wide Range ladder and a radiolabeled element-specific probe).

Syngenta also noted that choosing different enzymes would not eliminate the presence of any of the plasmid backbone sequences in the plasmid control lanes, because the plasmid controls on the Southern blots contain all the fragments of the digested plasmids.

EPA found that the registrant submitted a sufficient explanation for the non-specific binding in the Southern blot. Visual verification was needed, however, to confirm the lack of any unexpected hybridization band in the pZO1502 lane on the blot probed with the mcry3A-specific probe. The registrant should submit a Southern blot containing genomic DNA from Bt11, MIR604, Bt11 x MIR604 and a negative control as well as the plasmid controls pZO1502 and pZM26 hybridized with a combination of the mcry3A-specific and the newly employed Analytical Marker DNA, Wide Range ladder, which does not contain the COLE1 origin of replication vector sequence.

Syngenta submitted a supplemental study which showed Southern blot data with appropriate plasmid maps to explain the expected fragment banding patterns. The combination of several endonuclease digestions and probes gave the necessary background to judge the results from the single parental lines as well as the hybrid cross Bt11 x MIR604. There was no aberrant probe binding to the molecular weight markers. The control samples in each gel were free from probe reactivity except in the case of

positive controls. These data demonstrated stability and presence of both the Cry1Ab and mCry3A proteins and PAT and PMI marker genes in the parental line and the hybrid cross.

II. B. Human Health Assessment

1. Mammalian Toxicity and Allergenicity Assessment

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 Sec. 158.2130 and 158.2140).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 (MRID 461556-10, reviewed in Fellman, 2005a). 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 and known toxic proteins available in public protein data bases (MRID 461556-11, reviewed in Fellman, 2005a). According to the Codex Alimentarius guidelines, the assessment of potential toxicity also includes stability to heat (FAO/WHO Standards Programme, 2001). Further data demonstrate that mCry3A is inactivated against WCRW, when heated to 95 °C for 30 minutes (MRID 461556-08)

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 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 are 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* (MRID 461556-07, review in Fellman, 2005a). In a solution of simulated gastric fluid 1 mg/mL mCry3A test protein mixed with simulated gastric fluid (pH 1.2, containing 2 mg/mL NaCl, 14 *µL* 6 N HC1, and 2.7 mg/mL pepsin) resulting in 10 pepsin activity units/ µg 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 (MRID 461556-12). Further data demonstrate that mCry3A is not glycosylated and is present in low levels in corn tissue.

EPA also asked the March 14-15, 2006 FIFRA SAP to comment on EPA's conclusions regarding the lack of mammalian toxicity and allergenicity of the mCry3A protein- specifically the impact of the less potent mCry3A form on the results of the acute oral toxicity tests and the usefulness of *in vitro* digestibility studies and amino acid sequence homology analysis as part of the risk assessment (SAP, 2006 and reviewed in Fellman, 2006c). Overall, the Panel was more concerned with the quality of data, i.e. inadequately described methods and poor reproduction of data images. The Panel specifically noted that the amino acid sequence analysis to known toxins were missing the following data: a full, technical description of each specific toxin sequence compared to the mCry3A amino acid sequence in the NCBI database; specification of which version of NCBI database was utilized; descriptions of parameters utilized; and dates accessed for the BLAST search. EPA recognizes that these are important parameters to include in a description of an amino acid analysis, however, the parameters used and dates of the BLAST search were provided in the reference list and in a footnote, respectively, in the toxin homology report by the registrant. EPA is requiring submission of a detailed description of each amino acid sequence of known toxins compared to mCry3A by Syngenta Seeds, Inc. However, the Agency maintains that the conclusions of the amino acid sequence analysis are still valid for the purpose of the risk assessment. EPA reached this decision based on the following: 1) lack of mammalian toxicity of mCry3A protein as shown by the acute oral mouse study; 2) mCry3A protein is rapidly digested in SGF; 3) mCry3A protein originates from a non-allergenic source; 4) lack of sequence identity of mCry3A protein with 8 contiguous amino acids or more than 35% identity over 80 amino acids with known allergens; and 5) mCry3A protein is not glycosylated when expressed in corn.

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.

A summary of the toxicity and allergenicity data is presented in Table 3.

Table 3. Mammalian Toxicity and Allergenicity studies (2010 Update)

MRID	Title	Summary
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**
461556-07	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 M 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. 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**
461556-11	Modified Cry3A protein as expressed in transgenic maize event MIR604: assessment of amino acid homology with known toxins	The National Center for Biotechnology Information (NBI) GenBank Database (NBI, 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**

MRID	Title	Summary
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**
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**

MRID	Title	Summary
none	Information provided in a letter dated July 1, 2010, to supplement MRIDs 46155612 and 46155611.	In a review dated July 22, 2010, the Agency found that the letter provided the details of the NCBI database versions accessed, the parameters of the FASTA and BLASTP searches and the date of the searches themselves. This information supplements the risk assessments done for the mCry3A protein and fulfills the recommended additional information as reflected in the minutes of the FIFRA-Scientific Advisory Panel on the mCry3A corn PIP product.

2. 2010 Update: Terms and Conditions of the Event MIR 604 expressing mCry3A and Events MIR 604 x Bt11 expressing mCry3A and Cry1Ab proteins.

When products containing Event MIR 604 expressing mCry3A protein (EPA Reg. No. 67979-5), and events MIR 604 x Bt11 expressing mCry3A + Cry1Ab proteins (EPA Reg. No. 67979-8) were initially registered on October 03, 2006 and January 25, 2007 respectively, the Agency issued registration notices to Syngenta Seeds, Inc that contained the following requirement:

> "Submit the following data to augment the mCry3A amino acid sequence analysis to known toxins and allergens within six months of the date of registration: specification of which version of NCBI database was utilized; descriptions of parameters utilized; and dates accessed for the BLAST search."

Syngenta responded to the request for additional information in a letter dated July 1, 2010. In a review dated July 22, 2010, the Agency found that this letter provided the details of the NCBI database versions accessed the parameters of the FASTA and BLASTP searches and the date of the searches themselves. This information supplemented the risk assessments done for the mCry3A protein and fulfilled the recommended additional information requested.

3. 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 ranges, which makes the expected dietary exposure several orders of magnitude lower than the amounts of mCry3A protein shown to have no toxicity. Therefore, 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.

4. 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.

5. 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 toxin and was not efficacious against WCRW when inactivated by heat.

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 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.

6. Other Considerations

a) Endocrine Disruptors

2010 Updated Position. As required under FFDCA section 408(p), EPA has developed the Endocrine Disruptor Screening Program (EDSP) to determine whether certain substances (including pesticide active and other ingredients) may have an effect in humans or wildlife similar to an effect produced by a "naturally occurring estrogen, or other such endocrine effects as the Administrator may designate." The EDSP employs a two-tiered approach to making the statutorily required determinations. Tier 1 consists of a battery of 11 screening assays to identify the potential of a chemical substance to interact with the estrogen, androgen, or thyroid (E, A, or T) hormonal systems. Chemicals that go through Tier 1 screening and are found to have the potential to interact with E, A, or T hormonal systems will proceed to the next stage of the EDSP where EPA will determine which, if any, of the Tier 2 tests are necessary based on the available data. Tier 2 testing is designed to identify any adverse endocrine related effects caused by the substance, and establish a quantitative relationship between the dose and the E, A, or T effect.

Between October 2009 and February 2010, EPA issued test orders/data call-ins for the first group of 67 chemicals, which contains 58 pesticide active ingredients and 9 inert ingredients. This list of chemicals was selected based on the potential for human exposure through pathways such as food and water, residential activity, and certain post-application agricultural scenarios. This list should not be construed as a list of known or likely endocrine disruptors.

Modified Cry3A is not among the group of 58 pesticide active ingredients on the initial list to be screened under the EDSP. Under FFDCA § 408(p) the Agency must screen all pesticide chemicals. Accordingly, EPA anticipates issuing future EDSP orders/data call-ins for all Registration Review cases, including those for which EPA has already opened a Registration Review docket for a pesticide active ingredient.

For further information on the status of the EDSP, the policies and procedures, the list of 67 chemicals, the test guidelines and the Tier 1 screening battery, please visit our website: http://www.epa.gov/endo/.

b) Analytical Method(s)

A method for extraction and ELISA analysis of mCry3A protein in corn was submitted and found acceptable by the Agency.

2010 Updated Position. More recently, the Agency decided to allow this requirement to be satisfied when the Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) has verified the performance of a qualitative rapid test kit for detecting the presence of the biotechnology event in grains and oilseeds. In the case of mCry3A, the Agency has confirmed that a test kit has been verified by GIPSA and, therefore, the aforementioned requirement has been satisfied.

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.

7. Tolerance Exemptions

The data submitted and reviewed for Modified Cry3A support the petition for an exemption from the requirement of tolerance for *Bacillus thuringiensis* mCry3A protein and the genetic material necessary for its production in corn.

An exemption from tolerance was established for residues of mCry3Aprotein when used as plant-incorporated protectant in the food and feed commodities corn, field corn, sweet corn and popcorn. (40 CFR 174.505)

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II.C. Environmental Assessment

Background

Syngenta Ltd. has requested a registration for *Bacillus thuringiensis* mCry3A protein and the genetic material, which includes the PMI inert marker gene, necessary for its production in all corn lines and varieties. This protein is intended to control corn rootworm (CRW, *Diabrotica* spp.), a primary pest of corn in the United States. Corn rootworm larvae feed on corn roots, resulting in lodging and a reduction in a plant's ability to absorb water and nutrients from soil. In areas where the CRW is a pest

(*e.g.* Corn Belt), significant financial losses are realized from decreased corn yields and increased expenditures on chemical pest control agents, including organophosphate, carbamate and pyrethroid insecticides.

The EPA has conducted an environmental risk assessment of mCry3A when expressed in corn. General topics covered in this assessment include effects on wildlife, gene flow to related wild plants and its potential effects, and fate of mCry3A protein in the environment. This assessment is based on data submitted to EPA during the development of Event MIR604 corn lines, additional data submitted for registration, Federal Insecticide Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) recommendations, consultations with scientific experts, and public comments on Plant-Incorporated Protectant (PIP) regulation.

A. Environmental Hazard Assessment

I. The Hazard Assessment Process

1. Tiered Testing Hazard Assessment

To minimize data requirements and avoid unnecessary testing, risk assessments are structured such that risk is determined first from estimates of hazard under "worst-case" exposure conditions. A lack of adverse effects under these conditions would provide enough confidence that there is no risk and no further data would be needed. Hence, screening tests conducted early in an investigation tend to be broad in scope but relatively simple in design, and can be used to demonstrate acceptable risk under most conceivable conditions. When screening studies suggest potentially unacceptable risk additional studies are designed to assess risk under more realistic field exposure conditions. These later tests are more complex than earlier screening studies. Use of this "tiered" testing framework saves valuable time and resources by organizing the studies in a cohesive and coherent manner and eliminating unnecessary lines of investigation. Lower tier, high dose screening studies also allow tighter control over experimental variables and exposure conditions, resulting in a greater ability to produce statistically reliable results at relatively low cost.

Tiered tests are designed to first represent unrealistic worst case scenarios and ONLY progress to real world field scenarios if the earlier tiered tests fail to indicate adequate certainty of acceptable risk. Screening (Tier I) non-target organism (NTO) hazard tests are conducted at exposure concentrations several times higher than the highest concentrations expected to occur under realistic field exposure scenarios. This has allowed an endpoint of 50% mortality to be used as a trigger for additional higher-tier testing. Less than 50% mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic field exposure scenarios.

The EPA uses a tiered (Tiers I-IV) testing system to assess the toxicity of a Cry protein (*Bt* endotoxin) to representative non-target organisms that could be exposed to the toxin in the field environment. Tier I high dose studies reflect a screening approach to testing designed to maximize any toxic effects of the

test substance on the test (non-target) organism. The screening tests evaluate single species in a laboratory setting with mortality as the end point. Tiers II – IV generally encompass definitive hazard level determinations, longer term greenhouse or field testing, and are implemented when unacceptable effects are seen at the Tier I screening level.

Testing methods which utilize the tiered approach were last published by the EPA as Harmonized OPPTS Testing Guidelines, Series 850 and 885 (EPA 712-C-96-280, February 1996). These guidelines, as defined in 40 CFR 152.20, apply to microbes and microbial toxins when used as pesticides, including those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation. Therefore Bt Cry protein in corn, being a microbial toxin, is also covered by these testing guidelines.

The Tier I screening maximum hazard dose approach to environmental hazard assessment is based on some factor (whenever possible >10) times the maximum amount of active ingredient expected to be available to terrestrial and aquatic non-target organisms in the environment (EEC). Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels which increase the statistical power to test the hypotheses. Elevated doses, therefore, add certainty to the assessment, and such tests can be well standardized. The Guidelines call for initial screening testing of a single group or several groups of test animals at the maximum hazard dose level (MHD). The Guidelines call for testing of one treatment group of at least 30 animals or three groups of 10 test animals at the screening test concentration. The Guidelines further state that the duration of all Tier I tests should be approximately 30 days. Some test species, notably non-target insects, may be difficult to culture and the suggested test duration has been adjusted accordingly. Control and treated insects should be observed for at least 30 days after dosing, or in cases where an insect species cannot be cultured for 30 days, until negative control mortality rises above 20 percent.

Failing Tier I screening does not necessarily indicate the presence of an unacceptable risk in the field but it triggers the need for additional testing.[a] A less than 50% mortality effect at the MHD is taken to indicate minimal risk. However, greater than 50% mortality does not necessarily indicate the existence of unacceptable risk in the field, but it does trigger the need to collect additional dose-response information and a refinement of the exposure estimation before deciding if the risk is acceptable or unacceptable. Where potential hazards are detected in Tier I testing (i.e. mortality is greater than 50%), additional information at lower test doses is required which can serve to confirm whether any effect might still be detected at more realistic field (1X EEC)concentrations and routes of exposure.

When screening tests indicate a need for additional data, the OPPTS Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} and to quantify the hazard. In the definitive testing, the number of doses and test organisms evaluated must be sufficient to

[a] It is notable that that the 10 X EEC MHD testing approach is not equivalent to what is commonly known as "testing at a 10X SAFETY FACTOR" where any adverse effect at 10X must be considered significant.

determine an LD_{50} value and, when necessary, the Lowest Observed Effect Concentration (LOEC), No Observed Adverse Effect Level (NOAEL) , or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. In the final analysis, a risk assessment is made by comparing the LOAEC to the EEC; when the EEC is lower than the LOAEC, a no risk conclusion is made. Appropriate statistical methods, and appropriate statistical power, must be employed to evaluate the data from the definitive tests. These tests offer greater environmental realism, but they may have lower statistical power. Higher levels of replication, test species numbers or repetition are needed to enhance statistical power in these circumstances.

Data that shows less than 50 % mortality at the maximum hazard dosage level (i.e. LC_{50}, ED_{50}, or LD_{50} >10 X EEC) is sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. It is also notable that the recommended >10X EEC maximum hazard dose level is a highly conservative factor. The published EPA Level of Concern (LOC) is 50% mortality at 5X EEC (see footnotes 5 and 6).

The tiered hazard assessment approach was developed for the EPA by the American Institute of Biological Sciences and confirmed, in 1996, as an acceptable method of environmental hazard assessment by a FIFRA Scientific Advisory Panel (SAP) on microbial pesticides and microbial toxins. The December 9, 1999 SAP agreed that the Tiered approach was suitable for use with Plant Incorporated Protectants (PIP); however, this panel recommended that, for PIPs with insecticidal properties, additional testing of beneficial invertebrates closely related to target species and/or likely to be present in GM crop fields should be conducted. Testing of Bt Cry proteins on species not closely related to the target insect pest was not recommended, although it is still performed to fulfill the published EPA non-target species data requirements [40 CFR § 158.740 (d)]. In October 2000, another SAP also recommended that field testing should be used to evaluate population-level effects on non-target organisms. The August 2002 SAP, and some public comments, generally agreed with this approach, with the additional recommendation that indicator organisms should be selected on the basis of potential for field exposure to the subject protein.[b]

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[b] EPA-SAP. February 4, 2000. Characterization and non-target organism data requirements for protein plant-pesticides. SAP report No. 99-06A for FIFRA Scientific Advisory Panel Meeting held December 8, 1999, held at the Sheraton Crystal City Hotel, Arlington, VA.

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EPA-SAP. August 19, 2004. Product characterization, human health risk, ecological risk, and insect resistance management for *Bacillus thuringiensis* (Bt) cotton products. Transmittal of meeting minutes of the FIFRA Scientific Advisory Panel Meeting held June 8-10 at the Holiday Inn Ballston, Arlington, VA.

The tiered approach to test guidelines ensures, to the greatest extent possible, that the Agency requires the minimum amount of data needed to make scientifically sound regulatory decisions. The EPA believes that maximum hazard dose Tier I testing presents a reasonable approach for evaluating hazards related to the use of biological pesticides and for identifying negative results with a high degree of confidence. The Agency expects that Tier 1 testing for short-term hazard assessment will be sufficient for most studies submitted in support of plant-incorporated *Bt* Cry protein registrations. However, if long range adverse effects must be ascertained, then higher-tier longer-term field testing will be required. As noted above, the October 2000 SAP and the National Academy of Sciences (NAS 2000) recommended direct field testing to ascertain long range environmental effects. This approach, with an emphasis on testing invertebrates found in corn fields, was also recommended by the August 2002 SAP and was supported by several public comments.

Since delayed adverse effects and/or accumulation of toxins through the food chain are not expected to result from exposure to *Bt* Cry proteins, these protein toxins are not routinely tested for chronic effects on non-target organisms. The 30 day test duration requirement does, however, amount to subchronic testing when performed at field exposure test doses. *Bacillus thuringiensis* Cry endotoxins are proteins and proteins do not bioaccumulate. The biological nature of protein makes *Bt* Cry toxins readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports that Cry proteins bind to soil particles, it has also been shown that these proteins are degraded rapidly by microbes upon elution from soil. The same sources also report that *Bt* proteins present in soil collected from *Bt* corn fields have no detectable adverse effect on soil invertebrates or culturable microbial flora.

2. MIR604 Scientific Advisory Panel Comments

The FIFRA Scientific Advisory Panel (SAP) Meeting Held March 14 - 15, 2006 on Event MIR604 Modified Cry3A Protein Bt Corn had several panel members specifically assigned to evaluate the Agency's Environmental Risk Assessment. (These) "Panel members expressed a diversity of opinions concerning the adequacy of the Agency's analysis of the ecological studies submitted by the Registrant. These opinions ranged from nearly unqualified acceptance to qualified rejection." (p. 9, SAP Minutes).

The panel members who understood the Agency's concept of maximum hazard dose screening (limit) testing (outlined in Section A.I.1 above) concluded that … "If one takes the NTO (Non-Target Organism) toxicity assays of this Tier I hazard assessment at face value, none of them reported high acute toxicity. Rather, in most instances mortality was low or did not occur at the ETCs (Estimated Test Concentrations) achieved. Although the ETCs frequently did not meet the standard for maximum hazard dose testing, *these results do support the conclusion of the absence of a strong, toxic response in the NTOs*." (p. 19, SAP Minutes).

In reality, the absence of a strong (50%) mortality response at 5X the field concentration is the Agency standard (endpoint) for maximum hazard dose Tier I screening tests[c] (see Section A.I.1 above). The results of the testing performed for MIR604 were far below this published Agency standard (Level of Concern) for maximum hazard dose testing. There is an absence of 50% mortality in all of the non-target tests performed, and no mortality at all attributable to the test substance was noted.

Tiered tests are designed to first represent **unrealistic worst case scenarios** and ONLY progress to **field exposure scenarios** if the earlier tiered tests fail to indicate an adequate certainty of acceptable risk (i.e. show greater than 50% mortality of the test species). Many of the current Panel comments were made as if field exposure scenario testing was being reviewed, when in fact the worst case (screening) scenarios did not trigger a need for field exposure rate (sublethal/definitive) testing. Therefore many of the Panel's comments are superfluous to the established EPA hazard assessment process which is based on the results of screening tests (when these show less than 50% mortality of the test species).

The EPA risk assessment is centered only on adverse effects at the field exposure rates (1X EEC), and not on adverse effects at greater concentrations. "The Panel was *split* on the importance of the Registrant not adhering to the 10X (EEC test dose) standard. Several Panel members considered this a serious deficiency but a deficiency that easily could have been avoided. *Other Panel members considered the test concentrations/doses adequate to support the Agency's finding of no likely adverse ecological effects.*" (p. 16 SAP Minutes)[d] The dose margin can be less than 10x where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the concentration used must exceed 1X EEC.

The purpose of convening the SAP was to comment on the Agency's conclusion that the MIR604 corn would not cause unreasonable and/or irreversible adverse effects to the environment as determined by

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[c] See footnotes 5& 6

[d] It is notable those panel members who perceived any non-adherence to the 10X standard as a serious deficiency did so as a result of an impression that all testing must be done at a 10X "safety" factor. In reality the 10 X EEC screen testing approach is not equivalent to what is commonly known as "testing at a 10X SAFETY FACTOR" where any adverse effect at 10X must be considered significant. In addition, those panel members who perceived non-adherence to the 10X standard did so as a result of an impression that the purified, bacterially produced Cry protein test substance had half of the activity of the plant produced Cry protein. This impression may not be justified because the intrinsically variable insect bioassay results which appear to show a two fold difference in activity were in fact within the range of variability for insect bioassays. In addition, in the event that the test concentration was at half of the plant Cry protein activity level, the test concentrations are still at or above the EPA Level of Concern (5X EEC) and in all cases above the 1X field exposure rate (EEC) at which the assessment of adverse ecological effects is made. The 1X EEC (based on plant tissue content of Cry protein) is in turn still much greater than any amount which any given non-target organism may be actually ingesting in the field.

existing EPA risk assessment SOPs. As noted above, some panel members responded positively to this charge, whereas others chose to comment on the EPA testing and risk assessment SOPs. The MIR604 SAP was not asked to revise the EPA pesticide program's assessment system which has been developed through many years of evaluation both within the government and through outside experts. The methods have already undergone several layers of SAP, public, peer and EPA Science Advisory Board review and have been found to be adequate.[e] (See also Section A.I.1 above and footnote 2)

In addition to not commenting on the adequacy of the Agency's risk conclusions at field exposure rates (i.e. 1X EEC), some panel members were not prepared to accept the concept of screening (limit) testing. Some viewed the high dose screen tests designed to detect LD_{50}'s as if these were definitive determinations where any effect (at a limit test dose) is also a level of concern at field exposure concentrations. Tier I screening tests were also referred to as 10X "*safety factor"* tests by some Panel members (p.9, SAP Minutes). Tier I screen testing is not 'safety factor testing'. In a "10X safety factor" test any adverse effect noted is a "level of concern"[f], whereas in the EPA risk assessment scenario any adverse effect is viewed as a concern only at 1X the field exposure. *As a result the panel comments on the testing procedures were not addressing screening methods but the definitive (1X) testing procedures that are triggered only when the screening results show greater than 50% mortality.* This level of toxicity was not seen in any screening test performed for mCry3A protein. Therefore definitive testing (at 1X EEC) was not necessary and any comments made by the panel applicable only to the definitive (1X) testing methods are superfluous.[g] In addition, the EPA Guidelines require three replicates of 10 test animals, or one replicate of 30 for the tier I screening tests, therefore the SAP comments on insufficient replication and numbers of test species are also superfluous.

However, in principle the Agency is in agreement with these SAP comments and the OPPTS Testing Guidelines are already implementing them as much as they apply to definitive testing procedures (See Section A.I.1 above). The SAP methodological comments do not, however, apply to Tier I screening tests which in this case did not trigger additional, definitive testing for MIR604 mCry1A proteins There was no effort by some panel members to distinguish 'screening testing' (which is performed to

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[e] Environmental Protection Agency (USEPA) (1998). "Guidelines for Ecological Risk Assessment." EPA 630/R-95-002F. Washington, DC, USA. (Federal Register, May 14, 1998. 63(93): 26846-26924.]

[f] The established (see footnote 5 above), peer and USEPA Science Advisory Board reviewed guidance on screening test levels of concern is 50% mortality at 5X environmental concentration. The appropriate endpoints in high dose limit/screening testing are based on mortality of the treated, as compared to the untreated (control) non-target organisms. A single group of 30 test animals may be tested at the maximum hazard dose.

[g] The 1X EEC test dose is based on plant tissue content and is considered a high worst case dose of the Cry protein. This 1X EEC is in turn still much greater than any amount which any given non-target organism may be ingesting in the field. Therefore the actual exposure scenario would have to be further refined in the event that adverse effects are noted at the (1X) EEC based on plant tissue analysis.

determine intrinsic toxicity of an insecticidal protein) from 'definitive testing' triggered by positive screen test results and which is much more complicated and uses more realistic environmental exposure rates.

A tiered risk assessment as performed by the Agency is recognized as being the most appropriate and rigorous approach to assess non-target affects from both scientific and regulatory standpoints. This process is designed to optimize the use of resources and to identify and define sources of potential risk. Where no unreasonable hazard is detected at Tier I limit (screen/high dose) testing, costly and unnecessary testing at field exposure rates is prevented from taking place. Both hazard and exposure can be evaluated within different levels or "tiers" that progress from worst-case hazard and exposure to more realistic scenarios. Lower tier tests serve to identify potential hazards, and they are conducted in the laboratory at high dose levels which increase the statistical power to test the hypotheses and therefore do not require high replication or large numbers of test species. Where potential hazards are detected in Tier I testing (i.e. test species mortality is greater than 50%), additional information at lower test doses is required which can serve to confirm whether any effect might still be detected at more realistic field (1X EEC) concentrations and routes of exposure. The confirmatory tests offer greater environmental realism, but they may have lower statistical power. Higher levels of replication or repetition are needed to enhance statistical power in these circumstances. *Tier I high dose/screening tests do not require multiple replication and repetition as was recommended by some panel members*. In addition, screening tests without multiple replications are of publication quality, and numerous screening rests are published in peer reviewed literature.

Some panel members also listed deficiencies in the testing procedures of non-target wildlife other than insects. The precision of some assay methods was also criticized. As discussed above, these comments are valid, but they do not apply to screening testing, and additionally, testing of wildlife other than insects related to the target pest is not essential for an environmental risk assessment of mCry3A proteins. This fact was recognized by some members of the SAP who state that: "Cry3A is a protein endotoxin produced naturally by the *tenebrionis* strain *of Bacillus thuringiensis* subsp. *morrisoni* (H 8a8b) that upon ingestion and proteolytic activation exhibits a high level of insecticidal activity to certain species of coleopteran insects, but otherwise is *apparently non-toxic to other types of insects, as well as vertebrates*" (p.29, SAP minutes). Therefore any deficiencies as noted by the panel on testing of non-target organisms other than insects would not have had an adverse effect on the environmental risk assessment of mCry3A protein in corn because they were (a) superfluous tests for Bt Cry proteins and (b) none of the screening tests showed unacceptable effects according to the EPA risk assessment guidelines.

In summary, some panelists agreed with the Agency's risk assessment. Other panelists recommended a variety of refinements to field exposure rate testing protocols which do not apply to the high dose screening tests performed for MIR604 corn and therefore do not change the "no apparent environmental risk" assessment for MIR604 corn. The Panel's critical comments are valid for field exposure dose definitive testing which was not triggered by the screening rests performed with

mCry3A protein. However, the Panel's comments and recommendations are being reviewed for incorporation into the Agency's definitive testing protocols as may be necessary.

The following comments address additional observations found in the SAP Minutes.

