The following petition document, 04-362-01p, contains three parts: 1. the final revised petition for nonregulated status submitted by Syngenta Seeds, Inc. (Syngenta) on August 2, 2006, for the corn rootworm protected transformation event MIR604 in *Zea mays* (corn), USDA APHIS number 04-362-01p (which APHIS has deemed complete); 2. a letter of completeness sent to Syngenta by USDA APHIS Biotechnology Regulatory Services (BRS) on July 25, 2006, which identified deficiencies and items to correct in an earlier version of this same petition; and 3. the earlier version of the petition submitted by Syngenta on May 17, 2006. As both versions of the petition are required for readers to understand the questions and responses within the letter of completeness, USDA APHIS BRS has assembled this document with both versions of the petition included.

Petition for the Determination of Non-Regulated Status

Corn Rootworm Protected Transformation Event MIR604

Revised

The undersigned submits this petition under 7 CFR 340.6 to Request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by:
Henry-York Steiner
Regulatory Affairs Manager
Syngenta Seeds, Inc.
3054 East Cornwallis Road
Research Triangle Park, NC 27709-2257
Phone: (919) 541-8652
Fax: (919) 541-8535

Contributing Authors:

Robert Joseph[1], Linda Meyer[1], Alan Raybould[2], John Steffens[1], Jeff Stein[1] and Demetra Vlachos[1]

[1]Syngenta Seeds, Inc., 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257, USA; [2]Syngenta, Jealotts Hill International Research Center, Bracknell, Berkshire, RG42 6EY, UK

Date: August 2, 2006

CLAIM OF DATA CONFIDENTIALITY AND JUSTIFICATION FOR TREATMENT AS CBI

The information contained in Appendix 2 to this submission (hereinafter referred to as the "claimed information") is entitled to treatment as confidential business information ("CBI"), and should be protected against public disclosure. As discussed more fully below, the claimed information consists of valuable trade secret and confidential commercial information, the release of which would cause Syngenta substantial competitive harm. Consequently, this information should be protected against disclosure, pursuant to the Freedom of Information Act, 5 U.S.C. §§ 551 et seq. ("FOIA").

Nature of the Claimed Information

The claimed information consists of studies and data that have been developed by Syngenta in support of the commercialization of its new modified Cry3A (mCry3A) maize product. These data have been developed through extensive research, testing and analysis conducted and sponsored by Syngenta over several years, at a cost of several million dollars. These data provide essential product characterization information, key genetic sequence information, as well as information regarding the product's toxicological profile, its chemical profile, and its environmental fate and safety. Furthermore, these data provide an indispensable "road map" of the studies that are required in order to obtain the regulatory approvals necessary to market the company's new mCry3A maize product. Most of these studies have already been, or will be, submitted to the U.S. Environmental Protection Agency (EPA) under a claim of confidentiality, in order to obtain regulatory approvals for Syngenta's Cry 3A product under the Federal Insecticide, Fungicide, and Rodenticide Act, 7 U.S.C. §§ 136 et seq. ("FIFRA") and the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 321, et seq. ("FFDCA").

Basis for Confidentiality Claim

The Freedom of Information Act, in Exemption 4, specifically shields from public disclosure the following types of information:

> trade secrets and commercial or financial information obtained from a person and privileged or confidential.

See 5 U.S.C. § 552(b)(4). For purposes of this provision, the courts have defined "trade secret" information to mean a:

> secret, commercially valuable plan, formula, process, or device that is used for making, preparing, compounding, or processing of trade commodities and that can be said to be the end product of either innovation or substantial effort.

Public Citizen Health Research Group v. FDA, 704 F.2d 1280 (D.C.Cir. 1983). *See also, Sokolow v. FDA,* No. 1:97-CV-252, slip op at 7 (E.D. Tex. Feb 19, 1998), *aff'd* 162 F.3d 1160 (5th Cir. 1998) (information regarding the manner in which a drug is manufactured,

including "analytical methods employed to assure quality and consistency" and the "results of stability testing" are trade secret information for purposes of Exemption 4).

Similarly, APHIS, in its Policy Statement on the Protection of Privileged or Confidential Business Information, defines "trade secret" to include:

> production data, formulas, and processes, and quality control tests and data, as well as research methodology and data generated in the development of the production process.

50 Fed. Reg. 38561 (Sept. 23, 1985) (hereinafter referred to as APHIS's "Policy Statement on CBI").

The claimed information includes essential data pertaining to the formulation, characterization and stability of Syngenta's new mCry3A maize product. In addition, when examined as a whole, these studies provide a valuable road map of the studies needed to satisfy the data requirements for regulatory approval of the product under FIFRA and the FFDCA, which is an essential aspect of commercialization of the product. All of this information was developed at considerable expense and effort on the part of Syngenta. Accordingly, based on the standards enumerated by the courts and elaborated upon in APHIS's Policy Statement on CBI, the claimed information is eligible for treatment as "trade secret" information, and should be protected from disclosure under FOIA Exemption 4.

In addition, as discussed above, Exemption 4 shields from disclosure "commercial or financial information obtained from a person" that is "privileged or confidential." For purposes of this exemption, the courts have interpreted the term "commercial information" broadly, to include any information in which an entity has a commercial interest. *See Public Citizen Health Research Group v. FDA*, 704 F.2d 1280 (D.C. Cir. 1983). Moreover, the courts have found that such commercial information will be deemed "confidential" if its disclosure is "likely to cause substantial harm to the competitive position of the person from whom the information was obtained." *National Parks & Conservation Ass'n v. Morton*, 498 F.2d 765 (D.C. Cir. 1974). Similarly, APHIS has explained in its Policy Statement on CBI that:

> Documents containing commercial or financial information will be deemed confidential if review establishes that substantial competitive harm would result from disclosure.

50 Fed. Reg. 38561. According to the agency, confidential commercial information can include "safety data, efficacy or potency data, and environmental data." *Id.*

The courts have held that in order to demonstrate that information is confidential commercial information within the scope of Exemption 4, it is sufficient to show that there is actual competition and a "likelihood of substantial competitive injury" if the information is disclosed. *See CNA Financial Corp. v. Donovan*, 830 F.2d 1132 (D.C. Cir. 1987). Similarly, APHIS has explained in its interpretive guidance that a person seeking to protect confidential commercial information from disclosure must demonstrate that (i) the person faces active competition in the area to which the information pertains,

and (ii) release of the information would cause substantial competitive harm. 50 Fed. Reg. 38561.

There is no question that Syngenta faces active competition in its field of agricultural biotechnology, including competition from large multinational corporations such as Monsanto, Dow AgroSciences, Dupont, and others. Many of these competitors are developing or attempting to develop similar products that express insecticidal proteins. Thus, Syngenta satisfies the first criterion for protection of confidential commercial information under Exemption 4.

In addition, disclosure of the claimed information would likely result in substantial competitive harm to Syngenta. As alluded to previously, the claimed information consists of data that provide essential product characterization and composition information, as well as information regarding the product's toxicological profile, its chemical profile, and its environmental fate and safety. These data have been developed through extensive research, testing and analysis, at substantial cost to Syngenta. Access to this product information (including information pertaining to manufacturing and analytical protocols, methods and techniques) could provide our competitors with knowledge that would assist those competitors in developing similar products to compete with Syngenta's mCry3A product. Moreover, competitors would reap the benefits of Syngenta's research, thereby accelerating their ability to bring competitive products to market, without having to incur any of the costs of developing those data. In addition, access to the claimed information would provide competitors with commercially valuable insights into the product lines that Syngenta is intending to develop and bring to market, as well as the likely timetable for Syngenta's commercialization of those product lines.

Similarly, the claimed information, taken as a whole, provides a template of the studies that are needed to satisfy the data requirements necessary for regulatory approval of Syngenta's product under FIFRA and the FFDCA. In addition, the studies themselves have intrinsic value under these statutes, as reflected in the mandatory data compensation provisions in FIFRA Section 3(c)(1)(F) and FFDCA Section 408(i). Specifically, FIFRA Section 3(c)(1)(F) provides for a ten-year period of "exclusive use" of studies submitted to support the registration of new active ingredients. During this ten-year period, other manufacturers can rely on these "exclusive use" studies to support their own regulatory approvals under FIFRA only with the express permission of the original data submitter. 7 U.S.C. § 136a(c)(1)(F)(i). Following this period of exclusive use, other manufacturers are required to compensate the original data submitter if they wish to rely on those data to support their own (competitor product) registrations under FIFRA. *Id.* Similar data compensation protections are provided for studies submitted under Section 408(i) of the FFDCA. 21 U.S.C. § 346a(i). Consequently, if APHIS were to release these studies to the public, Syngenta would be deprived of potentially millions of dollars of compensation as a result of competitors being able to rely upon Syngenta's studies in order to obtain regulatory approvals their own products under FIFRA and the FFDCA, without having to compensate Syngenta for access to those studies.

Based on the foregoing, the claimed information is eligible for protection under Exemption 4 of FOIA.

Finally, Exemption 3 of FOIA protects from public disclosure information that is specifically exempted from disclosure by another statute. *See* 5 U.S.C. § 552(b)(3). Section 10 of FIFRA contains several provisions that restrict or prohibit the release of information submitted under the Act. Among other things, Section 10 of FIFRA strictly prohibits the release of such information to foreign or multinational companies. *See* 7 U.S.C. § 136h(g). Section 408 of the FFDCA provides similar protections against disclosure of data submitted under that statute. The restrictions on disclosure set forth in FIFRA and the FFDCA provide an independent basis for requiring APHIS to protect Syngenta's claimed information against disclosure, pursuant to Exemption 3 of FOIA.

Summary

Corn rootworm (*Diabrotica* spp) is a Coleopteran pest that costs farmers an estimated 1 billion dollars in crop damage and control costs each year. Rootworm larvae feed upon the root systems of corn plants causing damage that can lead to plant stress and/or lodging, resulting in yield loss. Crop rotation and insecticide application have been employed to reduce rootworm damage. However, corn rootworm variants and resistant phenotypes have reduced the effectiveness of these pest management strategies. Syngenta has developed a corn event, MIR604, that expresses a modified Cry3A (mCry3A) insect control protein which provides for excellent protection from rootworm larval damage during field trials conducted annually since 2001. Hybrids derived from Event MIR604 provide economic control of three major corn rootworm pest species in the U.S., western corn rootworm (*Diabrotica virgifera virgifera* Le Conte; WCRW), northern corn rootworm (*D. longicornis barberi* Smith and Lawrence; NCRW) and Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith; MCRW).

The amino acid sequence of the mCry3A protein corresponds to that of the native Cry3Aa2 protein from *B. thuringiensis* subsp. *tenebrionis (B.t.t.)*, except that (1) its N-terminus corresponds to methionine-48 of the native protein and (2) a consensus cathepsin G protease recognition site has been introduced. This cathepsin G recognition site is recognized by a chymotrypsin-like serine protease that is found in the midguts of CRW larvae providing greater toxicity towards certain species of CRW. mCry3A exhibits no toxicity toward either other corn pests (including the various Lepidoptera that attack corn) or against non-pest insect species.

Corn hybrids derived from Syngenta's transformation Event MIR604 contain two transgenes: (1) the modified *cry3A (mcry3A)* gene encoding the mCry3A insect control protein and (2) the *pmi* (*manA*) gene from *Escherichia coli,* which encodes the enzyme phosphomannose isomerase (PMI) as a selectable marker. Expression of the *mcry3A* gene in Event MIR604 is driven by the maize promoter from a metallothionein-like gene and *pmi* is driven by the maize polyubiquitin promoter. Data from Southern analysis and DNA sequencing demonstrate that MIR604 hybrids (1) contain a single copy of both the *mcry3A* and *pmi* genes, (2) do not contain any of the backbone sequences from the transformation plasmid pZM26, (3) the overall integrity of the intended insert and contiguousness of the functional elements have been maintained and (4) both *mcry3A* and *pmi* are inherited in the expected Mendelian ratio for a single locus.

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604. The *mcry3A* gene was transformed into a heterogenous genetic background selected for its suitability to transformation by *Agrobacterium*. Event MIR604 was crossed into a number of elite inbred lines and material generated from these crosses was used in molecular, expression, agronomic and other studies. Control germplasm was either near isogenic hybrids or negative segregants identified in field trials. To reduce the effect of insect damage to corn ears by lepidopteran pests, some agronomic experiments used MIR604 in a breeding stack with Bt11 which expresses the Cry1Ab protein (Bt). In these cases controls consisted of the corresponding Bt11-only hybrids.

MIR604 hybrids have been field tested for three years (2001-2003) at more than 25 locations in several states across the US cornbelt and have demonstrated statistically significant reductions in root damage relative to non-transgenic controls, equivalent or greater yields compared to near isogenic controls and no consistent differences in a number of agronomic traits examined. In addition, MIR604 hybrids are nutritionally equivalent compared to the near-isogenic controls, and to other commercial hybrids.

A comprehensive environmental safety assessment was conducted using bacterial and plant produced mCry3A test substances that showed mCry3A has a limited spectrum of activity within the Chrysomelidae family of coleopteran species, specifically to the major corn pest species WCRW, NCRW and MCRW. No pesticidal activity against other species tested was observed. Hazard and exposure assessments were performed on representative non-target species (bobwhite quail, rainbow trout, insidious flower bug, seven spot ladybird, rove beetle, ground beetle, honeybee, earthworm) and no harmful effects were detected in any of these tests.

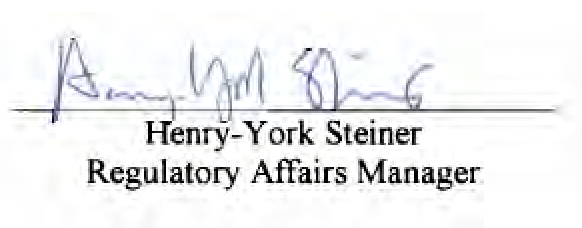
A thorough mammalian safety assessment was conducted for both mCry3A and PMI proteins as expressed in Event MIR604 hybrids. No adverse effects were observed for either protein. The large body of data and information described herein support the conclusion that the modified Cry3A protein as expressed in Event MIR604 corn will pose no hazard to humans or domestic animals upon commercial approval of the use of Event MIR604 corn. Additionally, the PMI protein has been granted a permanent exemption from tolerance in all crops from the U.S. EPA based on an extensive body of data that demonstrates this selectable marker presents no risk to humans, animals or the environment.

Event MIR604 hybrids have been field tested by Syngenta Seeds, under Notifications granted by USDA APHIS since 2001 in the primary corn growing regions of the midwestern US as well as in Hawaii and Puerto Rico. Information and data collected during these trials indicates that Event MIR604-derived hybrids exhibit no plant pathogenic properties, have no impact on biodiversity and are unlikely to harm other insects that are beneficial to agriculture. As corn does not establish weedy populations and has lost the ability to survive outside cultivation, MIR604 hybrids are no more likely to be a weed than non-transgenic maize. In addition, mCry3A is not expressed in pollen and thus MIR604 hybrids are unlikely to have an adverse ecological effect due to pollen exposure. Event MIR604-derived hybrids will lead to minimal exposure of the environment to mCry3A, will not promote gene flow increase the weediness or plant pest potential of any other cultivated plant or wild species or have an impact on non-target species, including humans.

Based on the information and data contained in this petition, Syngenta requests that USDA-APHIS/BRS make a determination of non-regulated status for Corn Rootworm Protected Transformation Event MIR604, any progeny derived from crosses between MIR604 and other corn varieties, and progeny derived from crosses of MIR604 with other transgenic corn varieties that have received non-regulated status under 7 CFR Part 340.

Statement of Grounds Unfavorable

The undersigned certifies, that to the best of their knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner that are unfavorable to the petition.



Syngenta Seeds, Inc.
3054 East Cornwallis Road
Research Triangle Park, NC 27709-2257
Phone: (919) 541-8652
Fax: (919) 541-8535

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Abbreviations Used in this Petition

aadA	Adenylytransferase gene
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service
Bp	Base pair
Bt	*Bacillus thuringiensis*
Bwt	Body weight
C	Concentration
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
ColE1	*E.coli* origin of replication
Cry	Crystal protein delta endotoxins
DDD	Daily dietary dose
DNA	Deoxyribonucleic acid
DT_{50}	Time to dissipation of 50% of the initial bioactivity
EEC	Estimated exposure concentration
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FIR	Food Intake Rate
HPLC	High Performance Liquid Chromatography
IFBC	International Food Biotechnology Council
ILSI	International Life Sciences Institute
IRM	Insect resistance management
Kb	Kilobase
kDa	Kilodalton
Kg	Kilogram
LB	Left border
LC_{50}	50% lethal concentration
LC_{90}	90% lethal concentration
LD_{50}	50% lethal dose
LOQ	Level of quantitation
manA	Phosphomannose isomerase gene from *Escherichia coli*
mcry3A	gene in event MIR604 encoding the modified Cry3A insecticidal protein
mCry3A	the modified Cry3A insecticidal protein expressed in Event MIR604
µg	Microgram
mg	Milligram
MOE	Margins of exposure
MRID No.	Master Record Identification Number
MTL	maize promoter from a metallothionein-like gene
NOEC	No observable effect concentration

NOEL	No observable effect level
nos	Nopaline synthase terminator
OECD	Organization for Economic Cooperation and Development
PBN	FDA Pre-market Biotechnology Notification
PCR	Polymerase chain reaction
PMI	Selectable marker protein phosphomannose isomerase
pmi	Gene in event MIR604 encoding the selectable marker protein
PPQ	Plant Protection and Quarantine
pZM26	Plasmid employed to create event MIR604
RB	Right border
RepA	Bacterial replication protein
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SOP	Standard operating procedure
spec	Spectinomycin resistance gene
T0	First generation transgenic
T1	Second generation transgenic
T-DNA	Transfer DNA
Ti-plasmid	Tumor inducing plasmid
ubq3	Ubiquitin-3 gene isolated from *Arabidopsis*.
Ubq3int	Promoter plus the first intron isolated from *Arabidopsis* ubiquitin-3 gene.
USDA	United States Department of Agriculture
US EPA	United States Environmental Protection Agency
VS1	*Agrobacterium* origin of replication
WHO	World Health Organization

Chapter 1. Syngenta Seeds Petition for the Determination of Non-Regulated Status of Corn Event MIR604

A. RATIONALE FOR THE DEVELOPMENT OF EVENT MIR604 CORN

Corn rootworm (*Diabrotica* spp), a Coleopteran pest ubiquitous to corn growing areas of the United States, costs farmers an estimated 1 billion dollars in crop damage and control costs each year (Ostlie, 2001; Payne *et al*., 2003). Rootworm larvae feed upon the root systems of young, developing corn seedlings. Moderate pruning of roots by rootworms can lead to plant stress during periods of inadequate moisture due to the inability of the corn plant to adequately translocate water and minerals from the roots to the rest of the plant. Corn plants suffering from moderate to severe root pruning are also susceptible to lodging during rain and wind storms (Wedberg, 1996). Corn ears on lodged plants may under develop or not be harvestable due to inaccessibility to harvest equipment.

The rootworm life cycle plays an integral role in the extent and severity of damage caused by this pest. Rootworm eggs over-winter in the top six inches of soil and begin hatching from May to mid-June depending upon the environmental conditions. The larvae go through three larval stages (instars) all of which feed on corn roots. The 1st instar larvae feed on the smaller, branching roots while the later instars feed on the inner root tissues and invade the brace roots (Wedberg, 1996). As the rootworm eggs will hatch over a long period of time, different larval stages will be present at any given time. After approximately 3 weeks of feeding the larvae enter the pupal stage whereupon the adult beetle will emerge after 6 to 10 days. Females reach sexual maturity slowly and do not begin laying eggs until 2 weeks after mating. A female corn rootworm beetle can lay up to 1000 eggs over a several week period. The eggs are in a diapause condition and must go through a cold incubation before the larvae will hatch the following spring.

Management practices employed to reduce rootworm damage generally fall into two categories: (1) crop rotation and (2) application of chemical insecticides. As the preferred diet of rootworm larvae is corn roots and corn rootworms beetles lay their eggs in a diapause condition, farmers have adopted a crop rotation strategy that can significantly interrupt the corn rootworm life cycle. By following a first year corn crop with soybeans or other non-corn crop in the second season’s planting, the population of emerging beetles is reduced as the primary food source (corn roots) for the hatching larvae is not available. With the pest population reduced, corn can then be planted again in the third season with reduced risk of economic impact. This crop rotation (primarily corn and soybeans) has been used since the mid-70s as a very effective non-chemical control strategy for corn rootworm.

In areas where crop rotation has been used extensively, rootworm variants have evolved which have minimized the effectiveness of the crop rotation strategy. The first adaptation is referred to as the ‘extended diapause’ variant and was first documented in Northwestern Iowa. In this instance, eggs laid by Northern CRW beetles remain in diapause 2 or more winters before hatching. In 2002, USDA-NASS (USDA, 2002)

estimated that this extended diapause variant is present in 9.8 million acres of corn in the U.S.

The second adaptation, referred to as the 'soybean variant', was first documented in east central Illinois. In this case, the Western CRW adult beetle migrates from the cornfield and deposits its eggs in neighboring soybean fields, which hatch in the following year's corn crop. According to USDA-NASS estimates, in 2002, 7.1 million acres of corn were planted in the heart of the soybean variant infestation zone that covers large portions of Illinois and Indiana. According to Payne *et al*. (2003), this new variant of CRW has also spread through most of northern Indiana, eastern Illinois, southern Michigan and western Ohio. Given historic movement patterns, the new variants may soon spread as far west as eastern Iowa (Onstad *et al*., 1999). This expanded area is equivalent to 16 million acres with the westward expansion being the greatest threat. Western CRW have also been detected in soybean fields located in counties extending to the Wisconsin border.

The second common practice of corn rootworm control is soil applied insecticides and seed treatments. Most chemical control practices target the larval stage, applying insecticide into the soil to protect roots from larval feeding. It is important that the insecticide provides effective control for at least 7-10 weeks, as eggs hatch over a 3-6 week period and feeding continues for an additional 3-4 weeks. There are several factors that affect insecticide performance including planting date, insecticide placement, insecticide characteristics and environment. Seed treatments are another form of chemical control available to corn growers. However their effectiveness is generally poor in circumstances where CRW pressure is moderate to high. In 2003, farmers applied nearly 6 million pounds of insecticide over 21 million acres at a cost of $216,000,000.

Syngenta scientists have developed a corn event, designated Event MIR604, that expresses a novel proteinaceous active ingredient that provides for excellent protection from rootworm larval damage during field trials conducted since 2001. Event MIR604 expresses a modified Cry3A insect control protein that provides economic control of the three major corn rootworm species in the U.S., western corn rootworm (*Diabrotica virgifera virgifera* Le Conte), northern corn rootworm (*D. longicornis barberi* Smith and Lawrence) (Chen and Stacy, 2003) and Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith). Farmers who plant this product will realize significant benefits of consistent performance and protection against yield loss from rootworm damage.

Syngenta Seeds, Inc. requests the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, make a determination that the article Event MIR604, described herein, should not be regulated under 7 CFR part 340.

B. BACKGROUND INFORMATION

B.1. Modified Cry3A Plant-Incorporated Protectant

Syngenta Seeds has developed a genetically improved line of corn, Event MIR604, which produces a modified Cry3A (mCry3A) insect control protein. The pesticidal mCry3A protein is related to a class of insecticidal proteins that are produced in crystalline inclusions during sporulation of the gram-positive soil bacterium *B. thuringiensis*. The source of the native, unmodified Cry3A protein is *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*) (Sekar *et al.*, 1987). mCry3A has a similar spectrum of activity to the native Cry3A, but with additional very markedly enhanced, commercially exploitable toxicity towards the Western corn rootworm (WCRW; *Diabrotica virgifera virgifera* Le Conte), Northern corn rootworm (NCRW; *D. longicornis barberi* Smith and Lawrence) and Mexican corn rootworm (MCRW; *Diabrotica virgifera zeae* Krysan and Smith), all of which are major Coleopteran pests of corn in the U.S. Importantly, mCry3A has no activity either against other corn pests including the various Lepidoptera that attack corn or against non-pest insect species.

The amino acid sequence of the mCry3A protein corresponds to that of the native Cry3A protein from *B.t.t.*, except that (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 (Rabe, 2004). This cathepsin G recognition site has the sequence alanine-alanine-proline-phenylalanine (AAPF), and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein. Cathepsin G is a chymotrypsin-like serine protease.

Syngenta scientists recently discovered that the midguts of WCRW larvae contain cathepsin G-like activity (Chen and Stacy, 2003). The mCry3A protein as expressed in Event MIR604 plants was designed to have significantly greater toxicity to WCRW by inserting the cathepsin G site that could be recognized by this protease (Garcia-Alonso and Vlachos, 2003).

B.2. Molecular Analysis of DNA Insert in Event MIR604

Event MIR604 corn was created via *Agrobacterium*-mediated transformation of plasmid pZM26 into embryonic corn tissue suitable for transformation. A complete description of the donor genes, regulatory sequences, transformation vector and transformation method is provided in Chapter 3, **Molecular Analysis of Event MIR604** (Rabe, 2004).

Maize (corn) derived from Syngenta's transformation Event MIR604 contains two transgenes: (1) the modified *cry3A (mcry3A)* gene encoding the mCry3A insect control protein and (2) the *pmi* (*manA*) gene from *Escherichia coli,* which encodes the enzyme phosphomannose isomerase (PMI) as a selectable marker. PMI has no pesticidal properties and is considered an 'inert ingredient' by the US EPA. PMI allows transformed corn cells to utilize mannose as a sole carbon source, while corn cells lacking the *pmi* gene fail to grow (Negrotto *et al.*, 2000).

Expression of the *mcry3A* gene in Event MIR604 is driven by the maize promoter from a metallothionein-like gene (MTL promoter; de Framond, 1991); it confers root-preferential gene expression in corn. Expression of the *pmi* gene in Event MIR604 is driven by the maize polyubiquitin promoter (Christensen *et al.,* 1992), which confers constitutive expression in monocots.

Data from Southern hybridisation analysis and DNA sequencing demonstrate that single copies of both the *mcry3A* and *pmi* genes are present in Syngenta's corn Event MIR604. Additionally, Event MIR604 does not contain any of the backbone sequences from the transformation plasmid pZM26. Sequence analysis of the entire T-DNA insert present in Event MIR604 confirms that the overall integrity of the intended insert and contiguousness of the functional elements have been maintained. A 43 base-pair truncation at the right border junction of the T-DNA insert and 44 base-pair truncation at the left border junction of the T-DNA insert were identified. Three single nucleotide changes were also identified relative to the intended DNA sequence. One of these changes occurred within a promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and give rise to two amino acid changes. The nucleotide changes identified in the *pmi* gene in Event MIR604 resulted in the substitution of (1) alanine in place of valine-61 and (2) histidine in place of glutamine-210 in the amino acid sequence of the PMI protein. These substitutions have not resulted in any apparent functional change in PMI as expressed in Event MIR604 (Hill, 2004).

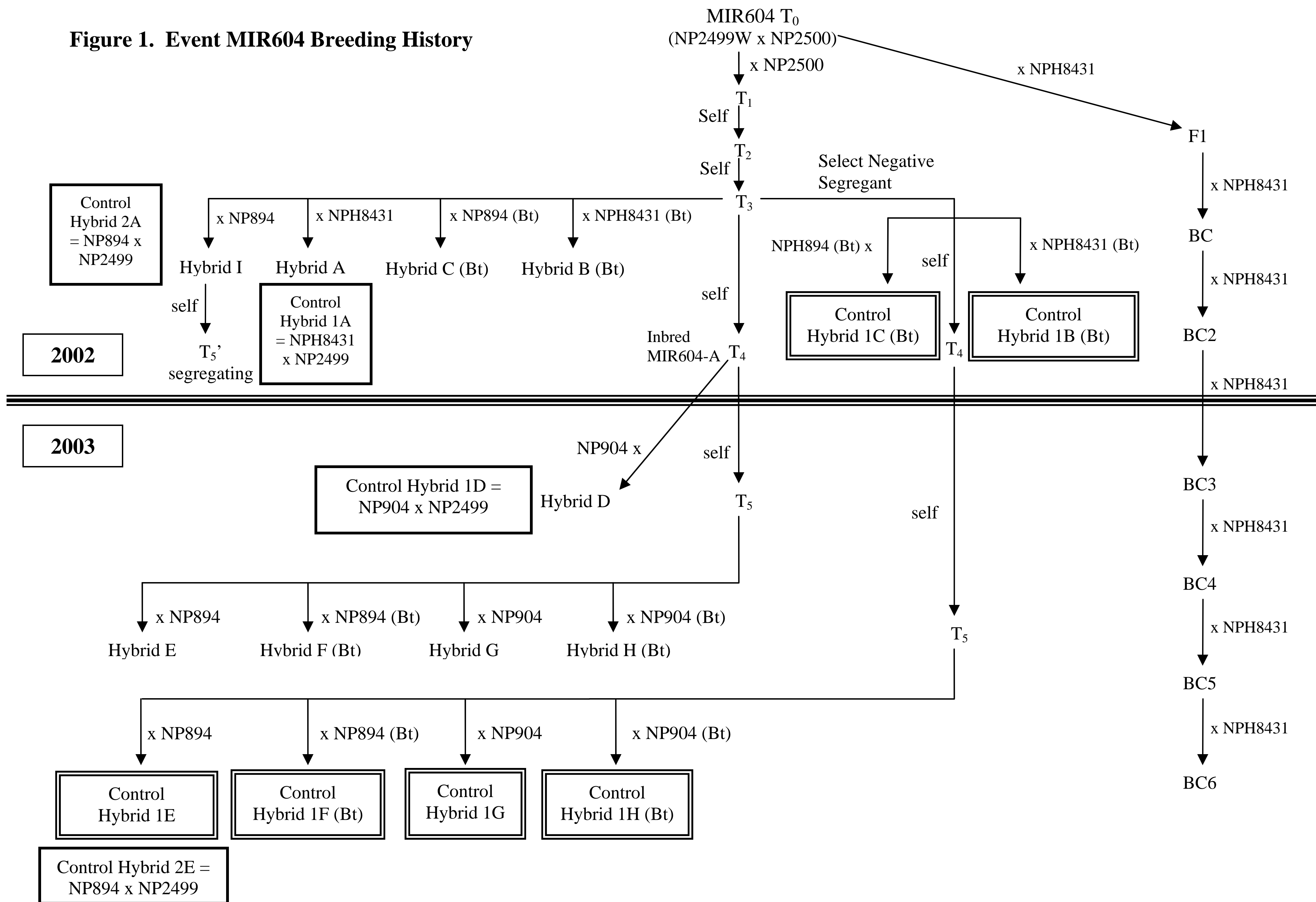
Statistical analysis confirmed the expected Mendelian inheritance ratio for both *mcry3A* and *pmi*. Additional information confirming the stability of expression of these genes in Event MIR604 is available in an accompanying data volume (Joseph and Hill, 2003). Levels of mCry3A and PMI proteins were determined to be stable in MIR604 plants over four successive backcross generations.

B.3. Breeding History of Event MIR604

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604 (see Figure 1). The pZM26 plasmid was transformed into a heterogenous genetic background (NP2499W x NP2500) derived from a cross between two dent corn inbred lines selected for its suitability to transformation by Agrobacterium. Once transformed, this line, designated Event MIR604, was crossed back into one of the parental inbred lines and then selfed 2 to 4 times to fix the transgene in the germplasm. A number of elite inbred lines were crossed into the Event MIR604 line and material generated from these crosses was used in agronomic and other studies (Figure 1). The early and late maturity MIR604 hybrids were made up by crossing different inbred corn lines which display varying days to maturity (maturity testers) into the fixed MIR604 germplasm.

Control germplasm was derived by one of two methods. In some experiments, the non-transgenic, near isogenic hybrids were constructed by using non-transformed germplasm closely matched to the genetic background of MIR604 for that particular stage of the breeding pedigree (indicated by single lined boxes in Figure 1). In other experiments, negative segregants were identified and used as breeding partners in crosses to elite inbred lines (indicated by double lined boxes in Figure 1).

Figure 1. Event MIR604 Breeding History



In order to reduce the confounding effect on yield of insect damage to the corn ears by lepidopteran pests, in some trials MIR604 was deployed in a breeding stack with Bt11 which expresses the Cry1Ab protein (indicated by '(Bt)' in Figure 1). In these cases controls consisted of the corresponding Bt11-only hybrids.

B.4. Agronomic Performance of Event MIR604

In 2002 and 2003, 22 different hybrids derived from traditional breeding of Event MIR604 were evaluated at 33 locations in the states of IA, IL, IN, KS, KY, MN, MO, NE, SD, TX, WI, for either insect efficacy evaluation or agronomic assessment, and yield (Chapter 4, **Agronomic Performance of Event MIR604 Hybrids**.). Event MIR604 hybrids consistently demonstrated statistically significant reductions in root damage relative to non-transgenic controls at all locations. Event MIR604 also provided statistically significant increases in harvestable yield compared to near isogenic controls under adverse growing conditions (e.g., drought), and was either equivalent or in some cases better in yield performance under non-adverse growing conditions. For all other agronomic traits, sporadic statistical differences were observed between Event MIR604 and its non-transgenic control but none were consistent over the two years of data collection or across locations.

B.5. Compositional Analysis of Event MIR604

Key nutritional components in maize grain and whole plants (forage) derived from Event MIR604 and near-isogenic control plants were compared. The whole plants and grain analyzed were from hybrid pairs (a hybrid pair consisting of transgenic and near-isogenic control plants) grown at 12 locations in the USA over two growing seasons (2002 and 2003). As would be expected from an analysis of this size, sporadic statistically significant differences were observed for some parameters between the MIR604 transgenic and near-isogenic controls. All components evaluated in this study were within the range of reported literature values for maize, with the exception of two components, potassium and phytosterols. At the time of analysis, a control range for concentrations of potassium in conventional corn forage was not available. Both control and transgenic values for phytosterols in grain were below the average concentration reported in the literature. For all other analytes, no consistent patterns emerged to suggest that biologically significant changes in composition or nutritive value of the grain or forage had occurred as an unintended result of the transformation process or expression of the transgene. The conclusion based on these data is that grain and forage from genetically modified MIR604 hybrids are substantially equivalent in composition to the near-isogenic controls, and other commercial hybrids. Further details of this study can be found in Chapter 5, **Compositional Analysis of Event MIR604.**

B.6. The Mode of Action of Modified Cry3A Protein

Syngenta scientists have investigated the solubilization, proteolytic processing, receptor binding, and pore forming properties of the mCry3A protein in side-by-side comparisons with native Cry3A. The results showed that the solubility, and pore forming properties of mCry3A protein were similar compared to native Cry3A protein. The introduction of the cathepsin G recognition site into the Cry3A protein results in a much more rapid and complete processing of the 67 kDa mCry3A protein to a 55 kDa product

as compared to native Cry3A protein. Also, binding of the 67 kDa mCry3A protein to first instar WCRW membranes is similar to that of native Cry3A, but binding of the 55 kDa product is enhanced in the case of the mCry3A protein. Thus, mCry3A would be expected to behave, from a mode of action standpoint, as other pore-forming Cry proteins characterized to date. Further details of these studies can be found in Garcia-Alonso and Vlachos (2003) and in Chen and Stacy (2003) and Steiner et. al. (2005).

B.7. Environmental Safety Assessment

A comprehensive environmental safety assessment has been conducted on mCry3A as the active principle in Event MIR604. This assessment included a literature and data review of the specificity of both native Cry3A and mCry3A for vertebrate and non-vertebrate species. Laboratory assessments of toxicity to vertebrate and non-vertebrate organisms as well as carefully chosen non-target organisms were also performed. Further details of these studies are provided in Chapter 7, **Environmental Safety**.

The data show that the toxicity of native Cry3A proteins is specific to certain Coleoptera of the families Chrysomelidae, Tenebrionidae and Curculionidae. No toxic effects have been reported on non-target insect species or on other invertebrates or vertebrates. The modified Cry3A (mCry3A) toxin as expressed in Event MIR604 maize (corn) has an extended spectrum of activity within the Chrysomelidae family of coleopteran species, such that it has enhanced toxicity to the major corn pest species WCRW, NCRW and MCRW, but its activity against other species tested to date appears to be unchanged.

Several non-target species were selected for individual laboratory safety assessment based on their potential for exposure in an agricultural setting, taxonomic similarity to the target pest, feasibility of testing and to meet certain U.S. EPA requirements. The test species included: bobwhite quail (*Colinus virginianus*), rainbow trout (*Oncorhynchus mykiss*), insidious flower bug (*Orius insidiosus*), seven spot ladybird (*Coccinella septempunctata*), a rove beetle (*Aleochara bilineata*), ground beetle (*Poecilus cupreus*), honeybee (*Apis mellifera*), earthworm (*Eisenia foetida*). Hazard and exposure assessments were performed on each test species to determine minimum marginal exposure limits at pre-selected, appropriate endpoints for each species. No harmful effects were detected in any of the tests performed on the non-target species (Raybould, 2004b). The large body of ecological toxicity data support the conclusion that the modified Cry3A protein as expressed in Event MIR604 corn will pose little to no risk to the environment.

B.8. Food and Feed Safety Assessment

A thorough assessment of the safety, including the potential for exposure, mammalian toxicity and allergenicity, of both the mCry3A and PMI proteins as expressed in Event MIR604 has been conducted. Details of the safety assessment and supporting studies can be found in Chapter 8, **Food and Feed Safety.** The data support the conclusion that neither the mCry3A nor the PMI protein as expressed in Event MIR604-derived corn will pose health hazards to humans or domestic animals upon commercial approval of the use of Event MIR604-derived corn.

Although prior dietary exposure to the mCry3A protein has not occurred, it is conceivable that a low level of dietary exposure to the native Cry3A protein has occurred, as it is registered for use in the U.S. in various *B.t.*-based microbial insecticides (Trident, Foil, Ditera, M-Trak, Novodor, Raven) and NewLeaf® Bt potatoes, and is permanently exempt from food and feed tolerances.

Expression levels of mCry3A in Event MIR604 plants were assessed across all growth stages. Mean mCry3A levels measured in leaves, roots and whole plants ranged from *ca.* 3 - 23 µg/g fresh wt. (4 - 94 µg/g dry wt.), *ca.* 2 - 14 µg/g fresh wt. (7 - 62 µg/g dry wt.), and *ca.* 0.9 - 11 µg/g fresh wt. (3 - 28 µg/g dry wt.), respectively (Joseph and Hill, 2003). Mean mCry3A levels measured in kernels at seed maturity and senescence ranged from *ca*. 0.6 – 1.4 µg/g fresh wt. (0.8 – 2.0 µg/g dry wt.).

Modified Cry3A protein (mCry3A) was analyzed in wet- and dry milled fractions generated from standard food processing procedures carried out on maize (corn) grain derived from Event MIR604, together with a corresponding non-transgenic control (Joseph and Kramer, 2003). Quantifiable levels of mCry3A were detected in various wet- and dry-milled fractions ranging from 2.12 µg mCry3A/g in flaking grits to below detectable levels in coarse fiber, germ, and starch. Although the concentration of mCry3A measured in the flour used in the preparation of corn chips was 0.32 µg mCry3A/g, no mCry3A was detected in the corn chips. Similarly, mCry3A was not detectable in oil, whereas the starting material, flaking grits, contained the highest level of mCry3A.

Both mCry3A and PMI recombinant proteins expressed in *E. coli*, and demonstrated as equivalent to their respective proteins as expressed in Event MIR604-derived corn tissue, have been evaluated in acute oral mouse toxicity studies (Johnson, 2003; Kuhn, 1999). No adverse effects were observed for mCry3A at a dose of 2377 mg/kg body weight and no adverse effects were observed for PMI at 3080 mg/kg body weight. Both mCry3A and PMI protein sequences were evaluated for similarity to known toxin and allergen sequences. Neither mCry3A nor PMI showed significant amino acid homology to any proteins identified as or known to be toxins. In addition, mCry3A did not show any significant homology to known or putative allergens. The PMI protein sequence was found to have one region of sequence homology of eight contiguous amino acids with a recently identified frog allergen α–parvalbumin (Hilger *et al*., 2002). Upon subsequent specific serum testing with patient serum, it was established that PMI does not cross react with antibodies which recognize the frog α–parvalbumin allergen. This indicates that the allergic patient’s serum IgE does not recognize any portion of the PMI protein as an allergenic epitope and, therefore, the low degree of amino acid sequence homology between the PMI protein and α–parvalbumin from edible frog is not biologically relevant or indicative of allergenic potential for PMI (Hart and Rabe, 2004d). Additionally, neither mCry3A nor PMI are derived from a known source of oral allergens, both are heat labile (Joseph, 2003; Hill, 2003), and both are readily digested in simulated mammalian gastic fluid (Joseph and Graser, 2003c; Privalle, 1999), further supporting the conclusion that neither mCry3A nor PMI are likely food allergens.

B.9. Environmental Consequences of Introduction of Event MIR604-derived Hybrids

Expression of mCry3A in Event MIR604-derived hybrids has been shown to occur in root and other tissues, and during the course of normal agricultural practice, the active principle mCry3A could *theoretically* enter neighboring environments through establishment of weedy populations of MIR604 plants outside fields, through transfer and expression of the mCry3A gene in other organisms via sexual hybridization or horizontal gene transfer, through off-crop movement of MIR604 pollen or via degradation of plant tissue and subsequent movement of mCry3A through soil.

The risk that mCry3A could persist or spread more widely because of gene flow or the establishment of weedy populations of MIR604 is very low given that species of *Zea* other than cultivated corn are not recorded outside botanical gardens in the USA and those related species which are widespread do not hybridize readily with cultivated corn. Corn has lost the ability to survive outside cultivation and is unlikely to form self-sustaining weedy populations in agriculture; it is easily controlled in subsequent crops with selective herbicides and seed dispersal is limited because seeds are held inside the husks of the cob. These properties indicate that exposure to mCry3A protein will be limited to the fields in which MIR604 corn will be grown and mCry3A is unlikely to persist in fields for a long period after grain from MIR604 hybrids have been harvested.

In addition to the low exposure risk, MIR604 is simpler to use and provides more consistent performance that results in superior efficacy and a positive yield impact compared to the chemical alternatives. In terms of reduction of root damage, the efficacy data show that MIR604 corn performs significantly better than non-treated and chemically treated controls. Yield data show an overall positive trend comparing MIR604 corn to both non-treated and chemically treated controls particularly in unfavorable growing conditions. To help insure the continued utility and long-term efficacy of MIR604 hybrids an appropriate insect resistance management (IRM) program has been proposed.

Introduction of Event MIR604-derived hybrids will lead to minimal exposure of the environment to mCry3A, will not promote gene flow or weediness in corn and provide yield and efficacy benefits, thus producing minimal consequence when introduced as a commercial product. Further detailed information can be found in Chapter 9, **Environmental Consequences of Introduction.**

C. REGULATORY PERMIT STATUS

An application for an Experimental Use Permit for mCry3A as a plant-incorporated protectant (Experimental Use Permit for Field Testing of the Modified Cry3A *Bacillus thuringiensis* Insect Control Protein as Expressed in Event MIR604-Derived Corn Plants (67979-EUP-U)) was submitted to the U.S. EPA on December 15, 2003 and approved on March 23, 2005. A permanent exemption from the requirement of tolerances for the inert marker protein, PMI, was recently granted for all crops (U.S. EPA, 2004a) on May 14, 2004. An amendment/extension to the existing Experimental Use Permit for MIR604 was submitted to the US EPA on August 16 2005 and approved by EPA on March 2 2006.

Syngenta Seeds, Inc. has also filed a U.S. EPA FIFRA Section 3 registration application on April 30, 2004 titled Plant-Incorporated Protectant Active Ingredient Modified Cry3A Protein as Expressed in Event MIR604-Derived Corn Plants (67979-L). The Section 3 application is expected to be approved by EPA in 2006. Accompanying this application was a request to the US EPA for a permanent exemption from the requirement of tolerances for mCry3A (US EPA, 2004b).

mCry3A corn event MIR604 also falls within the scope of the US Food and Drug Administration's (FDA) 1992 Statement of Policy: Foods Derived from New Plant Varieties, including genetically engineered varieties pursuant to 21 CFR Section 192.25 of the Federal Food, Drug, and Cosmetic Act. Syngenta has initiated a consultation with FDA and has filed a Pre-market Biotechnology Notification (PBN #0099) in 2005 and expects approval in 2006.

Syngenta is also pursuing field trial applications in the U.S. (see Table 1) to facilitate commercial development and regulatory approvals for Event MIR604 hybrids in the U.S. Event MIR604 hybrids have been planted in several states under USDA-APHIS comprehensive permit and notification since 2001.

Table 1. Summary of USDA-APHIS Notification/Permits and Field Trial Reports for Field Trials Planted with Event MIR604.

Year	USDA Notification or Permit No.	Planted Trial Sites by State[1]	Status of USDA field trial reports
2001	01-018-01n	HI	Submitted
	01-022-07 r/m	IL, PR	Submitted
2002	02-022-01 r/m	FL, IL, MN, TX	Submitted
	02-022-02 r/m	HI	Submitted
2003	03-021-01 r/m	FL,IA,IL,IN,KS,KY,MN,MO,MS,NE,PR,SD,TX,WI	Submitted
	03-021-02 r/m	HI	Submitted
	03-287-07n	PR	Submitted
	03-287-05n	FL, HI, IL	Submitted
	03-287-02n	HI	Submitted
	03-287-01n	HI	Submitted
	03-287-10n	HI	Submitted
	03-287-11n	HI	Submitted
	03-287-04n	IL	Submitted
	03-300-04n	PR	Submitted
2004	04-072-06n	AR,CA,CO,FL,HI,IA,ID,IL,IN,KS,KY,LA,MD,MN, MO,MS,NC,NE,NM,NY,OH,PA,PR,SD,TX,VA,WI	Submitted
	04-076-04n	FL,HI,IA,ID,IL,IN,KY,MN,MS,NE,PA,PR,SD,WI (IR/HT stacks)	Submitted
	04-085-08n	IA	Submitted
	04-086-03n	IL	Submitted
	04-140-01n	IA	Submitted
	04-203-09n	PR (IR/HT stacks)	Submitted

[1]If Status of USDA field trial reports listed as In Progress, then Trial sites may be approved, but not all trial sites planted.

Chapter 2. The Corn (Maize) Family

The following was excerpted, with minor edits from USDA APHIS Environmental Assessment 92-042-01 (authored by Dr. James Lackey), the Canadian Food and Inspection Authority (CFIA) Regulatory Directive Dir94-11 [http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.shtml] (CFIA, 1994) and the Organization for Economic Cooperation and Development (OECD) Consensus Document on the Biology of *Zea mays* subsp. mays (Maize) [http://www.oecd.org/document/9/0,2340,en_2649_201185_1889395_1_1_1_1,00.html] (OECD, 2003). Full descriptions and complete references can be obtained from these documents.

A. GENERAL DESCRIPTION OF *ZEA MAYS* L. (MAIZE/CORN)

Zea is a genus of the family Graminae (Poaceae), commonly known as the grass family. Maize (*Zea mays* L.) is a tall, monecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels) and pistillate inflorescences in the leaf axils, in which the spikelets occur in 8 to 16 rows, approximately 30 cm long, on a thickened, almost woody axis (cob). The whole structure (ear) is enclosed in numerous large foliaceous bracts and long styles (silks) protrude from the tip of the ear as a mass of silky threads (Hitchcock and Chase, 1971). Pollen is produced entirely in the staminate inflorescence and eggs, entirely in the pistillate inflorescence. Maize is wind pollinated and both self and cross-pollination are usually possible. Shed pollen usually remains viable for 10 to 30 minutes, but can remain viable for longer durations under favorable conditions (Coe *et al*., 1988). Cultivated maize is presumed to have been derived from teosinte (*Z. mexicana*) and is thought to have been introduced into the old world in the sixteenth century. Maize is cultivated worldwide and represents a staple food for a significant proportion of the world's population. No significant native toxins are reported to be associated with the genus Zea (International Food Biotechnology Council, 1990).

B. ORIGIN OF THE SPECIES *ZEA MAYS* L.

It is generally agreed that teosinte (*Z. mexicana*) is an ancestor of maize, although opinions vary as to whether maize is a domesticated version of teosinte (Galinat, 1988). Teosinte is an ancient wild grass found in Mexico and Guatemala. Because it has differentiated into various races, species and plant habits, taxonomic classification is still a matter of controversy. Doebley and Iltis (1980) and Iltis and Doebley (1980) classified the annual teosintes into two subspecies of *Z. mays*: ssp. *mexicana* (including races Chalco, Central Plateau and Nobogame) and ssp. *parviglumis*-var. *parviglumis* (race Balsas) and var. *huehuetenangensis* (race Huehuetenango) and the species *Z. luxurians* (race Guatemala). The perennial teosintes from Jalisco, Mexico are separated into two more species according to ploidy, *Z. perennis* and *Z. diploperennis*. The Meso-American region located within middle South Mexico and Central America is recognised as one of the main centres of origin and development of agriculture as well as centre of origin and diversification of more than one hundred crops (Vavilov, 1951; Smith, 1995; Harlan, 1992). At the present time, there is no agreement about where exactly maize was

domesticated and there are several proposals in this regard. Based on the findings of archaeological materials from the maize plant (pollen, cobs, husks, and other remnants) in the United States and Mexico, which are older than those found in South America, Randolph (1959) proposed that maize was domesticated, independently, in the southwestern United States, Mexico, and Central America. Mangelsdorf (1974) proposed that "corn had not one origin but several in both Mexico and South America", because the archaeological evidences are found in Mexico and several morphological characteristics of extant populations are found in the maize races of South America (Andes region) in comparison to those races of Meso-America.