A no "may effect" finding was made by the Agency to the endangered Hungerford's crawling water beetle due to low aquatic exposure from corn, a terrestrial crop. In addition, the submitted studies with mCry3A protein did not show any adverse effects at terrestrial field use rates to non-target insect species most closely related to the endangered aquatic beetle. The EPA Environmental Risk Assessment for MIR604 states: "There is no evidence for sensitivity of aquatic (including endangered) species to anti-coleopteran *Bt* Cry proteins. Furthermore, aquatic exposure to mCry3A is extremely small or non-existent since mCry3A is not expressed in MIR604 pollen." and: "… Since mCry3A protein has not been shown to have toxicity effects on … aquatic species, insects and other invertebrate species at the EEC, a 'may affect' situation for endangered land and aquatic species is not anticipated."; and "…Further, several of the federally listed insect species are aquatic and consequently, are unlikely to come in contact with MIR604 maize plant material." Therefore it is the Agency's position that the MIR604 Risk Assessment has adequately addressed the endangered aquatic beetle species.

Contrary to the perceptions of some panel members, the testing performed did meet, and in many cases actually exceeded the Agency's published Tier I testing standards. Where the 10X standard was not met it was due to insect biology (feeding habit) factors that did not permit exposure to 10X EEC concentrations.

The large variability in re-isolation of Cry protein in test soils or test diets is inherent in such testing, especially when insect bioassays are used. It is not realistic to expect precision or accuracy in such assays. For that reason the Agency relies on these tests primarily as a measure that the test material was actually added to, and is present in the test systems.

The determination of a slope (as was done in the soil degradation study) from experimental data points is a standard procedure in OPP and is not considered inadequate.

The maturation rates (weight gain) of male and female birds are naturally different in some avian species and therefore the Agency does not consider the different weight gain rates to be an effect of Cry protein ingestion.

The death of one test animal (fish or bird) in a screening high test dose study (where 50% mortality is the end point) is not considered by the Agency to be an indication of hazard at real world field exposure rates.

Criticism of the use of agar pour plates is not considered a critical issue by the Agency. Agar pour plates have been used for more than 50 years to enumerate microbial and viral particles, and the 50^{o}C

temperature used to prepare these plates does not affect the function of (protein) enzymes or viability of bacteria or virus particles. Therefore there is no reason to expect that the use of the pour plate method to prepare insect diets will have adverse effects on the functionality of Cry proteins.

3. 2010 Update: Terms and Conditions of the Event MIR 604 expressing modified Cry3A and Events MIR 604 x Bt11 expressing the proteins modified Cry3A and Cry1Ab.

When Event MIR 604 expressing mCry3A Corn (EPA Reg. No. 67979-5) and events MIR 604 x Bt11 expressing mCry3A and Cry1Ab (EPA Reg. No. 67979-8) were initially registered on October 03, 2006 and January 25, 2007 respectively, the Agency issued registration notices to Syngenta Seeds, Inc that contained the following requirement for further Environmental Assessment information:

> "Submit field degradation studies evaluating accumulation and persistence of mCry3A in several different soils in various strata. Representative fields must have been planted with mCry3A corn and include both conventional tillage and no-till samples and be harvested under typical agronomic conditions. Sampling must continue until the limit of detection is reached. Studies should include soils with high levels of a variety of clays. Both ELISA and insect bioassays need to be conducted and compared to determine if mCry3A is accumulating or persisting in soil samples. A protocol is due within 90 days of the date of registration. Should the registration expiration date be extended, a final report regarding data from fields that have had three continuous years of cultivation of Event MIR604 corn is due by January 31, 2011."

In response to this requirement, Syngenta Seeds, Inc. – Field Crops – NAFTA submitted a request to waive the conditionally-required soil persistence field study for the plant-incorporated protectant (PIP) *Bacillus thuringiensis* (*Bt.*) mCry3A insecticidal protein and the genetic material necessary for its production in Event MIR604 corn [EPA Reg. No. 67979-5] in support of the Section 3 registration.

Soil microorganism exposure to *Bt* proteins can occur through contact with corn plant roots (by direct feeding), corn plant exudates, incorporation of above-ground plant tissues into soil following harvest, or by soil-deposited pollen. Scientists have generated data on *Bt* proteins in crop residues and their persistence in soil for long periods of time following exposure to a wide range of environmental field conditions.

The results of these studies, summarized in Table 4, show that there is no detectable Cry protein accumulation in agricultural soils during commercial planting of currently registered Cry protein-producing crops (Icoz and Stotzky, 2007; Sanvido, *et. al*, 2007). Likewise, no unexpected accumulation of Cry proteins has been seen in numerous studies submitted directly to EPA for currently registered Cry proteins. There are various published studies that measured *Bt* protein degradation in a laboratory setting relating the dissipation time of the protein to bioactivity (DT_{50}); data predict that Cry proteins in the field degrade rapidly and do not persist or accumulate in the soil to

any extent that would affect nontarget organisms (NTOs). The DT_{50} (based on a simple first-order kinetic model) for mCry3A in an artificial soil environment was 7.6 days (MRID No. 462656-14). The results of this study demonstrate a similar rate of degradation to other Cry proteins in artificial soil systems (U.S. EPA, 2001) and, along with the literature cited below, indicate that mCry3A is likely to have a similar field degradation profile to other *Bt* proteins. In addition, data already exist to support the lack of effects of the mCry3A protein on NTOs (U.S. EPA, 2006; Raybould et al., 2007), which is the primary focus when considering soil fate data.

II. Non-Target Wildlife Hazard Assessment

Two separate SAP reports (October 2000 and August 2002) recommended that non-target testing of *Bt* Cry proteins should focus on invertebrate species exposed to the crop being registered. Following SAP recommendations, the EPA determined that non-target organisms with the greatest exposure potential to Cry protein in transgenic corn fields are beneficial insects, which feed on corn pollen and nectar, and soil invertebrates, particularly Coleoptera species. Therefore, maximum hazard dose toxicity testing on representative beneficial organisms from several taxa was performed in support of this Section 3 FIFRA registration. The toxicity of the mCry3A protein has been evaluated on several species of invertebrates including the lady beetle, Carabid beetle, rove beetle, flower bug, honey bee, and earthworm. Reproductive and developmental observations were also made in the lady beetle, rove beetle and honeybee studies.

Although the mCry3A protein is known to be very host specific, conferring toxic effects on corn rootworm, Colorado potato beetle, and closely related species, and despite the October 2000 and August 2002 SAP's recommendations against testing of non-target species not related to susceptible target pests, EPA has done a risk assessment on a range of non-target wildlife to comply with the Agency's published non-target data requirements (in the absence of PIP-specific risk assessment guidance, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins). These requirements include birds, mammals, plants and aquatic species. In addition, an earthworm study was voluntarily submitted to the Agency to ascertain the potential effects of mCry3A on beneficial decomposer species.

Test substances (*i.e.* source of mCry3A protein) used for studies submitted in support of the mCry3A registration included bacterially-produced purified mCry3A protein (referred to as mCry3A-0102) and corn grain. The October 2000 SAP recommended that while actual plant material is the preferred test material, bacterially-derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume corn plant tissue and where large amounts of Cry protein (Cry protein concentrations that exceed levels present in plant tissue) are needed for maximum hazard dose testing. An insect feeding study, which compared the relative potency of plant produced mCry3A protein to microbe produced mCry3A-0102, indicated that plant produced protein was twice as toxic as microbe produced protein (see Section A.II.1.d.vii). However, since exposure to mCry3A-0102 was at least several times the EEC for all submitted non-target studies, EPA determined that exposure to mCry3A protein was adequate despite the lower potency of microbe produced mCry3A-0102. In

accordance with OPPTS Harmonized Testing Guidelines, adult insect studies were generally conducted for 30 days or until mortality in the negative control reached, or exceeded, 20% and larval studies were carried out through pupation and adult emergence.

The results of ecological effects studies submitted in support of the MIR604 Section 3 FIFRA registration are summarized in Table 4 and presented in a more descriptive format in subsequent sections of this risk assessment document. Full reviews of each study can be found in the individual Data Evaluation Reports (DERs) and accompanying memos.

Table 4. Summary of environmental effects studies and waiver justifications submitted to comply with data requirements published in 40 CFR § 158.740 (d). (Update 2010).

Guideline	Study	Results	MRID
885.4150	Wild Mammal Testing, Tier I	Mammalian wildlife exposure to mCry3A protein is considered likely; however, mCry3A-0102 toxicity data indicate that, when tested at the maximum hazard dose level, there was no significant toxicity to rodents. Therefore no hazard to mammalian wildlife is anticipated and data on wild mammal testing is not required.	N/A
850.2100	Avian Acute Oral Toxicity Test, Tier 1 (Northern Bobwhite quail, *Colinus virginianus*)	Young adult northern bobwhite quail were administered a single nominal oral dose of 722 mg mCry3A-0102/kg body wt and observed for 14 days. There were no treatment-related adverse clinical signs or mortality. Body weight and feed consumption of the test birds were comparable to those of the controls. Classification: Acceptable	461556-16
885.4050	Avian Oral, Tier 1 (Broiler, *Gallus domesticus*)	In a 49-day feeding study, commercial broiler chickens were fed formulated diets containing one of the following ingredients: MIR604 corn (contained mCry3A protein); an MIR604 isoline (no mCry3A protein); or a non-transgenic commercial corn hybrid (no mCry3A protein). No adverse clinical signs were noted, and carcass yield and mortality were not significantly different among treatment groups. Classification: Acceptable	462656-15

Guideline	Study	Results	MRID
885.4200	Freshwater Fish Testing, Tier 1 (Rainbow trout, *Onchorhynchus mykiss*)	In a 28-day toxicity study, granular fish feed containing 50% by weight Event MIR604 corn grain did not produce statistically significant mortality or sublethal effects when fed twice daily to juvenile rainbow trout. Classification: Acceptable	461556-17 462656-02
885.4280	Estuarine and Marine Animal Testing, Tier I	Estuarine and marine animal studies are not required for this product, because mCry3A is not intended for direct application to estuarine or marine environments and there is very low potential that these ecosystems will be exposed to mCry3A protein in field corn.	N/A
885.4300	Nontarget Plant Studies, Tier I	The active ingredient is an insect toxin (*Bt* endotoxin) that is non-toxic to aquatic and terrestrial plants. Consequently, non-target plant studies have been waived for this product.	N/A
885.4340	Nontarget Insect Testing, Tier 1 (Lady beetle, *Coccinella septempunctata*)	Lady beetle larvae were fed live pea aphids that were dipped in a solution containing: 50µg mCry3A-0102/mL Agral 90 solution (a non-ionic surfactant); Agral 90 solution only (negative control); or 0.5 mL of Nemolt (teflubenzuron)/L Agral 90 solution (positive control). The rate of pupal development was not significantly different between the negative control and mCry3A-0102 treatments. However, the number of days to adult emergence was significantly lower in the mCry3A-0102 treatment. Classification: Acceptable	462656-03 462656-04
885.4340	Nontarget Insect Testing, Tier 1 (Carabid beetle, *Poecilus cupreus*)	Twenty-four to 48 hour-old Carabid beetle larvae were fed daily until pupation with blowfly pupae that had been injected with one of three treatments. Results showed no significant difference, in the percent of pre-imaginal mortality or mean weight of emerged adults, between the mCry3A treatment and the negative control group. Classification: Acceptable	462656-05 462656-06

Guideline	Study	Results	MRID
885.4340	Nontarget Insect Testing, Tier 1 (Rove beetle, *Aleochara bilineata*)	Rove beetles were provided approximately 0.2 g of minced beef treated with: 50 µg mCry3A protein/g meat; 10 mL deionized water/90 g meat (negative control); teflubenzuron at a rate of 0.01 mg a.i./g meat (positive control). Beetle mortality and reproductive capacity were not adversely affected by feeding on a test diet composed of 45.85 µg mCry3A/g diet for 35 days. Classification: Acceptable	462656-07 462656-08
885.4340	Nontarget Insect Testing, Tier 1 (Insidious flower bug, O*rius. insidiosis*)	Nymphal flower bugs were fed, on a daily basis, diet with one of three treatments: 50 µg mCry3A-0102 /g of diet; 20 mL deionized water/per 180 g diet (negative control); or teflubenzuron at a rate of 0.01 mg a.i./g diet. Mortality in the mCry3A-0102, negative control, and positive control treatments were 18, 23, and 98%, respectively. Average development time for all treatments was not significantly different. Classification: Acceptable	462656-09 462656-10
885.4380	Honey Bee Testing, Tier 1 (*Apis mellifera)*	Honeybees were exposed to sucrose solution containing 50 µg mCry3A protein/g sucrose solution, or a positive or negative control. Results suggest that incidental ingestion of mCry3A proteins would not adversely affect the hive condition, survival of larvae in brood cells, or exposed adult worker bees. Classification: Acceptable	461556-18
885.4240	Aquatic Invertebrate Acute Toxicity Test, Tier 1	The only plausible potential route of exposure of freshwater invertebrates to insecticidal proteins produced by transgenic corn plants is corn pollen drift into aquatic habitats. However, since the pollen of Event MIR604 corn plants has no detectable mCry3A protein, exposure of freshwater aquatic invertebrates to mCry3A protein will be negligible. Classification: Acceptable	Waiver justification
850.620	Earthworm Subchronic Toxicity Study (*Eisenia fetida)*	Earthworms were exposed to soil containing mCry3A-0102 at a nominal concentration of 370 µg/g dry soil for 14 days, or one of two control treatments. At test end, a mortality rate of 5% and a mean weight loss of 5.8% were recorded for mCry3A-0102 treated worms. For the negative control, mortality was 0%, and mean weight loss was 11.4%. Mortality was 100% for the positive control. Classification: Acceptable	462656-11 462656-12

Guideline	Study	Results	MRID
N/A	Insecticidal Activity Spectrum Study	The mCry3A protein has a similar spectrum of activity to native Cry3A, but with enhanced toxicity to NCRW and WCRW. Modified Cry3A produced in *E. coli* and maize were found to be active against WCRM with 144 hour LC_{50} values of 0.43 µg mCry3A-0102/mL diet and 0.20 µg mCry3A/mL diet surface, respectively.. Classification: Acceptable	461556-01 461556-03
885.5200	Expression in a Terrestrial Environment (Soil Fate)	A simple first-order kinetic model, based on CPB larvae feeding data, determined that the DT_{50} for mCry3A in this silty clay loam soil was 7.6 days. This finding suggests that soil incorporated mCry3A protein degrades over time. Classification: Acceptable	462656-14
NA	Environmental Fate Assessment	MIR604 corn plants have been shown to express mCry3A protein in leaves, kernels, roots, and silks, but the protein was not detected in corn pollen. Due to corn's lack of invasive characteristics and the low probability that the *mCry3A* gene from Event MIR604 would transfer to a wild relative of corn, it is unlikely that mCry3A will spread beyond cultivated sites and persist in weedy populations. It is also unlikely that genes present in MIR604 corn would be subject to horizontal gene transfer at a frequency that exceeds the rate of transfer in other plants. Classification: Acceptable	462656-13
NA	Endangered Species Assessment	The primary route of exposure to mCry3A protein in corn is through ingestion of corn tissue. There are no reports of threatened or endangered species feeding on corn plants, therefore such species would not be exposed to corn tissue containing Cry protein. Since mCry3A protein has not been shown to have toxicity effects on mammals, birds, plants, aquatic species, insects and other invertebrate species at the EEC, a "may affect" situation for endangered land and aquatic species is not anticipated. In addition, EPA does not expect that any threatened or endangered plant species will be affected by outcrossing to wild relatives or by competition with such entities. Hybrid corn does not exist in the wild, nor are there wild plants that can interbreed with corn in the United States. Because of the selectivity of mCry3A protein for	462656-01

Guideline	Study	Results	MRID
		coleopteran species, endangered species concerns are mainly restricted to the order Coleoptera. Examination of an overlay map showing the county level distribution of the 16 endangered/threatened coleopteran species (currently listed by the U.S. Fish and Wildlife Service) relative to corn production counties in the United States clearly indicated that any potential concern regarding range overlap with corn production was mainly restricted to the American burying beetle (*Nicrophorus americanus*). The American burying beetle is the largest carrion beetle in North America and is only found in limited areas of Rhode Island and portions of the Great Plains, including Arkansas and Georgia. Adults are nocturnal and feed on carrion and sometimes prey on other arthropods. Larvae feed exclusively on buried carrion provided by their parents. The American burying beetle's habitat is variable and often includes deciduous forest, grassland and agricultural areas. Considering that both larvae and adult insects feed exclusively on carrion, it appears that even if American burying beetles did occur in proximity to *Bt* corn fields, there would be little chance of exposure to *Bt* protein due to their feeding habits. After careful review of available data, the EPA determined that exposure of American burying beetle to harmful levels of MIR604 corn tissue is not expected. Likewise, a review of the preferred habitats of other coleopteran species listed as endangered by the U.S. Fish and Wildlife Service indicated that exposure to harmful levels of mCry3A protein would not take place. The main reasons for the lack of exposure are geographical and habitat limitations. These species are located in non-corn production areas and/or their habitat does not encompass agricultural areas. Likewise, other insect species in the orders Diptera, Hemiptera, Lepidoptera, Odonata and Orthoptera that are listed as endangered/threatened species are found in dune, meadow/prairie or open forest habitats and are not closely associated with row crop production, often times due to the specificity of the habitat of their host plants. The reviewed toxicological data shows the relative	

Guideline	Study	Results	MRID
		insensitivity of a range of insects in non-Coleopteran orders to the mCry3A proteins, indicating that MIR604 maize hybrids are not likely to have detrimental effects on non-Coleopteran insects included on the endangered/threatened species list. Further, several of the federally listed insect species are aquatic and consequently, are unlikely to come in contact with MIR604 maize plant material. Many of the endangered and threatened beetles occur in cave or aquatic habitats. Since movement into water bodies of soil containing mCry3A is expected to be negligible, pollen drift was considered the primary source of potential hazard to endangered aquatic Coleoptera. According to estimates based on published studies, if 100% of the pollen grains leaving a corn field were deposited in a 1 ha pond with 2 m depth and located ≥1 m from the edge of a corn field, <0.0001 µg mCry3A/mL of water would be expected. This is a few orders of magnitude below the toxic level to any insect.	

Guideline	Study	Results	MRID
NA	Long-term field degradation study on the accumulation and persistence of active ingredient	Soil microorganism exposure to *Bt* proteins can occur through contact with corn plant roots (by direct feeding), corn plant exudates, incorporation of above-ground plant tissues into soil following harvest, or by soil-deposited pollen. Scientists have generated data on *Bt* proteins in crop residues and their persistence in soil for long periods of time following exposure to a wide range of environmental field conditions. The results of these studies show that there is no detectable Cry protein accumulation in agricultural soils during commercial planting of currently registered Cry protein-producing crops (Icoz and Stotzky, 2007; Sanvido, *et. al*, 2007). Likewise, no unexpected accumulation of Cry proteins has been seen in numerous studies submitted directly to EPA for currently registered Cry proteins. There are various published studies that measured *Bt* protein degradation in a laboratory setting relating the dissipation time of the protein to bioactivity (DT_{50}); data predict that Cry proteins in the field degrade rapidly and do not persist or accumulate in the soil to any extent that would affect nontarget organisms (NTOs). The DT_{50} (based on a simple first-order kinetic model) for mCry3A in an artificial soil environment was 7.6 days (MRID No. 462656-14). The results of this study demonstrate a similar rate of degradation to other Cry proteins in artificial soil systems (U.S. EPA, 2001) and, along with the literature cited below, indicate that mCry3A is likely to have a similar field degradation profile to other *Bt* proteins. In addition, data already exist to support the lack of effects of the mCry3A protein on NTOs (U.S. EPA, 2006; Raybould et al., 2007), which is the primary focus when considering soil fate data. **RECOMMENDATION:** The data and rationales in support of the request to waive the need for a soil persistence field study are **ACCEPTABLE** for mCry3A protein, as expressed in MIR604 corn.	478477-01

1. Non-target Wildlife Testing and Hazard Assessment

a. Mammalian Wildlife

Mammalian wildlife exposure to mCry3A protein is considered likely; however, mammalian toxicology information gathered to date on *Bt* Cry proteins does not show a hazard to wild mammals. And an acute oral toxicity test, submitted to EPA in support of the MIR604 registration (see Human Health Risk Assessment), indicated that no significant toxicity was seen when rodents were exposed to mCry3A at the maximum hazard dose level. Therefore, no hazard to mammalian wildlife is anticipated and data on wild mammal testing is not required for this registration.

b. Avian hazard assessment

Published data and studies on file at EPA show that consumption of *Bt* corn has no measurable deleterious effects on avian species. However, to comply with published data requirements, the following studies were submitted to EPA in support of the MIR604 product registration. These studies were GLP compliant and, when considered together, meet EPA data requirements for avian species.

i. Northern Bobwhite Quail

This study meets current EPA Guideline requirements for acute toxicity testing of incidental exposures of plant incorporated Cry proteins to non-target birds in the wild.

Young adult (25 week old) northern bobwhite quail (*Colinus virginianus*) were administered a single nominal oral dose of 722 mg mCry3A-0102/kg body wt and observed for 14 days. There were no adverse treatment-related clinical signs or mortality. Body weight and feed consumption of the test birds were comparable to those of the negative control. The acute oral LD_{50} of mCry3A-0102 was shown to be greater than a nominal concentration of 722 mg mCry3A-0102/kg body wt (approximately 652 mg mCry3A protein/kg body wt). These data show that there will be no adverse effects on avian wildlife from incidental field exposure to mCry3A corn.

ii. Broiler study

The submitted study was not EPA GLP compliant, but was conducted according to accepted scientific methods.

In a 49-day avian feeding study, one day-old commercial broiler chickens (*Gallus domesticus*, Ross 344 males and feather-sexable Ross 308 females) were fed formulated diets containing one of the following ingredients: MIR604 corn (contained mCry3A protein); an MIR604 isoline (no mCry3A protein), or a non-transgenic commercial corn hybrid (no mCry3A protein). Starter, grower (days 16-31), and finisher diets (days 31-49) contained 57.5, 63.0 and 67.5% corn, respectively. The concentration of plant produced mCry3A in the transgenic diets was reported to be 0.04, 0.06, and 0.08 µg/g dry weight of the starter, grower, and finisher diets, respectively. Chicks were separated by sex and placed into single sex pens containing 25 birds each. Each treatment group contained 6 cages each

of males and females (12 cages x 25 birds/cage = 300 birds). Pen weights (25 birds/pen) were recorded at days 1 (hatch), 16, 31, and 49. On the later three dates, feed conversion ratios were determined. Feeding was terminated approximately16 hours before slaughter on day 51. Body weight, feed conversion, and survival data were recorded. Results indicate that sex had a significant effect on body weight and survival, with males weighing more and having higher mortality (which is normal for this species). No adverse clinical signs were noted, and carcass yield and mortality were not significantly different among treatment groups.

Table 5. Mean body weight of broiler chickens fed mCry3A positive or mCry3A negative corn grain.

Treatment	Body Weight (g)			
	Day 1 (hatch)	**Day 16**	**Day 31**	**Day 49**
MIR604 (mCry3A positive)	44.60 ± 0.19 a*	547.8 ± 9.6 a	1684.1 ± 50.6 a	3468.4 ± 129.5 a
MIR604 (mCry3A negative)	44.66 ± 0.18 a	563.0 ± 5.4 a	1690.5 ± 40.2 a	3469.4 ±113.1 a
Commercial hybrid (mCry3A negative)	44.70 ± 0.19 a	551.1 ± 9.6 a	1634.0 ± 44.3 b	3365.2 ± 117.6 b

* Within sampling days, means followed by different letters differ significantly ($p \leq 0.05$).

Table 6. Carcass and parts yield at day 51 for broiler chickens fed mCry3A positive or mCry3A negative corn grain.

Males				
Treatment	Dressed carcass*	Thighs	Pectoralis major	Pectoralis minor
	---------- g ----------			
MIR604 (mCry3A positive)	2947.2 ± 57.44 a**	526.3 ± 14.05 a	638.8 ± 19.18 a	150.8 ± 4.63 a
MIR604 (mCry3A negative)	2936.7 ± 56.88 a	484.3 ± 15.10 ab	609.6 ± 16.73 a	145.7 ± 4.57 a
Commercial hybrid (mCry3A negative)	2812.9 ± 61.49 a	454.6 ± 20.30 b	605.1 ± 23.09 a	151.3 ± 4.97 a
Females				
MIR604 (mCry3A positive)	2248.3 ± 45.53 a	380.8 ± 7.6 a	515.4 ± 18.9 a	126.2 ± 3.3 a
MIR604 (mCry3A negative)	2291.0 ± 41.4 a	361.9 ± 9.2 a	511.8 ± 13.8 a	128.0 ± 3.5 a
Commercial hybrid (mCry3A negative)	2204.2 ± 54.8 a	366.2 ± 11.3 a	487.1 ± 22.0 a	125.9 ± 3.4 a

* Fresh carcass without head, neck, feet, feathers, viscera, and blood
** Within carcass parts categories, means followed by different letters differ significantly ($p \leq 0.05$)

c. Aquatic species testing

There is no evidence for sensitivity of aquatic (including endangered) species to anti-coleopteran *Bt* Cry proteins. Furthermore, aquatic exposure to mCry3A is extremely small or non-existent since mCry3A was not detectable in MIR604 pollen.

i. Freshwater Fish

The Harmonized Testing Guidelines requirement for a static renewal freshwater fish toxicity study is usually waived for *Bt* corn PIPs due to the low potential for exposure to Cry protein produced in this crop. Nonetheless, a 28 day flow-through study was performed and submitted for review. This study is scientifically sound.

In this 28-day toxicity study, juvenile rainbow trout (*Onchorhynchus mykiss*) were fed fish feed containing 50% w/w Event MIR604 (0.09 µg mCry3A/g test diet) or non-transgenic (negative control) corn grain. Prior to test initiation, 40 fish were placed in each of two test vessels, the exposure tank and the control tank. Mortality and symptoms of toxicity were assessed on a daily basis and detailed

observations of symptoms and feeding responses were made on days 4, 7, 10, 15, and 22. No significant differences were detected in the weight of the control or test fish at 0, 14, or 28 days. No significant difference in length was seen at 14 or 28 days. In the MIR604 test group, transient discoloration, sounding, and surfacing were seen in one to three fish after day 15, and one fish (2.5% of test group) was found dead on day 21. No mortality was seen in the control group. Due to the lack of demonstrated toxicity of the mCry3A protein to juvenile rainbow trout and the low probability that aquatic systems will be exposed to the protein, no fresh water fish hazard is expected from commercial cultivation of Event MIR604 corn.

ii. Aquatic invertebrates

Update (July, 2010). In light of recently published laboratory studies showing reduced growth in shredding caddis flies exposed to anti-lepidopteran Cry1A protein corn litter (Rosi-Marshall, et al. 2007), additional aquatic invertebrate data are required. A 7 to 14 day *Daphnia magna* study as per the 885 series OPPTS Guidelines needs to be performed. The study may be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the *Daphnia* study.