C. CULTIVATION AND USE OF CORN

As discussed above, maize has been cultivated by the indigenous peoples of North America for thousands of years. The modern era of maize hybrid production began in the U.S. where research conducted in the early part of this century proved that hybrid maize could produce a yield superior to open-pollinated varieties (Sprague and Eberhart, 1977). Gradually, hybrid-derived varieties replaced the open-pollinated types in the 1930's and 1940's. Almost all corn grown in the United States now comes from hybrid seed that is obtained every planting season from private enterprises; the older open-pollinated varieties are virtually unknown in commerce (Hallauer et. al., 1988).

The production of hybrid seed requires the development and maintenance of inbred lines and subsequent controlled crosses to produce commercial seed. Self pollination is essential for inbred development while controlled cross pollination is mandatory for hybrid seed production. Mechanisms have been developed to ensure the correct form of pollination for each process and to prevent genetic contamination of seed stocks (Wych, 1988). Breeder or foundation seed is produced from self pollinated seed after the eighth or ninth generation of inbreeding. A high degree of self pollination is assured by planting in blocks that are isolated by a distance of at least 200 meters (~660 ft.) from any other contaminating source of pollen. Hybrid seed production is accomplished by interplanting rows of the male and female inbred parents (e.g., one row of male to four female rows). Hybrid seed production requires isolation similar to that for foundation seed. Self pollination of the female parent is prevented through detasseling prior to pollen shed or by the use of male sterile females. Genetic conformity of inbreds and hybrids is monitored and assured through grow-outs of representative seed lots and laboratory screening using such criteria as isozyme profiles.

Maize is planted when soil temperatures are warm (greater than or equal to 10°C) usually mid to late April through to mid-May in the US corn belt. Optimum yields occur when the appropriate hybrid maturity and population density are chosen. In addition, exogenous sources of nitrogen fertilizer are generally applied and weed and insect control measures are generally recommended. Choice of the appropriate hybrid for the intended growing area helps to ensure that the crop will mature before frost halts the growth of the plant at the end of the season; hybrids are categorized according to the amount of "heat units" that will be required for maturity. Therefore, a hybrid developed for a specific heat unit zone, will not mature in (cooler) areas that receive fewer "heat units". Traditional cultivation practices in maize often result in bare soil which is susceptible to erosion by wind or water; increasingly, "no till" maize is being grown in an effort to reduce this soil loss.

In 2003, there were more than 78 million acres planted to corn in the United States producing over 10 billion bushels of grain (USDA, 2004). Maize grown in the U.S. is predominantly of the yellow dent type, a commodity crop largely used to feed domestic animals, either as grain or silage. The remainder of the crop is exported or processed by wet- or dry-milling to yield products such as high fructose maize syrup and starch or oil, grits and flour. These processed products are used extensively in the food industry. For example, maize starch serves as a raw material for an array of processed foods, and in industrial manufacturing processes. Since the early 1980's a significant amount of grain has also been used for fuel ethanol production. The by-products from these processes are often used in animal feeds. For a full discussion of the uses of maize see Watson (1988).

D. POLLINATION OF CORN

Pollination, fertilization, and caryopsis development of corn follows the same pattern for chasmogamous wind-pollinated grasses, with the following exceptions:

1. Pollen is produced entirely in the staminate inflorescences. Eggs are produced entirely in the pistillate inflorescences.
2. Self-pollination and fertilization and cross-pollination and fertilization are usually possible and frequencies of each are usually determined by physical proximity and other environmental influences on pollen transfer. A number of complicating factors, such as genetic sterility factors and differential growth rates of pollen tubes may also influence the frequencies of self-fertilization versus cross-fertilization.
3. Corn styles and corn pollen tubes are the longest known in the plant kingdom.
4. Shed pollen typically remains viable for 10 to 30 minutes, but may remain viable of much longer under refrigerated conditions (Coe *et al*., 1988).
5. Pollen dispersal is limited due to its large size (0.1 mm diameter) and spherical nature. Numerous studies have shown that greater than 98% of pollen settles to the ground within 60 meters of its source (Raynor *et al*., 1972; Luna *et al*., 2001; Burris, 2002).
6. The staminate and pistillate inflorescences do not develop at the same time. The pistillate inflorescence is precocious. However, there is the appearance of slight protandry because the elongating styles (silks) are delayed for about seven days in emergence from the bracts of the pistillate inflorescence, while the later-developing staminate inflorescence is fully visible. The silks are receptive to pollen up to 10 days after emergence, but receptivity decreases rapidly thereafter (Walden and Everett, 1961).

Corn is primarily wind pollinated; insects are responsible for insignificant amounts of pollen dispersal (Russell and Hallauer, 1980).

E. INTER-SPECIES/GENUS HYBRIDIZATION

Maize and other species and subspecies of teosinte are sexually compatible and can produce fertile hybrids (Wilkes, 1977). Related *Zea* species are geographically restricted and occur only in Mexico and Guatemala. The closest known relative of *Zea* is

Tripsacum, a genus of eleven species, widely distributed between 42°N and 24°S latitude (de Wet *et al*., 1981). Three species occur in the United States, two of which, *Tripsacum floridanum* (Florida Gamagrass) and *Tripsacum lanceolatum* (Mexican Gamagrass), are confined to the southernmost states of the United States. Only one, *Tripsacum dactyloides* (Eastern Gamagrass), has a distribution that includes the northern (U.S.) maize belt (Gould, 1968).

F. POTENTIAL FOR INTROGRESSION FROM *Z. MAYS* INTO RELATIVES

An examination of the literature prior to 1980 would lead to the conclusion that there is constant gene flow between maize and teosinte, and that the weedy teosinte (*Z. mays* ssp. *mexicana*) is a hybrid of the two sub-species, and functions as a genetic bridge between the two (de Wet and Harlan, 1972; de Wet, 1975; Galinat, 1973). However, this premise has been re-evaluated using techniques of gene mapping, which failed to show any evidence of recent introgression between maize and teosinte (Smith *et al*., 1985). Moreover, *Z. mays* ssp. *mexicana* seems not to be a hybrid of the wild and cultivated forms of *Zea* and therefore probably does not serve as a genetic bridge as the physical similarities appear to be due to parallel adaptation to the same habitat (Doebley, 1984). There is evidence of highly restricted gene flow between *Zea* spp. that apparently occurs predominantly from teosinte into maize (Doebley *et al*, 1987). *Tripsacum* and *Zea* have different chromosome numbers (n = 9 versus n = 10). Crosses between *Z. mays* and *T. dactyloides* can be made, but only through human intervention and, even then, only with extreme difficulty. Moreover, the progeny are frequently sterile or genetically unstable (Manglesdorf, 1974). The process of transferring *Tripsacum* germplasm into maize is technically difficult. The transmission rate of the single extra *Tripsacum* chromosome added to the genome is so low and the rate of maize *Tripsacum* crossing over so reduced, as to practically exclude the general use of experimentally-introduced *Tripsacum* germplasm in maize improvement (Galinat, 1988).

G. VOLUNTEERS AND WEEDINESS IN CORN

Maize has lost the ability to survive in the wild due to its long process of domestication, and needs human intervention to disseminate its seed. Although corn from the previous crop year can over winter and germinate the following year, it cannot persist as a weed. The presence of corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with the hoe or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist during the following years. Volunteers are common in many agronomic systems, but they are easily controlled; however, maize is incapable of sustained reproduction outside of domestic cultivation. Maize plants are non-invasive in natural habitats (Gould, 1968). Some *Zea* species are successful wild plants in Central America, but they have no pronounced weedy tendencies (Galinat, 1988). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently seed dispersal of individual kernels does not occur naturally. Individual kernels of corn, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities (Hallauer, 2000).

Chapter 3. Molecular Analysis of Event MIR604

A. SUMMARY

Data from Southern analysis and DNA sequencing demonstrated that single copies of the modified *cry3A* (*mcry3A*) gene, phosphomannose isomerase (*pmi*) gene, MTL promoter and ZmUbiInt promoter were present in Syngenta's maize (corn) Event MIR604. Event MIR604 did not contain any of the backbone sequences from the transformation plasmid pZM26. Additionally, Southern analysis demonstrated that the T-DNA insert was stable over several generations of Event MIR604. Sequence analysis of the entire T-DNA insert present in Event MIR604 confirmed the overall integrity of the insert and that contiguousness of the functional elements had been maintained. A 43 bp truncation at the right border (RB) junction of the T-DNA insert and 44 bp truncation at the left border (LB) junction of the T-DNA insert were identified. Three single nucleotide changes were also identified in the T-DNA insert. One of these changes occurred within the MTL promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and give rise to two amino acid changes. These substitutions have not resulted in any apparent functional change in PMI as expressed in Event MIR604 (Hill, 2004). Statistical analysis confirmed the expected Mendelian inheritance ratio for both *mcry3A* and *pmi*.

B. INTRODUCTION

This chapter is being submitted to the USDA in support of a petition for non-regulated status for Syngenta Seeds' transformation Event MIR604. These plants express a modified Cry3A (mCry3A) protein that confers resistance to certain coleopteran pests. Additionally, the plants express phosphomannose isomerase (PMI), a selectable marker trait that is inert with regard to pesticidal properties (Negrotto, et. al., 2000; Reed, et. al., 2001; Wright, et. al., 2001). The present chapter presents a summary of data and information relevant to the molecular characterization of the T-DNA insert present in Syngenta's corn event MIR604. Included herein are data and information describing the genetic elements that have been introduced into Event MIR604, the process used for transformation, and a molecular and genetic characterization of Event MIR604-derived plants.

C. EVENT MIR604 MAIZE

Syngenta's maize Event MIR604 has been transformed with a synthetic, maize optimized, modified *cry3A* (m*cry3A*) gene whose expression produces a mCry3A insect control protein that is a member of a class of proteins which occur naturally in a gram-positive soil bacterium *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t*) (Sekar *et al.*, 1987). Additional changes in this maize-optimized gene were made, such that the mCry3A protein has enhanced activity against the western corn rootworm (WCRW; *Diabrotica virgifera virgifera*) and other related pests. The mCry3A amino acid sequence corresponds to that of the native Cry3A protein, except that (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-phenylalanine, and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein. The consensus recognition site for cathepsin-G was determined to be alanine-alanine-proline-phenylalanine by Blocker *et al.*, 1999 and Nakajima, *et al.*, 1979. Maize plants transformed with the synthetic modified *cry3A* gene from pZM26 (Figure 2) display resistance to these pests (Chen and Stacy, 2003).

Event MIR604 maize also contains the *pmi* gene, which was introduced *via* the same pZM26 transformation vector. This gene represents the *manA* gene from *Escherichia coli* and encodes the enzyme phosphomannose isomerase (PMI), which was employed as a selectable marker during the process of regenerating plant material following transformation (Negrotto *et al*., 2000). Maize cells expressing *pmi* can utilize mannose as a primary carbon source, whereas cells lacking *pmi* expression will fail to proliferate in a mannose-based culture medium. A complete description of the *pmi* gene and PMI protein is found in a separate report (Vlachos and Joseph, 2003).

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604. The *mcry3A* gene was transformed into a heterogenous genetic background selected for its suitability to transformation by *Agrobacterium*. Event MIR604 was crossed into a number of elite inbred lines and material generated from these crosses was used in the molecular and segregation analysis. The control germplasm in these experiments was negative segregants identified in the back cross populations.

D. DESCRIPTION OF THE TRANSFORMATION SYSTEM AND METHOD

Transformation of Syngenta's mCry3A-expressing maize Event MIR604 was conducted using immature maize embryos derived from a proprietary *Zea mays* line (Negrotto *et al*., 2000), via *Agrobacterium*-mediated transformation. By this method, genetic elements within the left and right border regions of the transformation vector are efficiently transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions are generally not. Immature embryos were excised from 8 - 12 day old ears and rinsed with fresh medium in preparation for transformation. Embryos were mixed with the suspension of *Agrobacterium* cells harboring the transformation vector pZM26 (Figure 2), vortexed for 30 seconds, and allowed to incubate for an additional five minutes. Excess *Agrobacterium* solution was aspirated and embryos were then moved to plates containing a non-selective culture medium. Embryos were co-cultured with the remaining *Agrobacterium* at 22°C for 2-3 days in the dark. Embryos were transferred to culture medium supplemented with ticarcillin (100 mg/ml) and silver nitrate (1.6 mg/l) and incubated in the dark for ten days. The phosphomannose isomerase gene, *pmi*, was employed as a selectable marker during the transformation process (Negrotto *et al*., 2000). Embryos producing embryogenic callus were transferred to cell culture medium containing mannose. After initial incubation with *Agrobacterium*, transformed tissue was transferred to, and grown for four months on selective media containing 500 mg/L of the broad-spectrum antibiotic cefotaxime insuring that the *Agrobacterium* was cleared from the transformed tissue. Regenerated plantlets were tested for the presence of both the *pmi* and *mcry3A* genes, as well as for the

absence of the spectinomycin (spec) antibiotic resistance gene, by TaqMan® PCR analysis. Plants positive for both genes, and negative for spec, were transferred to the greenhouse for further propagation.

E. THE DONOR GENES AND REGULATORY SEQUENCES

E.1. Active ingredient cassette:

MTL promoter (2556 bp): Derived from the *Zea mays* metallothionein-like gene (GenBank Accession number S57628). Provides root-preferential expression in *Zea mays* (de Framond, 1991).

***mcry3A* (1797 bp):** A modified version of a native Cry3A gene, which occurs in *Bacillus thuringiensis* subsp. *tenebrionis* (Sekar *et al.*, 1987). The gene was reengineered to incorporate a cathepsin-G serine protease recognition site within the expressed protein. The amino acid sequence of the expressed protein corresponds to that of the native Cry3A protein, except that (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 consensus recognition site for cathepsin-G was determined to be alanine-alanine-proline-phenylalanine by Blocker *et al.*, 1999 and Nakajima, *et al.*, 1979. This cathepsin-G recognition site has the sequence alanine-alanine-proline-phenylalanine, and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein. This modification increases the toxicity to target pests, particularly *Diabrotica virgifera virgifera* and *Diabrotica longicornis barberi* (Chen and Stacy, 2003). The entire coding region of the *mcry3A* gene was synthesized to accommodate the preferred codon usage for maize (Murray *et al*., 1989).

NOS (253 bp): Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (GenBank Accession number V00087). Its function is to provide a polyadenylation site (Depicker *et al*., 1982).

E.2. Selectable marker cassette:

ZmUbiInt (1993 bp): Promoter region from *Zea mays* polyubiquitin gene, contains the first intron comprising 1010 bp of the promoter (GenBank Accession number S94464). The entire promoter region provides constitutive expression in monocots (Christensen *et al*., 1992).

***pmi* (1176 bp):** *E.coli manA* gene encoding phosphomannose isomerase (GenBank Accession number M15380). Catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate (Negrotto *et al*., 2000).

NOS (253 bp): Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (GenBank Accession number V00087). Its function is to provide a polyadenylation site (Depicker *et al*., 1982).

E.3. Vector backbone components:

Spec (789 bp): Streptomycin adenylyltransferase, *aadA* gene from *E. coli* Tn7 (GenBank Accession Number X03043). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker (Fling *et al*., 1985).

VS1ori (405 bp): Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas* (similar to GenBank Accession Number U10487). Serves as origin of replication in *Agrobacterium tumefaciens* host (Itoh *et al*., 1984).

ColE1ori (807 bp): Origin of replication that permits replication of plasmid in *E. coli*. (similar to GenBank Accession Number V00268) (Itoh and Tomizawa, 1978).

LB (25 bp): Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline ti-plasmid (GenBank Accession number J01825). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski *et al*., 1982).

RB (25 bp): Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline ti-plasmid (GenBank Accession number J01826). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*., 1984).

***virG* (726 bp):** VirGN54D from pAD1289 (similar to GenBank Accession Number AF242881). The N54D substitution results in a constitutive *virG* phenotype. VirG is part of the two-component regulatory system for the vir regulon in *Agrobacterium* (Hansen *et al*., 1994).

***repA* (1074 bp):** pVS1 replication protein from *Pseudomonas*, which is a part of the minimal pVS1 replicon that is functional in gram-negative plant associated bacteria (GenBank Accession Number AF133831) (Heeb *et al*., 2000).

F. MOLECULAR ANALYSIS OF EVENT MIR604

F.1. Functional Element Copy Number Southern Analysis

Genomic DNA used for Southern analysis was isolated from pooled leaf tissue of ten plants representing the backcross six (BC6) generation (see Figure 3 for pedigree) of Event MIR604 using the method of Thomas *et al*., (1993). All plants used for DNA isolation were individually analyzed using TaqMan® PCR to confirm the presence of a single copy of the *mcry3A* gene and the *pmi* gene. For the negative segregant controls, DNA was isolated from pooled leaf tissue of plants representing either the backcross four (BC4) generation (used for the *mcry3A*, *pmi* and backbone Southern analysis) or the backcross six (BC6) generation (used for the MTL and ZmUbiInt Southern analysis) of Event MIR604. These plants were individually analyzed using TaqMan® PCR and the assays were negative for the *mcry3A* gene

and the *pmi* gene, but were as expected positive for the assay internal control, the endogenous maize *adh* gene.

Southern analysis was performed using state-of-the-art molecular biology techniques. Genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme, which has a single recognition site within the Event MIR604 T-DNA insert from plasmid pZM26 (Figure 2). This approach allows for determination of the number of copies of the elements, corresponding to the specific probe used for each Southern, which have been incorporated into Event MIR604. This results in one hybridization band per copy of the element present in Event MIR604. Following agarose gel electrophoresis and alkaline transfer to a Nytran® membrane, hybridizations were carried out using element specific full-length PCR generated probes (See Figures 4, 6, 8, 10, 12 and 14). The probes were labeled with ^{32}P *via* random priming using the Rediprime II™ system (Amersham Biosciences, Cat. No. RPN1633).

Included in each southern were three control samples:

1.) DNA from a negative (non-transformed) segregant used to identify any endogenous *Zea mays* sequences that may cross-hybridize with the element specific probe.
2.) DNA from a negative segregant into which is introduced an amount of digested pZM26 that is equal to one or less copy number based on probe length, to demonstrate the sensitivity of the experiment in detecting a single gene copy within the *Zea mays* genome.[1]
3.) Digested pZM26 plasmid that is equal to one copy number based on probe length, to demonstrate a positive control for hybridization as well as the sensitivity of the experiment.

These hybridization data provide confirmatory evidence to support the TaqMan® PCR analysis that Event MIR604 contains a single copy of the *mcry3A* gene and the *pmi* gene. Additionally, the data demonstrate that Event MIR604 contains a single copy of the MTL promoter and ZmUbiInt promoter and that Event MIR604 does not contain any of the vector backbone sequences present in pZM26. As expected for the *mcry3A* (Figure 5), *pmi* (Figure 7), MTL (Figure 9) and ZmUbiInt (Figure 11) probes the *Kpn*I digest resulted in a single hybridization band demonstrating that a single copy of each element is present in Event MIR604. Additionally for the full-length backbone probe (Figure 13) lack of hybridization demonstrates the absence of any pZM26 vector backbone sequences being incorporated into Event MIR604 during the transformation process.

F.2. Generational Stability Southern Analysis

[1] **Formula to determine one copy equivalent based on probe length**

(µg digested DNA loaded*1.00E+06 pg/µg*bp length of probe)/bp maize diploid genome =	pg for 1 copy
Example:	
Zea mays diploid genome size in bp:	5.34E+09
mcry3A probe length in bp:	1797
µg *Kpn*I-digested DNA loaded for Southern analysis:	7.5
Calculation for *mcry3A*: (7.5*1.00E+06*1797)/5.34E+09=	2.52 pg

Genomic DNA used for generational stability Southern analysis was isolated from pooled leaf tissue of ten plants per generation representing the backcross four (BC4), backcross five (BC5) and backcross six (BC6) generations of Event MIR604 using the method of Thomas *et al*., (1993). All plants used for DNA isolation were individually analyzed using TaqMan® PCR (data not shown) to confirm the presence of a single copy of the *mcry3A* gene and the *pmi* gene. For the negative segregant controls DNA was isolated from pooled leaf tissue of ten plants representing the BC6 generation of Event MIR604. These plants were individually analyzed using TaqMan® PCR (data not shown) and the assays were negative for the *mcry3A* gene and the *pmi* gene, but were as expected positive for the assay internal control, the endogenous maize *adh* gene.

As expected the hybridization pattern over several generations of Event MIR604 was identical using a *mcry3A* probe (Figure 15). The hybridization data demonstrates the T-DNA insert from pZM26 incorporated into Event MIR604 is stable over several generations.

F.3. T-DNA insert sequencing

The nucleotide sequence of the entire T-DNA insert present in Event MIR604 was determined to demonstrate overall integrity of the insert, contiguousness of the functional elements and to detect any individual basepair changes. The Event MIR604 insert was amplified from DNA derived from the BC5 generation as two individual overlapping fragments (Figure 16). Each fragment was amplified using one oligo homologous to plant genomic sequences flanking the Event MIR604 insert (data not shown) and one oligo homologous to the *mcry3A* gene. PCR amplification was carried out using the Expand High Fidelity PCR system (Roche, Cat. No. 1732650). Each sequencing fragment was individually cloned into the pCR® -XL-TOPO vector (Invitrogen, Cat. No. K4700-20) and three separate clones for each fragment were identified and sequenced. Sequencing was carried out using the ABI3730XL analyzer using ABI BigDye® 1.1 or Big Dye 3.1 dGTP (for GC rich templates) chemistry. The sequence analysis was done using the Phred, Phrap, and Consed package from the University of Washington and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998). The final consensus sequence was determined by combining the sequence data from the six individual clones (three for each sequencing fragment) to generate one consensus sequence of the Event MIR604 insert. To further validate any individual basepair discrepancies between the Event MIR604 insert and the pZM26 plasmid small (~300-500 bp) PCR products specific to any regions where a basepair discrepancy was seen in the initial consensus sequence were amplified using the same methodology above. For all putative basepair discrepancies in the Event MIR604 insert direct PCR product sequencing resulted in single clear peaks at all basepairs in question, indicating these discrepancies are likely present in the Event MIR604 insert (data not shown). Alignment was performed using the ClustalW program with the following parameters: scoring matrix blosum55, gap opening penalty 15, gap extension penalty 6.66 (Thompson *et al*, 1994).

The consensus sequence data for the Event MIR604 T-DNA insert demonstrate the overall integrity of the insert and that contiguousness of the functional elements within the insert as intended in pZM26 have been maintained (Rabe, 2004). Sequence analysis revealed that some truncation occurred at the RB and LB ends of the T-DNA insert

during the transformation process that resulted in Event MIR604. The RB portion of the T-DNA insert was truncated by 44bp and the LB end of the T-DNA insert was truncated by 43 bp (Rabe, 2004). These deletions have no effect on the efficacy of the T-DNA insert and this phenomenon has also been previously observed in *Agrobacterium* transformation (Tinland and Hohn, 1995). Additionally, three basepair changes were noted in the Event MIR604 T-DNA insert (Rabe, 2004). One change occurred within the MTL promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and did result in two amino acid changes; valine at position 61 has been substituted by alanine (V61A) and glutamine at position 210 has been substituted by histidine (Q210H) (Rabe, 2004). Alanine and valine are both aliphatic amino acids resulting in a conservative substitution. Replacement of glutamine with histidine results in the substitution of an acidic residue for a basic residue. These substitutions have not resulted in any apparent functional change in PMI as expressed in Event MIR604 (Hill, 2004).