Update (September 2010). Since the 2007 Rosi-Marshall et al. publication, numerous researchers have published peer-reviewed studies that identify issues with the scientific merit and relevance of the original caddis fly study (Swan et al. 2009, Jensen et al. 2010, summarized by Beachy et al. 2008, Parrott 2008, and Wolt and Peterson 2010). In response to comments received on the proposed terms and conditions for the extension of the 2010 expiring *Bt* corn registrations, EPA conducted a literature review of these recently published studies. Criticisms of the Rosi-Marshall et al. study included several findings: (1) adverse effects were not caused by toxicity of Cry1A but, rather, by other differences between plant test substances (Jensen et al. 2010); (2) the abundance of Trichoptera in streams containing residues of Cry1A was not reduced (Chambers et al. 2007); and (3) while post-harvest crop residue was identified as the most likely route of exposure (Carstens et al. 2010), aquatic exposure to biotech crops has been shown to be limited temporally and spatially with low to negligible exposure concentrations of Cry proteins in post-harvest crop tissues (Swan et al. 2009, Chambers et al. 2009, Jensen et al. 2010, Wolt and Peterson 2010, Carstens et al. 2010). In light of these results, EPA is not requiring additional aquatic invertebrate studies to assess hazard to aquatic shredder species for existing Cry protein PIP registrations.

iii. Estuarine and Marine Animals

Estuarine and marine animal studies were not required for this product, because of the low probability that aquatic systems will be exposed to the mCry3A protein produced in MIR604 corn plant tissues.

iv. Terrestrial and Aquatic Plants

Plant toxicity studies were not required for this product because the active ingredient is an insect toxin (*Bt* endotoxin) that has never shown any toxicity to plants.

d. Non-Target Insect Testing

The mCry3A protein specifically targets corn rootworm species, which are within the order Coleoptera (beetles). Since *Bt* toxins are known to have a limited host range, EPA requires that test species used for non-target insect evaluations should include several species that are related to the target pests (coleopteran species), since it is expected that these species will be most susceptible to the *Bt* toxin.

i. Ladybird Beetle

This study complies with the testing requirements outlined in OPPTS Series 885.4340 (Nontarget Insect Testing, Tier 1), the Organization for Economic Development (OECD) Principles of Good Laboratory Practice, and the UK Good Laboratory Practice Regulations.

Four–day old lady beetle larvae (*Coccinella septempunctata*) were fed live pea aphids that were dipped in one of three solutions for a period of 14 days. For each treatment, aphids were immersed for 30 seconds in a solution containing: mCry3A dissolved in a solution of Agral 90 (a non-ionic surfactant) at a concentration of 50 µg mCry3A protein/mL solution; Agral 90 solution only (negative control); or 0.5 mL of Nemolt (teflubenzuron)/L Agral 90 solution (positive control). Freshly-treated live aphids were provided to lady beetle larvae daily until pupation, and the number of pea aphids provided at each feeding time increased with larval age. Beetle larvae were assessed daily for developmental stage and mortality. Following adult emergence from pupae, beetles were fed 50 treated aphids 3 times a week for 14 days. Adults were assessed for mortality at each feeding.

There was no significant difference in the rate of pupal development between the negative control and mCry3A-0102 treatment. However, the mean number of days to adult emergence was significantly lower in the mCry3A treatment. There was no significant difference in pre-imaginal or adult survival among the negative control and mCry3A treatments. All larvae in the positive control died in the pre-imaginal stage. Since lady beetles are known to feed on corn pollen or insect prey, rather than corn leaves, and since mCry3A concentration in pollen (undetected) and prey is much lower than in corn leaves, it is not expected that lady beetles will be adversely affected by MIR604 corn in a field environment.

Table 7. Mean developmental time for lady beetles exposed to mCry3A-0102 or a control treatment.

Treatment	Concentration	Larvae to Pupae		Larvae to Adult	
		Mean	Standard Deviation	Mean	Standard Deviation
Negative control	Agral 90	5.48	0.50	9.8	0.68
mCry3A-0102	50 µg mCry3A/mL Agral solution	5.33	0.47	9.48*	0.59

*Significantly different from control ($p<0.05$)

Table 8. Mortality assessment for lady beetles exposed to mCry3A-0102 or a control treatment.

Treatment	Concentration	Pre-Imaginal Mortality		Adult Mortality	
		Mortality	Corrected Mortality	Mortality	Corrected Mortality
		---------- % ----------			
Negative control	Agral 90	0	-	7.5	-
mCry3A-0102	50 µg mCry3A/mL Agral solution	0	0	15.0	8.1
Nemolt	0.5 mL/L Agral solution	100*	100	-	-

* Treatment differed significantly from the negative control ($p<0.001$)

It is noted that the actual amount of mCry3A protein consumed by larvae and adult ladybird beetles was 9 µg mCry3A/g aphid, which is below the targeted concentration of 50µg/g aphid (10 x mCry3A concentration in corn leaves). However, since lady beetles are known to feed on corn pollen or insect prey, rather than corn leaves, and since mCry3A concentration in pollen (undetected) and prey is much lower than in corn leaves, it is not expected that lady beetles will be adversely affected by MIR604 corn in a field environment.

ii. Carabid Beetle

This study complies with the testing requirements outlined in OPPTS Series 885.4340 (Nontarget Insect Testing, Tier 1), the Organization for Economic Development (OECD) Principles of Good Laboratory Practice, and the UK Good Laboratory Practice Regulations.

Twenty-four to 48 hour-old Carabid beetle larvae (*Poecilus cupreus*) were fed daily with blowfly pupae until pupation. For each treatment, blow fly pupae were injected with: mCry3A-0102 at a rate of

50µg mCry3A/g pupa; deionized water only (negative control); or teflubenzuron at a rate of 0.664 ng a.i./g fly pupae (positive control). Fly pupae were replaced daily with freshly defrosted pupae until beetle larvae entered pupation. Beetle larvae were assessed three times per week for the first two weeks, and two times per week thereafter until day 32 of the study, at which time test containers were checked daily for emerging adult beetles. Adults were sexed, and overall weight and mortality were statistically analyzed. Results showed no significant difference, in the percent of pre-imaginal mortality or mean weight of emerged adults, between the mCry3A treatment and the negative control group. The positive treatment resulted in 100% pre-imaginal mortality (Table 9).

Table 9. Larval mortality and adult weight of Carabid beetles fed blowfly pupae treated with or without mCry3A-0102 protein.

Treatment	Percent Larval Mortality	Percent Corrected Larval Mortality	Mean Adult Weight
mCry3A-0102	10	0	82.9
Negative control	20	-	81.5
Positive control	100	100	-

Analysis of the test diet showed that the actual concentration of mCry3A in blowfly pupae was 12 µg/g pupae. This amount is less than 10X (50 µg/g) the expressed concentration in maize leaves. However, the most likely route of carabid exposure to mCry3A protein is through consumption of prey that has eaten MIR604 plant tissue and studies indicate that the concentration of mCry3A in prey is at least 1.4X lower than the protein concentration in plant tissue.

iii. Rove Beetle

This study complies with the testing requirements outlined in OPPTS Series 885.4340 (Nontarget Insect Testing, Tier 1), the Organization for Economic Development (OECD) Principles of Good Laboratory Practice, and the UK Good Laboratory Practice Regulations.

Rove beetles (*Aleochara bilineata*) were obtained from parasitized onion fly (*Delia antique*) pupae and adults were four days old (physiologically) at study initiation. During this 35 day feeding trial, beetles were provided one of three treatments of cooked minced beef. For each treatment, beetles were given approximately 0.2 g of minced beef treated with: 50 µg mCry3A protein/g meat; 10 mL deionized water/90 g meat (negative control); or teflubenzuron at a rate of 0.01 mg a.i./g meat (positive control). For the first 7 days, beetles (10 female and 10 male) were kept in round plastic pots. From days 7 to 35, test arenas were comprised of polystyrene boxes filled with at least 4 cm of quartz sand. On days 1, 7 and 35 living, moribund, dead, and missing beetles were noted. To assess beetle fecundity, approximately 500 onion fly pupae were incorporated beneath the sand surface in each test box on days 14, 21, and 28. After approximately seven days, fly pupae were removed from the sand and placed in plastic pots. F_1 beetles emerging from onion fly pupae were recorded every 1 to 4 days through day 76. The study was concluded when the mean number of beetles emerging per replicate declined to less than two per day in the control treatment. The mean number of F_1 progeny was 647 for

the negative control and 663 beetles for the mCry3A treatment and these results were not significantly different. At day 35, mortality in the mCry3A, negative treatment, and positive treatments were 31, 34, and 35%, respectively and did not differ significantly (Table 10). Results also indicate that the reproductive capacity of beetles feeding on the mCry3A test diet was not adversely affected.

Table 10. Mortality and number of progeny of rove beetles supplied mCry3A-0102 or a control treatment for 35 days.

Treatment	% Mortality at 35 days*	% Corrected Mortality	Mean no. of F_1 Progeny	% Effect on Reproduction
mCry3A-0102 (50 µg mCry3A/g diet)	31	0	663 ± 219	-2.5
Negative control	34	-	647 ± 169	-
Teflubenzuron (10 µg/g diet)	35	2	3 ± 5**	99.5

* Treatments did not differ significantly from the negative control ($p>0.05$)
** Treatment differed significantly from the negative control ($p<0.001$)

iv. Flower Bug

This study complies with the testing requirements outlined in OPPTS Series 885.4340 (Nontarget Insect Testing, Tier 1), the Organization for Economic Development (OECD) Principles of Good Laboratory Practice and the UK Good Laboratory Practice Regulations.

Nymphal *Orius insidiosis* were fed, on a daily basis, diet consisting of cooked beef, liver, yeast, honey, egg, sugar, water, and Nipagin (chemical preservative) for 21 days. The diet was treated with one of three treatments: 50 µg mCry3A/g diet; deionized water (negative control); or teflubenzuron at a rate of 0.01 mg a.i./g diet. For each treatment, approximately 0.2 g of diet was placed into a small plastic cup covered with parafilm; insects pierced the parafilm to reach the diet. Nymphs were assessed for mortality and vitality daily until adulthood, or until 21 days after test initiation. Bugs that were missing, squashed, or injured during the study were excluded from data analysis. Mortality in the mCry3A, negative control, and positive control treatments were 18, 23, 98%, respectively (Table 11). Average development time for all treatments was not significantly different ($p>0.5$). Analysis of the test diet showed that protein expression was 47.8 µg mCry3A/g diet, or 95.6% of the nominal concentration (50 µg/g diet). In addition, a bioassay in which Colorado potato beetle, CPB (*Leptinotarsa decemlineata*) larvae were fed a diet containing 10 or 20% mCry3A-treated diet resulted in mortality of 83 and 90%, respectively. Although control mortality was 23% (test guidelines state that negative control mortality should not exceed 20%), it is unlikely that study conclusions were affected by this high rate of mortality.

Table 11. Mortality of flower bugs supplied with diet containing mCry3A-0102 or a control diet.

Treatment	% Pre-imaginal Mortality	% Corrected Mortality
mCry3A-0102	18	0
Negative control	23	-
Teflubenzuron (10 µg/g diet)	98*	97

* Significantly different from negative control ($p<0.001$)

v. Honey Bee Larvae

An acceptable study was conducted based on OPPTS Series 885.4380 (Honey Bee Testing, Tier I), in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency in 40 CFR Part 160.

Honeybees (*Apis mellifera*) were exposed via in-hive feeders to sucrose solution containing 50 µg/g sucrose solution, a negative control of 50% w/v sucrose solution, or a positive control of 480 g/L diflubenzuron insect growth regulator (Dimilin Flo ™) in sucrose solution. Fresh treatment solutions were provided to each hive daily for five days. Egg cell mortality in the negative control, test, and positive control groups was 28.5, 27.3, and 100%, respectively. Larval cell mortality was 6.0, 6.8, and 100%, respectively. There was no significant difference in mortality between the test and negative control groups for cells with eggs or larvae. There was also no significant difference in pre- and post-test hive condition between the test and negative control treatments. Results for the positive control treatment were significantly different from the other treatments for both mortality and hive condition (Table 12). Adult bees were not affected by any of the treatments. These study results suggest that incidental ingestion of mCry3A proteins would not adversely affect the hive condition, survival of larvae in brood cells, or exposed adult worker bees.

Table 12. Honey bee egg and larval cell development and brood count per frame when exposed to mCry3A-0102 or a control treatment.

Treatment	Brood Development Assessments				Mean Brood per Frame	
	Egg Cells		Larvae Cells		Before Treatment	After Treatment
	Mortality	Corrected Mortality	Mortality	Corrected Mortality		
	----------%----------					
Negative control	28.5	-	6.0	-	36.4	49.6
mCry3A-0102	27.3	0	6.8	0.9	34.2	40.7
Dimilin Flo	100.0*	100.0	100.0	100.0*	34.5	27.7*

* Significantly different from negative control ($p<0.01$)

vi. Freshwater Aquatic Invertebrate (waiver justification)

The only plausible potential route of exposure of freshwater invertebrates to insecticidal proteins produced by transgenic corn plants is corn pollen drift into aquatic habitats. However, since the pollen of Event MIR604 corn plants has no detectable mCry3A protein, exposure of freshwater aquatic invertebrates to mCry3A protein will be negligible.

vii. Earthworm Toxicity Testing

This study complies with the testing requirements outlined in OPPTS Series 850.6200 (Earthworm Subchronic Toxicity Study), Good Laboratory Practice Standards as published by the EPA in 40 CFR Parts 160 and 792, and the Organization for Economic Development (OECD) Principles of Good Laboratory Practice.

In a laboratory test, earthworms (*Eisenia fetida*) were exposed to test substance mCry3A-0102, incorporated into artificial soil, at a nominal concentration of 370 µg/g dry soil (334 µg mCry3A protein/g dry soil) for 14 days. A negative control of deionized water and artificial soil and a positive control of 10, 20, 30 40, or 50 mg 2-chloroacetamide/kg dry soil were also used in the test. At test end, a mortality rate of 5% and a mean weight loss of 5.8% were recorded for mCry3A treated worms (Table 13). Worms included in the negative control had a mortality rate of 0% and a mean weight loss of 11.4%. Positive control worms exposed to ≥30 mg 2-chloroacetamide/kg of dry soil had a mortality rate of 100% and the LC_{50} value for earthworms exposed to 2-chloroacetamide was approximately 18 mg active ingredient/kg dry soil. The 14-day LC_{50} for earthworms exposed to mCry3A-0102 in an artificial soil substrate was determined to be greater than 370 µg test substance/g dry soil (equivalent to 334 µg/g mCry3A active ingredient/g dry soil, the highest concentration tested), or 67 times greater than the expected field concentration of 5.5 µg/g soil (based on mCry3A concentration in senescent MIR604 plant roots). These study findings, which are consistent with historical *Bt* cry protein feeding results, indicate that earthworms should not be adversely affected by MIR604 corn plants.

Although some public comments have questioned whether earthworm test organisms actually ingested the soil incorporated *Bt* Cry proteins, recently published data show that earthworms do ingest and excrete soil incorporated *Bt* Cry proteins.

Table 13. Mortality and weight loss of earthworms exposed to mCry3A-0102 or a control treatment.

Treatment	Cumulative mortality (%)		14-Day Weight loss (%)
	Day 7	Day 14	
mCry3A-0102	2.5	5.0	5.8
Negative control	0	0	11.4

viii. Insecticidal Activity Spectrum Study

Insect susceptibility studies showed that native Cry3A is primarily active against Colorado potato beetle and has minimal activity against northern corn rootworm (NCRW); both species are members of the Chrysomelidae family of beetles. The mCry3A protein has a similar spectrum of activity to native Cry3A, but with enhanced toxicity to NCRW and western corn rootworm (WCRW).

The bioactivity of the recombinant *E. coli* and maize event MIR604 mCry3A proteins were compared using a diet incorporation bioassay with first instar WCRW. Modified Cry3A from both *E. coli* and maize were found to be active against WCRW with 144 hour LC_{50} values of 0.43 µg mCry3A/mL diet and 0.20 µg mCry3A/mL diet surface, respectively (Table 14).

Table 14. **Toxicity of mCry3A protein derived from recombinant *E. coli* (mCry3A-0102) or from corn event MIR604 on WCRW larvae.**

Sample	LC50	
	µg mCry3A protein/mL diet	95% Confidence Interval
LPMIR604-0103 (corn-derived protein)	0.20	(0.09 - 0.41)
mCry3A-0102 (bacterial-derived protein)	0.43	(0.14 - 0.94)

2. Soil Fate

Soil organisms may be exposed to mCry3A protein through contact with corn plant roots (by direct feeding), corn plant root exudates, incorporation of above-ground plant tissues into soil following harvest, or by soil-deposited pollen. Some evidence suggests that acidic soils (pH 5.6), and those which are high in clays and humic acids, are more likely to bind Cry protein, and thus decrease the rate of protein degradation by soil microorganisms. It is noted, however, that the pH factor should not contribute to protein binding in corn fields, since maize is generally grown on neutral soils (above pH

5.6). And despite evidence that soils high in clay and humic acids may bind cry proteins, and thus interfere with the microbial degradation processes, the weight of evidence suggests that Cry proteins do not accumulate in soil to arthropod-toxic levels. Nonetheless, the Agency requires soil fate evaluations for each new insect protected crop.

This study was conducted in accordance with Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency, Office of Pesticide Programs in 40 CFR Part 160.

The test soil was a silty clay loam collected from a corn-growing region of Iowa. Treatments were the following: mCry3A test mixture (*Bt* cry protein and water) mixed with soil at a nominal dose of 230 µg mCry3A/g d/w soil, and sampled after 0, 1, 3, 7, 12, and 30 days of incubation; a negative control consisting of soil dosed with deionized water; and a positive control consisting of mCry3A mixed with test diet (no soil), at a concentration of 230 µg mCry3A/g diet (equivalent test dose level at Day 0).

For the CPB bioassay, mCry3A-treated soil collected at each incubation time point was incorporated into a stock diet at a concentration of 10% w/w, and the resulting suspension was poured into Petri dishes. Negative and positive controls were prepared in the same manner. Ten freshly-hatched CPB larvae were placed in each Petri dish, which were then covered and maintained under ambient laboratory conditions. Test material and negative control treatments were replicated 12 times (120 larvae/treatment) and the positive control was replicated four times (40 larvae). Larval mortality was assessed at 72 hours.

Mean CPB larval mortality in mCry3A- treated soil ranged from 48-54% during the first week, then declined rapidly to 9% on Day 30 (Table 15). The DT_{50} value (time for 50% of initial bioactivity to dissipate) for degradation of mCry3A in this soil was estimated to be 7.6 days. Biomass determinations of soil at study start and end showed that microbial activity was maintained during the study.

Table 15. mCry3A protein bioactivity in treated soil as measured by CPB larvae mortality.

Treatment	% CPB Mortality
Negative control	18
Positive control	53
Test Treatment	
Day 0	53
Day 1	54
Day 3	48
Day 7	51
Day 12	11
Day 30	9

Based on these results, it may be concluded that purified mCry3A-0102 insecticidal proteins degrade rapidly in silty clay loam soil. However, silty clay loam soil is just one of many soil classes used for corn production in the United States. A more useful study would evaluate protein degradation, accumulation, and/or persistence in a range of soil types, including those with high clay and humic acid content, due to their known binding affinity for proteins.

In addition, this study utilized field soil spiked with purified insecticidal protein. This approach is useful because dose responses can be easily quantified. However, the degradation and accumulation of Cry proteins found within decaying plant tissue may behave differently than proteins in artificially spiked soil. Thus, the relevance of these study results is unclear other than to show that degradation in soil does take place.

To account for the above concerns, it is recommended that additional studies should be conducted to evaluate insecticidal protein degradation, accumulation, and persistence in a variety of soil types, including those high in clay and humic acids, into which all non-harvested corn plant material is incorporated. Sampling should be conducted each year for three years in a field sown with continuous MIR604 corn. Soil should be monitored for a minimum of one growing season after harvest and monitoring should continue until mCry3A protein can no longer be detected. As noted in Table 16 below, the Agency has requested that the applicant submit this study as a condition of registration.

These recommendations are based in part on the August 27, 2002 SAP and public comments provided to EPA at that time. This Panel concluded that several different soils should be examined and monitored for a minimum of one growing season after corn harvest and that monitoring should continue until the Cry protein can no longer be detected. According to the Panel, at least three soil types should be evaluated for Cry protein persistence and these soils should be high in organic matter and clay, since the potential of persistence is highest in these soil types. The Panel also recommended that soil degradation studies may be conducted under less than optimum environmental conditions, such as high or low temperatures, since protein persistence may be affected by varying environmental conditions. The Panel further suggested that since corn roots grow into deep soil with reduced microbial activity, degradation rates in these zones should also be examined. With regards to protein source, the Panel recommended that studies should utilize plant material that is representative of actual field conditions. For example, soil samples should be collected from field sites where whole plant tissue has been incorporated into the field. And further, plant tissue should not be ground prior to incorporation, because grinding artificially increases the plant tissue surface area exposed to microorganisms, and increased surface area may lead to an increase in the rate of protein degradation. Finally, the SAP stated that "(r)eal life or true persistence (or Cry protein) is likely to be equal to or less than that measured with ELISA." So if an ELISA is conducted, results should be compared to a bioassay that uses a sensitive species such as the Colorado potato beetle.

3. Effects on Soil Microorganisms

2010 Update: Numerous published studies indicate that exposure to Cry protein produced in *Bt* PIP crop plants does not adversely affect soil microorganisms (Sanvido *et al*. 2007; Oliveira *et al*. 2008). In addition, *Bt* toxin released from root exudates and biomass of *Bt* corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil (Saxena and Stotzky 2001). Other research findings conclude no *Bt*-related risks have evolved from the decomposition of *Bt*-corn leaves for the meso- and macrofauna soil community (Hönemann *et al*. 2008). Although a minimal transient increase and shift in microbial populations may result from the presence of transgenic plant tissue in soil, no adverse effects have been attributed to the Cry protein.

In addition, there are several ongoing U.S. Department of Agriculture and EPA Office of Research and Development funded research projects evaluating the effects of *Bt* crops on soil microbial flora. If adverse effects are seen from this or any other research, the Agency will take appropriate action to mitigate potential risks.

With regard to the impact of genetically engineered crops on soil, it is important to note that agricultural practices themselves cause large changes in soil and soil microbial composition. Furthermore, factors such as variations in seasons and weather, plant growth stage, and plant varieties, independent of being genetically engineered, are also responsible for significant shifts in soil microbial communities. To date, most studies with genetically engineered crops have shown minor or no effects on soil microbes beyond the variation caused by the factors listed above.

4. Horizontal Transfer of Transgenes from *Bt* Crops to Soil Organisms

2010 Update: EPA has evaluated the potential for horizontal gene transfer (HGT) from *Bt* crops to soil organisms and has considered possible risk implications if such a transfer were to occur. Genes that have been engineered into *Bt* crops are mostly found in, or have their origin in, soil-inhabiting bacteria. Soil is also the habitat of anthrax, tetanus, and botulinum toxin-producing bacteria. Transfer of these genes and/or toxins to other microorganisms or plants has not been detected. Furthermore, several experiments (published in scientific journals), that were conducted to assess the likelihood of HGT, have been unable to detect gene transfer under typical environmental conditions. Horizontal gene transfer to soil organisms has only been detected with very promiscuous microbes under laboratory conditions designed to favor transfer.

As a result of these findings, which suggest that HGT is at most an artificial event, and the fact that the *Bt* toxins engineered into mCry3A corn are derived from soil-inhabiting bacteria, EPA has concluded that there is a low probability of risk from HGT of transgenes found in mCry3A-producing corn.

5. Gene Flow and Weediness Potential

Conclusions gathered from this review process are as follows:

- The potential for pollen-directed gene flow from corn to Eastern gamagrass is extremely remote (DeWald *et al*. 1999). This is evidenced by the difficulty with which *Tripsacum dactyloides* x *Z. mays* hybrids are produced in structured breeding programs. Additionally, the genus *Zea* does not represent any species considered as serious or pernicious weeds in the U.S., its possessions, and/or its territories (Holm *et al*. 1979). Any introgression of genes into this species as a result of cross fertilization with genetically modified corn is not expected to result in a species that is weedy or difficult to control. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the corn chromosomal complement in subsequent generations (DeWald, personal communication, 1999).

- Many of the *Zea* species loosely referred to as "teosintes" will produce viable offspring when crossed with *Zea mays* ssp. *mays*. However, none of these plants are known to harbor weedy characteristics and none of the native teosinte species, subspecies, or races are considered to be aggressive weeds in their native or introduced habitats (Schoper, personal communication, 1999). In fact, many are on the brink of extinction where they are indigenous and will be lost without human intervention (i.e., conservation measures). Further, none of the landraces or cultivated lines of *Z. mays* are considered to have weedy potential and are generally considered to be incapable of survival in the wild as a result of breeding practices (i.e., selection) during domestication of the crop.

The October 2000 Scientific Advisory Panel agreed that the potential for gene transfer between corn and any receptive plants within the U.S., its possessions, and/or its territories was of limited probability and nearly risk free. Based on these findings, the EPA has determined that there is no significant risk of gene capture and expression of the mCry3A protein by wild or weedy relatives of corn in the U.S., its possessions, or its territories.

2010 Update: Movement of transgenes from crop plants into weeds is a significant concern, due to uncertainty regarding the effect that a new pest resistance gene may have on plant populations in the wild. Under FIFRA, EPA has reviewed the potential for gene capture and expression of *Bt* endotoxins by wild or weedy relatives of corn, cotton, and potatoes in the U.S., its possessions, and/or its territories. To date, *Bt* plant-incorporated protectants have been registered for use in agronomic plant species that do not have a reasonable possibility of passing their traits to wild native plants. However, due to concern over the possibility that species related to corn (*Zea mays* ssp. *mays*), such as *Tripsacum* species and the teosintes, could be recipients of gene flow from genetically modified *Z. mays*, EPA conducted a thorough review of the scientific literature on what is known about the gene flow potential of *Z. mays* (U.S. EPA 2001).

6. Impacts on Endangered Species

The primary route of exposure to mCry3A protein in corn is through ingestion of corn tissue. There are no reports of threatened or endangered species feeding on corn plants, therefore such species would not be exposed to corn tissue containing Cry protein. Since mCry3A protein has not been shown to have toxicity effects on mammals, birds, plants, aquatic species, insects and other invertebrate species at the EEC, a "may affect" situation for endangered land and aquatic species is not anticipated. In addition, EPA does not expect that any threatened or endangered plant species will be affected by outcrossing to wild relatives or by competition with such entities. Hybrid corn does not exist in the wild, nor are there wild plants that can interbreed with corn in the United States.

Because of the selectivity of mCry3A protein for coleopteran species, endangered species concerns are mainly restricted to the order Coleoptera. Examination of an overlay map showing the county level distribution of the 16 endangered/threatened coleopteran species (currently listed by the U.S. Fish and Wildlife Service) relative to corn production counties in the United States clearly indicated that any potential concern regarding range overlap with corn production was mainly restricted to the American burying beetle (*Nicrophorus americanus*). The American burying beetle is the largest carrion beetle in North America and is only found in limited areas of Rhode Island and portions of the Great Plains, including Arkansas and Georgia. Adults are nocturnal and feed on carrion and sometimes prey on other arthropods. Larvae feed exclusively on buried carrion provided by their parents. The American burying beetle's habitat is variable and often includes deciduous forest, grassland and agricultural areas. Considering that both larvae and adult insects feed exclusively on carrion, it appears that even if American burying beetles did occur in proximity to *Bt* corn fields, there would be little chance of exposure to *Bt* protein due to their feeding habits. After careful review of available data, the EPA determined that exposure of American burying beetle to harmful levels of MIR604 corn tissue is not expected. Likewise, a review of the preferred habitats of other coleopteran species listed as endangered by the U.S. Fish and Wildlife Service indicated that exposure to harmful levels of mCry3A protein would not take place. The main reasons for the lack of exposure are geographical and habitat limitations. These species are located in non-corn production areas and/or their habitat does not encompass agricultural areas.

Likewise, other insect species in the orders Diptera, Hemiptera, Lepidoptera, Odonata and Orthoptera that are listed as endangered/threatened species are found in dune, meadow/prairie or open forest habitats and are not closely associated with row crop production, often times due to the specificity of the habitat of their host plants. The reviewed toxicological data shows the relative insensitivity of a range of insects in non-Coleopteran orders to the mCry3A proteins, indicating that MIR604 maize hybrids are not likely to have detrimental effects on non-Coleopteran insects included on the endangered/threatened species list.

Further, several of the federally listed insect species are aquatic and consequently, are unlikely to come in contact with MIR604 maize plant material. Many of the endangered and threatened beetles occur in cave or aquatic habitats. Since movement into water bodies of soil containing mCry3A is expected to

be negligible, pollen drift was considered the primary source of potential hazard to endangered aquatic Coleoptera. According to estimates based on published studies, if 100% of the pollen grains leaving a corn field were deposited in a 1 ha pond with 2 m depth and located ≥1 m from the edge of a corn field, <0.0001 µg mCry3A/mL of water would be expected. This is a few orders of magnitude below the toxic level to any insect.

2010 Update: Current ecological effects data and EPA reviews of mCry3A protein, as expressed in MIR 604 and in the stacked event MIR 604 x Bt11, expressing mCry3A x Cry1Ab, support the Agency's determination that adverse effects will not occur to nontarget organisms. Due to a demonstrated lack of toxicity and/or exposure, no effects from mCry3A protein, as expressed in MIR 604 and in the stacked events expressing Bt11 x mCry3A x Cry1Ab, are anticipated for any nontarget species, including federally-listed threatened and endangered ("listed") lepidopteran and coleopteran species and their designated critical habitats. EPA has also determined that there are no indirect effects on endangered and threatened plant species, such as impacts on lepidopteran pollinators that are important and/or essential to an endangered or threatened plant. The Agency is therefore upholding its determination that the registered uses of mCry3A protein as expressed in MIR 604 and in the stacked events expressing Bt11 x mCry3A x Cry1Ab will have "No Effect," direct or indirect, on endangered or threatened terrestrial or aquatic species as listed by the U.S. Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS).

B. Environmental Risk Assessment

The EPA uses a Maximum Hazard Dose Tiered system for biopesticide non-target wildlife hazard assessment. When no adverse effects at the maximum hazard dose are observed, the Agency concludes that there are no unreasonable adverse effects from the use of the pesticide.

At present, the Agency is aware of no identified significant adverse effects of mCry3A proteins on the abundance of non-target beneficial organisms in any population in the field environment, whether they are pest parasites, pest predators, or pollinators. Further, the EPA believes that cultivation of mCry3A corn may have fewer adverse impacts on non-target organisms than use of chemical pesticides for corn production, because under normal circumstances, mCry3A corn requires substantially fewer applications of chemical pesticides, compared to production of non-Bt corn. And fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers, in corn fields. In addition, no adverse effect on endangered and threatened species listed by the US Fish and Wildlife Service is expected from the proposed MIR604 CRW resistant corn registration (see Section A.II.6 above). Further, the EPA has determined that there is no significant risk of gene capture and expression of mCry3A protein by wild or weedy relatives of corn in the U.S., its possessions, or territories (see Section A.II.5 above), available data do not indicate that Cry proteins have any measurable adverse effect on microbial populations in the soil (see Section A.II.3 above), nor has horizontal transfer of genes from transgenic plants to soil bacteria been demonstrated (see Section A.II.4 above). In conclusion, this risk assessment

finds no hazard to the environment at the present time from cultivation of mCry3A protein expressing MIR604 corn for a time-limited registration.

C. Supplemental Studies Needed for Long Term MCry3A Non-Target Hazard Assessment

The Agency has sufficient information to believe that there is no risk from the proposed uses of mCry3A corn to non-target wildlife, aquatic, and soil organisms. However, in response to the August 2002 SAP recommendations, the Agency is requesting supplementary studies that will evaluate the persistence of mCry3A in the soil and the long range effects of cultivation of mCry3A on the invertebrate community structure in corn fields. This will facilitate identification of potential adverse effects which may result from long-term use of this product.

Table 16. Supplemental data requirements for Event MIR604 corn.

Testing Category	Type of Data
Ecosystem effects	Long range field studies should be conducted based on recommendations of the August, 2002 SAP.
Soil fate studies	Long range soil degradation field studies should be conducted. Studies should follow guidelines outlined by the August 2002 SAP, which are presented in summary form in the conclusion section of the soil fate review (see Section A.II.2 above).

Update September 2010. The soil fate studies have been waived, but the nontarget long range field studies data requirement is still outstanding.

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II. D. Insect Resistance Management

1. BACKGROUND

Syngenta submitted an amended insect resistance management strategy in support of their FIFRA Section 3 Registration for Event MIR604-derived transgenic maize that expresses modified Cry3A (mCry3A) protein (MRID# 465296-01, McCaffery et al., 2005). Modified Cry3A in MIR604 is expressed at a non-high dose against infestation by the Western corn rootworm (WCRW; *Diabrotica virgifera virgifera* Le Conte) and the Northern corn rootworm (NCRW; *D. longicornis barberi* Smith and Lawrence). A behavioral avoidance mechanism is also present. BPPD reviewed Syngenta's previously submitted IRM strategy and noted several deficiencies in the simulation modeling and evaluation of cross-resistance potential (BPPD, 2005). These deficiencies were addressed in the amended IRM strategy submission (McCaffery et al., 2005, MRID# 465296-01) and are discussed in this chapter.

2. SUMMARY OF SYNGENTA'S SUBMISSION (2004 - 2005)

Syngenta's submission is divided into three main parts: 1) Sustainable deployment of Event MIR604 maize through effective insect resistance management (IRM), 2) IRM plan for MIR 604, and 3) Justification for the proposed IRM plan for MIR604. The first two sections of the revised Syngenta's submission were originally submitted by Syngenta (McCaffery and Stein, 2004) and reviewed by BPPD (BPPD, 2005). There were significant deficiencies identified in the IRM modeling and evaluation of cross-resistance potential. Syngenta has addressed these deficiencies in the section entitled, "Justification for the proposed IRM plan for MIR604."

Syngenta's IRM plan for MIR604 maize contains the following elements:

1. 20% structured refuge and its deployment and management;
2. IRM as a component of an integrated pest management program; and
3. Product stewardship program.

The product stewardship program consists of the following aspects:

1. Grower education plans;
2. Grower obligations;
3. Grower compliance;
4. Annual resistance monitoring;
5. Action plan in the event of unexpected levels of CRW damage;
6. Remedial action plans; and
7. Annual IRM plan review.

Syngenta has provided the following areas of support for its proposed IRM plan for MIR604 maize:

1. Dose of mCry3A expressed in MIR604 (root expression assays, laboratory feeding assays using excised roots, field efficacy, and adult emergence studies);
2. Simulation models for development of resistance;
3. Impact on MIR604 maize on biology of CRW;
4. Cross-resistance potential.

3. BPPD REVIEW (BPPD, 2005; BPPD, 2006a)

a. Nature and Derivation of Syngenta's MIR604 CRW-Control Maize

A *cry3A* gene from *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) (Sekar *et al.,* 1987) was synthesized by Syngenta scientists for optimal expression in maize. The gene was then modified (hereinafter referred to as "mCry3A" or "modified Cry3A") to enhance activity against the *Diabrotica virgifera virgifera* (Western corn rootworm, WCRW) and *Diabrotica longicornis barberi* Smith and Lawrence (Northern corn rootworm, NCRW). The introduction of a particular serine protease recognition site (both chymotrypsin and cathepsin G are serine proteases) into the native Cry3A results in a much more rapid and complete processing of the 67 kDa protein to 55 kDa and thus increased activity against WCRW and NCRW. After binding to the receptor, the mCry3A protein inserts rapidly and irreversibly into the plasma membrane of the cell to form ion permeable pores in a similar fashion as other Cry toxins (Van Rie *et al.,* 1989; Garcia-Alonso and Vlachos, 2003). These ion channels cause a loss in membrane potential and the formation of these lesions leads to death resulting from septicaemia and/or starvation (Schnepf *et al.*, 1998).

b. Corn Rootworm Species Controlled

Native Cry3A is primarily active against Colorado potato beetle (*Leptinotarsa decemlineata*) and has minimal activity against NCRW, both members of the Chrysomelidae family of beetles. The mCry3A protein has a similar spectrum of activity to the native Cry3A, but with enhanced toxicity to NCRW and WCRW, both major coleopteran pests of maize in the USA. Modified Cry3A has some activity against *Diabrotica balteata* (Banded cucumber beetle). Trials were conducted in 2005 (data not available for this review) to determine the level of control of *Diabrotica virgifera zea* (Mexican corn rootworm; MCRW) by mCry3A expressing maize plants (Event MIR604). Modified Cry3A has no activity against non-coleopteran pests including various lepidopteran pests of maize. MIR604 maize is most effective against larvae, especially the first instar. Syngenta's IRM strategy focuses entirely on rootworms.

c. Pest Biology

A clear understanding of pest biology and ecology is essential to the development of a sound IRM plan. MIR604 Bt corn was developed to control two primary corn rootworm species: western corn

rootworm (*Diabrotica virgifera virgifera* LeConte, WCRW) and northern corn rootworm (*D. Barberi* Smith & Lawrence, NCRW). WCRW is the most prevalent target pest in the United States and throughout most of the Corn Belt[1]. NCRW, also found throughout the Corn Belt, is considered the second most prevalent rootworm pest in the United States, and is the primary target pest of the north-central region[2].

Key factors believed to influence CRW adaptation to MIR604 corn include distribution, univoltinism, adult dispersal among fields, adult dispersal within fields, larval dispersal across rows, larval mortality due to density-dependent processes, insecticide use, egg mortality, fecundity, and adult and larval population density. Syngenta has provided a sufficient summary of the biology and ecology of the corn rootworm target pest(s) both in the current IRM strategy submission (McCaffery et al., 2005, MRID# 465296-01) and the original IRM strategy submission (McCaffery and Stein, 2004, MRID# 462656-17). See BPPD (2005) for the review of Syngenta's original IRM strategy submission and pest biology information.

d. IRM Plan for MIR604

Syngenta's IRM plan for MIR604 maize contains three major components: 1) a 20% structured refuge; 2) IRM is part of an integrated pest management program; and 3) product stewardship. Each of these components will be discussed below.

1) 20% structured refuge and its deployment and management

There are two ways a grower can implement the refuge requirement: a non-Bt corn refuge can be planted as a continuous block adjacent to the MIR604 maize fields or as non-transgenic strips planted within transgenic field. Considering the limited movement of CRW larvae, planting refuges close to transgenic fields in large blocks is preferred to narrow strips (Gray 1999, Meinke et al. 2001). Syngenta will require growers to plant a minimum structured refuge of non-corn rootworm-control (CRW) maize on at least 20% of their maize acres. Refuge fields must not be planted with other transgenic corn used to control rootworm because neither acts as a refuge for the other. Use of a 20% refuge with MIR604 maize complements other technologies and provides a degree of uniformity for CRW control as well as with products with both CRW and lepidopteran control. This uniformity provides growers with a straightforward and understandable message about refuge requirements and promotes compliance. Justification of the 20% refuge requirement is discussed later in this review.

[1] WCRW is the primary rootworm pest in Colorado, Kansas, Nebraska, Ohio, Indiana, Illinois, Iowa, Missouri, and Michigan.

[2] NCRW is the primary rootworm pest in Wisconsin, Minnesota, South Dakota and northern Iowa.

Syngenta will require growers to plant the structured refuge as blocks, strips, perimeter borders or pivot corners. Encouragement will be given to growers to plant these non-CRW control corn acres within their CRW-control acres. Syngenta has proposed that in-field strips be at least 6 to 12 non-rootworm protected Bt corn rows, although this row width size may be larger than necessary based on the current understanding of rootworm biology. BPPD has reviewed larval movement data published by Hibbard et al. (2003). This study indicated that between 0.75% and 6% of larvae moved across corn rows. These results represent a relatively high-end estimate of the number of larvae that cross rows. This means that much narrower in-field strips should be sufficient to provide adequate protection from sub-lethal selection caused by CRW larval movement across rows and maintain low functional recessiveness. Any increase in sublethal selection would be offset by a greater probability that potentially resistant adults emerging from the Bt corn rows would be mated by susceptible adults from the refuge row. Single-row strips would likely be too narrow and allow too much larval movement across rows to sufficiently maintain low functional recessiveness. Therefore, seed mixes would not be a good refuge strategy for rootworms. In-field strips of ≥4 rows would provide the advantage of being more compatible with the current in-field strip width requirement for lepidopteran-protected Bt corn hybrids (≥4 row strips, with ≥6 row strips preferred). In-field strips of ≥4 rows would also be more practical and flexible for the grower because of the compatibility with split-planter designs. Because mCry3A will be stacked with Cry1Ab (submission pending), a recommendation of ≥4 row strips will provide the grower a more easily understandable and consistent message regarding the width of in-field strips. Overall, BPPD's recommendation is that Syngenta require growers to plant in-field strip refuges with widths of ≥4 rows (≥6 rows preferred) for rootworm-protected Bt corn hybrids. This will simplify refuge deployment and potentially increase grower compliance with refuge requirements. Use of an in-field strip refuge is not intended for fields planted to increase inbred seed since these fields need to be isolated from external corn pollen sources. An in-field or adjacent non-Bt corn refuge would be inconsistent with inbred seed production practices.

Management of the structured refuge must conform to the rotational and management practices to achieve synchronous CRW development in both refuge and MIR604 maize fields. Growers will have the option of treating the refuge with conventional insecticides (seed treatments, soil applications and foliar applications) to control severe damage by CRW and to control secondary pests. These treatments will impact the overall number of beetles that emerge from the refuge fields even though their efficacy is less than 100%. Syngenta's modeling study examines one scenario in which a nominal 50% of refuge insects are removed by insecticide control that impacted the potential development of resistance (see modeling section below). Syngenta indicates that there is no evidence for any cross-resistance, synergy, or antagonism between the mCry3A in MIR604 maize and Cry1Ab and other Bt toxins in Lepidoptera-control maize varieties. There are also no known interactions between mCry3A and insecticides.

Syngenta notes that the use of continuous corn acres for refuge fields is always to be encouraged. It is also permissible for growers to plant the refuge acres on first-year corn acres, but only where the CRW-control corn is also planted on first-year corn acres. This distinction is made for areas in which WCRW has adapted to crop rotation and sizable populations of these insects will emerge from first-

year corn. There is also a variant of NCRW in which there is extended diapause. Both rotation-resistant CRW variants impact the efficacy of crop rotation.

2) IRM is Part of an Integrated Program

Syngenta stressed that the IRM strategy for MIR604 maize is part of an overall package of integrated crop management techniques. These tactics include:

1. Crop rotation. Growers will be encouraged to maintain crop rotation as a vital part of CRW management. The presence of rotation-resistant phenotypes, however, may render such an approach ineffective in some areas.
2. Refuge quality. Growers will be educated on management practices to maximize the effectiveness of their refuges.
3. Insecticide use. When moderate to high CRW population pressures in the refuge, growers will be encouraged to treat the refuge with a soil insecticide or use seed treatments in a manner to maintain overall survival of CRW to function effectively as refuge mating partners.

3) Product Stewardship

Growers are crucial to the success of any IRM plan because they are responsible its implementation on the practical level. Syngenta has developed a product stewardship program that stresses the importance of implementation of the IRM plan for MIR604 maize. Syngenta's product stewardship program consists of the following aspects:

1. Grower education plans (e.g., Syngenta guide);
2. Grower obligations (grower agreements);
3. Grower compliance;
4. Annual resistance monitoring;
5. Action plan in the event of unexpected levels of CRW damage;
6. Remedial action plans; and
7. Annual IRM plan review.

Compliance programs are important in that they encourage growers to comply with IRM requirements, while providing mechanisms by which registrants can be held accountable for noncompliant growers. The compliance program presented in this submission mirrors those developed for existing lepidopteran-protected Bt maize registrations. The specific compliance assurance program components are: 1) Annual IRM Survey, 2) Mechanism for Handling Tips and Complaints, 3) On-Farm Visits, and 4) Phased Compliance Approach. Syngenta will respond to instances of non-compliance through the Phased Compliance Approach and will also use grower agreements and an annual affirmation scheme to reinforce grower understanding and compliance. Grower education programs give growers a clear understanding of the importance of IRM and its implementation. The IRM program will be communicated to growers by Syngenta through its authorized sales agents using both print and other media including workshops, educational pamphlets/brochures, and an assortment

of public relation activities. BPPD finds Syngenta's compliance assurance program to be "acceptable" with the following caveats. The EPA-approved changes (granted on June 16, 2006) to the annual IRM reporting requirements and the Compliance Assurance Program for the lepidopteran-protected Bt maize registrations should be incorporated into Syngenta's IRM Compliance Assurance Program for MIR604 Corn Products.

The need for proactive resistance detection and monitoring is critical to the survival of Bt technology. Consequently, the Agency mandates that a resistance monitoring plan must be implemented for all registered Bt maize products. Resistance can evolve regionally or as a local increase in resistance (*r*-) allele frequency. The resistance monitoring plan designed for MIR604 maize is an adaptation of the ABSTC program developed for lepidopteran-protected Bt maize. This program will attempt to detect either local or regional resistance early enough to initiate effective remedial action. As for all other Bt maize products, Syngenta's resistance monitoring program will be implemented through a two-pronged approach, including field reports of unexpected damage and population testing and sampling. The initial emphasis of their monitoring plan will be on establishing the baseline susceptibility for WCRW. Growers will be asked to report to Syngenta any unexpected field damage. Diagnostic bioassays of larvae from eggs laid by field-collected WCRW adults will be used to survey susceptibility of the insects to mCry3A. The monitoring efforts will focus on regions of the Corn Belt where WCRW populations are known to regularly reach high numbers and where the highest MIR604 adoption is expected. Detecting shifts in the frequency of resistance genes (i.e., susceptibility changes) through resistance monitoring can be an aggressive method for detecting the onset of resistance prior to widespread crop failure. As such, the utilization of sensitive and effective resistance monitoring techniques is critical to the success of an IRM plan. BPPD finds that Syngenta's basic resistance monitoring plan and remedial action plan for the mCry3A protein expressed in MIR604 maize are "acceptable," although certain issues need to be addressed. These are discussed below.

1. The monitoring plan only focuses on WCRW. CRW species are challenging to rear in the laboratory. Of the identified target pest species, scientists have had the most success rearing WCRW, and have had little success with NCRW. Consequently, BPPD agrees with Syngenta that initial monitoring through population sampling will focus on WCRW, but should include NCRW populations when available. No information exists on MCRW. These data should be provided to BPPD when they become available.
2. A diagnostic method cannot be developed until the dose-response relationships for WCRW to establish the baseline sensitivity to mCry3A. While BPPD agrees that a sensitive and reliable bioassay should be developed, these efforts are currently under development. Once this work is completed, the registrants must develop guidelines as to what level of root damage will be expected under various conditions, and what level of rootworm control is normally achieved. Growers will have to report any "unexpected damage." Without such guidelines, i.e., what is "acceptable" rootworm damage, then it will not be possible to determine what the "unexpected" rootworm damage is. Once these guidelines are established it will be possible to define what is "suspected resistance" as described under the "Remedial Action Plans" to mitigate the spread of putative resistant populations. Because of the importance of these guidelines, it should be

required that the registrant develops an interim rootworm damage guidelines by 2008 and final guidelines by 2010 and submits these to the Agency for review.

It is recommended that MIR604 maize be given the following resistance monitoring requirements:

1. The registrants should monitor for resistance and/or trends in increased tolerance for corn rootworm. Sampling should be focused in those areas in which there is the highest risk of resistance development. The registrants should submit to EPA an appropriate sampling protocol as part of its monitoring plan.
2. The registrants should provide EPA a description of its resistance monitoring plan by January 31, 2007. The description would include: sampling (number of locations and samples per locations), sampling methodology, bioassay methodology, standardization procedures (including QA/AC provisions), detection technique and sensitivity, and the statistical analysis of the probability of detecting resistance. A final resistance monitoring plan is required by January 31, 2008.
3. The registrants should develop an appropriate discriminating or diagnostic dose assay by January 31, 2008.
4. The registrants should follow-up on grower, extension specialist or consultant reports of unexpected damage or control failures for corn rootworm.
5. The registrants should provide EPA with an annual resistance monitoring report.

The remedial action plan is designed as a tiered approach for mitigating potential WCRW, NCRW, and MCRW resistance development to the Cry34/35Ab1 protein. BPPD agrees with the general framework for the Remedial Action Plan; however, because the "baseline sensitivity" has not been calibrated, this plan cannot be implemented. The submission states that mitigation measures will be initiated when unexpected levels of CRW damage occur. But, levels of "expected" damage cannot be identified until baseline sensitivity is determined (see discussion above "Resistance monitoring"). Consequently, it should be required that baseline sensitivity be established within two years of product commercialization, so that expected levels of crop damage and target pest resistance can be established, and a remedial action plan initiated when needed.

The following program summary describes, in order of events, the steps that should be taken to implement a remedial action plan if resistance to target pests is confirmed (this general process has been implemented for other lepidopteran and CRW Bt corn products).

1. **Definition of Suspected Resistance:** Resistance will be suspected if investigations of unexpected damage reports show that:

a. Implicated maize plant roots were expressing the mCry3A protein at the expected level;
b. Alternative causes of damage or lodging, such as non-target pest insect species, weather, physical damage, larval movement from alternate hosts, planting errors, and other reasonable causes for the observations, have been ruled out;
c. The level of damage exceeds guidelines for expected damage.

If resistance is "suspected", the registrants will instruct affected growers to use alternate pest control measures such as adulticide treatment, crop rotation the following year, or use of soil or seed insecticides the following year. These measures are intended to reduce the possibility of potentially resistant insects contributing to the following year's pest population.

2. **Confirmation of Resistance**: Resistance will be confirmed if all of the following criteria are met by progeny from the target pest species sampled from the area of "suspected resistance":

a. The proportion of larvae that can feed and survive on mCry3A roots from neonate to adult is significantly higher than the baseline proportion (currently being established);
b. The LC_{50} of the test population exceeds the upper limit of the 95% confidence interval for the LC_{50} of a standard unselected population and/or survival in the diagnostic assay is significantly greater than that of a standard unselected population, as established by the ongoing baseline monitoring program;
c. The ability to survive is heritable;
d. mCry3A plant assays determine that damage caused by surviving insects would exceed economic thresholds;
e. The identified frequency of field resistance could lead to widespread product failure if subsequent collections in the affected field area(s) demonstrated similar bioassay results.

3. **Response to Confirmed Resistance:** When resistance is "confirmed", the following steps will be taken:

a. EPA will receive notification within 30 days of resistance confirmation;
b. Affected customers and extension agents will be notified about confirmed resistance;
c. Affected customers and extension agents will be encouraged to employ alternative CRW control measures;
d. Sale and distribution of mCry3A maize in the affected area will cease immediately;
e. A long-term resistance management action plan will be devised according to the characteristics of the resistance event and local agronomic needs. [The details of such a plan should be approved by EPA and all appropriate stakeholders. This process may take longer than 30 days following confirmation of resistance.]

e. Support for Syngenta's IRM Plan for MIR604 Maize

1) Dose

Identifying the level of dose, as related to selection intensity, is crucial when determining size and structure of a refuge needed to delay CRW resistance to MIR604 maize. Syngenta conducted a series of field efficacy trials across the Corn Belt in 2003 and 2004 to assess dose. This information was used in the modeling study detailed later in this review. Additional data were obtained from root expression assays and *in vitro* feeding assays in the laboratory. Based on the review of Syngenta's data, it can be concluded that MIR604 maize does not provide a high dose for CRW control.

a) Root expression assays

Levels of expression in leaves, roots, and whole plants were determined at two developmental stages for two MIR604 hybrids. Levels of expression were numerically higher in leaves than in roots at both the whorl stage and at anthesis and somewhat higher in roots at the whorl stage than at anthesis, but there were few, if any statistically significant differences at $p<0.05$. Root expression was found to be uniform throughout the root architecture implying that there are no microhabitats. Therefore, larvae feeding on MIR604 roots should be uniformly exposed to the mCry3A toxin. Average values of mCry3A in roots range from 1.8 to 3.0 µg mCry3A/g fresh weight.

b) WCRW laboratory feeding assays

In the first laboratory feeding study, nine replicate groups of ten larvae were placed in plastic dishes containing 3 cm root sections excised from MIR604 hybrids. Five of nine replicates exhibited only minimal root damage (≤ 1 feeding hole) with an average of 22% mortality; while, three of nine replicates exhibited two to three feeding holes with an average of 13.3% mortality. Only one replicate had more than four or more feeding holes.

In the second laboratory feeding study, nine replicate groups of ten larvae were placed in plastic dishes were given a choice of two 3 cm root sections excised from MIR604 and non-transgenic (control) hybrids. In this second experiment, larvae preferred the non-transgenic hybrid to the MIR604 hybrid. Approximately 75% of non-transgenic hybrid roots were heavily damaged with more than four feeding holes per root section. As in the first experiment, the mortality levels on transgenic root sections were low, only 17%. A third experiment, using a similar choice protocol, produced similar results to the second experiment, but there was considerably more variation.

Following investigations by Syngenta and its co-operators, there is evidence for both a direct mortality mechanism of first instar CRW larvae as well as evidence that MIR604 hybrids have properties that might deter feeding. Thus, in terms of root protection, MIR604 plants exhibit a much higher dose than would otherwise be assumed from the consideration of the mortality data alone. The behavioral deterrence mechanism needs to be studied further by Syngenta.

c) Field efficacy

In 2003 and 2004 Syngenta conducted field-based studies of MIR604's efficacy against WCRW and NCRW and study results were used to develop models of MIR604. Trials included naturally and artificially infested sites and study endpoints were survival until adult stage, timing of adult emergence, sex ratio, and adult weight. Both artificial and natural infestation trials were conducted. A brief summary of the results is provided below.

Efficacy trials compared root damage among MIR604 and non-MIR604-derived corn hybrids. Experimental plots were artificially infested (400 to 1450 eggs/trial), located in fields with naturally occurring populations of corn rootworm, or both approaches to infestation were combined. When artificially infestation was used, at the V2 to V3 leaf stage rootworm eggs were mechanically placed 3 inches deep in soil, within 2 to 3 inches of the corn stalk base.

i. Artificial Infestation with WCRW:
The percentage of artificially infested CRW eggs known to survive to the larval stage is low, and even lower for adult survival. Consequently, at each trial location the ratio of viable eggs to larvae was assessed, and comparative estimates of mortality made.

Results indicate that MIR604 provided better control of WCRW than untreated non-transgenic controls. Emergence data from 2003 show that MIR604 controlled an average of 89.9% of CRW, with a low of 74% at Walcott, IA and a high of 97% at Bloomington, IL (Table 1). On an emergence per hectare basis, non-transgenic control plots averaged 740,410 beetles per hectare, while MIR604 plots averaged 72,019 beetles.

Table 1. Summary of artificial infestation trials that evaluate control of WCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2003 Walcott IA	2003 Stanton MN	2003 Bloomington IL	2003 Stanton MN	Artificial infestation totals	Artificial infestation averages
Plants/ha	61,507	61,507	61,507	61,507		61,507
Viable eggs/plant	350	428	405	224		352
Initial density eggs/ha	21.6 M	26.4 M	25.0 M	13.8 M		21.7 M
Emergence in MIR604 total (F+M)	38	48	14	18	118	30
Emergence in negative control total (F+M)	145	314	406	299	1164	291
Area evaluated per entry (ha)	0.00047904	0.00038323	0.00038323	0.00038323	0.0016287	0.00040718
MIR604 total (F+M)/ha	79,325	125,250	36,531	46,969	288,075	72,019
Negative cont. total (F+M)/ha	302,688	819,344	1,059,407	780,203	2,961,642	740,410
% control	73.8%	84.7%	96.6%	94.0%	90.3%	90.3%

ii. Natural Infestation with WCRW:

Field trials using natural infestation were implemented at six locations in 2004. Three of these locations (Clay Center, NE, Ames, IA, and Higginsville, MO) were lightly infested – characterized by few beetles and minimal plant root damage. The remaining three locations (Bloomington, IL, Urbana, IL, and Mead, NE) had a high level of infestation – characterized by high beetle population densities and a high level of plant root damage.