G. MENDELIAN INHERITANCE OF TRANSGENE INSERT

The inheritance pattern of the T-DNA insert derived from pZM26 in Event MIR604 was investigated. The initial Event MIR604 plant (T_0 generation) was bred to a non-transgenic inbred isoline, creating the T_1 generation. A single plant from this T_1 generation, identified by immuno-detection strip to be positive for PMI, was selfed to yield the T_2 generation. A single plant, putatively homozygous for *mcry3A* by TaqMan® PCR, from the T_2 seed was selfed to yield the T_3 generation. The zygosity of the T_3 generation was confirmed by conducting a progeny test by planting a 20 kernel sample of seed and assaying the resulting plants for *mcry3A* by TaqMan® PCR (data not shown). Progeny plants from the T_3 generation were crossed to a non-transgenic inbred to yield the T_4 generation (designated Hybrid I in Figure 3.). These progeny plants were selfed in the field and seed was collected in bulk, creating the T_5 seed. Individual T_5 plants were assayed for the presence of the trait by immuno-detection (ELISA) of mCry3A, as well as by TaqMan® PCR (data not shown) for both the *pmi* and *mcry3A* genes. The expected Mendelian inheritance ratio of positive and negative plants for a hemizygous trait in these populations is 3:1.

Genotypic data (Tables 2 and 3) were used to assess the goodness-of-fit of the observed genotypic ratio to the expected genotypic ratio using Chi Square analysis with Yates correction factor (Strickberger, 1976).

$$X^2 = \sum[\text{Observed-expected- } 0.5]^2/\text{expected}$$

Table 2. Observed vs. Expected* Genotype for T_5 Generation as determined by ELISA for mCry3A.

	Observed	Expected
Trait positive	317	313.5
Trait negative	101	104.5
Total	418	418

* X^2= 0.1148

This analysis tested the hypothesis that the genetic trait is segregating in a Mendelian fashion. The critical value to reject the hypothesis at the 5% level is 3.84 (Strickberger 1976). Since the Chi squared value is less than 3.84 the hypothesis that the genetic trait is behaving in a Mendelian fashion is accepted.

Table 3. Observed vs. Expected* Genotype for T_5 Generation as determined by Taqman® PCR Analysis for *pmi* and *mcry3a* genes.

	Observed	Expected
Trait positive	315	313.5
Trait negative	103	104.5
Total	418	418

* X^2= 0.0128

The critical value to reject the hypothesis at the 5% level is 3.84 (Strickberger 1976). Since the Chi squared value is less than 3.84 the hypothesis that the genetic trait is behaving in a Mendelian fashion is accepted[2].

H. CONCLUSIONS

Data from Southern analysis and DNA sequencing demonstrate that single copies of the modified *cry3A* (*mcry3A*) gene, phosphomannose isomerase (*pmi*) gene, MTL promoter and ZmUbiInt promoter are present in Syngenta's maize (corn) Event MIR604. Event MIR604 does not contain any of the backbone sequences from the transformation plasmid pZM26. Additionally, the T-DNA insert is stable over several generations of Event MIR604. Sequence analysis of the entire T-DNA insert present in Event MIR604 confirms that the overall integrity of the insert and contiguousness of the functional elements has been maintained. A 43 bp truncation at the right border (RB) junction of the T-DNA insert and 44 bp truncation at the left border (LB) junction of the T-DNA insert was identified. Three single nucleotide changes were also identified. One of these changes occurred within the MTL promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and give rise to two amino acid changes which have not resulted in any apparent functional change in PMI as expressed in Event MIR604. Statistical analysis confirmed the expected Mendelian inheritance ratio for both *mcry3A* and *pmi*.

[2] The same 418 samples were used in both the PCR and ELISA experiments. The difference in the number of observed scores was attributed to two plants which were scored negative in the Taqman assay while scoring positive by ELISA assay. It is not possible to determine whether this difference is due to false postives in the ELISA or false negatives in the Taqman PCR. Regardless, the data clearly demonstrate that the transgene is inherited in a Mendelian fashion.

Figure 2. Plasmid map of pZM26. Map identifies restriction sites used for Southern analysis.

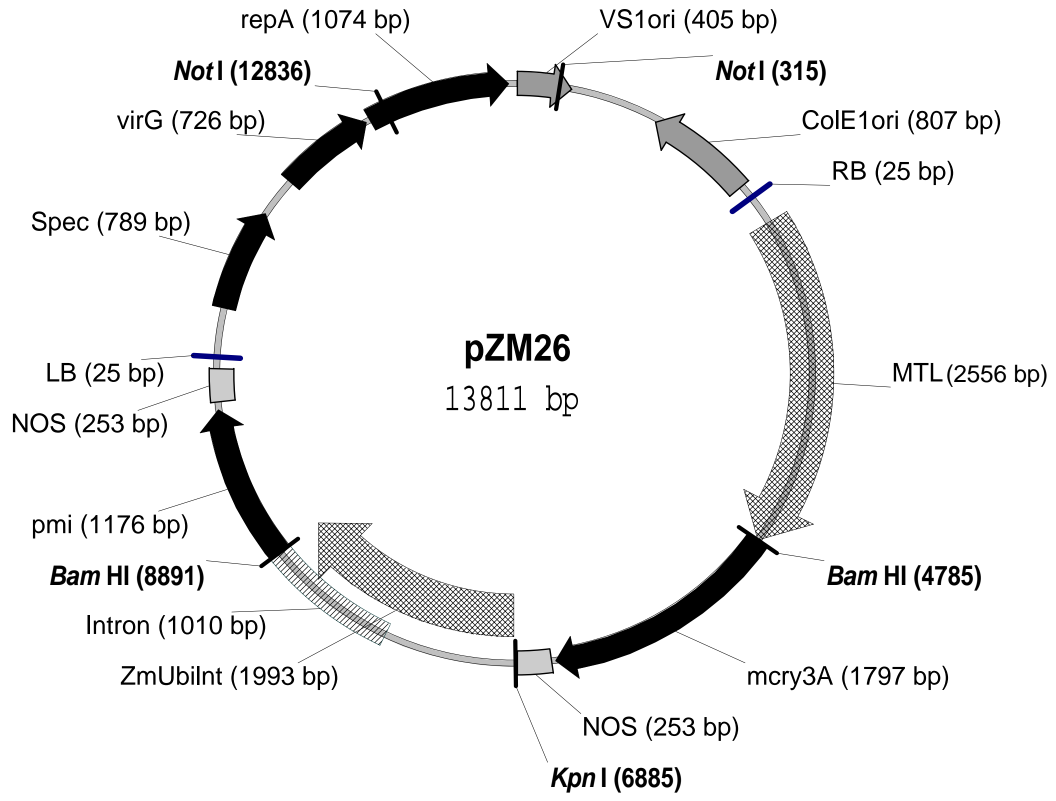


Figure 3. **MIR604 breeding history indicating generations used in the molecular analysis of Event MIR604.** The partial breeding history is extracted directly from Figure 1.

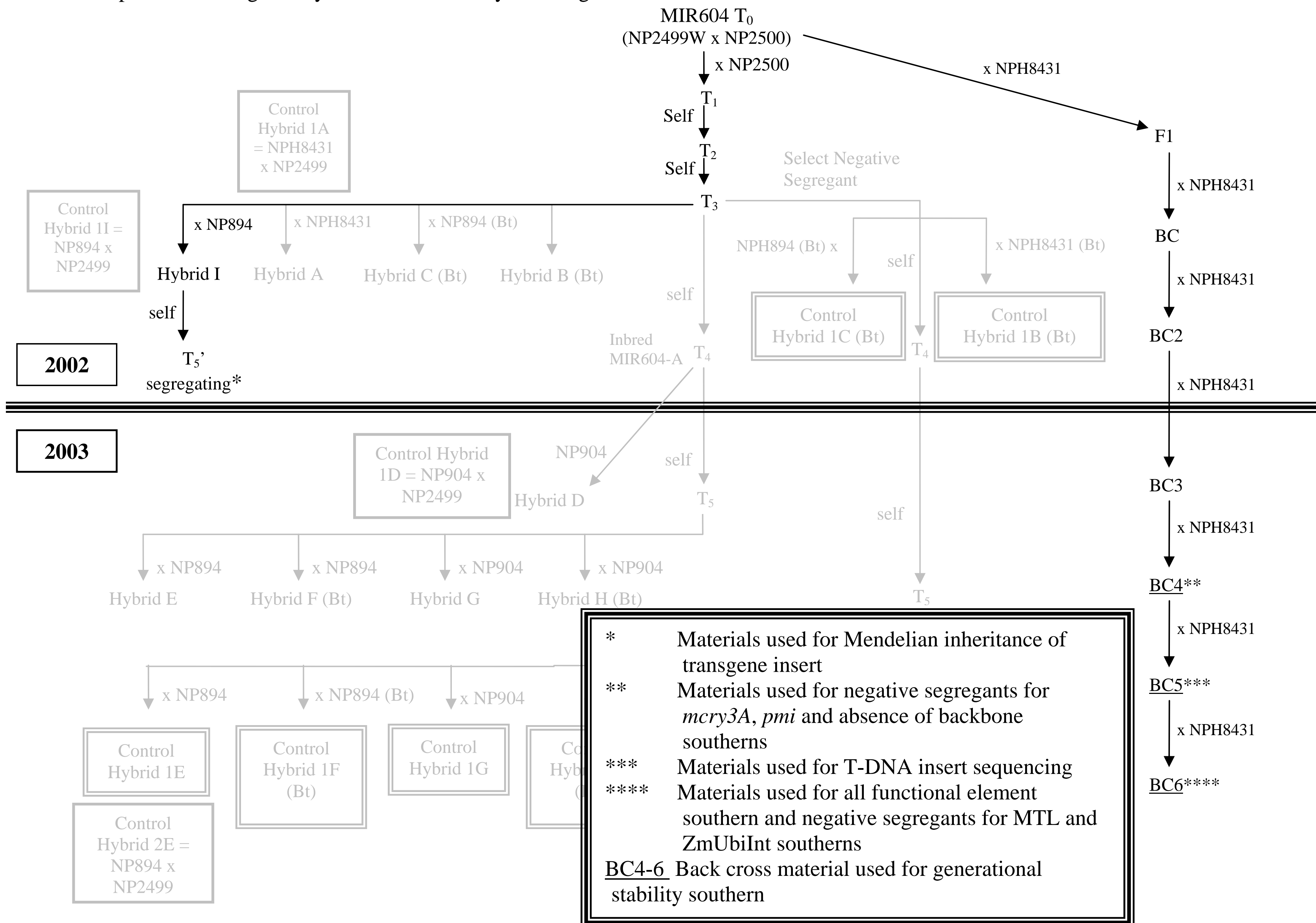


Figure 4. Location of *Kpn*I restriction site and position of *mcry3A* probe in the transformation vector pZM26 introduced into Event MIR604.

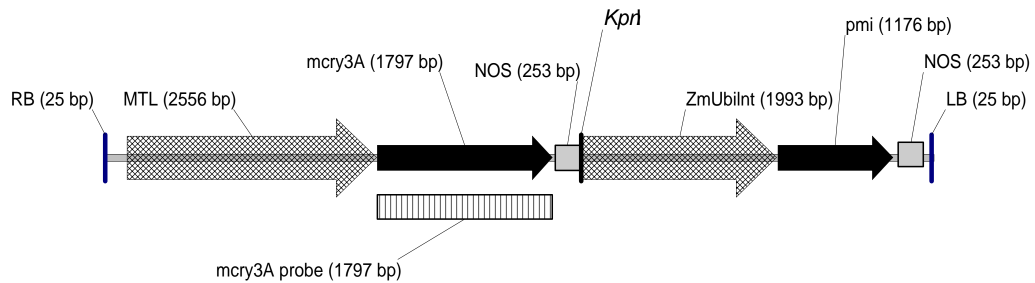


Figure 5. Southern analysis of Event MIR604 with *mcry3A* specific probe. Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *mcry3A* specific probe (1797 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC4 generation of Event MIR604; Lane 5: Negative segregants from BC4 generation of Event MIR604 spiked with 2.52 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 2.52 pg *Kpn*I digested pZM26 DNA.

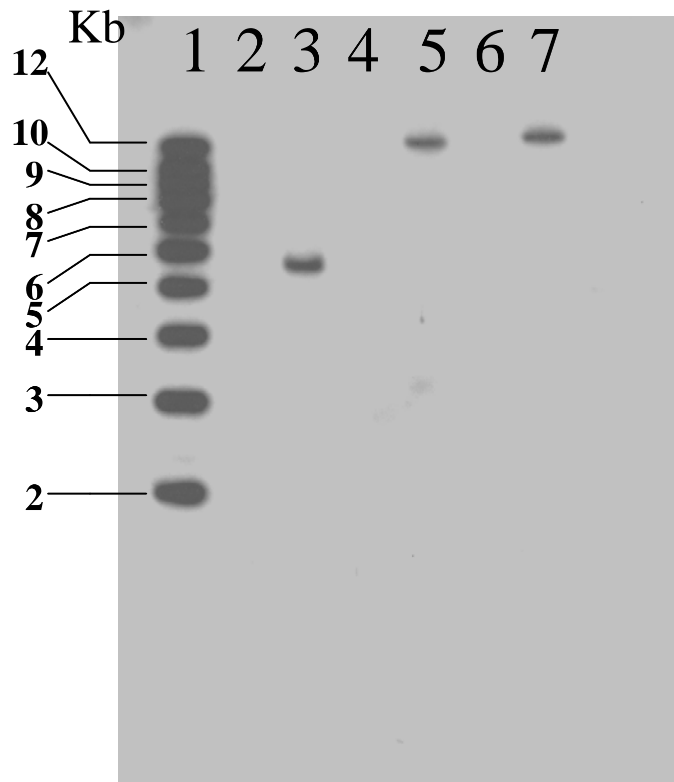


Figure 6. Location of *Kpn*I restriction site and position of *pmi* probe in the transformation vector pZM26 introduced into Event MIR604.

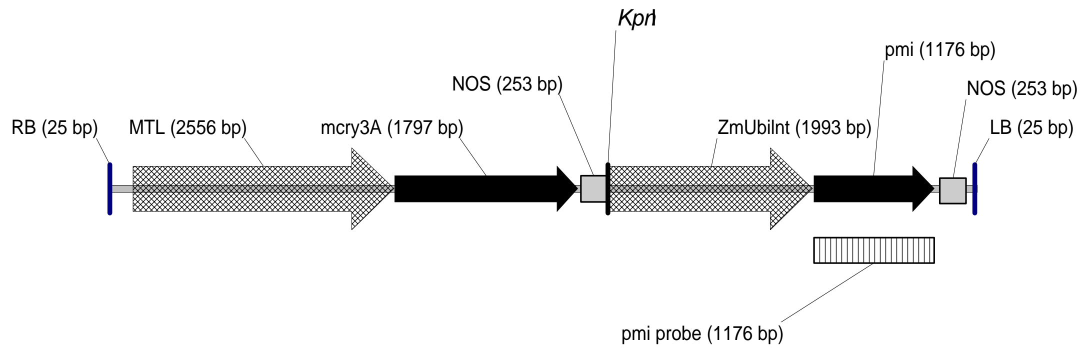


Figure 7. Southern analysis of Event MIR604 with *pmi* specific probe.
Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *pmi* specific probe (1176 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC4 generation of Event MIR604; Lane 5: Negative segregants from BC4 generation of Event MIR604 spiked with 1.65 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 1.65 pg *Kpn*I digested pZM26 DNA.

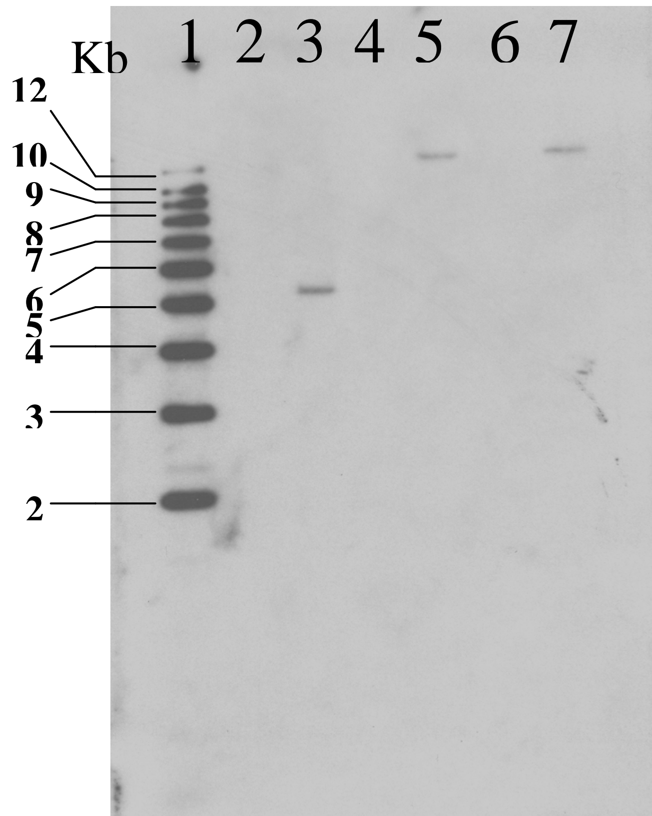


Figure 8. Location of *Kpn*I restriction site and position of MTL probe in the transformation vector pZM26 introduced into Event MIR604.

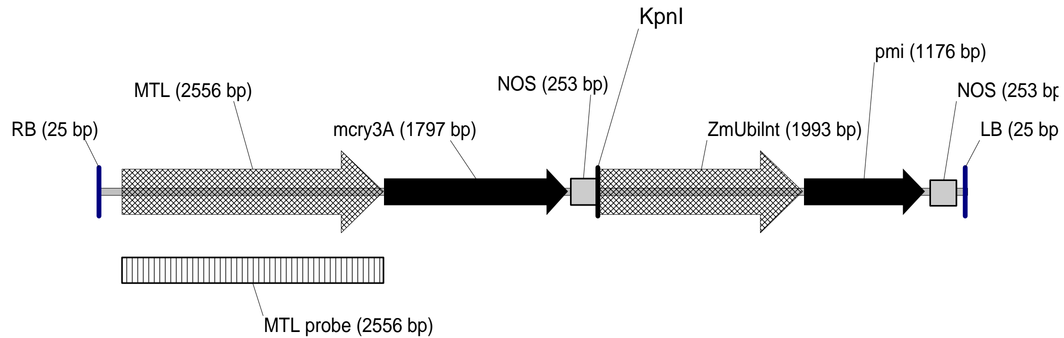


Figure 9. Southern analysis of Event MIR604 with MTL specific probe.
Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a MTL specific probe (2556 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC6 generation of Event MIR604; Lane 5: Negative segregants from BC6 generation of Event MIR604 spiked with 3.59 pg *Bam*HI/*Not*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 3.59 pg *Bam*HI/*Not*I digested pZM26 DNA. Note: *Bam*HI/*Not*I were used to digest pZM26 due to a preliminary experiment in which a *Kpn*I digest of pZM26 resulted in comigration with an endogenous *Zea mays* band that cross-hybridized with the MTL probe.

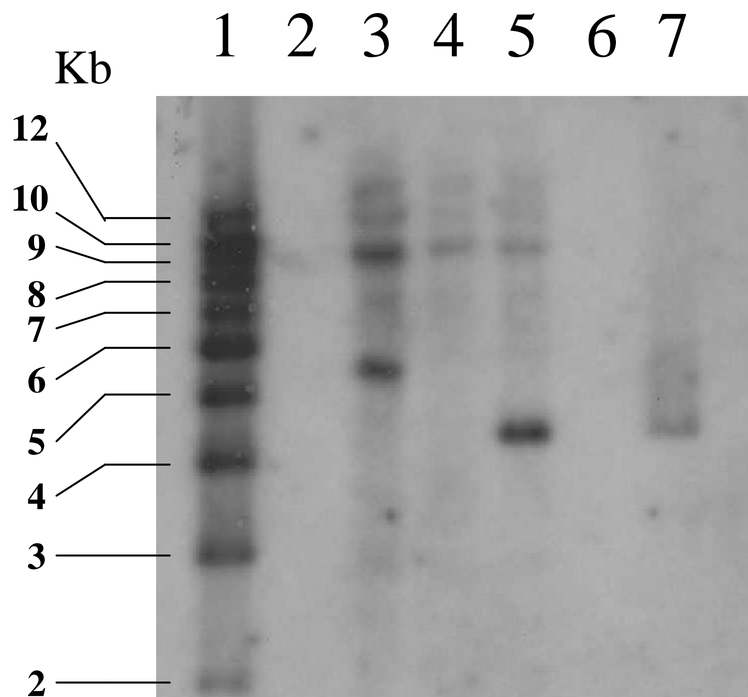


Figure 10. Location of *Kpn*I restriction site and position of ZmUbiInt probe in the transformation vector pZM26 introduced into Event MIR604.

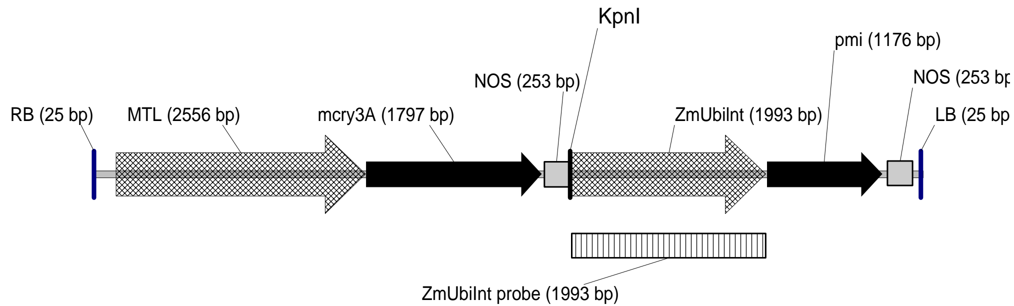


Figure 11. Southern analysis of Event MIR604 with ZmUbiInt specific probe. Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a ZmUbiInt specific probe (1993 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC6 generation of Event MIR604; Lane 5: Negative segregants from BC6 generation of Event MIR604 spiked with 2.80 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 2.80 pg *Kpn*I digested pZM26 DNA.

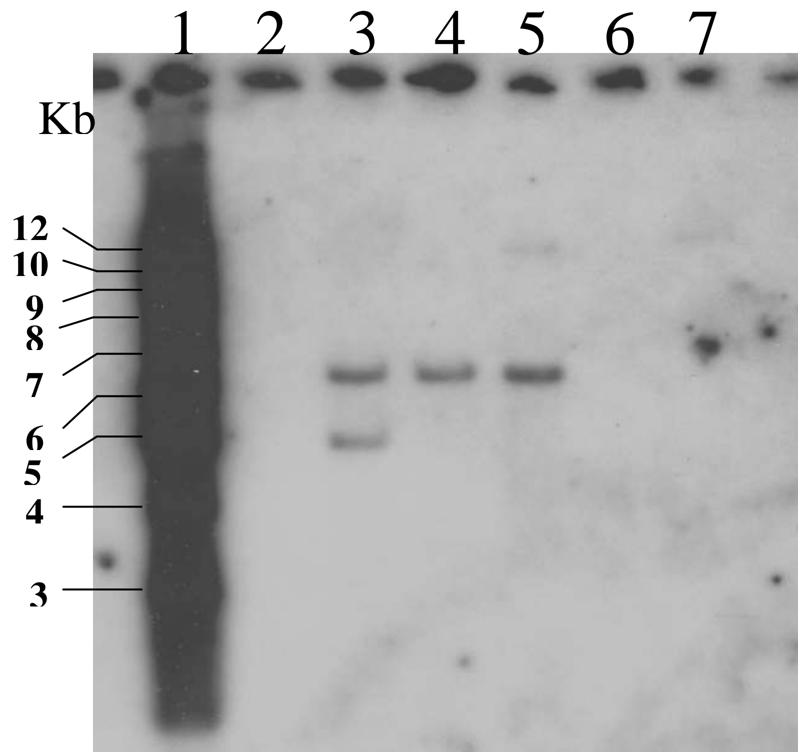


Figure 12. Location of *Kpn*I restriction site and position of backbone probe in the transformation vector pZM26.

Encompasses all basepairs outside of the LB and RB regions.

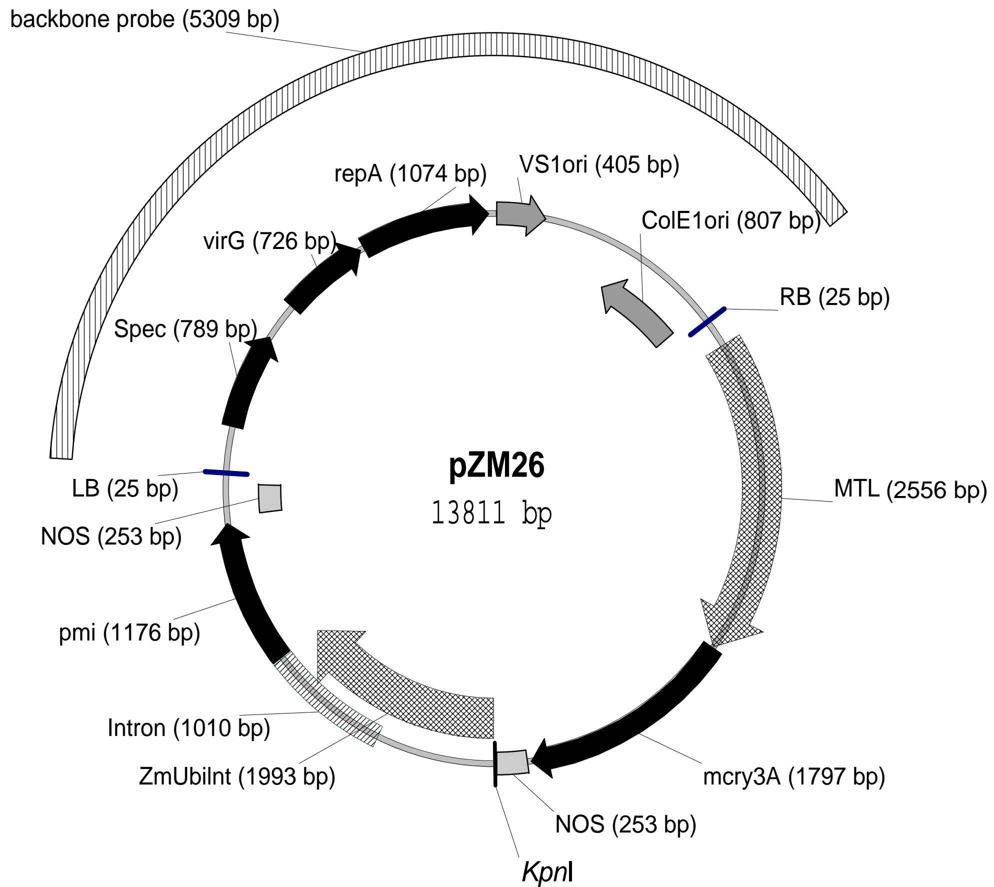


Figure 13. Southern analysis of Event MIR604 with backbone specific probe. Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a backbone specific probe (5309 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC4 generation of Event MIR604; Lane 5: Negative segregants from BC4 generation of Event MIR604 spiked with 7.46 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 7.46 pg *Kpn*I digested pZM26 DNA.

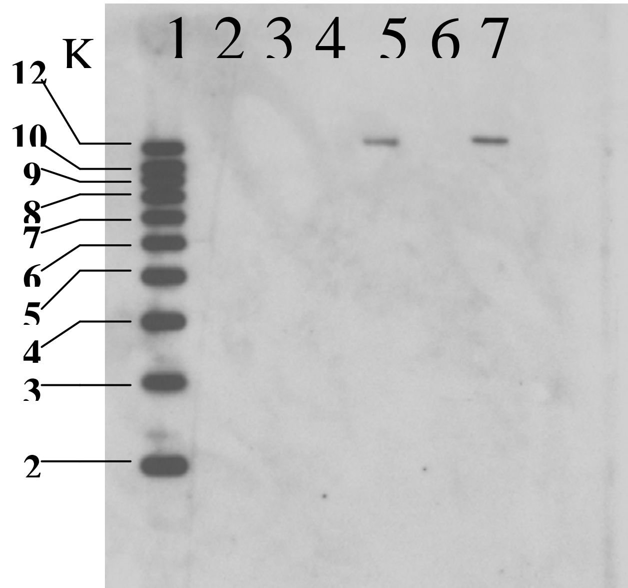


Figure 14. Location of *Kpn*I restriction site and position of *mcry3A* probe in the transformation vector pZM26 introduced into Event MIR604.

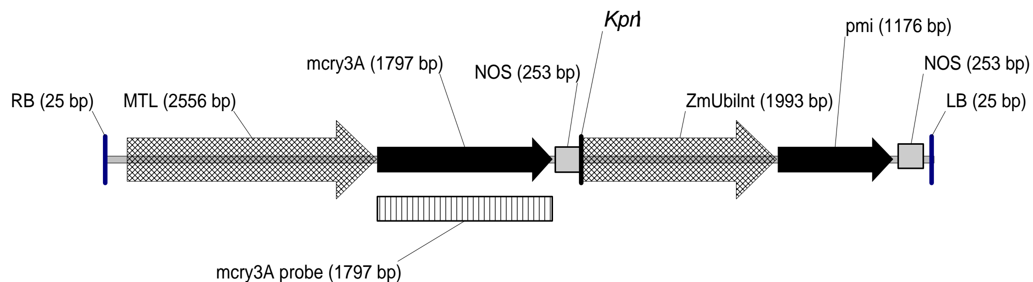


Figure 15. Generational stability southern analysis of Event MIR604 with *mcry3A* specific probe.

Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *mcry3A* specific probe (1797 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC4 generation Event MIR604; Lane 4: BC5 generation Event MIR604; Lane 5: BC6 generation Event MIR604; Lane 6: Negative segregants from BC6 generation of Event MIR604; Lane 7: Negative segregants from BC6 generation of Event MIR604 spiked with 2.52 pg *Kpn*I digested pZM26 DNA; Lane 8: Blank; Lane 9: 2.52 pg *Kpn*I digested pZM26 DNA.

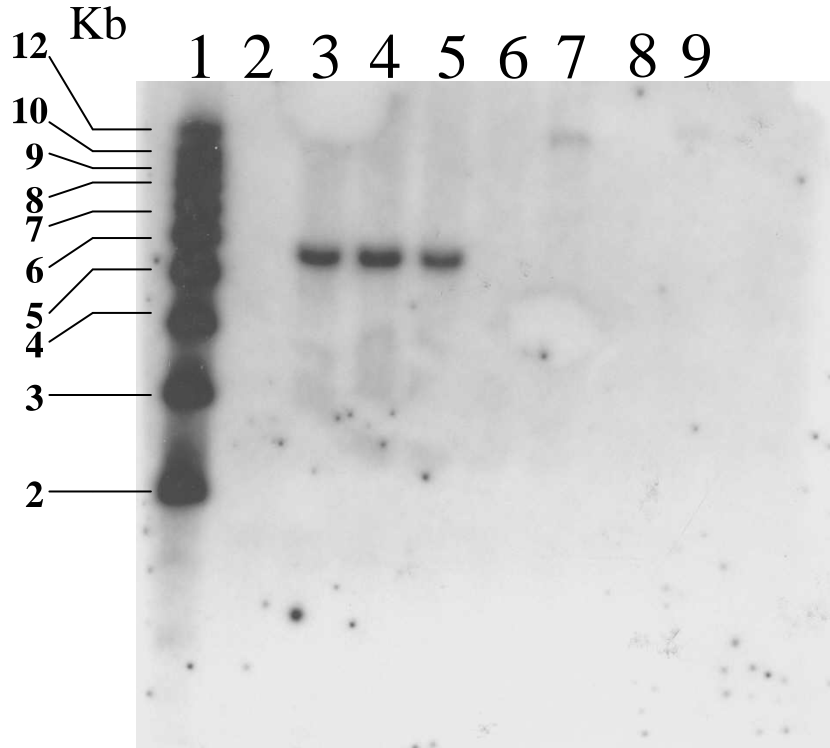
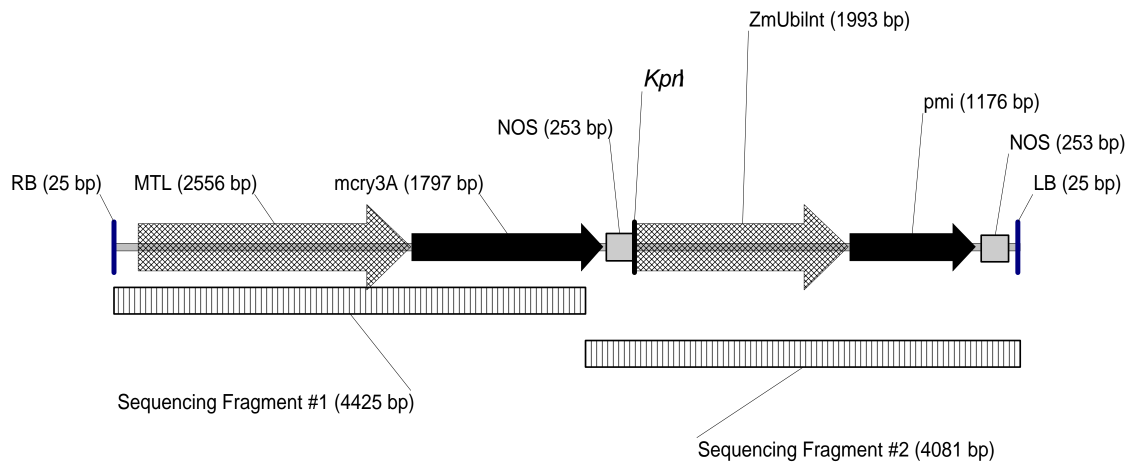


Figure 16. Location of fragments amplified from Event MIR604 to determine insert sequence.



Chapter 4. Agronomic Performance of Event MIR604 Hybrids

A. SUMMARY

Efficacy, yield, and agronomic performance of Event MIR604-derived field corn hybrids (MIR604 hybrids) were evaluated during 2002 and 2003 in 32 field trial locations across the U.S. corn belt (USDA Comprehensive Permit numbers 02-022-01 r/m, 02-022-02 r/m, 03-021-01 r/m, 03-021-02 r/m). Insect efficacy trials were conducted in field plots that were infested with naturally occurring populations of corn rootworm, with populations enhanced by use of trap crops the preceding season, or artificially infested with rootworm eggs. In addition to regular inspections for disease and insect pests, qualitative and quantitative comparisons for a number of morphological and agronomic traits were made between the transgenic and non-transgenic negative segregants or non-transgenic near isolines. Yield and agronomic trials were conducted both in the presence and absence of pest pressure, and in conjunction with an in-furrow insecticidal treatment. The traits chosen for agronomic comparison are those that are typically monitored by professional breeders and agronomists in the seed industry, and cover a broad range of characteristics that encompass the entire lifecycle of the maize plant.

The outcome of these studies, based upon data presented below, indicate that except for reduced damage to roots caused by corn rootworm the agronomic performance of MIR604-derived hybrids is similar, and for most traits, equivalent to their non-transformed near isogenic counterparts. In contrast to the similarity in most agronomic parameters, the yield performance of MIR604-derived hybrids in the presence of corn rootworm pressure is significantly increased relative to corn hybrids lacking this trait. Data referred to in this chapter are presented in Appendix 1, pp. 153-178.

B. AGRONOMIC METHODS

B.1. General Experimental Methods

The majority of experimental field trials were conducted as a simple random complete block design (RCBD). The experimental plots were divided into blocks of a size that would accomodate four rows 17 ft. long at a minimum. After blocking the plots, treatments were assigned at random within each block such that each treatment occurred once in every block. Within each block, control or test material was homogenously planted and agronomic, maintenance and havesting methods were applied as uniformly as possible. Comparisons of treatments and the computation of experimental error were performed within blocks. Any deviations from the basic trial design are footnoted in the trial data tables.

For efficacy trials, individual plants in field trials were artificially infested with an aqueous suspension of as few as 400 to as many as 1450 viable Western corn rootworm eggs (actual numbers of eggs per plant used in each trial are indicated as a footnote in the data tables). However, within an individual trial to maintain experimental integrity, the number of eggs per plant was standardized across plots. In artificially infested trials, as

plants reached the V2 to V3 leaf stage, eggs were mechanically placed 3 inches deep into the soil within 2-3 inches of the base of the stalk of each plant. Egg viability was assessed by immobilizing eggs onto a solid support, incubating in the laboratory and observing the proportion of emerged neonates versus unhatched eggs. Field infestations were performed with egg numbers normalized to account for differences in viability among egg populations. In cases where it was not possible or appropriate to artifically infest with Western corn rootworm eggs, the trial design included planting a trap crop with continuous corn or cucurbits the previous year to enhance the natural population of CRW. Egg densities within these plots were not assessed due to the technical complexities of such an analysis. However, root damage observed in the control corn plants within these plots was uniformly more severe compared to non-trap crop plots indicating that the CRW egg density was indeed enhanced.

To assess root damage by CRW feeding, corn plants at the VT to R1 stage (tasseling silking stage), were manually dug from the ground, soil washed from the roots and the roots examined and rated for corn rootworm damage according to the recent Iowa State 0-3 node-injury rating scale (http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html). Most trials included various chemical insecticide treatments as internal controls for comparison with the MIR604-derived hybrid entries. In some cases a chemical insecticide was applied in addition to the MIR604-derived hybrids to assess synergies between the two technologies and to serve as an experimental control. The chemical insecticide treatment included an in-furrow soil-applied granular treatment; Force 3G® (tefluthrin, Syngenta). This chemical formulation represents a highly popular and widely used CRW insecticide used on farms today. Application rates are indicated in the data tables.

B.2. MIR604 Pedigree

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604 (see Figure 17). Control germplasm consisted of near isogenic hybrids using non-transformed germplasm closely matched to the genetic background of MIR604 for that particular stage of the breeding pedigree (indicated by single lined boxes in Figure 17) or negative segregants used as breeding partners in crosses to elite inbred lines (indicated by double lined boxes in Figure 17). Further breeding details can be found in Chapter 1.

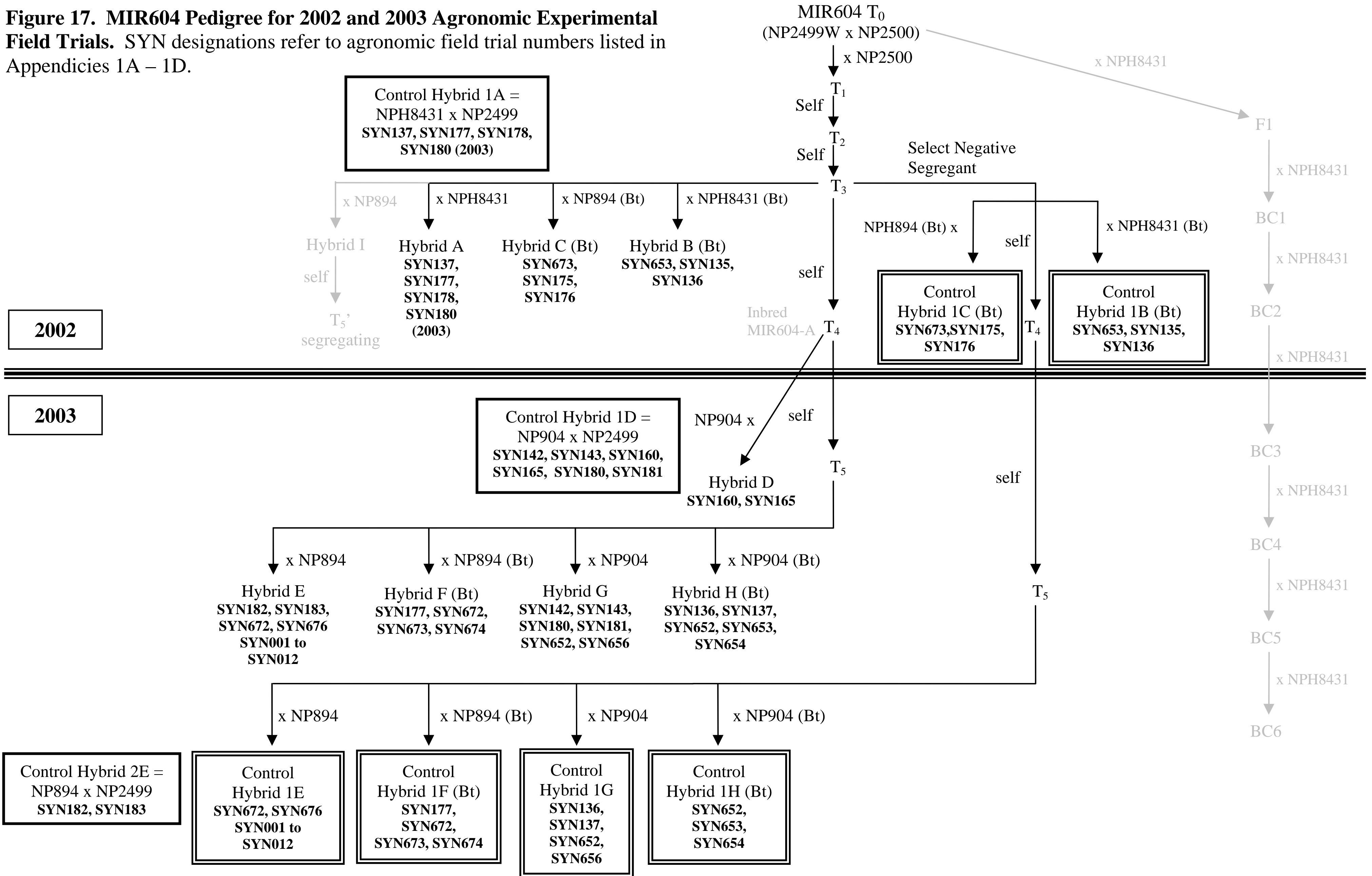


Figure 17. MIR604 Pedigree for 2002 and 2003 Agronomic Experimental Field Trials. SYN designations refer to agronomic field trial numbers listed in Appendicies 1A – 1D.

In order to reduce the confounding effect on yield of insect damage to the corn ears by lepidopteran pests, in some trials MIR604 was deployed in a breeding stack with Bt11 which expresses the Cry1Ab protein (indicated by '(Bt)' in the data tables and Figure 17). In these cases controls consisted of the corresponding Bt11-only hybrids.

C. CORN ROOTWORM CONTROL EFFICACY

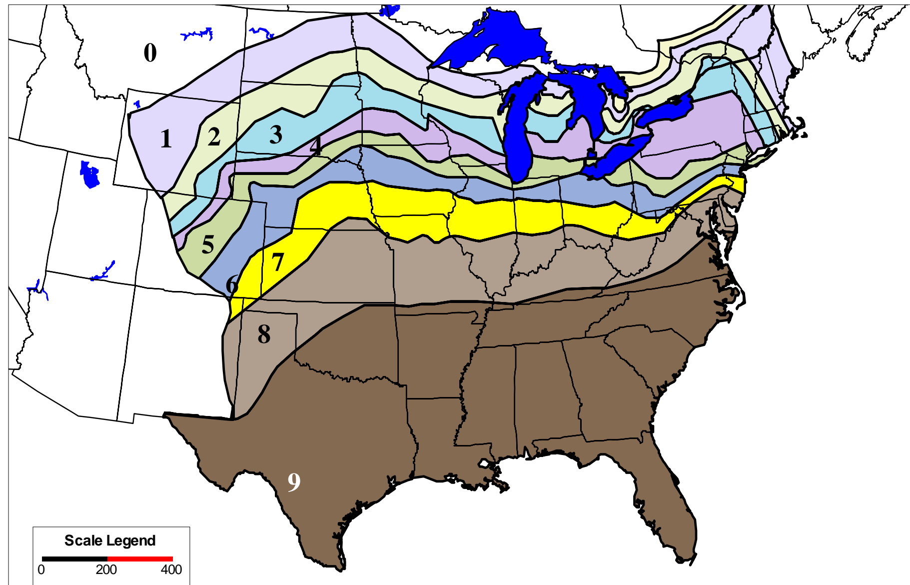
C.1. Western Corn Rootworm.

Western CRW is the most widespread and most damaging rootworm species affecting corn in the U.S. Root damage caused by CRW larval feeding is assessed by digging, examining and rating according to the Iowa State University 0-3 node-injury rating scale (Appendix 1A, Table 1A). According to the upper range of this scale, a rating of 3.00 denotes '*three or more nodes eaten'*, a high degree of root damage, whereas a rating of 0.0 is no damage. In some instances of extreme corn rootworm pressure, total root crown destruction can occur such that no roots are left on the corn plant. Depending on the degree of root crown destruction, this level of root damage is assigned by Syngenta a rating as high as 3.9.

In 2002, MIR604-derived hybrids were evaluated (see Appendix 1A, Table 1A) for insect efficacy at 3 locations in MN and IL (see Appendix 1A, Table 2A). MIR604-derived hybrids showed statistically significant reductions in root damage ratings relative to non-transgenic controls at all locations.

To assess MIR604 hybrids across hybrid maturity zones, a total of eight MIR604 hybrids were assessed for efficacy at 12 locations in 2003 (Appendix 1A, Table 3A). Mean root damage was significantly lower in the MIR604 hybrids compared to the non-transgenic negative isoline controls at all locations. MIR604 hybrids used in these studies included early and late maturity groups (Figure 18). Conventional corn hybrids are developed for optimal performance in specific maturity zones defined by the length of time from planting to harvesting grain. Early maturity hybrids are primarily adapted for growth in maturity zones 5 and lower while late maturity hybrids can be grown in maturity zones 5-8. In some cases efficacy was assessed in early maturity hybrids in trials outside their intended maturity zone. In these trials, the early maturity MIR604 hybrids demonstrated similar, significantly lower root damage as compared to controls when the same hybrid material was grown in their intended maturity zone (Appendix 1A, Table 3A, e.g., trial SYN180 conducted in Bloomington, IL). The same lower root damage ratings observed in the early maturity MIR604 hybrids were also seen in late-maturing MIR604 hybrids grown in the same area indicating that hybrid maturity group had little impact on the overall efficacy of MIR604 hybrids. Between different MIR604 hybrids, no statistical difference in root damage was seen at any of the locations, similar to data acquired in 2002. In two cases where corn rootworm pressure was very low, no statistically significant difference in root damage rating was seen between MIR604 derived hybrids and negative segregant controls (Appendix 1A, Table 3A, trials SYN008 and SYN010).

Figure 18. Designation of Syngenta Seeds Hybrid Corn Maturity Zones



B.2. Northern Corn Rootworm.

The Northern CRW is geographically not as widely distributed as the WCRW and is responsible for less damage to the U.S. corn crop. However, NCRW infestations can be severe in the northern maturity zones and often occur in the presence of WCRW. Due to the difficulties in rearing Northern corn rootworm, a limited number of Northern corn rootworm efficacy trials have been conducted on MIR604 hybrids. Mean root damage ratings for MIR604-derived and non-transgenic control hybrids in glasshouse trials artificially infested with northern corn rootworm show significantly greater efficacy compared to the negative control (Appendix 1A, Table 4A).

MIR604 hybrids were also exposed to natural populations of Northern corn rootworm at some of the locations listed in Appendix 1A, Tables 2A-3A. In particular, the Willmar, MN location (Trial SYN143, Appendix 1A, Table 3A) was populated primarily with Northern corn rootworm (NCRW) while the rest of the 2003 locations were a mixture of Western and Northern corn rootworms. Significantly less root damage was observed in MIR604 hybrids grown under natural NCRW pressure at Willmar, MN relative to near isogenic control hybrids. These results combined with those obtained in the artificially infested glasshouse trial indicate that MIR604 hybrid roots sustain significantly less NCRW feeding damage compared to the near isogenic controls.

C.3. Mexican Corn Rootworm.

Though not as prevalent as Western and Northern CRW, Mexican CRW can cause yield impacting root damage primarily in the southern maturity zones of 8 and 9 (Figure 16). MIR604 hybrids showed efficacy toward Mexican corn rootworm (MCRW) (Appendix 1A, Table 5A). MCRW trials were conducted at two Texas locations in 2003. Mean root damage ratings varied somewhat across the two locations. However, at both

locations, mean root damage in MIR604 hybrids was significantly lower than in the non-transgenic negative isoline controls.

D. YIELD EVALUATION

MIR604-derived hybrids were evaluated for yield (bushels per acre) at five field trials in MN and IL in 2002 (Appendix 1B, Table 1B). According to breeders and agronomists conducting the field trials, growing conditions were considered normal for MN in 2002 while IL field trial locations experienced drought and significant CRW feeding pressure. The impact on yield derived from MIR604 hybrids between the two growing conditions was significant. The average yield of MIR604 hybrids grown at the MN locations was statistically equivalent to the negative segregant controls. However, at the IL locations, the average yield increase of MIR604 hybrids was 61 bu/acre or a 233 % increase in yield over the negative controls (Appendix 1B, Table 1B).

Yield from MIR604 hybrids was also assessed in 2003 at 20 locations (Appendix 1B, Table 2B). In contrast to 2002, growing conditions for 2003 were typical for most of the trial sites. Yield loss associated with moderate corn rootworm damage largely depends upon environmental conditions which can lead to root lodging (high winds) or poor cob development and grain fill (drought stress). If the trial plots do not experience these conditions (as was the case in 2003), then yield losses can often be minimal despite corn rootworm feeding pressure. The average yield of MIR604-derived hybrids was statistically equivalent to or greater than the negative segregant controls at nearly all trial sites in 2003.

In the absence of high corn rootworm pressure and drought stress, MIR604-derived hybrids yield an equivalent amount of grain compared to their negative isoline controls over a wide geographical range. When MIR604-derived hybrids are grown under adverse conditions of heavy corn rootworm pressure and drought stress, a significant positive yield impact is observed which can provide significant yield preservation.