Percent control was determined by comparing emergence in MIR604 plots against the non-transgenic control. This method assumed that the natural infestation of WCRW was evenly distributed across treatments (an unlikely scenario), although the procedure may provide a rough estimate of the efficacy of MIR604. At the lightly infested locations, MIR604 provided greater control of WCRW than non-transgenic controls, with a mean of 92.2% beetle control compared to the controls (Table 2). At heavily infested locations MIR604 provided an average of 11.1% beetle control, with a low of -13.5% and a high of 44.5%, compared to non-transgenic control treatments (Table 3).

Table 2. Summary of light pressure natural infestation trials that evaluate control of WCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2004 Clay Center NE	2004 Ames IA	2004 Higginsville MO	Light pressure natural infestation totals	Light pressure natural infestation averages
Plants/ha	61,507	61,507	61,507		61,507
Emergence in MIR604 total (F+M)	23	35	16	74	25
Emergence in negative control total (F+M)	263	276	412	951	317
Area evaluated per entry (ha)	0.00038323	0.00038323	0.00038323	0.0011497	0.00038323
MIR604 total (F+M)/ha	60,016	91,328	41,750	193,094	64,365
Negative cont. total (F+M)/ha	686,266	720,188	1,075,063	2,481,517	827,172
% control	91.3%	87.3%	96.1%	92.2%	92.2%

Table 3. Summary of heavy pressure natural infestation trials that evaluate control of WCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2004 Bloomington IL	2004 Urbana IL	2004 Mead NE	Heavy pressure natural infestation totals	Heavy pressure natural infestation averages
Plants/ha	61,507	61,507	61,507		61,507
Emergence in MIR604 Total (F+M)	126	386	345	857	286
Emergence in negative control total (F+M)	227	340	397	964	321
Area evaluated per entry (ha)	0.00038323	0.00038323	0.00038323	0.0011497	0.00038323
MIR604 total (F+M)/ha	328,781	1,007,219	900,235	2,236,235	745,412
Negative cont. total (F+M)/ha	592,328	887,188	1,035,922	2,515,438	838,479
% control	44.5%	-13.5%	13.1%		11.1%

iii. Natural Infestation with NCRW:

High natural infestations of NCRW were seen at one location in 2003 and six locations in 2004. Infestations were light at Stanton, MN, Cavour, SD, Bloomington, IL, and Mead, NE, while pressure was heavier in Walcott, IA, Ames, IA, and Higginsville, MO. Percent control was determined by comparing emergence in MIR604 plots against the non-transgenic control. This method assumed that the natural infestation of NCRW was evenly distributed across treatments (an unlikely scenario), although the procedure may provide a rough estimate of the efficacy of MIR604. At the Ames and Higginsville locations NCRW emergence reached one third of total CRW adult emergence. For these locations, control of NCRW ranged from 45 to 95%. Control ranged from 34.7 to 100% in areas with lower NCRW pressure (Table 4).

Table 4. Summary of natural infestation trials that evaluate control of NCRW by MIR604 corn. Product efficacy is estimated by the reduction in adult emergence from MIR604 plants relative to untreated, non-transgenic control plants.

Location	2003 Walcott IA	2004 Stanton MN	2004 Cavour SD	2004 Bloomington IL	2004 Ames IA	2004 Higginsville MO	2004 Mead NE
Plants/ha	61,507	61,507	61,507	61,507	61,507	61,507	127,859
Emergence in MIR604 Total (F+M)	28	2	0	5	63	10	32
Emergence in negative control total (F+M)	73	22	53	12	115	218	49
Area evaluated per entry (ha)	0.00047904	0.00038323	0.00038323	0.00038323	.00038323	0.00038323	0.00038323
MIR604 total (F+M)/ha	58,450	5,219	0	13,047	164,391	26,094	83,500
Negative cont. total (F+M)/ha	152,388	57,406	138,297	31,313	300,078	568,844	127,859
% control	61.6%	90.9%	100.0%	58.3%	45.2%	95.4%	34.7%

d) Adult emergence

To make realistic judgments regarding the likelihood that a resistant CRW beetle will pass on resistance alleles to the next generation, adult fitness and timing of emergence must be understood. Both the fitness of the surviving beetles and the timing of their emergence from the crops have a major impact on their ability to mate with other WCRW adults that will have an impact on their ability to contribute genetically to the subsequent generation. Based on all MIR604 replicate treatments, there was a delay in the emergence of adults from the crop when compared to the untreated negative isoline control treatments.

Over all locations, a mean delay of 7.7 days was seen for adult emergence in all MIR604 treatments when compared to non-transgenic controls. No discernable differences were seen between treatments with light or heavy pressure and between male and female beetles.

i) Artificial infestation with WCRW:

In trials that used artificial infestation, delays of 6 to 10 days compared to non-transgenic controls were commonly seen among male and female beetles (Table 5).

Table 5. Emergence delay times (days) for adult male and female WCRW in emerging from artificially infested MIR604 corn fields in 2003 and 2004.

Location	2003 Walcott IA	2003 Stanton MN	2003 Bloomington IL	2004 Stanton MN	Artificial infestation averages
Emergence parameter	Emergence (days)				
5% male emergence	6.1	4.2	0.1	18.4	7.2
50% male emergence	4.6	9.8	0.9	11.0	6.6
80% male emergence	2.6	14.3	1.6	10.2	7.1
5% female emergence	14.8	3.5	1.3	20.4	10.0
50% female emergence	6.9	8.4	-0.3	9.0	6.0
80% female emergence	5.0	8.1	6.6	8.5	7.0
Mean emergence delay for M+F	6.7	8.0	1.7	12.9	7.3

ii) Natural infestation with WCRW:
Emergence delays of over 10 days were seen among female CRW beetles in locations with light natural infestation (Table 6). Few males were identified in these plots, so emergence time could not be reliably measured. Emergence delays in locations with heavy natural infestation were shorter than delays seen in sites with light emergence (Table 7). This finding was heavily influenced by the Urbana IL results, which were atypical and for efficacy purposes, considered an outlier. Removing the Urbana results from the heavily infested data set brings the mean value for emergence to 6.5 days, which is more similar to results seen in the artificial infestation trial.

Overall, statistical analysis showed no significant difference in rates of emergence among sexes, locations or treatments. The most reliable data were collected from three trials in 2004 that recorded over 100 adults in emergence cages. For these sites, a mean delay of 5.8 days was calculated for males and females. Consequently, the modeling study (described in modeling section) uses a standard delay of 6 days for males and females emerging from MIR604 corn fields.

Table 6. Emergence delay times (days) for adult male and female WCRW in emerging from light pressure naturally infested MIR604 corn fields in 2003 and 2004.

Location	2004 Clay Center NE	2004 Ames IA	2004 Higginsville MO	Light pressure natural infestation averages
Emergence parameter	Emergence (days)			
5% male emergence	N/A*	N/A*	N/A*	N/A*
50% male emergence	N/A*	N/A*	N/A*	N/A*
80% male emergence	N/A*	N/A*	N/A*	N/A*
5% female emergence	8.6	12.3	11.5	10.8
50% female emergence	15.4	12.1	7.0	11.5
80% female emergence	19.4	12.5	5.8	12.6
Mean emergence delay for M+F	14.4	12.3	8.1	11.6

N/A* - < / = 3 beetles emerged from this entry at this location, and therefore data are excluded from delay calculations

Table 7. Emergence delay times (days) for adult male and female WCRW in emerging from heavy pressure naturally infested MIR604 corn fields in 2003 and 2004.

Location	2004 Bloomington IL	2004 Urbana IL	2004 Mead NE	Heavy pressure natural infestation averages
Emergence parameter	Emergence (days)			
5% male emergence	1.1	6.9	9.5	5.8
50% male emergence	3.4	4.8	5.0	4.4
80% male emergence	11.2	-3.2	5.9	4.6
5% female emergence	2.4	4.5	10.6	5.8
50% female emergence	7.0	-4.9	9.4	3.8
80% female emergence	8.7	-6.5	4.1	2.1
Mean emergence delay for M+F	5.6	0.3	7.4	4.4

iii) Artificial infestation with NCRW:

In 2003 and 2004 data on NCRW emergence was collected from four locations. Although there was some variability among locations, emergence delays ranged from 5.5 to 11 days. As noted with WCRW emergence data, beetle sex was not shown to affect rate of emergence.

Table 8. Emergence delay times (days) for adult male and female NCRW in emerging from naturally infested MIR604 corn fields in 2003 and 2004.

Location	2003 Walcott IA	2004 Ames IA	2004 Higginsville MO	2004 Mead NE	NCRW natural infestation averages
Emergence parameter	Emergence (days)				
5% male emergence	7.5	0.0	N/A*	12.9	6.8
50% male emergence	2.9	6.1	N/A*	9.5	6.2
80% male emergence	1.4	3.9	N/A*	19.6	8.3
5% female emergence	18.4	6.4	13.1	14.4	13.1
50% female emergence	9.7	10.5	5.6	6.3	8.0
80% female emergence	6.3	6.1	-0.5	3.2	3.8
Mean emergence delay for M+F	7.7	5.5	6.1	11.0	7.6

N/A* - < / = 3 beetles emerged from this entry at this location, and therefore data are excluded from delay calculations

iv) Adult body weight

The body weights of adult WCRW beetles were recorded at the artificially infested trial in Stanton, MN. Results show that the mean body weight of adult beetles emerging from MIR604 corn fields was significantly greater than the weight of those emerging from non-transgenic plots.

Table 9. Mean dry weight of adult WCRW emerging from artificial infestation field trial at Stanton, MN in 2003.

Treatment	% Control w.r.t. untreated control	Mean adult weight (g x 10-5)
MIR604-3-11	87.0	206.5 bc
MIR604-3-12	90.3	183.4 bc
Untreated, non-transgenic, control	0.0	155.4 c
Non-transgenic + FORCE 3G	85.9	222.8 ab

2) Simulation Models for Development of Resistance

Simulation modeling was used by Syngenta to predict the evolution of WCRW resistance to the mCry3A toxin expressed in MIR604 maize. Models have been useful tools in the development of IRM strategies. Previously, Monsanto modified a Caprio model for cotton bollworm (*Helicoverpa zea*) to predict the risk of CRW developing resistance to MON 863 corn (U.S. EPA, 2002). Andow and Alstad (2002), Onstad et al. (2001), and Onstad et al. (2003) used deterministic models to predict the evolution of WCRW resistance using a variety of management strategies. The Andow and Alstad model predicted that, under certain conditions, a 20% refuge would probably delay resistance for greater than 15 generations when the dose was low, but would be ineffective for resistance management of a high dose (U.S. EPA, 2003). In Onstad et al. (2001), the model showed that when resistance was dominant, the resistance allele frequency exceeded 3% within 2-5 years as refuge size ranged from 5 to 30% for all doses of toxin, but the resistance allele frequency never exceeded 3% when resistance was recessive. This model was further modified to examine the impact of landscape on WCRW resistance evolution (Onstad et al., 2003). Both of the Onstad models were further expanded to evaluate the risk of resistance by WCRW to both transgenic crops and crop rotation in areas with or without rotation-resistant phenotypes (Crowder et al., 2005). Storer (2003) developed a stochastic, spatially explicit model to simulate adaptation (resistance) of WCRW in much of the US Corn Belt. In this model, the relative rate of adaptation was affected by the refuge size and the manner in which the non-transgenic refuge maize was deployed. Specifically, the adaptation rate was lowest if the non-transgenic refuge maize was planted in the same fields each year (Storer, 2003). This model was further modified to predict the evolution of resistance to Cry34Ab1/Cry35Ab1 and considered the influence of rotation-resistant phenotypes (U.S. EPA, 2005).

3) Syngenta's Specific Modeling for MIR604 Maize

a) Methods

Syngenta worked with Dr. David Onstad of the University of Illinois to customize the Crowder et al. (2005) model using the value for efficacy (i.e., dose) generated by Syngenta (see section on "Dose" above). The model includes patches of crops without explicit spatial structure. Each patch had a basic spatial unit of a 100 ha farm and was in a homogeneous region consisting of similar farms. The typical model landscape had four crops and MIR604 transgenic maize planted to both continuous and rotated cornfields. The crops include continuous corn, rotated corn, rotated soybean, and extra vegetation. Six scenarios were studied. Scenario I consisted only of 100% continuous maize fields in the landscape with no rotation-resistant WCFW in this area. Scenarios II-V had landscapes with 5% extra vegetation, 10% continuous corn, 42.5% soybean, and 42.5% rotated corn based on the standard set in the work of Crowder et al. (2005). In these four scenarios the area was inhabited by rotation-resistant WCRW. Scenario VI was similar to scenario I, but in this case a soil insecticide was used in the refuges, giving 50% survival of refuge insects. In scenarios IV-V, transgenic corn was planted only in rotated maize fields. Crowder et al. (2005) demonstrated that planting transgenic maize only in continuous maize fields in these kinds of landscapes was an inferior strategy so this scenario was not evaluated. In areas with rotation-resistant WCRW (scenarios II-V), there were two kinds of landscapes

evaluated: one with an initial resistance allele frequency of 0.0001 of the rotation-resistance allele (Y) in the rootworm population (scenarios II and IV) and the second had an initial Y-allele frequency of 0.1 (scenarios III and V). In both cases, the expression of the rotation resistance allele was dominant. Previously, Crowder et al. (2005) and Crowder and Onstad (2005) demonstrated that the simulation of realistic evolution of rotation resistance required either additive or dominant expression.

Three refuge levels were evaluated: 10%, 20%, and 30%. The refuge fields and MIR604 fields were assumed to be planted together in either continuous maize or in rotated maize. The refuge was assumed to be planted in the MIR604 field or adjacent to it. If the refuge was continuous maize, the refuge had to remain in the same place every year; otherwise, the refuge in rotation maize fields could change location from year to year.

The time horizon used was 15 years after introduction of MIR604 transgenic maize. This model had a daily time step during July-September to simulate adult activity.

Resistance to MIR604 transgenic maize and rotation was modeled as dominant, recessive, or additive. Precise additive expression for resistance to transgenic maize was used. Resistance to crop rotation was modeled as dominant (Y allele).

The initial adult density was assumed to be 50,000/ha of maize for all types. The sex ratio of adults was 50:50. The initial allele frequency for resistance to MIR604 transgenic maize was 0.0001. The initial allele frequency for resistance to rotation was 0, 0.0001, or 0.1.

A seed insecticide treatment was not included in most scenarios except for Scenario VI, which included soil insecticide treatment in refuge in continuous corn. Larval survival was 50% before density-dependent survival.

The model used a range of MIR604 transgenic maize mCry3A doses derived from the adult emergence data provided by Syngenta (see discussion above) and calculated using two larval survival functions described by Onstad et al. (2001, 2003). These calculations are not shown in this review, but can be found in McCaffery et al. (2005, MRID# 465296-01). Homozygous resistant individuals (RR) were assumed to always have 100% survival to MIR604 maize. Heterozygous individuals with R dominant (sR) were assumed to always have a 100% survival to the transgenic crop. The survival of homozygous susceptible individuals (SS) and heterozygote individuals with R recessive (Sr) were assumed to have a survival of 0, 0.001, 0.05, and 0.20 to represent a theoretical high, practical high, medium, or low toxin dose, respectively.

The standard density-dependent survival of larvae per state is 0.21 X exp (-0.058 EGG), where EGG is the density of eggs in millions per ha. The maximum larval survival based on this function was 21%. It was assumed that density-dependent mortality occurred after mortality due to overwintering and toxin exposure. In a second approach, it was assumed that mortality is density-independent based on the field data collected by Hibbard et al. (2004); thus, 5% of larvae survived after overwintering and

toxin exposure. The Urbana, IL field data collected in 2004 was omitted from the analyses because it was considered an outlier. The data from 2003 and 2004 were combined. Assuming that density-dependent survival occurs, the overall mean proportion of survival by susceptibles to the mCry3A toxin in the nine trials was 0.093. For the five trials with natural infestations, the mean survival was 0.14. For the four trials with artificial infestations, the mean survival was 0.038. For density-dependent survival, a range of 0.038, 0.093, to 0.14 for the survival rate of susceptible neonates encountering MIR604 transgenic maize was simulated.

The mean larval survival based on the assumption of density-independent mortality after surviving toxin was calculated based on proportional differences between emergence of adults in control cages and treated cages. The overall mean survival by susceptibles to the toxin in the nine trials was 0.24. For the five trials with natural infestations, the mean survival was 0.34. For the five trials with natural infestations, the mean survival was 0.34. For the four trials with artificial infestations, the mean survival was 0.125. Larval WCRW survival on MIR604 maize was simulated as 0.125, 0.24, and 0.34.

Delays in the emergence of adults were determined from data supplied by Syngenta (see discussion on adult emergence above). In the modeling done by Crowder et al. (2005), there was no significant effect of early mortality and delays of 10 and 14 days. For all cases in 2003-2004 in which there were at least 38 adults emerging in the treatment cages, there observed delays ranged from no delay to a delay of almost 15 days. A mean delay of 5.8 days was calculated for both males and females for three situations in which over 100 adults emerged from the treatment cages (most reliable data). The model used a standard delay of 6 days for both susceptible males and females emerging in MIR604 transgenic maize fields. Susceptible geneotypes are SS when R is dominant and SS and rS when R is recessive. For the additive case, simulations were run with only SS having early mortality, and then other simulations were run with both SS and RS having early mortality.

Sensitivity analyses were conducted. In all sensitivity analyses a refuge size of 20% was simulated. In the sensitivity analysis all variables were set to standard conditions (unless otherwise noted) except for the function being tested. The effect of initial population size, effect of initial R allele frequencies (resistance to transgenic corn) and the effect of lower fecundity by susceptible adults in transgenic maize were also included in the sensitivity analyses. The sensitivity analysis also used a 12-day delay (in addition to the 6-day delay) to determine how the results were affected by a longer delay.

Table 10. Landscape Scenarios Modeled.

I. No rotation resistance (Y allele frequency = 0); only continuous maize
II. Rotated maize with Y allele frequency = 0.0001 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize in both rotated and continuous maize fields
III. Rotated maize with Y allele frequency = 0.1 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize in both rotated and continuous maize fields
IV. Rotated maize with Y allele frequency = 0.0001 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize only in rotated maize fields
V. Rotated maize with Y allele frequency = 0.1 5% extra vegetation, 10% continuous maize, 42.5% soybean, 42.5% rotated maize Transgenic MIR604 maize only in rotated maize fields
VI. No rotation resistance (Y allele frequency = 0); only continuous maize Annual soil insecticide use in refuge with larval survival of 50%

b) Results from Syngenta's Specific Modeling for MIR604 Maize

Syngenta's modeling results are found in Appendix 1 of their submission (McCaffery et al., 2005, MRID# 465296-01).

1. Recessive resistance allele. If resistance to transgenic MIR604 maize is recessive, then WCRW never became resistant within 15 years in all simulations for all scenarios.
2. Dominant resistance allele. For scenarios I-III, and VI, either when larval survival was density-dependent or density-independent, resistance to transgenic MIR604 maize evolved in less than 13 years with a 20% refuge (all three toxin scenarios). For scenarios IV and V, either when larval survival was density-dependent or density-independent, resistance to transgenic MIR604 maize did not evolve within the 15 years of the simulation. Resistance evolution using the 10% refuge was worse for scenarios I-III, and VI than using the 20% refuge. The 30% refuge delayed evolution of resistance several years beyond 15 in Scenario III, IV, V when larval survival was density-dependent and density-independent, but resistance still evolved in less than 15 years in Scenarios I, II, and VI.
3. Additive resistance expression. Two simulations representing two possible effects of additive expression on early mortality were run for each combination of toxin survival (3 combinations) and refuge level (3 levels) for a total of 18 simulations. Like the results for dominant resistance

expression, the results for scenarios I-II were much different from those for scenarios IV-V when expression was additive.

Density-Dependent. For Scenarios I and II, with density-dependent survival, resistance evolved in less than 15 years using both the 10% and 20% refuge options for all but one simulation that for additive expression case #1 in which the toxin survival was 0.14 using a 20% refuge. No resistance was predicted if a 30% refuge (with density-dependent survival) was used in any of the six landscape scenarios with additive expression (both case #1 and #2) during the 15 years of the simulation. No resistance evolved during the 15 years of the model simulations using scenarios IV and V (MIR604 maize was planted only in rotated maize fields and rotation-resistance existed).

Density –Independent. Resistance did not evolve during the 15 years of the simulation under scenarios I-V using a 20% and 30% refuge if there was density-independent survival except in additive expression case #2 when toxin survival was 0.125 for larvae in scenarios I-III. In these three simulations, resistance evolved in 13 years. A 10% refuge delayed resistance for at least 10 years in scenarios I-III. Resistance did evolve in less than 15 years using all refuge sizes and all toxin combinations with scenario VI (continuous maize treated with soil insecticide). In this case, the 20% refuge with an annual soil insecticide causing 50% mortality will effectively become a 10% refuge. No resistance evolved during the 15 years of the model simulations using scenarios IV and V.

c) Conclusions from Syngenta's Specific Modeling for MIR604 Maize

The sensitivity analysis indicated that changes in the initial allele frequency had the greatest effect on results. Results were insensitive to recessive expression in MIR604 maize. Simulations using scenario V were insensitive to changes in initial adult density, R-allele frequency, and emergence delay. For scenario I and dominant expression of resistance to transgenic MIR604 maize, the results were generally not sensitive to increases in initial pest density, lengthening of the early-mortality period, no reduction in fecundity of survivors in transgenic MIR604 maize fields. For scenario I and additive expression or dominant expression, results were sensitive to changes in initial allele frequency.

If rootworm resistance to the mCry3A toxin as expressed in MIR604 maize is recessive (this is thought to be the case), then the modeling study suggests that an IRM plan with a 20% refuge, as proposed by Syngenta, will be adequate for delaying the evolution of resistance for at least 15 years. If MIR604 maize is planted in areas with observable rotation-resistance in the WCRW population, then the simulations indicate that planting the transgenic maize only in rotated maize fields is a good IRM plan that will delay resistance evolution to the mCry3A toxin expressed in MIR604 maize by at least 15 years regardless of gene expression. This strategy will also counteract the rotation resistance in WCRW (see Crowder et al., 2005).

The most complicated cases are for areas without rotation-resistance or for framers who want to plant continuous corn. The simulations show that resistance to MIR604 maize may occur in 9-12 years with a 20% refuge, a toxin survival of 0.093 (the overall mean) and with dominant expression of resistance.

If expression is additive, the evolution of resistance is delayed a bit more than for dominant expression by 1-2 years. If the initial resistance allele frequency is higher than assumed then resistance can occur a few years earlier. If soil insecticides are used on an annual basis in the refuge, then a 20% refuge effectively becomes a 10% refuge when resistance expression is dominant or additive and resistance evolves more quickly.

4) Impact of MIR604 on Biology of CRW

In order to develop an effective IRM plan and appropriate deployment strategies for MIR604, Syngenta submitted information describing CRW biology, ecology, behavior and toxicology related to MIR604. This information is especially important for this product because some individuals survive after feeding on the event. Not only is it important to understand the relative fitness of adults that emerge after feeding on MIR604, it is also important to understand the behavior of surviving larvae. For the most part Syngenta submitted detailed information to address these issues. Syngenta considers this research ongoing, however, and BPPD anticipates additional information when it comes available.

Evidence and observations made by Syngenta show that corn rootworm males normally start to emerge before females and this emergence period generally continues for over a month (Hein at al., 1998; Elliot and Hein, 1991; Meinke, 1995). Experiments conducted by Syngenta in 2003 and 2004 indicate that WCRW adults emerging from MIR604 emerge an average of 6-7 days later than WCRW adults emerging from non-transgenic isoline corn. A similar delay in emergence was observed with NCRW. Syngenta found no evidence that there is a difference in the delay in emergence between males and females (although there was some variability and the emergence profiles did vary). This last observation is especially important because it implies that normal mating patterns are not likely to be disrupted by any shift in the sex ratios normally found in the field. Given that the emergence period of WCRW is over a month, the observed delays of 6-7 days for WCRW adults emerging from MIR604 plots should not impact their availability to mate with adults from refuge plots. Syngenta also investigated the impact of density-dependent and density-independent mortality with respect to delays in emergence. These factors were evaluated for their impact on the evolution of resistance in Syngenta's model (discussed above).

It is clear from Syngenta's submission that the development of surviving larvae is significantly delayed, which is reflected in the delay of emerging adults. Therefore, it is also necessary to establish that adults emerging from MIR604 are not physiologically compromised in a way that would prevent them from mating with refuge adults. Syngenta is currently working with collaborators to fully understand the relative fitness of adults that emerge from MIR604. Currently little is known about sub-lethal effects of toxicants on specific *Diabrotica* species. WCRW females mate soon after they emerge and need to feed for about two weeks before they can lay eggs (Hein and Tollefson, 1985; Hein et al., 1988). Observations have shown early emerging adults survived longer and were more fecund than later emerging adults, conferring reduced fitness to the later emerging results (Boetel and Fuller, 1997). In most cases, the fitness of insects that are exposed to sub-lethal doses of an insecticide is decreased and the number of offspring is reduced (Haynes, 1988). The reduced relative fitness of

insecticide-resistant genotypes is also common among insects (Crow, 1957), which has been documented for strains of insects resistant to synthetic insecticides (e.g., Ferrari and Geoghiou, 1981; Roush and Plapp, 1982) and strains resistant to *Bacillus thuringiensis* (Groeters et al., 1994; Alyokhin and Ferro, 1999). Syngenta's continued research regarding the biological impact of MIR604 on CRW adults is important.

It is evident from submitted information that the prevention of damage to MIR604 corn roots is not necessarily accompanied by high levels of larval mortality. Syngenta and collaborators conducted studies to better understand the interactions between CRW larvae and MIR604 roots and the means by which roots are protected. To investigate these interactions, both MIR604 and negative isoline plants were from seed and infested with newly hatched WCRW. At several intervals (1, 2, 3, 7 and 14 days) after infestation estimates of root weight, larva number, larval wet and dry weights and larval feeding activity were recorded. Results showed that the weight of MIR604 roots increased significantly after day 3 when compared with the control roots, showing that MIR604 roots were protected from WCRW damage. With regard to larval numbers, the numbers of WCRW larvae on both negative isoline and MIR604 roots declined after infestation, but on MIR604 this decline occurred more quickly and by day 7 no larvae were alive. This finding contrasts with the negative isoline roots where significant survival was observed. Larval feeding was recorded on each assessment day and significantly fewer larvae were observed on the MIR604 roots compared to the control. For example, on days 1 and 2 less than 10% of the larvae were present on MIR604 roots and none were feeding on day 3, whereas about 50% of larvae on the control roots were observed feeding at any one time. As expected, this difference in feeding resulted in significant differences in larval weight between the two groups. Both wet and dry weights of larvae feeding on MIR604 roots were significantly lower than those feeding on negative isoline roots. Possibly the most importing information from Syngenta's research were the behavioral findings. It was observed that the larvae feeding on MIR604 roots became sick and either died within the first day or survived until day 2 but did not continue to feed on root tissue. Larvae on the MIR604 roots appeared to have one of two behaviors: 1) feeding without inhibition followed by death a short time later, or 2) movement through the root zone, not feeding on the root tissue, but sampling root hairs and continuing to search for food. Applying these finding to a field setting, it is possible that larvae can survive to the 2nd instar by feeding on root hairs of MIR604 plants, taking in small amounts of root tissue, and supplementing their diet with surrounding grassy weeds and plant roots (Wilson and Hibbard, 2004; Clark and Hibbard, 2004). By surviving to the 2nd instar stage, which is less susceptible to mCry3A than the 1st instar stage, WCRW could re-establish on MIR604. Although a re-establishment of the pest is possible, it is documented that natural loss of WRCW between root penetration and adult emergence is high (Elliot et al., 1989).