E. AGRONOMIC AND MORPHOLOGICAL CHARACTERISTICS

MIR604-derived hybrids and their non-transgenic controls were grown in 22 locations in 8 states during 2002 and 2003. Up to 18 separate agronomic traits were assessed at each trial; not all traits were recorded at each location (see Table 4). For the great majority of the agronomic traits compared in the two years of data, no statistically significant differences between MIR604-derived hybrids and their negative segregant control counterparts were observed. While some differences between transgenic and control plants were found to be significant, there were no consistent trends in the data across locations or across years that would indicate that any of these differences were due to the presence of the transgene. For example, agronomic traits HU5SN (heat units to 50% silking) and HUPSN (heat units to pollen shed) in the 2002 Stanton, MN trial (Appendix 1C, Table 1C) were significantly different (lower) for both MIR604-derived hybrids compared to their controls. However, these observations did not repeat themselves in the 2003 field trial held at Stanton, MN (Appendix 1C, Table 3C). These

differences also did not occur at other field trial sites in 2003, which were performed in the presence of natural Western and Northern CRW populations. Likewise, significant differences between agronomic traits (e.g. intactness rating, INTLR) observed between MIR604-derived hybrids and their negative isoline controls in 2002 field trials (Appendix 1C, Table 1C) were not observed in corresponding trials in 2003 (Appendix 1C, Table 2C and 4C).

Although instances of statistically different measurement between MIR604 and non-transgenic control hybrids were observed for some traits, the variation observed is within historical ranges derived from agronomic field trials conducted with transgenic events by Syngenta over the past decade. As MIR604 hybrids experience less root feeding damage than control hybrids, some significant agronomic and morphological differences are to be expected depending upon the trait being assessed and combination of pest pressure and environmental conditions experienced at each trial location.

TABLE 4. List and Definition of Traits Assessed in Agronomic Field Trials

Trait Code	Description
DROPP	Percent dropped ears just prior to harvest
EMRGP	Percent plants emerged. (Taken within approx. 14 days post-planting)
ERHTN	Ear height in cm. Taken at R2-R6 stage of corn development.
GMSTP	Grain moisture % measured at harvest time
HAVPN	Harvest population (plants per acre)
HU5PN	Heat units to 50% pollen shed
HU5SN	Heat units to 50% silking
HU9PN	Heat units to 90% pollen shed
HU9SN	Heat units to 90% silking
INTLR	Intactness rating (Late Season integrity of the plant above the ear; 1= completely intact, 9 = 100% of the plants are broken at the ear node)
LRTLP	Late root lodging % (recorded after anthesis)
PLHTN	Plant height in cm. Taken at R2 – R6 stage of corn development.
POL5N	Days to 50% plants pollen shedding
PSTSP	Push test for stalk/root quality on erect plants (data taken just prior to harvest)
SLK5N	Days to 50% plants silking
STKLP	Percent stalk lodging (data taken just prior to harvest)
TWSMN	Grain test weight in lbs/Bu at 15.5% moisture
YGSMP	Grain yield at standard 15.5% moisture

Specific disease trials were conducted in 2002 and 2003 where Syngenta Seeds agronomists, breeders and pathologists observed the disease susceptibility of MIR604-derived hybrids and negative segregant controls to a number of maize pathogens, including Northern corn leaf blight (*Helminthosporium turcicum*), Southern corn leaf blight (*Helminthosporium maydis*), Eyespot (*Kabatiella zeae*) and Gray leaf spot (*Cercospora zeae-maydis*). Disease susceptibility was measured by a 1-9 foliar disease

rating scale where 1 equals no lesions on a leaf and a score of 9 represents highly abundant lesions on all leaves (Appendix 1C, Table 5C). No indication of differential disease susceptibility was observed between negative segregant controls and MIR604-derived hybrids (Appendix 1C, Table 6C).

MIR604-derived hybrids have been grown under a number of USDA permits and notifications in 2002 and 2003 (see Chapter 1, Table 1). As part of the USDA field trial process, Syngenta is required to record any abnormal agronomic occurences observed during the course of the trial. For the 2002 and 2003 growing seasons, no adverse effects relating to non-target and pest insects were recorded. Similarly, there were no reports of enhanced susceptibility to insect pests in either 2002 or 2003.

F. PERFORMANCE OF MIR604 IN CONJUNCTION WITH INSECTICIDAL TREATMENT

A limited number of 2002 and 2003 field trials designed to (1) compare the insect control efficacy of MIR604-derived hybrids with currently practiced chemical insecticide treatments and (2) quantify any added efficacy, yield or agronomic performance afforded by the combination of applying low rates of chemical insecticides with MIR604 (Appendix 1D, Tables 1D-4D). MIR604 hybrids treated with Force 3G insecticide were assessed for efficacy in 2002 (Appendix 1D, Table 1D). During typical growing conditions experienced in Minnesota, root damage ratings for Force 3G treated MIR604 hybrids were not significantly different from similarly treated near isogenic control plants, though the values for root damage were numerically lower for both MIR604 hybrids (Appendix 1D, Table 1D). Under adverse growing conditions in Illinois for 2002, the mean root damage ratings for MIR604 hybrids were significantly lower compared to the negative controls. The difference between the two trials is most likely due to the inability of the insecticide treatment to protect the control roots during the drought conditions in Illinois.

Agronomic performance, including yield, of MIR604 hybrids compared to their near-isoline controls in the presence of Force 3G treatment was also assessed (Appendix 1D, Table 2D). Yield data across both 2002 and 2003 indicated no statistical difference between MIR604-derived hybrids and their negative segregant controls. The application of Force 3G on the negative isoline controls resulted in yields (in 2002) that were equivalent to those of Force 3G treated MIR604 hybrids despite the adverse growing conditions in IL. Clearly, the addition of an insecticide treatment helped protect against yield loss in the negative controls across the majority of locations in 2002 and 2003. One exception was trial (SYN673) in which the Force 3G treatment did not adequately protect the negative control hybrids due to adverse environmental conditions (wet early spring followed by drought) at both of the Bloomington locations in 2002. In this trial, the negative isoline control yield was significantly less than the MIR604 hybrid yield, a result which parallels 2002 yield data for a non-insecticide treated trial showing significant yield loss in the negative isoline control (see Appendix 1A, Table 2B). In 2003 trials, applying Force 3G insecticide to MIR604 hybrids does not appear to produce a synergistic effect on yield as the overall yield values are similar to the untreated non-transgenic controls (Appendix 1D, Tables 2D-4D).

Comparison of agronomic data clearly showed an absence of interaction between insecticidal treatment and performance of MIR604-derived hybrids (Appendix 1D, Tables 2D-4D). Some significant differences were observed between MIR604-derived hybrids and negative isoline controls (see Appendix 1D, Table 3D). However, these differences were not consistent between years, trials and hybrids.

G. CONCLUSIONS

Field trials conducted in 2002 and 2003 demonstrated that MIR604 hybrids provide consistent and statistically significant rootworm control (decreased root damage ratings) compared to non-transgenic controls. As a result, yield performance of MIR604-derived hybrids was statistically significantly greater compared to non-transgenic controls grown under adverse environmental conditions (e.g., drought) and equivalent to non-transgenic controls under normal growing conditions.

Agronomic data generated in 2002 and 2003 clearly show that MIR604-derived hybrids do not display statistically significant differences in agronomic characteristics that would render them phenotypically different than their near isogenic controls, except for traits (such as yield) that may be directly attributed to the root protection afforded by the inserted transgene.

MIR604-derived hybrids, in combination with an insecticide treatment, can provide significantly lower root damage than non-transgenic hybrids similarly treated during adverse growing conditions, and no adverse effects were observed in trials combining MIR604-derived hybrids and Force 3G insecticide.

Chapter 5. Compositional Analysis of Event MIR604

A. SUMMARY

Key nutritional components in maize grain and whole plants (forage) derived from Event MIR604 and near isogenic non-transgenic control plants were compared (Kramer, 2004; see Table 5 and 6 below). The whole plants and grain analyzed were from hybrid pairs (a hybrid pair consisting of transgenic and near isogenic control plants; see Figure 19) grown at 12 locations in the USA over two growing seasons (2002 and 2003). As would be expected from an analysis of this size, sporadic statistically significant differences were observed for some parameters between the MIR604 transgenic and near isogenic controls as represented in the summary of proximate analytes in Tables 7-10 below. All components evaluated in this study were within the range of reported literature values for maize with the exception of potassium in forage and phytosterols in grain.

At the time the forage potassium data were generated, Syngenta was unable to identify and provide a control range for concentrations of potassium in conventional corn forage. Syngenta subsequently conducted a study to measure potassium concentrations in non-transgenic forage using the same methodology that was employed in the original analysis of the MIR604 forage (de Fontes and Kramer, 2006; Table 11). In addition, further literature investigations revealed historical data that were previously overlooked. Forage potassium concentrations for MIR604 and its controls were compared and found to be within both the newly measured and literature ranges (Table 11).

Average phytosterol levels in both control and transgenic grain samples were below the average concentration reported in the literature (Table 12). Syngenta subsequently conducted a study to measure campesterol and stigmasterol concentrations in non-transgenic grain using the same methodology that was employed in the original analysis of the MIR604 grain (de Fontes and Kramer, 2005). Grain campesterol and stigmasterol concentrations for MIR604 and its controls were compared and found to be within the newly measured ranges (Table 12).

For all other analytes, no consistent patterns emerged to suggest that biologically significant changes in composition or nutritive value of the grain or forage had occurred as an unintended result of the transformation process or expression of the transgene. The conclusion based on these data is that there is strong evidence that the genetically modified MIR604 hybrids are substantially equivalent in composition to the isogenic controls, and other commercial hybrids.

Figure 19. **MIR604 Breeding history indicating generations used in the composition analysis of Event MIR604.** The partial breeding history is extracted directly from Figure 1.

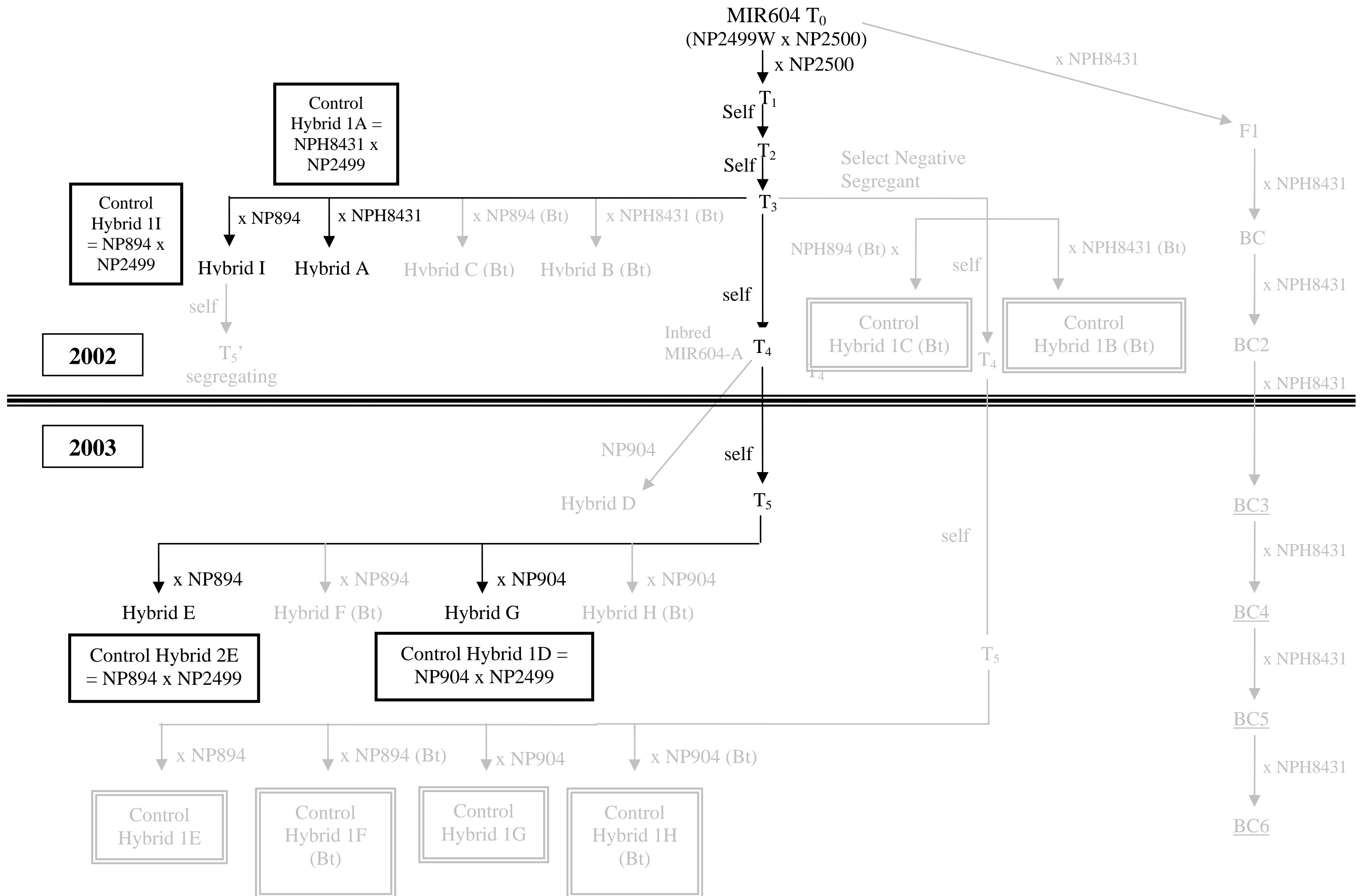


Table 5. Analytes Measured in Grain in 2002 and 2003 Growing Seasons[1]

Analyte	2002	2003
Proximates:		■
ash	■	■
fat	■	■
moisture	■	■
protein	■	■
carbohydrate		■
crude fiber	■	
Acid Detergent Fiber (ADF)		■
Neutral Detergent Fiber (NDF)		■
Total Dietary Fiber (TDF)		■
Minerals: Ca, Cu, Fe, Mg, Mn, P, K, Na, Zn, Cr or Se	■	■
Beta Carotene	■	■
Cryptoxanthin		■
Folic Acid	■	■
Vitamin B_1 (Thiamine)	■	■
Vitamin B_2 (Riboflavin)	■	■
Vitamin B_3 (Niacin)	■	■
Vitamin B_5 (Pantothenic Acid)		■
Vitamin B_6	■	■
Vitamin C		■
Vitamin E (Tocopherols)	■	■
Amino Acid Composition	■	■
Fatty Acid Profile (5 abundant)	■	■
Ferulic and p-Coumaric Acids		■
Furfural		■
Inositol		■
Phytic Acid		■
Raffinose		■
Trypsin Inhibitor		■
Phytosterols:		■
cholestrol		■
campesterol		■
stigmasterol		■
beta-sisterol		■

[1]shaded boxes indicate analytes that were measured in that year

Table 6. Analytes Measured in Forage in 2002 and 2003 Growing Seasons[1]

Analyte	2002	2003
Proximates:		■
ash	■	■
fat	■	■
moisture	■	■
protein	■	■
carbohydrate		■
crude fiber	■	
Acid Detergent Fiber (ADF)	■	■
Neutral Detergent Fiber (NDF)	■	■
Total Dietary Fiber (TDF)	■	■
Minerals: Ca, Cu, Fe, Mg, Mn, P, K, Na, Zn, Cr or Se	■	■

[1]shaded boxes indicate analytes that were measured in that year

Table 7. Summary of Proximate Analysis for 2002 MIR604 Grain. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids[1]. Hybrid designations correspond to those in Figure 19.

Analyte[2]	2002						Literature Values				
	MIR604 Hybrid A	Control Hybrid 1A	Std. Dev.	MIR604 Hybrid I	Control Hybrid 1I	Std. Dev.	OECD (2002)	ILSI (2004)	USDA (2004)	Watson (1987)	Souci (1994)
Moisture	9.18	8.90	0.33	8.93	9.37	0.43	7.0-23	6.1-26.2	10.37	7-23	12-13.2
Protein	10.44	10.02	0.49	11.21	11.41	0.39	6-12.7	6.15-15.01	9.42	6-12	7.61-9.84
Total Fat	2.65	2.63	0.08	3.09	2.89	0.24	3.1-5.8	1.742-5.564	4.74	3.1-5.7	3.20-4.30
Ash	1.56	1.55	0.10	1.59	1.62	0.07	1.1-3.9	0.616-6.282	1.2	1.1-3.9	N/A[4]
Starch	68.70	68.90	1.40	67.99	69.09	1.44	N/A	67.8-73.8	N/A	61-78	60.98-63.80
Crude Fiber[3]	3.49	3.39	0.21	3.49	3.46	0.25	N/A	N/A	N/A	N/A	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid A = Hybrid D, Control Hybrid 1A = Control Hybrid C, Hybrid I = Hybrid F, Control Hybrid 1I = Control Hybrid E.

[2]Percent dry weight except for Moisture (% fw).

[3]Literature values for crude fiber were determined by taking the cumulative low and high values from NDF, ADF and TDF (1.82 – 25.63 % dw for grain).

[4] Data not available.

Table 8. Summary of Proximate Analysis for 2003 MIR604 Grain. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids[1]. Hybrid designations correspond to those in Figure 19.

Analyte[2]	2003						Literature Values				
	MIR604 Hybrid G	Control Hybrid 1D	Std. Dev.	MIR604 Hybrid E	Control Hybrid 2E	Std. Dev.	OECD (2002)	ILSI (2004)	USDA (2004)	Watson (1987)	Souci (1994)
Moisture	9.84	9.84	0.40	9.90	10.32	0.35	7.0-23	6.1-26.2	10.37	7-23	12-13.2
Protein	10.88*	10.42	0.61	11.8*	11.00	0.70	6-12.7	6.15-15.01	9.42	6-12	7.61-9.84
Total Fat	3.53	3.38	0.37	3.88	3.46	0.32	3.1-5.8	1.742-5.564	4.74	3.1-5.7	3.20-4.30
Ash	1.55	1.51	0.15	1.50	1.48	0.11	1.1-3.9	0.616-6.282	1.2	1.1-3.9	N/A[4]
Carbohydrate[3]	84.0*	84.7	0.7	82.8*	84.0	0.6	82.2-82.9	77.4-89.5	74.26	N/A	N/A
Starch	55.2	56.7	2.5	54.5	55.7	2.0	N/A	67.8-73.8	N/A	61-78	60.98-63.80
NDF	13.4	12.9	1.2	12.9	13.2	2.0	8.3-11.9	5.59-22.64	N/A	8.3-11.9	N/A
ADF	5.5	4.9	0.8	5.2	4.9	0.9	3.0-4.3	1.82-11.34	N/A	3.3-4.3	N/A
TDF	13.4	14.1	0.9	13.1	13.5	0.8	11.1	11.8-25.63	N/A	N/A	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, Control Hybrid 1D = Control Hybrid E1, Hybrid E = Hybrid E4, Control Hybrid 2E = Control Hybrid E2.

[2]Percent dry weight except for Moisture (% fw)

[3]Carbohydrates are calculated as the percentage of dry weight = 100% - % protein - % fat - % ash.

[4]Data not available.

*An F-Test probability of <5% was observed indicating a significant difference between transgenic and control. However, all values are within historical ranges demonstrating that hybrids derived from Event MIR604 are not materially different in composition from near isogenic controls.

Table 9. Summary of Proximate Analysis for 2002 MIR604 Forage. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids. Hybrid designations correspond to those in Figure 19.

Analyte[1]	2002						Literature Values	
	MIR604 Hybrid A	Control Hybrid 1A	Std. Dev.	MIR604 Hybrid I	Control Hybrid 1I	Std. Dev.	OECD (2002)	ILSI (2004)
Moisture	64.21	64.63	2.59	71.62	71.94	2.05	62-72	55.3-80.4
Protein	7.27	7.46	0.27	8.02	8.24	0.55	4.7-9.2	3.14-11.56
Total Fat	1.54	1.92	0.38	1.37	1.68	0.37	1.5-3.2	0.373-4.570
Ash	3.46	3.76	0.15	4.01	3.82	0.29	2.9-5.7	1.997-9.638
NDF	37.41	38.20	3.82	39.43	37.63	1.76	40.0-48.2	20.29-63.71
ADF	20.84	21.78	1.63	21.77	21.30	1.23	25.6-34	16.13-41.92
Crude Fiber[3]	19.52	20.38	1.82	20.52	20.30	1.30	N/A[4]	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid A = Hybrid D, Control Hybrid 1A = Control Hybrid C, Hybrid I = Hybrid F, Control Hybrid 1I = Control Hybrid E.

[2]Percent dry weight except for Moisture (% fw)

[3]Literature values for crude fiber were determined by taking the cumulative low and high values from NDF, ADF and TDF (1.82 – 25.63 % dw for grain).

Table 10. Summary of Proximate Analysis for 2003 MIR604 Forage. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids[1]. Hybrid designations correspond to those in Figure 19.

	2003						Literature Values	
Analyte[2]	MIR604 Hybrid G	Control Hybrid 1D	Std. Dev.	MIR604 Hybrid E	Control Hybrid 2E	Std. Dev.	OECD (2002)	ILSI (2004)
Moisture	66.89*	65.10	2.13	72.75*	70.61	2.32	62-72	55.3-80.4
Protein	9.03	8.31	0.96	8.46	8.60	0.85	4.7-9.2	3.14-11.56
Total Fat	2.15	2.12	0.48	1.46	1.64	0.46	1.5-3.2	0.373-4.570
Ash	4.43	4.24	0.55	4.23	4.08	0.56	2.9-5.7	1.997-9.638
Carbohydrate[3]	84.4	85.3	1.1	85.9	85.7	1.1	N/A[4]	76.4-91.5
NDF	41.2	42.4	3.4	44.3	45.2	5.3	40.0-48.2	20.29-63.71
ADF	28.1	28.8	4.0	27.7	29.4	3.4	25.6-34	16.13-41.92
TDF	48.0	47.8	3.2	54.1	53.0	3.7	N/A	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, Control Hybrid 1D = Control Hybrid E1, Hybrid E = Hybrid E4, Control Hybrid 2E = Control Hybrid E2.

[2]Percent dry weight except for Moisture (% fw)

[3]Carbohydrates are calculated as the percentage of dry weight = 100% - % protein - % fat - % ash.

[4]Data not available.

*An F-Test probability of <5% was observed indicating a significant difference between transgenic and control. However, all values are within historical ranges demonstrating that hybrids derived from Event MIR604 are not materially different in composition from near isogenic controls.

Table 11. Potassium Levels Measured in Forage from Conventional and MIR604 Maize Hybrids. Hybrid designations correspond to those in Figure 19.

Analyte[1]	Conventional Corn		Transgenic Hybrids[2]		Literature Values					
	Mean	Range	MIR604 Hybrid G	MIR604 Hybrid E	Adams (1974)	Ballard *et al.* (2001)	Berger (1990)	Buchman-Smith *et al.* (1974)	Kappel *et al.* (1985)	Thomas *et al.* (2001)
Potassium	0.854	0.437-1.45	1.196	1.271	0.02-3.28	1.10-1.33	1.08	0.172-1.87	0.25-1.94	1.06-1.30

[1]Data for conventional corn are derived from 95 samples from 5 non-transgenic commercial hybrids. Data for transgenic corn are derived from 10 samples averaged across a miminum of 10 locations. All units are % dw.

[2]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, and Hybrid E = Hybrid E4.

Table 12. Summary of Phytosterol Analysis in Conventional Field Corn and MIR604 Grain. Hybrid designations correspond to those in Figure 19.

Analyte[1]	Conventional Corn		Transgenic Hybrids[2]		Literature Values[3]
	Mean	Range	MIR604 Hybrid G	MIR604 Hybrid E	
Campesterol	12.1	6.9-18.4	14.0	12.4	32
Stigmasterol	6.9	3.7-10.3	6.33	5.28	21
Beta-sitosterol	50.4	38.3-62.9	45.5	42.6	120

[1]Data for conventional corn are derived from 93 samples from 10 non-transgenic commercial hybrids. Data for transgenic corn are derived from 10 samples averaged across a miminum of 10 locations. All units are mg/100g dw.

[2]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, and Hybrid E = Hybrid E4.

[3]Souci (1994).