5) Cross-Resistance

It is important to consider the impact of cross-resistance on the evolution of corn rootworm resistance to MIR604 maize. Cross-resistance occur when a pest becomes resistant to one Bt toxin, for example, and by virtue of this resistance, it confers resistance to another distinct Bt toxin. The degree to which this cross-resistance might occur depends largely on the mechanism of resistance characterizing the

original resistance and the degree to which the two (or more) toxins are independently compromised by those mechanisms. For example, Cry1Ac and Cry1F, two Bt Cry toxins that target Lepidoptera, have a least one midgut receptor in common and if target site modification is responsible for resistance then a degree of cross-resistance is probably (Granero et al., 1996; Ballester et al., 1999).

Syngenta discussed the potential for cross-resistance involving the mCry3A toxin in MIR604 maize in the IRM submission. To date, no receptor for Cry3 toxins has been definitely isolated or characterized. Recently, a cadherin-link protein has been identified in the midgut of WCRW (Siegfried et al., 2005).

Syngenta's event MIR604 CRW-control maize will be deployed into a landscape consisting of conventional corn and other CRW-control varieties. At present there are two commercially available CRW-control varieties that must be included in the evaluation of cross-resistance: Monsanto's YieldGard® Rootworm (MON863) maize that expresses the Cry3Bb1 toxin and Dow AgroSciences Herculex® RW maize that expresses the Cry34Ab1/Cry35Ab1 toxins. The IRM plan for MIR604 must therefore consider the likelihood and possible consequences of the evolution of resistance in CRW that confers cross-resistance to multiple transgenic varieties.

The mode of action of mCry3A expressed in MIR604 CRW-control maize is similar to that of all known Cry toxins (see discussion earlier in this review). The degree of sequence homology may influence the potential for cross-resistance, but this depends on the specificity of the mechanism of resistance that might arise. For Bt Cry toxins, only two modes of resistance have been observed: 1) altered detoxification by protease enzymes in the midgut lumen that cleave the toxin and 2) modification of the target site receptor on the brush border membrane of the midgut epithelium so that binding of the toxin is prevented or hindered (Ferré and Van Rie, 2002). The latter mechanism was observed in diamondback moth (*Plutella xylostella*) that were selected with formulated microbial Bt products that resulted in field resistance as described in Tabashnik et al. (1997). Other non-target site mechanisms of Bt resistance have also been described: reduced or impaired gut proteolytic activity in *Plodia interpunctella* (Indian meal moth) (Oppert et al., 1994, 1997; Herrero et al., 2001) and alternation of toxin processing or some other metabolic step in laboratory-selected strains of *Heliothis virescens* (tobacco budworm) (Jurat-Fuentes *et al.*, 2003). A non-target site mechanism of resistance would be detrimental to IRM strategies based around reduced selection resulting from stacked Cry toxin genes or mosaic plantings of varieties expressing different toxins. The degree to which such non-target site mechanisms of resistance might impact resistance to Cry toxins in CRW is currently unknown.

As described in Ballester et al. (1999), modifications of the target site binding protein resulting from selection are likely to confer a very specific spectrum of resistances to closely related Cry proteins that also have some affinity with the binding site in question. Between mCry3A and native Cry3Bb1 there is a 61.7% identity and 69.1% similarity in amino acid sequences. Taking into consideration the size difference between the native and modified Cry3A proteins (mCry3A starts at residue #48), the identity is 74.3% and similarity is 66.5%. This level of amino acid homology is not considered to be particularly high and such differences indicate that there may be a differential recognition of these two

proteins in the insect midgut. Galitsky et al. (2001) note that there are differences between Cry3A and Cry3Bb1 in certain oligomeric structures in domain II and domain III that affect pore formation in the midgut membrane, regulation of channel function, and specificity towards target pests. These differences may affect the behavior of each of these proteins and reduce the likelihood of target-site cross-resistance, although this is unknown.

The Cry34Ab1 and Cry35Ab1 toxins expressed in the Dow AgroScience's CRW product, Herculex® RW represent a new family of insecticidal crystal proteins (Ellis et al., 2002). Although the target site for these CRW-active proteins is completely unknown, it can be argued that cross-resistance at the target-site between them and the Cry3 toxins is extremely unlikely give the marked divergence in structure of these proteins from the Cry3 group. Therefore, cross-resistance between MIR604 and Herculex RW is considered to be unlikely and need not be considered further.

By in large, the mechanisms of resistance that might evolve to Bt toxins in Coleoptera like the CRW are largely unknown. Based on the review of the literature, the only genuine cases of resistance to Bt toxins suggest that target site resistance is the most common, and probably is responsible for the majority of resistance cases. Whether this would actually be the case for CRW is unknown. Work by Siegfried *et al.* (2005), however, suggested that there is similarity of the target sites in Coleoptera and Lepidoptera so that if resistance to Cry3 toxins evolves in CRW, it might involve a target site modification. For a brief period when there was a commercially available Cry3A-expressing potato in the US, potential resistance mechanisms were studied in the laboratory. Based on these experiments, Colorado potato beetle was shown to survive for a short period of time on transgenic plants (Rahardja and Whalon, 1997). No further characterization of this resistance was ever undertaken as NatureMark stopped marketing these Bt potatoes in 2001.

More recent studies by Rausell et al. (2004) compared the toxin-binding capacities of proteolytically-processed Cry3A, Cry3B and Cry3C toxins to midgut brush border membranes of Colorado potato beetle. *In vitro* heterologous competition binding experiments showed that the three proteoloytically-activated Cry3 toxins all shared a common binding site, but Cry3Aa and Cry3Ca have an extra binding site that is not shared with the Cry3Ba toxin. This means that there could be some differences in the binding of mCry3A in MIR604 and that of Cry3Bb1 in YieldGard RW. The mCry3A has been modified to promote processing so that the availability of active toxin for interaction with the binding site may be different for different Cry3 toxins in the natural situation in CRW midguts.

Receptors for Cry3 toxins have never been isolated and characterized. To date, cadherin and aminopeptidase-N are most frequently associated with Cry toxin binding in Lepidoptera (e.g., Gahan et al., 2001; Luo et al., 1996), although actin (McNall and Adang, 2003), alkaline phosphatase (McNall and Adang, 2003), and glycolipids (Griffith et al., 2005) have also been identified more recently in binding. Only insect cadherins have been proven to mutate to give resistance to Cry toxins in Lepidoptera (Gahan et al., 2001; Morin et al., 2003). Siegfried et al. (2005) used an expressed sequence tag to identify the first Coleopteran cadherin gene in CRW. Cadherin could be a receptor for Bt proteins in CRW, but further studies are necessary to confirm this possibility. Genetic and

biochemical studies with different insect species have shown that resistance mechanisms based on target-site genes such as cadherin are inherited as recessive or incompletely recessive traits (Ferré and Van Rie, 2002). On the other hand, non-target site mechanisms, such as altered metabolism or processing, are more likely to be inherited as incompletely dominant traits. One would therefore predict that a recessively-inherited target site mechanism is the most likely to evolve if resistance occurs to mCry3A in CRW. The modeling studies (discussed above) include varying degrees of dominance.

Rausell et al. (2004) found that CryBb1 might confer some cross-resistance to Cry3A (mCry3A) through modification of the shared receptor. The reverse, however, is not necessarily true. That is, resistance to mCry3A could occur through modification of a unique binding site with which Cry3Bb1 does not interact. Thus, CRW developing resistance to mCry3A expressed in MIR604 maize may not confer complete resistance to Cry3Bb1 maize. While Rausell et al. (2004) did demonstrate specific binding of processed toxin they did not demonstrate functional receptor binding. Because there is a lack of real information on the nature of resistance, especially in CRW, it is best to assume complete cross-resistance between Cry3Bb1 and mCry3A. This "worst-case" was assumed in the modeling studies discussed above. It is more likely, however, that if CRW resistance does occur to mCry3A, only partial cross-resistance to Cry3Bb1 is expected.

To study these issues further, it is recommended that Syngenta conduct cross-resistance studies using CRW colonies resistant to mCry3A. Experiments should be conducted to investigate the nature, inheritance, and fitness costs of specific mechanisms of resistance to the mCry3A protein expressed in MIR604 maize.

4. CONDITIONALLY REQUIRED DATA FOR MIR604 CORN (2010 UPDATE)

As part of the terms and conditions of the MIR604 (mCryA) registration, the registrant was required to submit programs for resistance monitoring and refuge compliance. Annual reports summarizing the data collected under these requirements must also be submitted to EPA. Further, data were required to address aspects of potential resistance to mCry3A. The following terms and conditions were required for resistance monitoring, compliance, and additional research:

- Resistance Monitoring
    - Description of the steps to be taken to establish corn rootworm baseline sensitivity and damage guidelines (due within 90 days of registration -- January 31, 2007);
    - Submission of a detailed resistance monitoring plan. Description of the program including baseline sensitivity, sampling (number of locations and samples per locations), sampling methodology, bioassay methodology, standardization procedures, detection technique, sensitivity, and the statistical analysis of the probability of detecting resistance, and an interim description of rootworm damage guidelines (due January 31, 2008);

- Submission of rootworm damage guidelines (for unexpected pest damage) (due January 31, 2010);
- Development and validation of an appropriate discriminating or diagnostic dose assay (due January 31, 2010);
- Annual report of the insect resistance monitoring program. Results of monitoring and investigations of damage reports (due August 31st each year, beginning in 2008).

- Compliance
  - Written description of Compliance Assurance Program (due within 90 days of registration -- January 31, 2007);
  - Grower Agreements: proposed system to assure growers sign grower agreements and affirmation system to assure annual affirmation by growers of their IRM obligations (due within 90 days of registration -- January 31, 2007);
  - Annual report on Compliance Assurance Program (CAP) activities and results: third-party grower survey, on-farm visitation program, phased-compliance report, tips and complaints, and grower education programs (due January 31st each year, beginning in 2008);
  - Grower education program for MIR604 corn. Subsequent changes to the grower education program must be included in the annual compliance assurance program report (due January 31, 2008).
- Additional IRM Data
  - Initiate establishment of CRW strains that are resistant to mCry3A and investigate the nature, inheritance, and fitness costs of specific mechanisms of resistance to the mCry3A protein expressed in MIR604 maize (due January 31, 2010);
  - Study the behavioral deterrence (avoidance) mechanism further and submit appropriate results (due January 31, 2010);
  - Continue studies on the biological impact of adults surviving on MIR604 maize and submit these results (due January 31, 2010).

Syngenta has submitted all of the required reports as mandated under the terms of registration. A discussion of the reports and data for the resistance monitoring and compliance programs for MIR604 corn follows in the next sections.

a) Resistance Monitoring

Monitoring Plan for mCry3A Corn (2008)

A resistance monitoring program for MIR604 (mCry3A) was submitted to EPA in 2008 (MRID 473401-01; reviewed in BPPD, 2009a). This submission described the monitoring protocols for mCry3A corn as well as progress to establish baseline susceptibility data for mCry3A, develop a diagnostic bioassay, create a response protocol for unexpected pest damage, and develop a mCry3A-resistant colony.

Like other Bt corn registrations, the monitoring plan for MIR604 is based on the framework of the Agricultural Biotechnology Stewardship Technical Committee's (ABSTC) document titled "Updated Monitoring Plan for Bt Corn." Specifically, Syngenta considered the following elements of monitoring: spatial patterns of resistance, geographic and species focus, baseline susceptibility data, and an annual sampling plan.

The monitoring program for MIR604 relies on two main components: reports of unexpected pest damage and bioassays of field collected Western corn rootworm (*Diabrotica virgifera virgifera,* WCRW). With respect to bioassays and insect collections, WCRW are considered the only reliable species for annual monitoring although the Bt toxin is also active against other CRW pests. The regions from which Syngenta plans to collect WCRW are listed below. Modifications to the sampling program may occur based on MIR604 corn adoption and changes in pest importance.

- Region 1: Illinois – soybean variant of WCR
- Region 2: Nebraska – organophosphate resistant WCR
- Region 3: Iowa – wild type WCR

After baseline susceptibility has been established for mCry3A, diet bioassays will continue to be conducted for the three regions described above. Any unusually low sensitivity to mCry3A will be investigated as soon as practical and tied to field relevance. 'Unusually low' sensitivity would be investigated by considering the following questions:

- Is low sensitivity heritable?
- Is the proportion of field collected individuals with unusually low sensitivity that can feed and survive on MIR604 corn significantly lower than the baseline proportion?
- Did plant assays with mCry3A determine that damage exceeded economic threshold?

Based on 2007 baseline susceptibility data (MRID 480470-02), Syngenta proposed that only population samples with LC_{50} values >8,500 ng/cm^2 would be considered for further investigation. This value would be 10-times higher than the mean value observed in past assays while still below the maximum concentration used to test field collections. EPA would be informed by Syngenta within one month when bioassay results exceeding 8500 ng/cm^2 would be obtained.

BPPD Analysis of mCry3A Resistance Monitoring Plan

BPPD's review of the mCry3A monitoring plan (BPPD, 2009a and 2010) concurred with Syngenta that it is appropriate to focus the current monitoring efforts on WCRW only. Because of WCRW's widespread distribution and abundance, but similarity in life cycles compared to NCRW and MCRW, it may be more likely that they evolve resistance first to mCry3A. BPPD, however, also has concerns for NCRW and recommends that Syngenta begin to develop baseline susceptibility data for this target

pest. Past difficulties with rearing of NCRW and arguments of limited geographic distribution should not be the driving factors for neglecting to monitor for resistance in this important corn pest.

Syngenta proposed that it would focus its annual monitoring for resistance on 1) using dose response curves, 2) field reports of unexpected pest damage, and the 3) development of resistant WCRW colonies in the lab. For CRW monitoring and dose-response curves, Syngenta proposed that only population samples with LC_{50} values >8,500 ng/cm^2 would be considered for further investigation. BPPD notes that although this value may be 10-times higher than the mean value observed over the years, it still appears somewhat arbitrarily chosen. It is not clear how sensitive and reliable this particular value will be; LC values may be misleading because larvae can survive on Bt diets for some time without feeding. BPPD recommends that Syngenta pursue a sublethal measure to monitor for shifts in susceptibility to mCry3A and explore the use of the Sublethal Seedling Assay (SSA) developed by Nowatzki et al. (2008).

Unexpected Pest Damage Guidelines (2010)

Syngenta submitted finalized protocols for monitoring unexpected pest damage in 2010 (MRID 480470-02). The following strategy was proposed for MIR604 corn:

In cases of unexpected root damage on MIR604 corn, Syngenta proposed a threshold level of > 2.0 on the Node Injury Scale (NIS; Oleson et al., 2005) to trigger investigation. Syngenta set this level based on their experience with MIR604 corn. When growers and extension specialists, or consultants report to seed dealers that there was unusually high CRW damage (>2.0), then:

- Syngenta representatives will test for presence of mCry3A in root tissue of damaged plants and confirm hybrid and lot number. If tests confirm that mCry3A was present, then additional roots will be dug and rate the root damage. At this point it will be determined if other causes were responsible for excessive root damage. If damage is again >2.0 and due to CRW, then Syngenta will be notified. If confirmed, the representative will dig additional plants and determine if the root damage was caused by other corn pests not susceptible to the toxin, weather conditions, hybrid genetics, or planting error. If the root node injury in the field is >2 due to CRW feeding, then Syngenta will be notified.
- Syngenta will perform more flow strip assays to assess the % of plants expressing mCry3A. If more than 95% of the plants tested express the toxin, then Syngenta will collect WCRW for analysis.

BPPD Analysis of Unexpected Pest Damage Guidelines

BPPD did not concur with Syngenta that the threshold level for unexpected damage for MIR604 should be >2.0 (see review in BPPD, 2010). BPPD believes that a damage threshold level of 1.0 (NIS) may be more appropriate and conservative under moderate insect pressure for single, non-high dose CRW products such as are currently registered by Syngenta (MIR604). When insect pressure is

exceedingly high, then both Bt protected and refuge corn will incur greater damage. Under these circumstances, a threshold level of 1.5 may be more suitable. In addition, for any mCry3A pyramided products (i.e., with two toxins expressing a functional high dose), the damage threshold should be lower than 1.0 and based on the efficacy against the target pest.

BPPD recommended that the following be considered when Syngenta follows up with incidents of unexpected damage reports (BPPD, 2010):

1) The inherent dose of the toxin to control CRW (high dose vs. non-high dose control);
2) Prior use and crop history in the Bt field where excessive damage was observed;
3) Damage on non-Bt plants in the same field or immediately adjacent to the Bt plants;
4) Insect pressure during that corn growing season (low vs. moderate vs. high); and
5) Weather pattern during the corn growing season and possible effects on Bt protein expression and pest population dynamics.

When greater node injuries are observed on refuge corn than on Bt corn, then this could be an indication that damage is not due to resistance but some other factors. Likewise, if Bt plants have less damage than refuge plants that were treated with insecticides, then the cause of damage is not likely due to resistance. But, if damage levels on Bt plants exceed the level observed on non-Bt plants, then field resistance should be suspected.

Syngenta's response to unexpected damage reports is protracted but could be made more efficient by collecting CRW immediately after unexpected damage reports are received (if adults are still flying). After sampling, Syngenta representatives should still be able to proceed with flow strip assays and root damage analysis, as well as putative causes for damage. Furthermore, BPPD recommended that monitoring samples for potentially resistant insects should be taken from within and adjacent fields where the pest damage has occurred. Studies by Naranjo (1990) and Coats et al. (1986) suggest that a significant fraction of the female WCRW population engages in longer distance and pre-ovipositional dispersal. Hence, BPPD recommended that collections of putative resistant individuals should also extend passed a ½ mile radius from the Bt field with unexpected damage (BPPD, 2010).

Baseline Susceptibility and Diagnostic Assays for mCry3A (Monitoring Results 2006-2008)

Syngenta collected baseline susceptibility data for mCry3A from WCRW populations sampled in 2006 through 2008. DM Crop Research Group, Inc. (a separate company working with Bt corn registrants to conduct CRW sampling) collected adult samples of WCRW populations from the three sampling regions (IA, IL, and NE) during the 2006 to 2008 growing seasons. Between 11 and 15 populations were sampled each year.

Custom BioProducts, Inc. (IA) (another independent company that conducts the susceptibility testing) maintained the WCRW colonies and resulting eggs and performed the subsequent bioassays on the populations using neonate larvae (< 24 hours old). Neonates were transferred into 96-well plates

containing artificial diet developed for CRW covered with surface applied Bt dilutions containing mCry3A at eight different concentrations: 0 (0.1% Triton-X 100 control), 25, 100, 500, 1000, 2500, 5000, and 10,000 ng mCry3A/cm^2. The protein used in the tests consisted of mCry3A in a lyophilized powder at a concentration of 900 µg/mg (supplied by Syngenta). Larvae were allowed to feed for five to seven days depending on when fungal contamination appeared. Mortality was recorded when larvae did not respond or move when probed. Six replicates of dose response bioassays were conducted for every population sample. A replication was considered valid if control mortality was ≤ 25%. Results from the assays were used to determine LC_{50}, LC_{90}, and LC_{99} values for the collected populations.

Data from the mCry3A susceptibility bioassays are summarized (range of mean susceptibility values for the sampled populations) in Table 11 below. Overall, the baseline susceptibility data for mCry3A were variable as was evident by the LC_{50} and LC_{90} results, although the LC_{50} ranges were more consistent year-to-year than the LC_{90} values. One population (from McLean Co., IL) collected during 2008 showed much higher tolerance to mCry3A (LC_{50}: 4822.6 ng/cm^2; LC_{90}: 37,820,880.0 ng/cm^2) than the other sampled populations (LC_{50}: 191.9 - 1518.4 ng/cm^2; LC_{90}: 1430.0 - 225,779.9 ng/cm^2). Based on the susceptibility testing, Syngenta proposed that only population samples with LC_{50} values > 8,500 ng/cm^2 would be considered for further investigation. BPPD noted that although this value may be 10-times higher than the mean value observed in the testing through 2008, it still appears to be somewhat arbitrarily chosen. It is not clear how sensitive and reliable this particular value will be; LC values can be misleading because larvae can survive on Bt diets for some time without feeding (see review in BPPD, 2010).

LC_{99} values and ranges were submitted for each year but are not shown in Table 11 because the extrapolated values were exceedingly high for most populations. Chi square testing resulted in high probabilities values that indicated that the extrapolated mean LC_{99} values were not a reliable estimate for the actual LC_{99}. This suggests that the use of 'lethal concentrations' will not be useful to monitor for changes in CRW susceptibility to mCry3A and an effective diagnostic concentration (LC_{99}) can not be realistically achieved for this toxin. Given these difficulties, Syngenta may need to consider sublethal measures (i.e., molting inhibition) to estimate the mCry3A activity against CRW. BPPD (2010) recommended that Syngenta use a sublethal measure for detecting shifts in susceptibility to mCry3A and/or consider the use of the Sublethal Seedling Assay (SSA) developed by Nowatzki et al. (2008) to monitor for shifts in CRW susceptibility. Nowatzki et al. (2008) found that the SSA, which measured survival and age structure of larval populations in three potential instar groups, was able to detect shifts in susceptibility of CRW at a much smaller scale than the diet bioassay.

Table 11. Cumulative Results of mCry3A Susceptibility Testing for WCRW (2006 - 2008; Table created from data submitted by Syngenta).

Year	LC_{50} range (ng/cm^2)	LC_{90} range (ng/cm^2)	MRID Citation
2006	357.9 - 1923.6	3788.7 - 28,766.6	473401-01
2007	209.8 - 1172.3	2470.5 - 52,515.0	480470-02
2008	191.9 - 4822.6	1430.0 - 37,820,880.0 [1]	480470-02

[1] One population in 2008 exhibited high tolerance to mCry3A. The other sampled populations had susceptibility ranges of 191.9 - 1518.4 (LC_{50}) and 1430.0 - 225,779.9 (LC_{90}).

b) Compliance

The compliance program for MIR604 (mCry3A corn) is aligned with that developed by ABSTC (in consultation with the EPA) for corn-borer protected Bt corn products. Specific elements of the program and additional reports with annual survey data are discussed below.

MIR604 Compliance Assurance Program

As a term of the MIR604 (mCry3A) registration, Syngenta was required to develop and submit to EPA a compliance assurance program (CAP) to ensure grower adherence to IRM requirements. The terms of registration mandated a number of components for the compliance program including:

- Grower Agreements: Contractual arrangement between the registrant and grower to obligate adherence to IRM requirements.
- Annual IRM survey: A survey (conducted anonymously by an independent research firm) intended to provide a statistically representative sample of growers from various corn-growing regions in the U.S. Results from the survey should assess levels of grower compliance with refuges as well as grower motivations, attitudes, and reasons for non-compliance.
- On-farm assessments: The registrant is required to develop an on-site assessment program in which trained personnel from each company make visits to farms growing Bt corn. During these visits, compliance with refuge requirements is assessed and growers not in compliance are identified for corrective action under the Phased Compliance Approach.
- Tips and complaints: The registrant must establish a means for the reporting and investigation of incidences of refuge non-compliance.
- Phased Compliance Approach (PCA): A consistent set of procedures (for all Bt corn registrants) to be employed to address non-compliance among growers and seed dealers.

The Agricultural Biotechnology Stewardship Technical Committee (ABSTC), a consortium of Bt corn registrants, previously developed and submitted a CAP for lepidopteran Bt corn PIPs in 2002. Subsequently, ABSTC submitted revised versions of the CAP in 2004 and 2005 in response to EPA

reviews of annual growing season reports (see discussion in the Cry1Ab/Cry1F Bt corn Biopesticide Registration Action Document). MIR604 (and other registered products with mCry3A) have been included as part of this existing program, though data are tabulated separately for lepidopteran, rootworm, and stacked (lepidopteran + rootworm) Bt corn PIPs (and not by toxin). EPA reviews of compliance data for rootworm-protected PIPs (some of which predates the registration of MIR604) can be found in BPPD (2004), BPPD (2007), and BPPD (2009b).

Annual Grower Surveys

As a condition of each individual Bt corn registration, the registrant must perform an annual third-party survey of a statistically representative sample of Bt corn growers. The grower survey functions to measure compliance adherence to refuge size and distance requirements at a regional level and to identify educational opportunities to increase grower compliance with IRM requirements. More than 500 growers from four separate regions are anonymously surveyed annually. The methodology for conducting the grower survey has remained virtually unchanged since it was first conducted by Marketing Horizons, Inc in 2000 for the lepidopteran Bt corn registrations. But, starting in 2007 due to an increasing complexity of growers' Bt corn planting practices and a need to standardize the grower survey across insect-protected traits, Marketing Horizons utilized an internet-based survey approach.

Surveys for the corn rootworm PIPs encompasses all growers planting rootworm-protected traits (Cry3Bb1, mCry3A, and Cry34/35Ab1). Cumulative results of the surveys are summarized in Table 12 below. Results from the stacked (lepidopteran + rootworm) Bt corn surveys are tabulated separately (Table 13) and also include all registered rootworm PIP traits.

Table 12. Summary of Telephone (2005-2006) and Online (2007-2008) Survey Results for Rootworm-Protected Bt Corn Growers

Survey Question	2005[1] % Respondents	2006[2] % Respondents	2007 % Respondents	2008 % Respondents
Adherence to Refuge[3] Size	93	89	80	74
Adherence to Distance Requirements[3]	87	82	79	63
Awareness of IRM Requirements	97	93	97	96
Unaided recall of refuge size	51	57	63	72
Unaided recall of refuge distance	58	55	33	34

[1] Includes only YieldGard RW and YieldGard Plus corn growers
[2] Includes only YieldGard RW, YieldGard Plus, Herculex RW, and Herculex XTRA corn growers
[3] Weighted averages across all four regions surveyed

Table 13. Summary of Telephone and Online Survey Results for Stacked (Rootworm + Corn Borer) Bt Corn Growers (2006-2008)

Survey Question	2006 % Respondents	2007 % Respondents	2008 % Respondents
Adherence to Refuge Size[1]	78	70	72[3]
Adherence to Distance Requirements[1]	92	66	66[4]
Awareness of IRM Requirements	95	96	97
Unaided recall of refuge size	59	62 and 55[2]	81
Unaided recall of refuge distance CRW	48	39	36
Unaided recall of refuge distance ECB	32	77	86

[1] Weighted average across all four regions surveyed
[2] First number listed is for ECB and the second number for CRW refuge compliance.
[3] On a per field basis, adherence was 73%
[4] On a per field basis, adherence was 76%

Overall compliance (per grower) with refuge requirements for both single trait and stacked rootworm-protected PIPs has declined from 2005 to 2008. Grower adherence to the necessary refuge size fell to

below 75% in 2008 for single trait and stacked rootworm PIPs. Compliance with refuge proximity was lower; in 2008, ≤ 66% of rootworm PIP growers deployed refuges within the required distance to the Bt field. The percent of growers who were able to recall the correct refuge distance requirements (unaided) for rootworm PIPs drastically declined to the below 40% in 2008. Refuge distance requirements for rootworm-protected Bt corn products may be more challenging for growers because the refuge must be deployed either within or immediately adjacent to the Bt field. Stacked products present additional challenges due to the need to plan either two refuges (for lepidoptera and rootworm) or a combined refuge for both pest complexes. Nevertheless, grower awareness of the distance requirements has been poor and likely explains much of the reported non-compliance.

On-Farm Assessments

The on-farm assessment program is the portion of the CAP that identifies individual non-compliant growers (regardless of farm size) for remedial IRM education, follow-up reassessments, and other activities as part of the phased compliance approach (PCA). It can also serve as a tool to enhance the registrant's understanding of the obstacles growers face in implementing IRM requirements. The mandatory on-farm assessment program was fully implemented for the first time in 2003 (for lepidopteran registrations) and has typically encompassed more than 2,000 growers per seasons (for all types of Bt corn). On-farm assessments for rootworm-protected PIPs (including mCry3A products) began in 2006.

Data from the on-farm assessments (2006 through 2008) of rootworm-protected Bt corn PIPs are summarized in Table 14 below. These on-farm assessments encompass all growers planting rootworm-protected PIPs including varieties expressing the Cry3Bb1, mCry3A, and Cry34/35Ab1 toxins. Results for the on-farm assessments of stacked (lepidopteran + rootworm) PIPs are detailed in Table 15. The assessments do not have the statistical power associated with the consistently stratified and randomized telephone/on-line surveys and are not used to measure representative rates of non-compliance. In 2007 and 2008, no information was provided regarding specific non-compliance with refuge size and distance. This information should be provided in future reports to be consistent with previously collected data and to illustrate how growers are out of compliance (see EPA review in BPPD, 2009b).

Table 14. Cumulative Results for the On-Farm Assessments of Coleopteran-Protected Bt Corn Growers (2006-2008)[1]

	2006	2007	2008
Growers assessed	395	247	134
Refuge distance deviations[2]	13	N/A	N/A
Refuge size deviations	7	N/A	N/A
Significant deviations	11 (2.8%)	16 (6.5%)	12 (9.0)
Insignificant deviations	10 (4.0%)	8 (3.2%)	7 (5.2%)
Compliant growers	374 (94.7%)	223 (90.3%)	115 (85.8%)
Non-compliant growers	21 (5.3%)	24 (9.7%)	19 (14.2%)

[1]Table adapted from page 12 of MRID 470444-01
[2] Some growers had compliance deviations other than refuge size or distance; thus, the total of refuge distance and size deviations does not equal the number of non-compliant growers.

Table 15. Cumulative Results of the On-Farm Assessments of Stacked Bt Corn (Rootworm + Corn Borer) Growers (2006-2008)[1]

	2006	2007	2008
Growers assessed	600	1069	1799
Refuge distance deviations	51[2]	N/A	N/A
Refuge size deviations	8	N/A	N/A
Significant deviations	45 (7.5%)	77 (7.2%)	**86** [3]
Insignificant deviations	16	33 (3.1%)	**36** [3]
Compliant growers	539 (89.8%)	959 (89.7%)	1546 (85.9%)
Non-compliant growers	61 (10.2%)	110 (10.3%)	253 (14.1%)

[1] Table adapted from page 12 of MRID 470444-01
[2] Some growers had compliance deviations other than refuge size or distance; thus, the total of refuge distance and size deviations does not equal the number of non-compliant growers.
[3] The numbers of deviations do not add up to the 253 non-compliant cases reported.

Tips and Complaints

As required by the terms of registration, Bt corn registrants must have a "tips and complaints" system as a mechanism for individuals (e.g., growers, sales representatives, etc.) to report alleged instances of IRM noncompliance. The number of tips and complaints (summarized for all Bt corn registrations including lepidopteran and rootworm varieties) received through 2008 is summarized in Table 16

below. Each of these growers identified through the tips and complaints mechanism were visited as part of the on-farm assessment program. It is not possible, however, to determine whether any of the non-compliant growers identified via the tips and complaints route were subject to the Phased Compliance Approach.

Table 16. Anonymous Tips and Complaints about Non-Compliance with IRM requirements (Data from ABSTC annual reports 2003 through 2008)

Year	Number of Tips and Complaints
2003	0
2004	0
2005	5
2006	3
2007	14
2008	5

Phased Compliance Approach

ABSTC's CAP for lepidopteran and rootworm-protected PIPs includes a standard set of procedures (shown in Table 17), known as the Phased Compliance Approach (PCA), which is to be used by registrants when responding to instances of grower noncompliance with the IRM requirements. The PCA also established a tiered approach for non-compliance with "significant" deviations and "other" deviations. For a 20% CRW refuge requirement (Corn Belt), a significant size deviation is defined as a Bt grower planting less than 15% non-Bt corn refuge. This definition is also applicable to "combined" refuges planted for both lepidoptera and CRW for stacked Bt corn PIPs. On the other hand, a significant deviation based on refuge proximity has not been clearly defined for CRW refuges and as of the 2008 CAP report it is unclear what standards are being used by ABSTC. For lepidopteran Bt corn, a significant deviation is triggered if fewer than 2/3 of the Bt corn fields are planted within ½ mile of a non-Bt corn refuge. This definition is not compatible with CRW refuge because the distance requirement mandates that refuges be placed adjacent to or within the Bt corn field. A reasonable extension of the lepidopteran definition for CRW could be "less than 2/3 of the non-Bt refuge is deployed adjacent or within the Bt field" and "fewer than two-thirds (2/3) of the in-field strips are at least four rows wide" (see discussion in BPPD, 2006b). This formula would also be applicable to combined refuges for stacked PIPs.

Table 17. Phased Compliance Approach (PCA) – Standards for Bt Corn Refuge Non-Compliance (submitted with the ABSTC 2002 CAP)

	Mandatory Responses	Additional Responses
Significant Deviations	• IRM education. • Warning letter. • Compliance assistance contact prior to planting. • Compliance assessment contact for the following growing season. • Deny access to the Bt corn product for any significant deviation two years in a row.	• Invoice monitoring. • Technical assistance. • Grower IRM training. • Reaffirmation of IRM obligations. • Deny access to the Bt corn product for other deviations that are repeated over a period of years.
Other Deviations	• IRM education. • Letter and/or compliance assistance contact prior to planting. • Compliance assessment contact in the following growing season.	

Under the PCA, sales are to be suspended to individual growers for one year after two years of significant deviations. Following the one-year suspension, growers will need to be requalified to purchase seeds. Growers identified as non-compliant (significant or other deviations) are required to receive a "compliance assessment contact" the following year under the PCA. Non-compliant growers are typically identified through the on-farm assessment program (see discussion in the on-farm assessment section above). Table 18 summarizes the numbers of non-compliant growers reassessed under the PCA and the growers still found to be out of compliance. As of the 2008 growing season, one grower was denied access to Bt corn technology due to a refusal to be reassessed in the following season after significant non-compliance. Compliance data, including results of on-farm assessments and PCA activities, are detailed in BPPD (2007) and BPPD (2009b).

Table 18. Reassessment of Rootworm-Protected and Stacked Bt Corn Growers Under the Phased Compliance Approach (taken from ABSTC annual CAP reports) [1]

Year	Reassessments [2]	Significant Deviations [3]	Loss of Access to Technology
2006	62	0	1 [4]
2007	82	0	0
2008	134	0	0

[1] The data in this table includes both growers planting single trait rootworm PIPs and stacked (lepidopteran + rootworm) PIPs. The data in the table has been summed for both groups.
[2] Reassessments of growers identified with deviations (significant and other) to refuge requirements the previous growing season.
[3] Significant deviations recorded the following season. Two successive years of significant deviations results in loss of access to Bt corn technology.
[4] One grower refused to be reassessed in 2006 and was denied access to Bt corn

c) Additional IRM Data

As a condition of registration, Syngenta was required to address three areas of research related to the nature of potential resistance to mCry3A in CRW. Syngenta addressed these requirements in a submission to EPA (MRID# 480470-01; reviewed in BPPD, 2010). The specific requirements and responses are described below.

i) "Initiate establishment of CRW strains that are resistant to mCry3A and investigate the nature, inheritance, and fitness costs of specific mechanisms of resistance to the mCry3A protein expressed in MIR604 maize."

Since 2006, Syngenta has worked with Dr. Bruce Hibbard (USDA-ARS, Columbia, MO) to establish a mCry3A-resistant (non-diapausing) WCRW strain. Nine generations of selection have occurred since the beginning of the bioassay experiment. A control strain was reared on non-Bt maize as a comparison (see Table 19).

After three generations of selection, the mean LC_{50} of the treatment colony was 0.259 µg/cm^2 (95% C.I.: 0.0906 - 0.5380 µg/cm^2) and the LC_{50} of the unselected control colony was 0.552 (95% C.I.: 0.318 - 0.917 µg/cm^2). Since the 95% C.I, did overlap, the results did not differ significantly.

After six generations of selection, the mean LC_{50} of the treatment colony was 5.541 µg/cm^2 (95% C.I.: 2.361 - 26.429 µg/cm^2) and the LC_{50} of the unselected control colony was 1.301 (95% C.I.: 0.871 - 2.003 µg/cm^2). The 95% C.I, did not overlap, hence the results differed significantly.

After nine generations of selection, the mean LC_{50} of the treatment colony was 11.25 µg/cm^2 (95% C.I.: 5.70 - 32.69 µg/cm^2) and the LC_{50} of the unselected control colony was 0.73 (95% C.I.: 0.51 - 1.05 µg/cm^2). The 95% C.I, did not overlap, hence the results differed significantly.

Table 19. LC_{50} Values of a mCry3A-Selected Colony and Lab Reference Strain (Table created from data submitted in MRID# 480470-01).

Generations of Selection	Mean LC_{50} Value with 95% C.I. (µg/cm^2)	Significance between Selected and Lab Strain based on overlapping 95% C.I.
Three generations on Bt (treatment colony)	0.259 (0.0906-0.5380)	not significant
Three generations on non-Bt (lab reference strain)	0.552 (0.318-0.917)	
Six generations on Bt (treatment colony)	5.541 (2.361-26.429)	significant
Six generations on non-Bt (lab reference strain)	1.301 (0.871-2.003)	
Nine generations on Bt (treatment colony)	11.25 (5.70-32.69)	significant
Nine generations on non-Bt (lab reference strain)	0.73 (0.51-1.05)	

To determine the field relevance of differences in LC_{50}s, field evaluations were performed and damage to MIR604 and non-Bt corn was assessed.

Despite the differences in the LC_{50}s between selected and non-selected colonies, no statistically significant differences in damage, beetle emergence, or larval weight were observed when larvae were exposed to MIR604. When the selected colony was placed on Bt and non-Bt plants, no difference in emergence, weight, and root damage were observed. At this point, it is unclear whether similar performance is related to increasing fitness of the selected colony on MIR604 plants, decreased fitness of the selected colony on isoline plants, or a combination of both.

Additional steps to be performed include maintenance of the colony and further selection in upcoming years to investigate the nature, inheritance, and fitness costs of specific mechanisms of resistance to mCry3A.

ii) "Study the behavioral deterrence (avoidance) mechanism further and submit appropriate studies."

Syngenta worked with Dr. Hibbard (USDA-ARS, Columbia, MO) to further study any potential avoidance mechanisms in CRW larvae to mCry3A expressing corn. The methods by Strnad and Dunn (1990) were employed to evaluate host recognition of WCRW and MIR604, non-Bt corn, oat roots, and filter paper (negative control).

Neonates exposed to MIR604 or isoline diet had moved significantly lower distances and exhibited lower velocities than larvae exposed to the negative control or oats. Larvae exposed to isoline treatments had significantly higher angular velocities and turn angle as well as lower distances moved than neonates exposed to MIR604. These results were consistent with observations of local search behavior described for larvae feeding on host roots.

These results suggest that CRW larvae recognize MIR604 corn as a host and that larvae engage in short range searching behavior when exposed to mCry3A in corn diet. Hence, it is unlikely that there is an avoidance mechanism unique to mCry3A and WCRW. Syngenta suggested that any avoidance behavior may be due to the intoxication from exposure to Bt.

iii) "Continue studies on the biological impact of adults surviving on MIR604 maize and submit results."

Syngenta worked with Dr. Lance Meinke (University of Nebraska) to measure potential effects of adult WCRW sublethal exposure to MIR604 (field and greenhouse experiments).

The study design was set up as a 2 x 2 factorial experiment of all possible larva/adult diet combinations. The impacts of larval and adult diet on fecundity and longevity were measured.

Treatments:

- Larvae reared on MIR604 – adults fed on MIR 604 tissue
- Larvae reared on MIR604 – adults fed on non-Bt tissue
- Larvae reared on non-Bt corn – adults fed on MIR604 tissue
- Larvae reared on non-Bt corn – adults fed on non-Bt tissue

Results of the study showed no difference in longevity and fecundity between adults of the different treatments. Since adult CRW do not seem to detect the difference between Bt and non-Bt corn (no repellency to adults), Syngenta concludes that diet switching should not negatively impact the effectiveness of any IRM refuge strategy in place.

Syngenta's report on these three studies adequately addresses the condition of registration. The selection experiment is still ongoing, and BPPD recognizes that it may take significant additional time to fully address that condition of registration.

5. CONCLUSIONS

The original proposed IRM strategy (submitted in 2005) and data to support it were found to be "acceptable" except that the in-field strip refuge must be at least 4 rows wide based on recent larval movement data (see EPA reviews in BPPD, 2005 and 2006a). If resistance is recessive, then the proposed IRM plan using a 20% structured refuge should be adequate to delay resistance for at least 15 years given the assumptions of Syngenta's model. If MIR604 maize is planted in areas with known

rotation-resistant WCRW, then planting transgenic corn only in rotated maize fields should be a sound IRM strategy that will delay the evolution of resistance by at least 15 years regardless of gene expression.

In accordance with the terms and conditions of registration, Syngenta has submitted additional data regarding resistance monitoring, the nature of potential resistance to mCry3A, and refuge compliance. These data (as discussed in section 4 above), support the original IRM determination including the use of 20% non-Bt corn refuge to mitigate resistance development by CRW to mCry3A.

6. IRM TERMS AND CONDITION OF REGISTRATION

The terms and conditions for each of the mCry3A registrations contain a complete description of the IRM requirements for the product. Details are provided on the requirements for refuge (size and structure), resistance monitoring, remedial action, compliance assurance, grower education, and annual IRM reports. For additional information, please refer to the following document:

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II. **E. Benefits and EPA Public Interest Finding**

1. PUBLIC INTEREST FINDING

The criteria for determining whether registration of a pesticide chemical is in the public interest are set forth in a Federal Register Notice dated March 5, 1986 volume 51, No. 43 (OPP-32500; FRL-2977-2) titled *Conditional Registration of New Pesticides*. There is a presumption that registration of a pesticide chemical is in the public interest if one of the following criteria is met: i) the use is for a minor crop; (ii) the use is a replacement for another pesticide that is of continuing concern to the Agency; (iii) the use is one for which an emergency exemption under FIFRA Section 18 has been granted for lack of an alternative pest control method, or (iv) the use is against a pest of public health significance. Further, EPA may determine that such a registration is in the public interest on the basis of the following criteria: i) there is a need for the new chemical that is not being met by currently registered pesticides; ii) the new pesticide is comparatively less risky to health or the environment than currently registered pesticides; or iii) the benefits (including economic benefits) from the use of the new active ingredient exceed those of alternative registered pesticides and other available non-chemical techniques.

Syngenta has provided data to support its claim that MIR604 CRW-protected corn is in the public interest. EPA's analysis supports the following conclusions:

1. Results of efficacy trials conducted in 2002, 2003, and 2004 indicate that MIR604 corn provides effective control of key rootworm pests of field corn.

2. MIR604 corn has unique biochemical properties which may benefit insect resistance management for this and other CRW-protected corn products.

3. If MIR604 corn is registered, it will be the third CRW-protected *Bt* corn product on the market. The availability of multiple CRW-protected corn products will increase grower choice and price competition, resulting in lower seed prices for consumers and higher adoption rates.

4. Registration of MIR604 corn is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for CRW-control are highly toxic to humans and the environment, while mCry3A-expressing corn poses no foreseeable human health or environmental risks.

2. BACKGROUND

Corn is the most widely cultivated U.S. crop, in terms of acreage planted and net value. In 2004, U.S. corn acreage totaled 80.9 million, yielding 11.8 billion bushels. Corn rootworm (CRW, *Diabrotica* spp.), one of the most damaging pests of field corn, can cause yield losses in the range of 8 to 16% if left uncontrolled.

Prior to the advent of insect protected field corn, CRW was controlled through the use of crop rotation and insecticides. Although crop rotation is regarded as an effective CRW control tool (Levine and Sadeghi, 1991), behavior changes in northern corn rootworm (NCRW, *Diabrotica barberi*) (extended diapause) and western corn rootworm (WCRW, *D. virgifera virgifera*) (soybean rotation) have reduced the effectiveness of this management option in some corn growing regions. Insecticidal control, a pest management alternative to crop rotation, employs chemicals that are highly toxic to fish, birds, and other wildlife species. In addition, resistance to some CRW insecticides, such as methyl parathion and carbaryl, (Meinke et al., 1998; Scharf et al., 1999; Zhu et al., 2001), may result in increased chemical use.

Since 2003, a third method of CRW control, CRW-protected *Bt* corn, has been available to farmers. The first and second *Bt* field corn products registered for CRW control were Monsanto's event MON863 (expressing the Cry3Bb1 protein) and Dow/Pioneer's DAS 59122-7 (expressing the Cry34Ab1 and Cry35Ab1 proteins) field corn products, respectively. This review concerns the third CRW-protected *Bt* corn product submitted for registration, Syngenta's MIR604, which produces the mCry3A insecticidal protein. Similar to event MON863 and DAS 59122-7 corn hybrids, event MIR604 Bt corn is targeted against the WCRW, NCRW, and Mexican corn rootworm (MCRW, *D. virgifera zeae*).

3. EFFICACY AND YIELD TRIALS

Early and late maturity MIR604-derived non-commercial, experimental hybrids were evaluated in northern, Midwest, and southern U.S. corn-belt locations in 2002, 2003, and 2004. Small plot trials utilizing either randomized complete block or split plot designs were conducted. Most trials included application of chemical insecticides to assess synergies between MIR604 corn and chemical insecticides. Chemical insecticide treatments included one of two seed treatments, ProShield ST® (tefluthrin) and Cruiser® (thiamethoxam), plus one of three in-furrow soil-applied granular treatments, Force 3G® (tefluthrin), Aztec 2.1G® (Tebupirimiphos + cyfluthrin) and Lorsban 15G® (chlorpyrifos).

a. Efficacy

Efficacy trials compared root damage among MIR604 and non-MIR604-derived corn hybrids. Experimental plots were artificially infested (400 to 1450 eggs/trial), located in fields with naturally occurring populations of corn rootworm, or both approaches to infestation were combined. When artificially infestation was used, at the V2 to V3 leaf stage rootworm eggs were mechanically placed 3 inches deep in soil, within 2 to 3 inches of the corn stalk base. At the VT to R1 stage, corn plants were manually dug from the ground, soil was washed from the roots and roots were examined and rated for corn rootworm damage according to the Iowa State 0-3 node-injury rating scale.

1) 2002 Field Season

In 2002, results from trials located in Minnesota and Illinois showed that MIR604 derived hybrids had significantly less root damage compared to non-transgenic controls, with and without insecticide

applications. In all but one case, MIR604 seed treated with Cruiser or ProShield ST (ProShield is an older seed treatment that has been replaced by Cruiser), or plants treated with Force 3G soil-applied insecticide, had significantly reduced root damage ratings relative to non-transgenic control hybrids with the same chemical treatment. The exception was in Stanton, MN, trial SYN137 which included a Force 3G insecticide application, where similar reductions were seen but the difference was not statistically significant.

2) 2003 Field Season

Eight event MIR604 hybrids were assessed for efficacy at over a dozen locations in Illinois, Iowa, Kansas, Minnesota, Missouri, Nebraska, South Dakota, Texas, and Wisconsin in 2003. At all locations, MIR604 corn had significantly lower mean root damage, compared to non-transgenic controls with the same chemical treatment. And for most trials, untreated Event MIR604 hybrids demonstrated significantly greater efficacy compared to negative isoline controls treated with Cruiser at low or high rates. No significant difference could be detected between rates of Cruiser applied to MIR604 hybrids; however, efficacy of Cruiser seed treatment on negative control plants did appear to increase with increasing application rates. Overall, Event MIR604 hybrids seem to provide similar protection against CRW, compared to nontransgenic controls treated with Lorsban 15G, Force 3G, or Aztec 2.1 granular soil-applied insecticides. In general, MIR604 hybrids delivered equivalent or better efficacy than chemical controls currently employed to control root damage from corn rootworm.

Due to difficulties in rearing NCRW, a limited number of efficacy trials were conducted with this rootworm species. In greenhouse trials that employed artificial infestation of NCRW, mean root damage ratings show significantly greater efficacy among MIR604 hybrids compared to negative controls. In these trials, no significant difference was seen among MIR604 hybrids. The study author stated that MIR604 hybrids were exposed to natural populations of NCRW at the majority of locations. However, the Willmar, MN location was populated primarily with NCRW and results from this trial indicate that MIR604 hybrids provided greater CRW control, compared to negative hybrids with and without chemical treatment.

At two locations in Texas, Event MIR604 corn hybrids showed efficacy toward MCRW, but not to the same extent as WCRW and NCRW. At both locations, application of Cruiser seed treatment to Event MIR604 hybrids enhanced the efficacy in a rate dependent manner.

3) 2004 Field Season

In 2004, root damage ratings were significantly different among untreated MIR604 hybrids and negative controls. More specifically, at all locations, except Champaign, IL, MIR604 hybrids treated with Cruiser® seed treatment had significantly less root damage than untreated controls. Further, at all locations, except Urbana, IL, root damage ratings were not significantly different between untreated MIR604 hybrids and MIR604 hybrids treated with Cruiser® insecticide at 0.125 or 0.75 mg AI/seed. These findings suggest that negative interactions between mCry3A protein and Cruiser® insecticide did not result from use of the seed treatment on MIR604 hybrids. The efficacy of MIR604 corn for rootworm protection was also compared to Force 3G®, a granular soil insecticide that is used to control

CRW. Results of these trials showed that untreated MIR604 corn, MIR604 corn treated with Cruiser® insecticide, and a negative control hybrid treated with Force 3G® insecticide provided statistically equivalent root protection. These results suggest that the efficacy of MIR604 corn, with or without Cruiser® insecticide, is similar to that of Force 3G®, a commercially available corn rootworm insecticide.

Significant natural populations of northern corn rootworm (NCRW, *Diabrotica barberi*) were identified at three trial locations in 2004. At the Redwood Falls, MN location, adults seen in the field before and after root evaluations were said to be primarily NCRW. At university trials in Ames, IA and Higginsville, MO, adult emergence counts indicated that the NCRW population in these sites was 35% and 32%, respectively, of the total rootworm population. Results from the three locations identified above were similar to results seen for the overall experiment. Mean root damage ratings for MIR604 hybrids were found to be significantly lower than damage ratings for the untreated negative control hybrid and damage ratings for MIR604 hybrids with or without Cruiser® insecticide applications were statistically equivalent to the negative control hybrid treated with Force 3G® insecticide.

An MIR604 hybrid, identified as "late hybrid 25", was also shown to be effective at the Texas site, which contained large populations of MCRW. As seen in the trials described above, rootworm damage on the untreated MIR604 corn hybrid was statistically equivalent to the negative control hybrid treated with Force 3G® insecticide. In addition, root damage ratings were not significantly different between the MIR604 hybrid treated with and without Cruiser® insecticide. Based on limited data collected during the 2003 and 2004 cropping seasons, the registrant concluded that MIR604 corn hybrids provide moderately high levels of root damage protection against MCRW and that, on average, greater control was provided against WCRW than MCRW. Additional data will be collected in 2005 to confirm these findings.

b. Yield

Yield trials compared grain production among MIR604 and non-MIR604-derived corn hybrids. Experimental plots were located on sites with or without natural CRW pressure and chemical insecticides were applied to some sites, but not to all.

1) 2002 Field Season

In 2002, Event MIR604 derived hybrids were evaluated for yield (bu/acre) at several field trials in Minnesota and Illinois. Growing conditions were normal at the Minnesota locations, while some Illinois sites experienced drought and significant CRW feeding pressure. At the Minnesota locations, the average yield of MIR604-derived hybrids was statistically equivalent to the negative isogenic controls (with and without insecticide). However, at the Illinois locations, MIR604-derived hybrids produced greater yields than negative controls with and without chemical insecticide applications.

2) 2003 Field Season

MIR604 yield trails were repeated in 2003 at field sites in Illinois, Indiana, Iowa, Kentucky, Minnesota, Nebraska, South Dakota and Wisconsin. In contrast to 2002, growing conditions were good

at most trial sites. Mean yields from untreated (no chemical insecticides) MIR604 hybrids were, on average, statistically equivalent to control hybrids, regardless of maturity group and insecticide treatment. This data suggests that where CRW pressure exists, MIR604 has a positive effect on yield, and this effect may be magnified under adverse environmental conditions; however, where CRW pressure does not exist, yield among MIR604 and non-transgenic plots will be similar.

3) 2004 Field Season

Grain yield for MIR604 plots that were untreated or treated with Cruiser® insecticide produced an average of 27.5 bushels per acre more than the untreated negative control hybrid. In addition, results at Bloomington-2, IL, which experienced extreme rootworm pressure and stressful growing conditions, suggest that MIR604 corn is most beneficial where rootworm pressure is greatest. The submission states that although some of these trials lacked the precision to show statistical differences among treatments, the numerical trends in grain yield were positive for MIR604 corn.

A multiple location yield trial conducted in the absence of CRW feeding pressure (granular insecticide applied to all plots within trial) showed no significant difference in yield between MIR604 hybrids and the negative isogenic control at all trials and locations except SYNAM445, where MIR604 hybrid 22 yielded 10.5 bushels more per acre than the isogenic negative control hybrid. These results suggest that, under low stress conditions, MIR604 hybrids perform as well as negative isogenic hybrids.

c. Conclusions

Results from the 2002, 2003, and 2004 efficacy trials suggest that MIR604 hybrids provide greater protection against CRW than negative control hybrids. Further, the efficacy of MIR604 hybrids appears to be similar to that of negative control hybrids treated with Force 3G® insecticide and the mCry3A protein that is present in MIR604 hybrids does not seem to interact with Cruiser® seed treatment. Conclusions regarding the relative yield from 2002 and 2003 field trials data were obscured by poor experimental design and extreme weather conditions. Data from 2004 yield trials indicated that under high stress conditions, MIR604 hybrids (±Cruiser® seed treatment have greater yields than untreated negative controls, although there was no statistical difference among treatments. Under low stress conditions, there was no statistical difference in yield between MIR604 hybrids (±Cruiser® seed treatment) and the negative controls (± insecticide). Yield data suggest that MIR604 hybrids perform about the same as the isoline hybrids.

4. BENEFITS

EPA has completed an independent analysis of the potential grower benefits associated with *Bt* CRW-protected corn hybrid technologies. Grower demand for CRW control technologies is influenced by the level of CRW infestation (acreage and degree of infestation), comparative cost-benefit of competing CRW control technologies, U.S. and global market acceptance and approval of a technology, and other regulatory constraints (*e.g.* refuge requirements). In the year 2000, almost 8 million pounds of CRW insecticide, costing $172 million ($12.29/acre), were applied to 14 million acres, or 17% of U.S. corn acreage. And between the years 2005 to 2013, CRW infested acreage is projected to increase from

approximately 31.8 million acres to 39 million acres (Table 36). BPPD anticipates, through evaluation of USDA/NASS and DOANE databases on CRW damage and control costs, that the market for transgenic in-plant CRW protection will increase by 2.6% per year, reaching 18 to 19 million acres by the year 2013.