Chapter 6. Quantification of mCry3A and PMI Proteins in Event MIR604

A. SUMMARY

To characterize the range of expression of the mCry3A and PMI proteins in maize (corn) plants derived from Event MIR604, the concentrations of mCry3A protein (the active insecticidal principle) and PMI (the selectable marker) were determined by ELISA in several plant tissues and whole plants at four growth stages (whorl, anthesis, seed maturity and senescence; Joseph and Hill, 2003) in two field maize hybrids (Hybrid A and Hybrid I[3]) and one maize inbred (Inbred MIR604-A; see Figure 20). Hybrids A and I were derived from crossing the elite inbred MIR604-A into the T_3 generation of MIR604 (hemizygous for the transgenes) as depicted in the MIR604 breeding tree in Figure 20. Elite inbred MIR604-A was derived from recurrent selection followed by identification of a homozygous line. The quantity of mCry3A protein was also estimated on a per-acre and a per-hectare basis. Additionally, mCry3A and PMI levels in silage were measured 15, 29 and 75 days after ensiling the plant material from one maize hybrid (Hybrid A).

Quantifiable levels of mCry3A protein were detected in all Event MIR604-derived plant tissues analyzed except pollen. Across all growth stages, mean mCry3A levels measured in leaves, roots and whole plants ranged from *ca.* 3 - 23 µg/g fresh wt. (4 - 94 µg/g dry wt.), *ca.* 2 - 14 µg/g fresh wt. (7 - 62 µg/g dry wt.), and *ca.* 0.9 - 11 µg/g fresh wt. (3 - 28 µg/g dry wt.), respectively. Mean mCry3A levels measured in kernels at seed maturity and senescence ranged from *ca*. 0.6 – 1.4 µg/g fresh wt. (0.8 – 2.0 µg/g dry wt.). Mean mCry3A levels measured in kernels from the MIR604 hybrids at senescence (corresponding to the stage closest to grain harvest) were *ca*. 0.7 µg/g fresh wt. (0.9 µg/g dry wt.) across both hybrid genotypes. Mean mCry3A levels measured in silk tissue at anthesis were below the lower limit of quantification (LOQ), <0.1 µg/g fresh wt. (<1.0 µg/g dry wt.). Mean mCry3A levels measured in silk tissue at seed maturity ranged from *ca*. 0.6 – 1.9 µg/g fresh wt. (1 – 3 µg/g dry wt.). No mCry3A protein was detectable in pollen from neither the inbred MIR604-A nor the hybrids A and I (limit of detection (LOD) = 0.07 µg/g fresh wt., 0.15 µg/g dry wt.).

The levels of mCry3A were generally similar between hybrids for each tissue type at each time point. For the inbred line, mCry3A expression was generally higher than in the hybrids in leaves, roots and whole plants at whorl and anthesis stages and in roots at seed maturity. Over the growing season and across genotypes, estimates of mCry3A in MIR604-derived plants ranged from mean levels of *ca.* 8 g mCry3A/acre (21 g mCry3A/hectare) at senescence stage to *ca.* 240 g mCry3A/acre (592 g mCry3A/hectare) at seed maturity, assuming a planting density of 26,500 plants per acre (65,500

[3] The hybrids listed are designated differently in the Joseph and Hill 2003 report: Hybrid A = MIR604-B and Hybrid I = MIR604-C.

plants/hectare). In silage, the average mCry3A level measured was 2.5 µg/g fresh wt. (7.3 µg/g dry wt.) over 15, 29 and 75 days. By comparison, the level of mCry3A measured in the chopped plant material prior to ensiling was *ca*. 8 µg/g fresh wt. (20 µg/g dry wt).

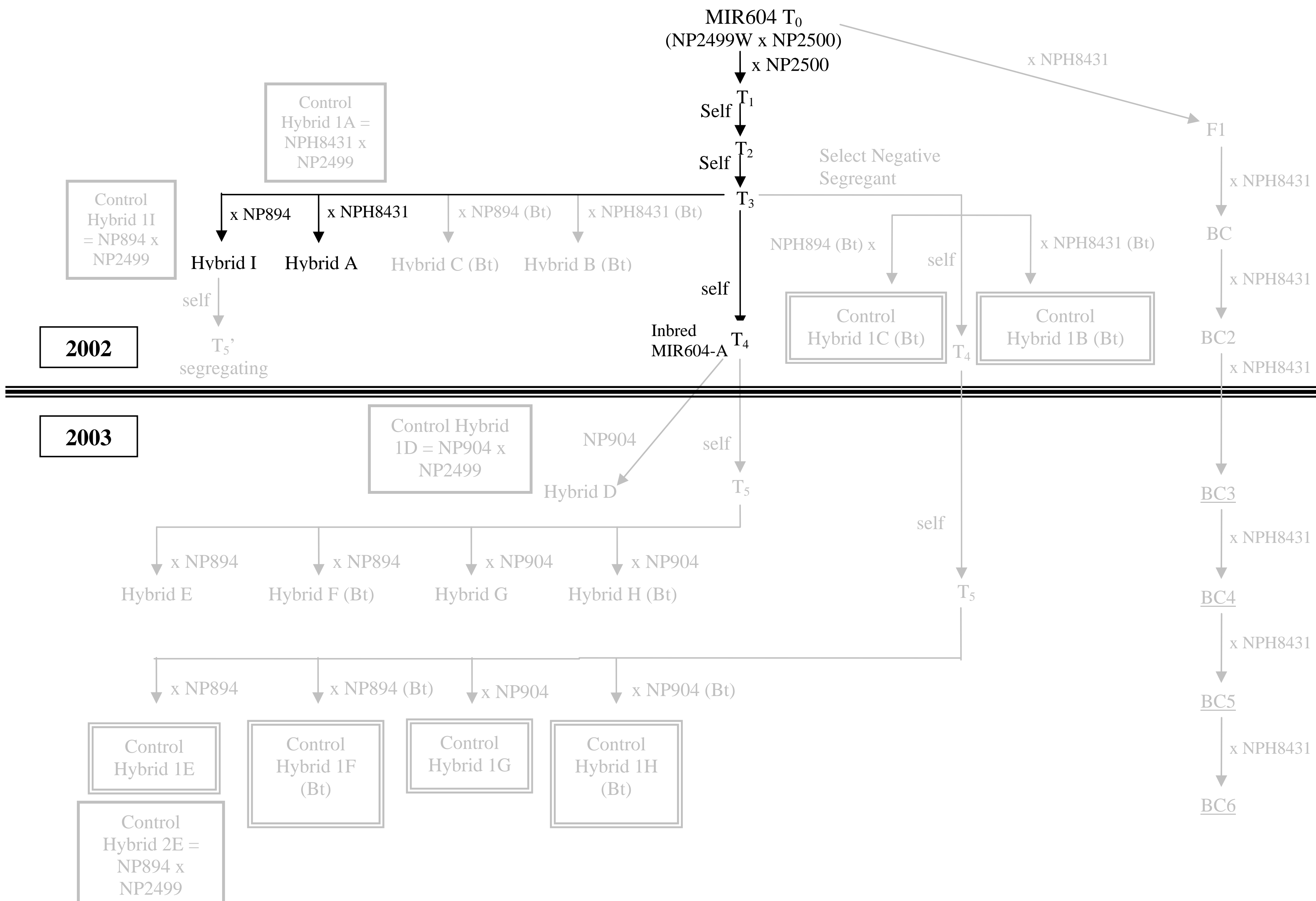
PMI protein was detected in most of the Event MIR604-derived plant tissues analyzed, albeit at low levels. Across all plant stages, mean PMI levels measured in leaves, roots and whole plants ranged from not detectable (ND) to *ca.* 0.4 µg/g fresh wt. (ND – 2.1 µg/g dry wt.), below the LOQ (<0.03 µg/g fresh wt.) to *ca*. 0.2 µg/g fresh wt. (<0.1 – 1.0 µg/g dry wt.), and below the LOQ (<0.02 µg/g fresh wt.) to *ca.* 0.3 µg/g fresh wt. (<0.04 – 2 µg/g dry wt.), respectively. Mean PMI levels measured in kernels at seed maturity and senescence ranged from below the LOQ (<0.06 µg/g fresh wt.) to *ca.* 0.4 µg/g fresh wt. (<0.07 – 0.5 µg/g dry wt.). Mean PMI levels measured in kernels from the MIR604 hybrids at senescence (corresponding to the stage closest to grain harvest) were <0.14 µg/g fresh wt. (<0.17 µg/g dry wt.) across both hybrid genotypes. Mean PMI levels measured in silk tissue at anthesis and seed maturity ranged from below the LOQ (<0.1 µg/g fresh wt.) to *ca.* 0.8 µg/g fresh wt. (<0.2 – 6.8 µg/g dry wt.). PMI in pollen ranged from *ca.* 1.9 – 2.6 µg/g fresh wt. (3.9 – 5.2 µg/g dry wt.).

The levels of PMI were generally similar among the inbred and hybrid genotypes for each tissue type at each time point. PMI was not detectable in silage at all three sampling times (day 15, 29 and 75). By comparison, the level of PMI measured in the chopped plant material prior to ensiling was *ca*. 0.3 µg/g fresh wt. (0.7 µg/g dry wt.).

The stability of mCry3A and PMI protein expression over multiple generations was evaluated. Seed from four successive backcross generations (representing genotypes that were hemizygous for the Event MIR604 transgenes) was grown under greenhouse conditions and leaf material was collected at anthesis for analysis of mCry3A and PMI protein levels. Mean mCry3A protein levels across the four generations were *ca*. 2.3 – 3.1 µg/g fresh wt. (*ca*. 11.8 – 15.5 µg/g dry wt.). Overall, levels were similar across the four generations analyzed and there was no evidence of any significant trend either up or down, indicating that the expression of mCry3A protein is stable.

A similar result was seen for the PMI protein. Mean PMI protein levels across the four generations were *ca*. 0.2 – 0.3 µg/g fresh wt. (*ca*. 1.1 – 1.3 µg/g dry wt.). Overall, levels were similar across the four generations analyzed and there was no evidence of any significant trend either up or down, indicating that the expression of PMI protein is stable. Therefore, both mCry3A and PMI appear to be stably expressed in Event MIR604 maize across multiple generations.

Figure 20. **MIR604 Breeding history indicating generations used in the quantification analysis of Event MIR604.** The partial breeding history is extracted directly from Figure 1.



Chapter 7. Environmental Safety of mCry3A

A. INTRODUCTION

Three sources of data are used to evaluate the environmental safety of the modified Cry3A (mCry3A) *Bacillus thuringiensis*-related insect control protein expressed in the transgenic corn Event MIR604. First, we present a summary of data collected by Syngenta to establish how the spectrum of activity of mCry3A differs from native Cry3A. These data suggest that the toxicity of mCry3A differs from native Cry3A only in the intended increase in activity against certain pest species in the genus *Diabrotica* (Coleoptera: Chrysomelidae). Secondly, we review data on the spectrum of activity of native Cry3A. These data are not used directly to evaluate the risks to non-target organisms from mCry3A in MIR604 because quantitative comparisons of the activity of native Cry3A in the studies and mCry3A in MIR604 are not possible. The purpose of citing these data is to provide a weight of evidence about the likely spectrum of activity (*i.e.*, the hazard) of mCry3A: as Syngenta's laboratory studies show that mCry3A and native Cry3A differ only in the intended increase of activity of mCry3A to certain *Diabrotica* species, the toxicity of native Cry3A is a good predictor of the hazard of mCry3A to non-target organisms (NTOs).

From the native Cry3A data we predict that mCry3A is unlikely to be hazardous except to certain species in 3 families of Coleoptera: the Chrysomelidae (leaf beetles, flea beetles and rootworms), the Curculionidae (weevils and snout beetles) and the Tenebrionidae (darkling beetles). The third source of data which was then used to test this hypothesis, was single-species laboratory studies exposing representative NTOs to concentrations of mCry3A in excess of the expected environmental concentrations (EECs) of mCry3A resulting from the proposed cultivation of MIR604 corn hybrids (hereafter MIR604). Test species were chosen to represent taxa that might be exposed to mCry3A *via* tissues of Event MIR604, or taxa related to the western corn rootworm (*Diabrotica virgifera virgifera*) and northern corn rootworm (*D. longicornis barberi*), the larvae of which are the main target pests of MIR604. In addition, test species were selected to include functional groups found in agricultural fields and other habitats into which mCry3A might spread: birds, freshwater fish, predators and parasitoids of crop pests, soil invertebrates and pollinators.

To ensure an adequate margin of exposure (*i.e.,* a "safety margin") under field conditions, studies on invertebrates were designed to expose test organisms to at least 10 times the EEC of mCry3A using conservative assumptions, yet using likely routes of exposure for the species in question. The margin of exposure for the bird study was calculated in terms of a dose rather than a concentration; bobwhite quail were exposed to about 1000X the worst-case daily dietary dose of mCry3A for a seed-eating bird.

The principal route of exposure to mCry3A for most non-target organisms will be through ingestion of plant material containing mCry3A, or through consumption of prey that has fed on plants containing mCry3A. Therefore to achieve a margin of exposure, most of the test species were exposed to mCry3A *via* a preparation of microbially

expressed mCry3A (Test Substance MCRY3A-0102; 90.3% pure) incorporated into artificial diets. For fish, the principal route of exposure to mCry3A will be consumption of fish feed formulated with corn grain; hence rainbow trout were given feed prepared from MIR604 grain. The safety margin was achieved by preparing the feed using the highest possible proportion of corn grain and the mildest pelleting to minimize heat degradation of mCry3A. No adverse effects of exposure to mCry3A were observed in any of the non-target organism studies.

For each of the tests, a summary of the study design and results is provided, together with a description of the test materials and, where relevant, studies to characterize mCry3A in the diet or test substrate. Final reports of the non-target organism studies (see Table 13) have been submitted to the US Environmental Protection Agency (US EPA) as part of US EPA Experimental Use Permit (67979-EUP-U) and Section 3 (67979-L) applications for Event MIR604 corn.

TABLE 13. List of Environmental Safety Reports Previously Submitted to U.S. EPA

Study Name	MRID #	Reference
Summary of Data Demonstrating the Environmental Safety of Modified Cry3A *Bacillus thuringiensis* Insect Control Protein and Event MIR604-Derived Corn (Maize) to Non-Target Organisms	46155615	Raybould, 2003
An Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Northern Bobwhite	46155616	Gallagher *et al.*, 2003
A 28-Day Laboratory Study to Evaluate the Effects of Modified Cry3A Maize Fish Feed (FFMIR604-0103) on the Growth of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)	46155617	Hutchings and Caunter, 2003
A semi-field test to evaluate the effects of the modified Cry3A protein (MCRY3A-0102) on brood development of the honeybee, *Apis mellifera* (Hymenoptera: Apidae)	46155618	Halsall, 2003
Environmental Safety Assessment of Modified Cry3A Protein and Event MIR604 corn to Non-Target Organisms	46265601	Raybould, 2004b
Characterization of Fish Feed Test Substance (FFMIR604-0103) Prepared From Event MIR604-Derived Maize Grain	46265602	Graser, 2004a
A Laboratory Test of the Toxicity of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae)	46265603	Waterman, 2003
Analysis of Test Diet Used to Expose *Coccinella septempunctata* to Modified Cry3A Protein: Supplement to a Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle *Coccinella septempunctata* (Coleoptera: Coccinellidae)'	46265604	Graser, 2004c
A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Ground-Dwelling Beetle, *Poecilus cupreus* (Coleoptera: Staphylinidae)	46265605	Vinall, 2004a
Analysis of Test Diet Used to Expose *Poecilus cupreus* to Modified Cry3A Protein: Supplement to a Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) Larvae of the Ground-Dwelling Beetle, *Poecilus cupreus* (Coleoptera: Carabidae)'	46265606	Graser, 2004e
A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylinidae)	46265607	Vinall, 2003b
Analysis of Test Diet Used to Expose *Aleochara bilineata* to Modified Cry3A Protein: Supplement to a Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylindae)'	46265608	Graser, 2004d

Study Name	MRID #	Reference
A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)	46265609	Vinall, 2003a
Analysis of Test Diet Used to Expose *Orius insidiosus* to Modified Cry3A Protein: Supplement to a Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)'	46265610	Graser, 2004b
Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the Earthworm *Eisenia foetida* in an Artificial soil Substrate	46265611	Vinall, 2004b
Analysis of Soil Used to Expose *Eisenia foetida* to Modified Cry3A Protein: Supplement to a Report Titled 'Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the earthworm *Eisenia foetida* in an Artificial Soil Substrate'	46265612	Graser, 2004f
Environmental Fate Assessment of Modified Cry3A Protein in Event MIR604 Corn	46265613	Raybould, 2004a
Laboratory Soil Degradation of Modified Cry3A Protein (MCRY3A-0102)	46265614	Kramer and Joseph, 2004

B. HAZARD ASSESSMENT OF MODIFIED CRY3A

B.1. Syngenta studies comparing mCry3A and native Cry3A

Lepidopteran and coleopteran pests of corn, and a dipteran pest of fruit crops, were tested at Syngenta for sensitivity to mCry3A (see Table 14). In most cases, native Cry3A was also included in the tests for comparison. These laboratory bioassays were conducted as screening tests, using a concentration from 500-600 μg of either mCry3A or native Cry3A protein/ml diet. In most cases, first instars (10 larvae per replicate, 3 replicates) were fed artificial diet with an overlay of microbially expressed mCry3A or native Cry3A protein solution. The microbially expressed mCry3A protein was produced using the same modified *cry3A* gene that was used in the maize transformation that resulted in event MIR604. Due to problems with quarantine and commercial availability of Mexican corn rootworm (*Diabrotica virgifera zeae*; MCRW), this species was not tested in laboratory bioassays. However, Event MIR604 was tested for efficacy against this species in the field. Field trial data indicate that Event MIR604 derived corn plants have significant resistance against root damage caused by MCRW larval feeding when compared to negative controls (see **Agronomic Performance of Event MIR604 Hybrids**, Chapter 4).

Table 14. Susceptibility of insect pest species to native Cry3A and mCry3A proteins via direct exposure in laboratory studies conducted at Syngenta (unpublished data)

Order/ Species	Common name	Family	Cry3A	mCry3A
Coleoptera				
Leptinotarsa decemlineata	Colorado potato beetle	Chrysomelidae	Active	Active
Diabrotica virgifera virgifera	Western corn rootworm	Chrysomelidae	Not Active	Active
Diabrotica longicornis barberi	Northern corn rootworm	Chrysomelidae	Inconsistent Activity	Active
Diabrotica undecimpunctata	Southern corn rootworm Spotted cucumber beetle	Chrysomelidae	Not Active	Not Active
Diabrotica balteata	Banded cucumber beetle	Chrysomelidae	LowActivity[1]	Active
Anthonomus grandis	Cotton boll weevil	Curculionidae	Not Active	Not Active
Lepidoptera				
Agrotis ipsilon	Black cutworm	Noctuidea	Not Active	Not Active
Helicoverpa zea	Corn earworm	Noctuidae	Not Active	Not Active
Ostrinia nubilalis	European corn borer	Pyralidae[2]	Not Active	Not Active
Spodoptera frugiperda	Fall armyworm	Noctuidea	Not Active	Not Active
Pectinophora gossypiella	Pink bollworm	Gelechiidae	Not Active	Not Active
Heliothis virescens	Tobacco budworm	Noctuidea	Not Active	Not Active
Diptera				
Drosophila melanogaster	Fruit fly	Drosophilidae	Not tested	Not Active

[1] From Herrnsdtadt *et al.,* 1987
[2] Alternatively classified as Crambidae

These results confirmed that native Cry3A is primarily active against CPB and has minimal activity against NCRW, both members of the Chrysomelidae family of beetles. The results also showed that 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 corn in the USA.

The mCry3A as expressed in Event MIR604 corn showed moderate activity against *Diabrotica balteata*, whereas a previous report indicated that native Cry3A had low activity against these chrysomelid beetles (Herrnstadt *et al*., 1987). However, native Cry3A was not included in this Syngenta test so a direct comparison is not available.

In summary, these data show that the mCry3A toxin expressed in Event MIR604 has enhanced toxicity towards some species of chrysomelid beetles (NCRW and WCRW) when compared with native Cry3A, but has no apparent altered activity to the other insect species tested, with the possible exception of *D. balteata*.

B.2 Published literature and data reports

This section collates studies on the toxicity of native Cry3A from the scientific literature and regulatory reports submitted to the US EPA. The purpose is to delimit the taxonomic range of sensitivity to native Cry3A. Because studies in Section B.1.a suggest

that mCry3A has the same spectrum as native Cry3A, apart from intended enhanced activity against particular *Diabrotica* species, the limit of toxicity of native Cry3A predicts the taxonomic limits of the toxicity of mCry3A. The data in this section are not intended for use directly in a quantitative risk assessment of MIR604; they are intended to contribute to the weight of evidence that the hazard of mCry3A is restricted to species in 3 families of Coleoptera.

The effects of native Cry3A protein on many insect pests have been reported in the literature (Table 15). Results from different studies are sometimes difficult to compare, as the levels of activity are often qualitative (low, high, or intermediate activity); there are no established guidelines that define these categories in reference to mortality observed in the tests. However, the results obtained to date show that only pest species from the order Coleoptera are susceptible to the Cry3A toxin. Among the coleopteran pest species tested, only some species belonging to the Chrysomelidae, Curculionidae and Tenebrionidae families were shown to be susceptible to Cry3A in laboratory assays.

Additionally, transgenic plants expressing Cry3A protein have demonstrated efficacy in controlling coleopteran pests in the Chrysomelidae family. These include potatoes (Perlak *et al*., 1993; Adang *et al*., 1993) and tomatoes (Rhim *et al.*, 1995) for control of CPB; poplar plants for control of *Chrysomela tremulae* (Génissel *et al*., 2003); and eucalyptus plants for control of *Chrysophtharta bimaculata, Chrysophtharta agricola* and *Chrysophtharta variicolis* (Harcourt *et al*., 2000).

Table 15. Susceptibility of insect pest species to native Cry3A proteins via direct exposure in laboratory studies (van Frankenhuyzen and Nystrom, 2002)

ORDER/Family *Species*	Gene	Stage/Instar[1]	Activity	Reference
COLEOPTERA **Chrysomelidae**				
Chrysomela scripta	cry03Aa1	L2	highly active	Federici and Bauer, 1998
Diabrotica balteata	cry03Aa1	larva	low activity	Herrnstadt *et al.,* 1987
Diabrotica balteata	cry03Aa1	adult	low activity	Herrnstadt *et al.,* 1987
Diabrotica undecimpuctata	cry03Aa1	adult, L1, L2	low activity	Herrnstadt *et al.,* 1986
Diabrotica undecimpunctata	cry03Aa4	neonate	not active	Macintosh *et al.,* 1990
Diabrotica undecimpunctata	cry03Aa5	adult	not active	Johnson *et al.,* 1993
Diabrotica undecimpunctata	cry03Aa5	L1	low activity	Johnson *et al.,* 1993
Haltica tombacina	cry03Aa1	adult,L2, L3	highly active	Herrnstadt *et al.,* 1986
Leptinotarsa decemlineata	cry03Aa1	L1, L2	highly active	Herrnstadt *et al.,* 1986
Leptinotarsa decemlineata	cry03Aa2	neonate	active	Sekar *et al.,* 1987
Leptinotarsa decemlineata	cry03Aa2	L2	active	Wu and Dean., 1996
Leptinotarsa decemlineata	cry03Aa4	L1	active	MacIntosh *et al.,* 1990
Leptinotarsa decemlineata	cry03Aa4	neonate	active	McPherson *et al.,* 1988
Leptinotarsa decemlineata	cry03Aa5	neonate	active	Donovan., 1988
Leptinotarsa decemlineata	cry03Aa5	adult	not active	Johnson *et al,* 1993
Leptinotarsa decemlineata	cry03Aa5	L1	active	Johnson *et al.,* 1993
Phaedon cochleariae	cry03Aa	L2	highly active	Carroll *et al.,* 1989
Phyllotreta armoraciae	cry03Aa4	L1	not active	MacIntosh *et al.,* 1990
Pyrrhalta luteola	cry03Aa1	not specified	highly active	Herrnstadt *et al.,* 1986

ORDER/Family *Species*	Gene	Stage/Instar[1]	Activity	Reference
<u>COLEOPTERA</u>				
Tenebrionidae				
Tenebrio molitor	cry03Aa	not specified	low activity	Carroll *et al*., 1989
Tenebrio molitor	cry03Aa1	L1, L2, L3	intermediate activity	Herrnstadt *et al.,* 1986
Tribolium cataneum	cry03Aa1	adult, L3	intermediate activity	Herrnstadt *et al.,* 1986
Tenebrio molitor	cry03Aa2	L7	low activity	Wu and Dean., 1996
Curculionidae				
Anthonomus grandis	cry03Aa1	adult, L2, L3	highly active	Herrnstadt *et al.,* 1986
Anthonomus grandis	cry03Aa4	neonate	not active	Macintosh *et al.,* 1990
Hypera brunneipennis	cry03Aa1	adult	not active	Herrnstadt *et al.,* 1987
Hypera brunneipennis	cry03Aa1	larva	highly active	Herrnstadt *et al.,* 1987
Hypera postica	cry03Aa4	L1	not active	Macintosh *et al.,* 1990
Otiorhynchus sulcatus	cry03Aa1	L2, L3	intermediate activity	Herrnstadt *et al.,* 1986
Premnotrypes vorax[2]	cry03Aa	L1	active	Gomez *et al.,* 2000
Dermestidae				
Attagenus unicolor	cry03Aa1	L3	not active	Herrnstadt *et al.,* 1986
Bruchidae				
Callosobruchus maculatus	cry03Aa1	adult	low activity	Herrnstadt *et al.,* 1987
Callosobruchus maculatus	cry03Aa1	larva	low activity	Herrnstadt *et al.,* 1987
Nitidulidae				
Carpophilus hempterus	cry03Aa1	larva	not active	Herrnstadt *et al.,* 1987
Ptinidae				
Gibbium psylloides	cry03Aa1	adult	not active	Herrnstadt *et al.,* 1986
Scarabeidae				
Popillia japonica	cry03Aa4	L1	not active	Macintosh *et al.,* 1990
<u>DIPTERA</u> **Cuclidae** *Aedes aegypti*	cry03Aa1	L1	not active	Herrnstadt *et al.,* 1986
Muscidae *Musca domestica*	cry03Aa1	larva	not active	Herrnstadt *et al.,* 1987
<u>LEPIDOPTERA</u> **Noctuidae**				
Spodoptera exigua	cry03Aa1	L1	not active	Herrnstadt *et al.,* 1986
Trichoplusia ni	cry03Aa1	L1	not active	Herrnstadt *et al.,* 1986

[1] L1, L2, L3, etc. refer to instars 1, 2, and 3, etc.