Data on product efficacy suggests that Event MIR604 corn provides greater protection against CRW damage than does unmodified corn (see Efficacy section above). However, submitted yield data was inconclusive and should be supported by a third year of field trials (see Yield section above).

Table 36. Projected acreage with corn rootworm infestation and breakdown of associated CRW control practices for the years 2000 to 2013. Information presented for 2000 and 2002 reflect actual infestation and insect control tactics.

Year	Acres Infested	Acres Treated	CRW-Protected Bt Corn Acreage	Conventional Treatments
	----------acreage x 10^6----------			
2000	28.0	14.0	0.0	14.0
2001[1]	-	-	-	-
2002	29.5	14.7	0.0	14.7
2003	30.2	15.1	1.0	14.1
2004	31.0	15.5	2.5	13.0
2005	31.8	15.9	4.0	11.9
2006	32.6	16.3	6.0	10.3
2007	33.5	16.7	7.2	9.5
2008	34.3	17.2	8.6	8.5
2009	35.2	17.6	10.4	7.2
2010	36.1	18.1	11.9	6.1
2011	37.1	18.5	13.7	4.8
2012	38.0	19.0	15.8	3.2
2013	39.0	19.5	16.8	2.7
Annual Growth Rate	2.58%	2.58%	16.8%	-14.36%

[1] EPA does not have data for the 2001 growing season.

a. Human Health Benefits

Event MIR604 CRW-protected corn is expected to be safer for handlers, applicators, farmers, and the public than chemical pesticides in current use. Twenty-five of the 39 registered conventional insecticides used to control CRW are classified as "Restricted Use", 12 have the "Danger" label classification, and several are in Agency Special Review (*e.g.*, dimethoate, phorate, and terbufos). Further, each year there are confirmed reports of human illness associated with these registered

chemical insecticides (See EPA's Incident Data Base, http://www.opp.gov/pesticides). Adoption of mCry3A corn hybrids has the potential to reduce occupational, farmer, and public health risks associated with the manufacture, transport, storage, handling, application, and disposal of conventional insecticides, by providing a safer alternative for CRW control.

Increased adoption of transgenic CRW-protected corn products is of special importance to EPA, because many of the chemical insecticides registered for CRW control are highly toxic to humans and the environment, are in Special Review, and have restricted use labels. The chemical insecticides (larvicides and adulticides) subject to the greatest use reduction following adoption of transgenic CRW-protected hybrids are: organophosphates (chlorpyrifos, tebupirimphos, tebufos, methyl parathion, and chlorethoxyfos), pyrethroids (tefluthrin, cyfluthrin, bifenthrin, lambda cyhalothrin), carbamates (carbofuran), and pyrazoles (fipronil) (Appendix A). Adoption of transgenic CRW-protected corn products is not expected to result in reduced use of seed treatments (nicotinoids); however seed treatment products are generally less toxic than at-plant and post-plant products.

In contrast to the registered chemical insecticide alternatives, EPA has concluded that there is reasonable certainty of no harm from aggregate exposure to the mCry3A protein as expressed in corn. See mCry3A Human Health and Product Characterization reviews for more information on potential human health effects [Fellman, 2005(a,b); 2006(c)].

b. Environmental Benefits

Chemicals commonly used for CRW control can cause adverse environmental effects when used according to label instructions. Of the most commonly used CRW control products, 15 are labeled as "toxic," six as "highly toxic," one as "very highly toxic," and 14 as "extremely toxic" to birds, fish, and other wildlife. Several of the synthetic insecticides (e.g. organophosphates, carbamates, and synthetic pyrethroids), are moderately to highly toxic to terrestrial non-target species. Of special concern are methyl parathion and carbofuran, both of which are implicated in bird kills (Appendix A, Table 1A). In contrast, the ecological risk assessment and characterization of the mCry3A protein, as expressed in corn, suggests that these proteins pose no significant ecological risk to non-target species, including endangered and beneficial species (Milofsky, 2006).

c. Insect Resistance Management Benefits

There is concern about the ability of target pests to evolve resistance to CRW control mechanisms, including crop rotation, chemical insecticides, and CRW-protected Bt corn products. Currently, the favored management practice for CRW control is crop rotation, specifically corn-soybean rotations, complimented by application of chemical insecticides – pyrethroids, organophosphates, carbamates, pyrazoles, and more recently, neonicotinoids as seed treatments. CRW has developed resistance to a number of chemical control products used as adulticides. The recent development of the NCRW extended diapause and WCRW soybean resistant variants have further reduced the efficacy of crop rotation and chemical control options.

Since mCry3A CRW-protected corn appears to have good efficacy against CRW, introduction of this product is expected to extend the durability of other commercially available CRW-protected *Bt* corn products (*e.g.*, Cry3Bb1 PIPs and Cry34/35Ab1 PIPs). To ensure the long-term efficacy of mCry3A CRW-protected corn hybrids, an insect resistance management plan should be implemented (Matten et al. 2006).

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Appendix A

Table 1A. Insecticide end-use products registered by EPA for use on corn for control of corn rootworm species.

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Ambush® Insecticide – Syngenta	Permethrin – 25.6%	SP	0.2 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Asana®XL Insecticide 0.66 Emulsifiable Concentrate - DuPont	Esfenvalerate – 8.4%	SP	0.05 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Aztec 2.1% Granular Insecticide – Bayer Corp.	Tebupirimfos – 2.0% cyfluthrin – 0.1%	OP SP	0.15 lb/ac 0.01 lb/ac	Larval control	**WARNING. Restricted Use**; toxic to fish and wildlife
Baythroid 2 Emulsifiable Pyrethroid Insecticide - Bayer	Cyfluthrin – 25%	SP	0.04 lb/ac	Adult control	**DANGER. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees, may cause allergic skin reactions
Capture 2EC Insecticide/Miticide – FMC Corp.	Bifenthrin – 25.1%	SP	0.3 lb/ac	Larval control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Chlorfos 15G Insecticide Granular – Griffin LLC	Chlorpyrifos – 15%	OP	2.02 lb/ac	Larval control	**CAUTION.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Chlorfos® 4E Insecticide – Griffin LLC	Chlorpyrifos – 42%	OP	2.52 lb/ac	Adult & Larval control	**WARNING.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms
Counter® CR Systemic Insecticide-Nematicide – American Cyanamid Company	Terbufos – 20%	OP	1.30 lb/ac	Larval control	**DANGER. Restricted Use**; fatal if swallowed, inhaled or absorbed through skin, extremely toxic to fish and wildlife
D-z-n® diazinon AG500 Insecticide - Syngenta	Diazinon – 48%	OP	0.48 lb/ac	Adult control	**CAUTION. Restricted Use**; highly toxic to birds, fish and other wildlife, highly toxic to bees
D-z-n® diazinon AG600 WBC Insecticide - Syngenta	Diazinon - 56%	OP	0.45 lb/ac	Adult control	**CAUTION. Restricted Use;** highly toxic to birds, fish and other wildlife, highly toxic to bees
Declare® Emulsifiable Insecticide Concentrate – Griffin LLC	Methyl parathion – 45.11%	OP	0.22 lb/ac	Adult control	**DANGER. Restricted Use**: fatal if swallowed, inhaled or absorbed through skin, highly toxic to aquatic invertebrates and wildlife, highly toxic to bees
Diazinon 500-AG Organophosphate Insecticide – UAP	Diazinon – 48%	OP	0.48 lb/ac	Adult control	**CAUTION. Restricted Use;** highly toxic to birds, fish and other wildlife, highly toxic to bees
Dimethoate 4 EC Systemic Insecticide - Helena	Dimethoate – 44.8%	OP	0.45 lb/ac	Adult control	**WARNING.** Toxic to wildlife and aquatic invertebrates, highly toxic to bees

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Dimethoate 400 Systemic Insecticide-Miticide - UAP	Dimethoate – 43.5%	OP	0.44 lb/ac	Adult control	**WARNING.** Toxic to wildlife and aquatic invertebrates, highly toxic to bees
5 lb. Dimethoate Systemic Insecticide - Helena	Dimethoate – 57%	OP	0.46 lb/ac	Adult control	**DANGER.** Toxic to wildlife and aquatic invertebrates, highly toxic to bees
Force® 3G Insecticide - Syngenta	Tefluthrin – 3%	SP	0.17 lb/ac	Larval control	**CAUTION. Restricted Use**; very highly toxic to freshwater and estuarine fish and invertebrates
Fortress® 2.5G granular insecticide - DuPont	Chlorethoxyfos – 2.5%	OP	0.16 lb/ac	Larval control	**DANGER. Restricted Use**; toxic to wild mammals, birds, fish and aquatic invertebrates
Fortress® 5G granular insecticide – DuPont	Chlorethoxyfos – 5%	OP	0.16 lb/ac	Larval control	**DANGER. Restricted Use**; toxic to wild mammals, birds, fish and aquatic invertebrates
Furadan® 4F insecticide/ nematicide – FMC Corp.	Carbofuran – 44%	C	0.88 lb/ac	Adult & larval control	**DANGER. Restricted Use**; poisonous if swallowed or inhaled, toxic to fish, birds and other wildlife, highly toxic to bees, can seep or leach through soil and can contaminate groundwater
Lannate® LV insecticide – DuPont	Methomyl – 29%	C	0.65 lb/ac	Adult control	**DANGER. Restricted Use**; fatal if swallowed, toxic to fish, aquatic invertebrates and mammals, highly toxic to bees, known to leach through soil into groundwater

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Lannate® SP insecticide - DuPont	Methomyl – 90%	C	0.45 lb/ac	Adult control	**DANGER. Restricted Use**; fatal if swallowed, may cause blindness, toxic to fish, aquatic invertebrates and mammals, highly toxic to bees, known to leach through soil into groundwater
Lorsban® 15G Granular Insecticide – Dow Agrosciences	Chlorpyrifos – 15%	OP	2.03 lb/ac	Larval control	**CAUTION.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms
Lorsban® -4E Insecticide– Dow Agrosciences	Chlorpyrifos – 44.9%	OP	2.69 lb/ac	Adult & larval control	**WARNING.** Toxic to birds and wildlife, extremely toxic to fish and aquatic organisms
Mocap® 10% Granular Nematicide Insecticide – Aventis CropScience	Ethoprop – 10%	OP	3.53 lb/ac	Larval control	**WARNING.** Toxic to aquatic organisms and wildlife
Mocap® *EC Nematicide-Insecticide* – Aventis CropScience	Ethoprop – 69.6%	OP	3.34 lb/ac	Larval control	**DANGER. Restricted Use**; toxic to aquatic organisms and extremely toxic to birds
Penncap-M® Microencapsulated Insecticide – Elf Atochem	Methyl Parathion – 22%	OP	0.44 lb/ac	Adult control	**WARNING. Restricted Use**; highly toxic to aquatic invertebrates and wildlife
Phorate 20 G Organophosphate Insecticide - UAP	Phorate – 20%	OP	1.3 lb/ac	Adult & larval control	**DANGER. Restricted Use**; extremely toxic to fish and wildlife

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Pounce® WSB Insecticide – FMC Corporation	Permethrin - 24.7%	SP	0.2 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Pounce® 3.2 EC Insecticide – FMC Corporation	Permethrin – 38.4%	SP	0.2 lb/ac	Adult control	**CAUTION. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Pounce® 25 WP Insecticide – FMC Corporation	Permethrin – 25%	SP	0.2 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic invertebrates, highly toxic to bees
Regent 4 SC Insecticide – Aventis CropScience	Fipronil – 39.4%	PP	0.13 lb/ac	Larval control	**WARNING. Restricted Use**; toxic to birds, fish and aquatic invertebrates
Sevin brand 80S Carbaryl Insecticide – Aventis CropScience	Carbaryl – 80%	C	2.0 lb/ac	Adult control	**WARNING.** Extremely toxic to aquatic and estuarine invertebrates, highly toxic to bees
Sevin brand XLR PLUS Carbaryl Insecticide – Aventis CropScience	Carbaryl – 44.1%	C	1.76 lb/ac	Adult control	**CAUTION.** Extremely toxic to aquatic and estuarine invertebrates, highly toxic to bees
Thimet 20-G Soil and Systemic Insecticide – American Cyanamid	Phorate – 20%	OP	1.3 lb/ac	Larval control	**DANGER. Restricted Use**; extremely toxic to fish and wildlife

Product	Active Ingredients	Type [a]	Use Rate [b]	Use	Classification [c]
Thimet 20-G Soil and Systemic Insecticide – American Cyanamid	Phorate – 20%	OP	1.3 lb/ac	Larval control	**DANGER. Restricted Use**; extremely toxic to fish and wildlife
Warrior® Insecticide with Zeon Technology - Syngenta	Lambda-cyhalothrin – 11.4%	SP	0.03 lb/ac	Adult control	**WARNING. Restricted Use**; extremely toxic to fish and aquatic organisms and toxic to wildlife, highly toxic to bees
Cruiser® 5FS - Syngenta	Thiamethoxam	N	0.1 lb/ac (seeds)	Larval control	**CAUTION**. toxic to aquatic organisms
Poncho® 1250 - Gustafson LLC	Clothianidin	N	0.1 lb/ac (seeds)	Larval control	**CAUTION.** This product is toxic to aquatic invertebrates
Prescribe® - Gustafson LLC	Imidacloprid	N	0.11 lb/ac (seeds)	Larval control	**CAUTION**. Highly toxic to birds and aquatic invertebrates

Table reproduced from MON 863 registration and updated with data provided by DAS in MRID # 46123921.
a - OP: organophosphate; SP: synthetic pyrethroid; C: carbamate; PP: phenyl pyrazole; N: nicotinoid
b – maximum labeled use rate expressed in pounds of active ingredient per acre (assume that 1 liq pt 1 lb)
c – precautionary language as stated on label.

III. **Regulatory Position for Modified Cry3A Protein and the Genetic Material Necessary for its Production (Via Elements of pZM26) in Event MIR604 Corn SYN-IR604-8**

A. Initial Regulatory Position

In 2006 EPA conditionally registered Syngenta Seeds Inc.'s new active ingredient, modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8. The Agency determined that the use of this pesticide was in the public interest and that it would not cause any unreasonable adverse effects on the environment during the time of conditional registration.

Results of efficacy trials conducted in 2002, 2003, and 2004 indicate that MIR604 corn provides effective control of key rootworm pests of field corn. MIR604 corn has unique biochemical properties that may benefit insect resistance management for this and other CRW-protected corn products. MIR604 contains the third CRW-active Bt corn protein on the market. The availability of multiple CRW-protected corn products will increase grower choice and price competition, resulting in lower seed prices for growers and higher adoption rates. Registration of MIR604 corn is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for CRW-control are highly toxic to humans and the environment, while mCry3A-expressing corn poses no foreseeable human health or environmental risks.

The new corn plant-incorporated protectant, Event MIR604 corn, produces its own insecticide within the corn plant. This protectant, mCry3A protein, is derived from *Bacillus thuringiensis* (Bt), a naturally occurring soil bacterium. The mCry3A protein used in this product controls corn rootworm, a highly destructive pest responsible for the single largest use of conventional insecticides in the United States.

In order to reduce the possibility of corn rootworm developing resistance to Bt, EPA required Syngenta Seeds, Inc. to ensure that 20 percent of the planted acreage of this product be set aside in which non-CRW-protected corn will be grown to serve as a "refuge." These refuge areas will support populations of corn rootworm not exposed to the CRW-protected corn. The insect populations in the refuges will help prevent resistance development when they cross-breed with insects in the CRW-protected fields. This resistance management strategy was developed as a condition of the registration, and EPA will require routine monitoring and documentation that these measures are followed. The submitted insect resistance management data support a registration until 2010.

A tolerance exemption at 40 CFR Part 174.456 was established for residues of *Bacillus thuringiensis* modified Cry3A protein and the genetic material necessary for its production in corn.

Pursuant to FIFRA section 3(c)(7)(C), EPA may conditionally register a new pesticide active ingredient for a period of time reasonably sufficient for the generation and submission of required data that are lacking because insufficient time has elapsed since the imposition of the data requirement for those data to be developed. EPA may grant such conditional registration only if EPA determines that (1) the use of the pesticide product during the period of the conditional registration will not cause any unreasonable adverse effect on the environment, and (2) the registration and use of the pesticide during the conditional registration is in the public interest. EPA determines that all of these criteria have been fulfilled.

The first criterion under FIFRA Section 3(c)(7)(C) mentioned above has been met because insufficient time has elapsed since the imposition of the data requirements for:

1) Field degradation studies evaluating accumulation and persistence of mCry3A in several different soils in various strata.

2) Three (3) year full-scale field or semi-field studies for evaluation of mCry3A Event MIR604 corn exposure on non-target invertebrates.

3) Data to augment the mCry3A amino acid sequence analysis to known toxins and allergens within six months of the date of registration: specification of which version of NCBI database was utilized; descriptions of parameters utilized; and dates accessed for the BLAST search.

4) Insect resistant management data: a) Specific cross-resistance studies. Establish strains of CRW that are resistant to mCry3A and investigate the nature, inheritance, and fitness costs of specific mechanisms of resistance to the mCry3A protein expressed in MIR604 maize; b) Study the behavioral deterrence (avoidance) mechanism further and submit appropriate results; c) Continue studies on the biological impact of adults surviving on MIR604 maize and submit these results.

The applicants submitted or cited data sufficient for EPA to determine that conditional registration of modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8 under FIFRA 3(c)(7)(C) will not result in unreasonable adverse effects to the environment, as discussed above. The applicants submitted and/or cited satisfactory data pertaining to the proposed use. The human health effects data and non-target organism effects data are considered sufficient for the period of the conditional registration. These data demonstrate that no foreseeable human health hazards or ecological effects are likely to arise from the use of the product and that the risks of resistance developing to mCry3A protein during the conditional registrations are not expected to be significant.

Registration of modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8 is in the public interest because:

1. Results of efficacy trials conducted in 2002, 2003, and 2004 indicate that MIR604 corn provides effective control of key rootworm pests of field corn.

2. MIR604 corn has unique biochemical properties which may benefit insect resistance management for this and other CRW-protected corn products.

3. If MIR604 corn is registered, it will be the third CRW-protected *Bt* corn product on the market. The availability of multiple CRW-protected corn products will increase grower choice and price competition, resulting in lower seed prices for consumers and higher adoption rates.

4. Registration of MIR604 corn is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for CRW-control are highly toxic to humans and the environment, while mCry3A-expressing corn poses no foreseeable human health or environmental risks.

In view of these minimal risks and the clear benefits related to modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8, EPA believes that the use of the product during the limited period of the conditional registration will not cause any unreasonable adverse effects.

Although the data with respect to this particular new active ingredient are satisfactory, they are not sufficient to support an unconditional registration under FIFRA 3(c)(5). Additional data are necessary to evaluate the risk posed by the continued use of this product. Consequently, EPA is imposing the data requirements specified earlier in Section III.

EPA has determined, as explained in section II.E., that the third criterion for a FIFRA 3(c)(7)(C) conditional registration has been fulfilled because the use of modified Cry3A protein and the genetic material necessary for its production (via elements of pZM26) in event MIR604 corn SYN-IR604-8 under this registration is in the public interest.

The submitted data in support of this registration under section 3(c)(7)(C) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) have been reviewed and determined to be adequate. Studies mentioned above are included in the terms, conditions, and limitations of these registrations. This registration will not cause unreasonable adverse effects to man or the environment and is in the public interest.

The expiration date of the registrations has been set to September 30, 2010.

B. 2010 Update: Regulatory Position

Section 3(c)(7)(A) of FIFRA provides for the registration or amendment of a pesticide when the pesticide and proposed use "…are identical or substantially similar to any currently registered pesticide and use thereof, or differ only in ways that would not significantly increase the risk of unreasonable adverse effects on the environment, and (ii) approving the registration or amendment in the manner proposed by the applicant would not significantly increase the risk of any unreasonable adverse effect on the environment." Unreasonable adverse effects on the environment are defined under section 2(bb) of FIFRA as "… any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide…" Thus, pursuant to section 3(c)(7)(A), EPA may conditionally register a pesticide if (1) the pesticide and its proposed use are identical or substantially similar to a currently registered pesticide; or (2) the pesticide and its proposed use differ only in ways that would not significantly increase the risk of unreasonable adverse effects; and (3) approving the registration would not significantly increase the risk of any unreasonable adverse effect.

The Agency concludes that the following mCry3A corn product registrations, set to expire in September 2010 and described in-depth throughout this BRAD, meet both criteria (1) and (2):

(1) Agrisure RW Rootworm-Protected Corn (EPA Reg. No. 67979-5)
(2) Agrisure CB/LL/RW (EPA Reg. No.67979-8)

All of these mCry3A corn products are identical in both composition and use (corn) to plant-incorporated protectants that are currently registered. Thus, criterion (1) has been fulfilled.

With regard to criterion (2), the Agency maintains, as was previously determined for the original registration of these particular products, that cultivation of mCry3A-containing corn will not cause unreasonable adverse effects on the environment. The conditional environmental effects data, submitted in response to terms and conditions of registration, strengthen the Agency's initial position and also confirm that long-term effects on non-target organisms are not anticipated. Lastly, the continued use of these products will likely still provide many of the benefits as were evaluated in section II(E) of this BRAD to support the 2006 registration of Event MIR604 corn (e.g., reduction in use of conventional insecticides that are highly toxic to both humans and the environment).

In conclusion, as the expiring mCry3A products (i.e., Agrisure RW Rootworm-Protected Corn, (MIR604 Corn, EPA Reg. No. 67979-5) and Agrisure CB/LL/RW (Bt11 x MIR604 Corn, EPA Reg. No.67979-8)) have met the required criteria under section 3(c)(7)(A) of FIFRA, the Agency is amending these registrations to extend their respective expiration dates as follows:

Product Name (EPA Reg. No.)	Expiration Date
Agrisure RW Rootworm-Protected Corn (EPA Reg. No. 67979-5)	September 30, 2015
Agrisure CB/LL/RW (EPA Reg. No. 67979-8)	September 30, 2015

Although data provided were satisfactory to make the determinations required by section 3(c)(7)(A) of FIFRA, they were not sufficient to support an unconditional registration under FIFRA section 3(c)(5). Additional data, specific in relation to long term nontarget testing and insect resistance management, are necessary for a finding of registrability under FIFRA section 3(c)(5) and remain as terms or conditions for the purposes of the amendments.

C. Period of Registration

In the 2001 *Bt* Corn reassessment, EPA determined that it was appropriate to amend the then-existing registrations to extend the period of registration of those products to an expiration date of October 15, 2008. All of the products being assessed at that time were efficacious against lepidopteran pests. EPA based this action on the finding that use of Cry1Ab or Cry1F expressed in corn will not significantly increase the risk of unreasonable adverse effects on the environment "for the limited time period of 7

additional years (to October 15, 2008)." These registrations were later amended to extend the period of registration to an expiration date of September 30, 2010. EPA subsequently granted time-limited registrations to products efficacious against coleopteran corn rootworm pests. For example, EPA registered Cry3Bb1 on February 24, 2003, to May 1, 2004, and extended that registration twice, to February 24, 2008, and September 30, 2010.

As set forth elsewhere in this document, EPA's primary concern for the *Bt* protected transgenic corn products is the possibility that target pests will develop resistance to one or more of the plant-incorporated protectant (PIP) toxins. Development of resistance to a *Bt* toxin would be a grave adverse effect, and, for over 15 years, EPA has imposed stringent requirements intended to countermand the potential development of resistance. Registrants similarly have been busily developing various products, product mixes (i.e., so-called "pyramids" and "stacks"), and resistance strategies, to maximize agronomic benefits and address resistance management issues. The result has been a vast array of product combinations and, occurring over the past couple of years, a re-emergence of varying refuge requirements for different products.

As discussed in the 2001 *Bt* PIP BRAD (at IID13), the earliest *Bt* corn registrations did not include mandatory refuge requirements. There was a lack of scientific consensus as to what the appropriate refuge requirement should be, and, it was assumed that the limited market penetration of these early crops would be so low as to guarantee that adequate natural refuges would be available from neighboring non-*Bt* corn fields. From 1995 to 1997, *Bt* corn registrations included voluntary refuge requirements of 0% to 20% in the Corn Belt. In 1999, the Agricultural Biotechnology Stewardship Technical Committee (ABSTC), in conjunction with the National Corn Growers Association, proposed uniform insect resistance management (IRM) requirements for *Bt* corn registrations. With some modifications, this proposal, put in place for the 2000 growing season, formed the baseline IRM requirements for almost all *Bt* corn registrations for the better part of a decade: farmers were required to plant a 20% refuge that could be treated for insects, or a 50% treated refuge in cotton-growing areas; all refuges to be planted within one-half mile of the *Bt* corn field.

These uniform requirements brought certainty and consistency to the market after the initial period where many *Bt* corn products had different refuge requirements. Recently, however, as product developers have begun to conceive of products with different combinations of "pyramided" products (i.e., products containing two or more toxins efficacious against the same pest) and "stacked" products (i.e., products combining toxins efficacious against different pests), the refuge requirements have begun to vary. For example, certain products require a 20% external refuge; some products permit a 5% external refuge; one product incorporates a 10% seed blend refuge; we have applications in process for products that propose to incorporate a 5% seed blend refuge; and other permutations are possible.

Given the profusion of various toxin combinations and refuge options, we can no longer proceed on the basis that, as concerns insect resistance management, all products are equal. It was a relatively simple proposition when the default requirement of a 20% sprayed refuge applied to almost all of the *Bt* corn crops in the market. Under those circumstances, the relative durability of products against the

development of resistance was functionally equivalent, and, as a consequence, imposing functionally equivalent registration periods was appropriate. That is now no longer the case.

As part of our continually evolving regulatory approach to the continually evolving product mix wrought by developers, we think it appropriate to revise our regulatory requirements in scientifically defensible ways to reflect the comparative level of risks posed by the products that we regulate. Here, for example, where we've determined that a particular product, or category of products, likely will pose less risk of insect resistance developing to a particular PIP protein, we think it appropriate to grant that particular product, or category of products, a registration for a period greater than that granted a corresponding product that poses a greater risk of insect resistance developing. This approach is reflective of complementary principles: first, to ensure that we apply our limited resources to the products that pose greater risk of adverse effects to the environment; and, second, to conserve the resources that registrants and applicants must expend in amending the registrations of products that pose less risk of adverse effects to the environment.

The scheme that we are following includes registration periods of five, eight, and twelve years; a fifteen-year registration period will also be available, if adequately supported by our science assessment. In this scheme, (i) a product with a single PIP toxin, and a 20% external refuge, qualifies for a five-year registration; (ii) a product with pyramided PIP toxins (i.e., two or more toxins with distinct, non-cross reacting modes of action), that are non-high dose (the definition for a high dose product remains unchanged), with either a seed blend or external refuge, qualifies for an eight-year registration; (iii) a product with pyramided PIP toxins (i.e., two or more toxins with distinct, non-cross reacting modes of action), that are **high-dose**, with either a seed blend or external refuge, qualifies for a twelve-year registration; (iv) a product with pyramided PIP toxins (i.e., two or more toxins with distinct non-cross reacting modes of actions), with either a seed blend or external refuge, that has been determined by EPA's science assessment to be 150% as durable as the baseline single toxin product with a 20% external refuge, would qualify for a fifteen-year registration. Products determined by EPA's science assessment to be less than 100% as durable as the baseline single toxin product with a 20% external refuge would not qualify for a five-year registration and the registration period for such products will be determined on a case-by-case basis consistent with the level of risk they pose. Similarly, instances where other risk issues may arise, or where novel resistance concerns may be present, would also be determined on a case-by-case basis, as will novel refuge configurations that may present unique durability profiles.