[2] Entry is from the literature and does not appear in van Frankenhuyzen and Nystrom database

In addition to these studies, data supporting the registration of a Cry3A plant-incorporated protectant in Monsanto's NewLeaf® Bt potato include tests of the sensitivity of selected insect pest species to Cry3A. These included three rootworm species; four lepidopterans (European corn borer, tobacco hornworm, corn earworm, and tobacco budworm); one dipteran (yellow fever mosquito); one orthopteran (German cockroach); and one hemipteran (green peach aphid) (US EPA, 2001a). Among the tested species, only the Colorado potato beetle (*Leptinotarsa decemlineata*: Chrysomelidae) displayed significant mortality.

In addition to the tests conducted to establish the toxicity of Cry3A protein to insect pest species, there are a number of studies in the literature that show that native Cry3A does not have any detectable toxicity to non-target insect species and other invertebrates (see Table 16).

Table 16. Non-target invertebrate species shown to be insensitive to native Cry3A protein as expressed in Bt potato via direct exposure in laboratory studies

Order/ Test Species	**Common name**	**Reference**
Coleoptera		
Hippodamia convergens	convergent ladybeetle	US EPA, 2001a
Hymenoptera		
Apis mellifera	honeybee	US EPA, 2001a
Nasonia vitripennis	parasitic wasp	US EPA, 2001a
Isotomidae		
Folsomia candida	springtail (collembola)	Sims and Martin, 1997
Xenylla grisea	springtail (collembola)	Sims and Martin, 1997
Neuroptera		
Chrysoperla carnea	green lacewing	US EPA, 2001a
NON-INSECT SPECIES		
Phylum/Subphylum/ *Genus + species*		
Annelida		
Eisenia foetida	earthworm	US EPA, 2001a

There are many reports in the literature demonstrating that Bt δ-endotoxins do not have adverse toxic effects on mammalian or avian species, or freshwater fish (US EPA, 2001a; Mendelsohn *et al.,* 2003). Studies supporting the registration of a native Cry3A plant-incorporated protectant in Bt potato showed that this protein is not toxic to mammals or birds (Table 17).

Table 17. Tests of native Cry3A on vertebrate species.

Species	Study type	Treatment	Results	References
bobwhite quail	Dietary toxicity	50,000 ppm in Cry3A potato tubers	No treatment related adverse effects	US EPA, 2001a
mouse	Acute oral toxicity	5220 mg Cry3A/kg body weight	No treatment related adverse effects	US EPA, 2001a

B.3 Conclusions from Studies of Cry3A and mCry3A Among Target and Non-Target Species

The data described in this summary show that the toxicity of native Cry3A proteins is limited to certain species of Coleoptera in the families Chrysomelidae, Tenebrionidae and Curculionidae. No toxic effects have been recorded on non-target insect species or on other invertebrates or vertebrates. As described herein (see Table 14), data generated by Syngenta demonstrate that the mCry3A protein as expressed in Event MIR604 corn has an extended spectrum of activity against certain species of *Diabrotica*, so it is now highly active towards the major pest species western corn rootworm and northern corn rootworm, but its activity against other species tested appears to be unchanged; therefore we predict from these studies that mCry3A is hazardous only to certain species of Chrysomelidae, Tenebrionidae and Curculionidae.

The next section describes studies that test the hypothesis that the hazard of mCry3A is limited to certain species of Chrysomelidae, Tenebrionidae and Curculionidae. Estimates of exposure in these toxicity studies are compared with estimated environmental concentrations (EECs) or daily dietary doses (DDDs) of mCry3A that will result from the proposed cultivation of MIR604. The ratio of the exposure in the study to the EEC or the DDD is the margin of exposure ("safety margin"). High safety margins (>10X EEC) provide confidence that the results of the hazard studies are predictive of the effects of mCry3A on species that have not been tested; they also reduce the likelihood of type II statistical errors, that is the study fails to detect an effect when mCry3A is hazardous at the EEC.

C. LABORATORY SAFETY ASSESSMENT OF MODIFIED CRY3A PROTEIN AND EVENT MIR604 CORN TO NON-TARGET ORGANISMS

C.1 Applicability of Non-Target Organism Studies with Purified mCry3A Protein to the Environmental Safety of Corn Plants Derived from Event MIR604

C.1.a. Test Substances Used for Safety Studies and Margins of Exposure

All non-target organism tests with mCry3A, apart from the fish study, were conducted with a test substance prepared from cells of recombinant *Escherichia coli* over-expressing the gene for mCry3A that is present in MIR604. Purification of the *E. coli* culture yielded a white powder, designated MCRY3A-0102, containing 90.3% w/w mCry3A. The mCry3A in MCRY3A-0102 was shown to be chemically and biologically equivalent to mCry3A produced in MIR604 (Joseph and Graser, 2003a). MCRY3A-0102 was presented to the test organisms in appropriate artificial diets as judged by low mortality in negative control treatments and high mortality in positive control treatments.

Microbially expressed protein was chosen in preference to plant material containing mCry3A so that non-target organisms could be exposed to at least ten times the estimated exposure concentration of mCry3A. Exposure to ≥10X EEC provides a margin of safety to allow for the possibility that the EEC may be exceeded in certain environments where corn derived from Event MIR604 is grown. The margin of safety also allows for the possibility of different sensitivity to mCry3A within the groups of non-target organisms represented by the chosen test species. In all non-target arthropod studies, the test substance was presented in diets or other media at 50 µg mCry3A/g. Preliminary measurement of mCry3A concentrations in leaves of hybrids derived from Event MIR604 suggested a value of about 4 µg/g fresh weight (other plant tissues contained less than this value). Assuming an extraction efficiency of approximately 80%, leaves of MIR604 hybrids were estimated to contain about 5 µg mCry3A/g averaged over the life of the plant. As non-target arthropods tend not to consume corn leaf tissue, 5 µg/g represented a preliminary extreme worst-case EEC of mCry3A, and therefore the test organisms were exposed to 50 µg mCry3A/g to ensure an exposure of at least 10X EEC.

Confirmed values of concentrations of mCry3A in MIR604 hybrids were higher than original estimates. Leaves contained about 5.1 µg/g fresh weight averaged over four time points; the highest concentration was at seed maturity (7.8 µg/g fresh weight) and the lowest at anthesis (3.8 µg/g fresh weight) (Joseph and Hill, 2003). The extraction efficiency for MIR604 leaves was determined to be 77.1% (Joseph and Hill, 2003), giving a corrected mean value of 6.6 µg mCry3A/g fresh weight.

Although the concentration of mCry3A in test diets represents a clear margin of exposure based on the concentration of mCry3A protein in the leaves of MIR604, leaf concentration is not a realistic EEC because (by definition) non-target organisms do not consume significant quantities of crop tissues. When realistic routes of exposure are considered, test materials containing 50 µg mCry3A/g represent at least 10X EEC for the non-target arthropods tested. The rationale for setting the EEC for each test species is given in the study summaries in Part C.3. below.

Studies of the toxicity of mCry3A to earthworms and birds also used MCRY3A-0102 as the test substance. The calculation of the EEC for earthworms was based on the concentration of mCry3A in senescent leaf tissue of MIR604 hybrids (Joseph and Hill, 2003). The amount of protein used in the bird study was calculated from concentrations

in kernels of MIR604 hybrids (Joseph and Hill, 2003), and in this case the margin of exposure relates to a dose rather than a concentration of mCry3A. The calculation of an EEC for earthworms and a measure of dose for birds are described in the respective study summaries in Part C.3. below.

The assessment of toxicity of mCry3A to freshwater fish was the single exception to the use of MCRY3A-0102 as the test substance in non-target organism studies. The main anticipated route of exposure to fish will be the consumption of feed prepared from grain of MIR604 (Raybould, 2004b). Therefore fish feed was prepared using MIR604 grain in a manner intended to provide a margin of exposure. It is possible that fresh water fish could be exposed during flooding of corn fields. However, the exposure to mCry3A *via* this route is likely to be minimal compared with exposure through feed because of degradation and dilution of the protein; also it is not certain whether fish migrate into flooded fields when rivers are in spate.

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C.1.b. Selection of test species

The rationale for species selection was based on the requirement to test the hypothesis that modification of native Cry3A to enhance toxicity to western and northern corn rootworm larvae has not changed its spectrum of activity to non-target organisms. Several criteria were employed to select test species:

- Potential for exposure: common in cornfields, preys upon corn pests, eats corn tissue or processed corn, etc.
- Taxonomy: similarity to target pest or to exposed species for which no test exists.
- Test practicalities: availability of artificial diet, laboratory culture, sensitive endpoint, etc.
- Testing of particular functional groups as required by EPA (US EPA, 1996).

The species chosen for testing, along with the main reasons for their selection, are summarized in Table 18. A more detailed summary is given below:

C.1.b.1. Bobwhite quail - *Colinus virginianus* (Galliformes: Phasianidae)

Birds may be exposed to mCry3A through eating grain of MIR604 plants. Bobwhite quail is a standard representative bird species for testing the environmental effects of crop protection products and PIPs.

C.1.b.2. Rainbow trout – *Oncorhynchus mykiss* (Salmoniformies: Salmonidae)

The main route of exposure of freshwater fish to mCry3A is likely to be through feed prepared from MIR604 grain (Raybould, 2004b). The Rainbow trout was chosen for testing because it is a common freshwater fish often raised in fish farms and is a standard organism for laboratory tests.

C.1.b.3. Insidious flower bug – *Orius insidiosus* (Hemiptera: Anthocoridae)

O. insidiosus is a very common beneficial insect in cornfields in the United States, where it is useful in controlling pests such as corn earworm, corn leaf aphid, European corn borer, southwestern corn borer, spider mites and thrips (Steffey *et al*., 1999). In addition to importance in its own right, *O. insidiosus* is also a representative of the Hemiptera, an order that contains beneficial insects such as damsel bugs, assassin bugs and stink bugs, and also plant pests and even ectoparasites of birds and mammals.

Of all the non-target species tested, *O. insidiosus* is most likely to be exposed to mCry3A through consumption of tissue of MIR604. *Orius* adults lay eggs in corn leaves and the nymphs burrow through leaf tissue to reach the leaf surface to begin feeding. In later life stages, *Orius* bugs are generalist predators; they also eat pollen and have been observed apparently feeding on leaves. A closely related species, *O. laevigatus*, is used widely as a representative non-target arthropod in toxicity tests of crop protection products in the European Union. Experience with testing *O. laevigatus* provides useful information on the robustness of endpoints and expected levels of control mortality for the purpose of setting validity criteria for the *O. insidiosus* study.

C.1.b.4. Seven spot ladybird beetle – *Coccinella septempunctata* (Coleoptera: Coccinellidae)

The seven spot ladybird beetle is not a native of the USA, but has been widely introduced to control corn leaf aphids. It is the 'predominant' ladybird in the eastern United States (Steffey *et al*., 1999). Like the target rootworm pests, ladybirds are Coleoptera and therefore provide a good test of the hypothesis that the effect of modifying Cry3A is limited to the intended enhancement of toxicity to western and northern corn rootworm. *C. septempunctata* is widely used in the European Union as a representative non-target arthropod for testing the effects of crop protection products. *C. septempunctata* was chosen in preference to *Coleomegilla maculata* because the latter species consumes corn pollen, which is not a realistic route of exposure to mCry3A because the protein is undetectable in MIR604 pollen (Joseph and Hill, 2003).

C.1.b.5. Rove beetle – *Aleochara bilineata* (Coleoptera: Staphylinidae)

Aleochara bilineata has been recorded from the USA, though it is not a native species, and it was selected as a representative rove (staphylinid) beetle. Like ground beetles, rove beetles are common soil invertebrates in cornfields (Steffey *et al*., 1999) and have been recorded as eating eggs and larvae of corn rootworm species. Rove beetles are generalist predators, although some species will eat decaying plant tissue; larvae of *Aleochara* are parasitoids of fly pupae. The most likely route of exposure of rove beetles to mCry3A is through consumption of insects that have fed on MIR604 or by eating decayed tissues of MIR604. It is also possible that developing larvae could be exposed inside their host if it had eaten MIR604 tissue prior to pupating. As with *Coccinella* and *Poecilus*, one of the main reasons for selecting *Aleochara* was to investigate further the

spectrum of activity of mCry3A within the Coleoptera. *Aleochara* is a commonly used species for testing the safety of crop protection products in the European Union, and therefore there is extensive information on test endpoints for studies involving this species.

C.1.b.6. Carabid (ground) beetle – *Poecilus cupreus* (Coleoptera: Carabidae)

Poecilus cupreus is not recorded from the USA, but *P. chalcites* is common in US cornfields (Steffey *et al*., 1999). *P. cupreus* was selected for testing because it is a representative ground (carabid) beetle. Ground beetles are common generalist predators in the soil of cornfields (Steffey *et al*., 1999) and could be exposed to mCry3A through eating pest species that have eaten MIR604 tissue. Ground beetles are Coleoptera and are therefore another important taxon for indicating the spectrum of activity of mCry3A to non-target species. Historically, *Poecilus cupreus* adults were used in safety assessments of crop protection chemicals in the European Union. More recently, they have been used less because of a perceived lack of sensitivity (e.g. Candolfi *et al*., 1999). To overcome this drawback, larvae were used as they are expected to be more sensitive than the adults.

C.1.b.7. Honeybee – *Apis mellifera* (Hymenoptera: Apidae)

In general, corn is not an important source of food for honeybees, mainly because corn does not produce nectar. Honeybees will forage for corn pollen, although it is not a favored source of food. However, mCry3A is not detectable in pollen of MIR604 plants (Joseph and Hill, 2003) and therefore exposure of honeybees to mCry3A will be negligible. Nevertheless, *A. mellifera* is a useful test organism because it is a species of Hymenoptera for which methods exist to expose both larval and adult stages to test substances. Larval exposure is particularly valuable because there is potential for larvae of parasitic Hymenoptera to be exposed to mCry3A while they are developing within the body of an organism that has fed on MIR604 plants. At present there is no validated method for exposing parasitoid larvae to high doses of PIPs, although there are methods for exposing adults (*e.g.*, Romeis *et al*., 2003). Insect larvae are usually more sensitive to toxins than are adults of the same species; therefore larval honeybee exposure was considered to be a better predictor than exposure of adult parasitoids to assess the toxicity of mCry3A to parasitic Hymenoptera.

C.1.b.8. Earthworm - *Eisenia foetida* (Haplotaxida: Lumbricidae)

The earthworm *Eisenia foetida* is a standard soil invertebrate for testing the effects of crop protection chemicals and PIPs. Earthworms may be exposed to mCry3A through ingestion of soil containing fragments of MIR604 plant tissue, most likely derived from roots or senescent leaf material.

Table 18. Ecotoxicology studies: species selection

Test species	Common name	Order: family	Functional group	Comments
Colinus virginianus	bobwhite quail	Galliformes: Phasianidae	Bird	Representative
Oncorhynchus mykiss	rainbow trout	Salmoniformies: Salmonidae	Freshwater fish	Possible exposure *via* grain in feed
Orius insidiosus	insidious flower bug	Hemiptera: Anthocoridae	Predator (foliar)	Common beneficial insect in corn
Coccinella septempunctata	seven spot ladybird	Coleoptera: Coccinellidae	Predator (foliar)	Common beneficial insect in corn
Aleochara bilineata	rove beetle	Coleoptera: Staphylinidae	Predator (soil)	Representative
Poecilus cupreus	ground beetle	Coleoptera: Carabidae	Predator (soil)	Representative
Apis mellifera	honeybee	Hymenoptera: Apidae	Pollinator	Representative (e.g. for parasitoids)
Eisenia foetida	earthworm	Haplotaxida: Lumbricidae	Soil invertebrate	Representative

C.1.b.9. Functional groups not tested

EPA testing requirements specify that the effects of PIPs on freshwater invertebrates should be assessed. Historically, this has involved two-day exposures of *Daphnia magna* to pollen from the Event expressing the PIP, because dispersal of pollen into ponds and other water bodies is the most likely route of exposure for freshwater invertebrates. There is no detectable expression of mCry3A in MIR604 pollen (Joseph and Hill, 2003) and therefore minimal exposure of freshwater invertebrates to mCry3A is expected *via* this route. Another possible route of exposure of mCry3A to freshwater invertebrates is during flooding of corn fields. However, exposure outside corn fields is expected to be negligible *via* this route due to the degradability of the protein and its likely large dilution. Accordingly, a request to waive this test was made to the US EPA (Vlachos, 2004) as part of a FIFRA Section registration application; this application has been deemed complete by the US EPA, indicating that the request for a waiver was successful.

EPA also requires that the risk of PIPs to wild mammals be assessed. No test solely for this purpose was carried out; however, a 14-day mouse acute oral toxicity study (Johnson, 2003), undertaken to assess the safety of mCry3A to humans and livestock, was used to estimate the toxicity of mCry3A to wild mammals. The estimate of toxicity and data on exposure allow the risk to wild mammals from the cultivation of MIR604 corn to be assessed.

C.1.c. Test designs and endpoints

To provide maximum predictive power, the non-target organism studies attempted to achieve the following results within the constraints of test design:

- Exposure to ≥ 10X EEC mCry3A for as long as practicable
- Exposure of the potentially most sensitive life stage of the test species
- Measurement of sensitive endpoints
- Minimal effects on negative control populations
- Verification of route of exposure by use of appropriate toxic reference items

These objectives were met using a variety of techniques (see Table 19 for a summary). Where possible, fresh batches of artificial diet prepared with the test substance were presented daily to minimize any degradation of mCry3A during the test. Meat-based diets were necessary for some test species, and these were cooked before addition of the test substance to denature proteases that might degrade mCry3A; analysis of mCry3A in the diet confirmed that bioactive mCry3A was present in these diets. For non-target insects apart from *Aleochara*, early instars were used to start the test and, where possible, insects were reared through to adult emergence rather than pupation. Finally, teflubenzuron, an insect growth regulator that is active orally, was used as the toxic reference substance in all insect tests apart from the honeybee.

Table 19. Ecotoxicology studies: exposure routes, margins of exposure and endpoints

Test species	Exposure route to mCry3A	Minimum MoE	Supply of fresh mCry3A	Endpoints
bobwhite quail	Microbial protein in gelatin capsule	1400X DDD	Single oral dose	14d mortality, body weight, feed consumption
rainbow trout	Fish feed prepared from MIR604 grain	37.0X EEC	Daily for duration of test	28d mortality and growth
flower bug	Microbial protein in artificial diet	10.6X EEC	Daily for duration of test	Pre-imaginal mortality
ladybird beetle	Aphids dipped in solution of protein	12.3X EEC	Daily until pupation and after adult emergence	Pre-imaginal mortality and development; adult mortality
rove beetle	Microbial protein in artificial diet	15.6X EEC	Daily for first 35d of test	Fecundity
carabid beetle	Blowfly pupae injected with protein sol'n	11.2X EEC	Daily until pupation	Pre-imaginal mortality
honeybee	Microbial protein in sugar solution	36X EEC	Daily for first 5d of test	Brood development; adult and larval mortality
earthworm	Microbial protein in artificial soil	46X EEC	Single application	14d mortality and body weight
mouse[†]	Microbial protein in methycellulose sol'n	2600X DDD	Single oral dose	14d mortality, body weight, organ weights, feed consumption

MoE, Margin of Exposure; EEC, Estimated Environmental Exposure; DDD, Daily Dietary Dose
[†]Mammalian toxicology study

C.2. mCry3A Test Materials Used in Studies on Non-Target Organisms

Two types of test material were used in the studies that evaluated the safety of mCry3A protein for non-target species. As explained in section B.1 above, most studies were carried out with the test substance MCRY3A-0102 to achieve exposures to mCry3A that were at least 10X EEC for the test species concerned. The study of toxicity of mCry3A to freshwater fish was not carried out with MCRY3A-0102 because mCry3A is extremely unlikely to enter rivers, streams, lakes or ponds (Raybould, 2004b). Therefore, rainbow trout were fed a specially formulated fish feed, test substance FFMIR604-0103, made from grain of MIR604 (designated Hybrid I and the corresponding negative control hybrid 1I in Figure 1, Chapter 1: Syngenta Seeds Petition for the Determination of Non-regulated Status of Corn Event MIR604. Agronomic Performance of Event MIR604 Hybrids).

C.2.a. mCry3A protein produced in a microbial expression system (Test Substance MCRY3A-0102)

Test substance MCRY3A-0102 was prepared by expressing the modified *cry3A* gene used to generate corn Event MIR604 in an *E. coli* over-expression system. The modified *cry3A* gene was linked to the *Bacillus thuringiensis cry1Ac* native promoter in a Bluescript™ (Strategene, La Jolla, CA, USA) vector and transformed into *E. coli* strain DH5α. The *cry1Ac* promoter permits expression of the modified *cry3A* gene in the stationary phase of bacterial cell growth.

MCRY3A-0102 was prepared in two batches, both by Apex Bioscience, Inc., Research Triangle Park, NC, USA. Briefly, mCry3A protein was purified from *E. coli* following cell disruption using a grinding mill containing glass beads. Insoluble material was removed by centrifugation and inclusion bodies containing mCry3A were isolated, washed and solubilized in 50mM sodium carbonate. The solubilized proteins were subjected to anion exchange chromatography. MCry3A bound to the matrix was eluted with increasing sodium chloride concentration and then dialyzed *vs*. 20 mM ammonium bicarbonate, pH 9.5. The dialyzed fractions were lyophilised at Syngenta. The resulting lyophilised protein was designated as test substance MCRY3A-0102, and stored at –20°C. Upon characterization, this test substance was demonstrated to be *ca*. 90.3% pure mCry3A protein and to retain insecticidal activity against coleopteran larvae known to be sensitive to mCry3A. The preparation and characterization of test substance MCRY3A-0102 are described in detail in a separate report (Joseph and Graser, 2003b).

C.2.b. Fish feed prepared from MIR604 grain (Test Substance FFMIR604-0103)

Fish feed test substance FFMIR604-0103 was prepared by Zeigler Brothers, Inc. (Gardners, Pennsylvania, USA). The feed was formulated to contain 50% w/w of corn grain from an Event MIR604 hybrid; this is the maximum proportion of corn grain that can be used in fish feed, while maintaining a nutritionally balanced diet for juvenile trout. To minimize degradation of mCry3A protein by exposure to the typical heat and pressure conditions used in diet preparation, 'cold' pelleting was used to formulate the

feed. Grain from hybrid corn plants isogenic to Event MIR604 plants and grown concurrently under the same conditions was used to formulate the control fish feed, FFMIR604-0103C, in the same manner. A detailed description of the formulation of the test and control fish diets, as well as an analysis of the presence of mCry3A protein in the test diet is provided in a separate report (Graser, 2004a).

Exposure to mCry3A *via* FFMIR604-0103 provides a margin of safety in two ways. First, 50% w/w is higher than the standard amount of corn grain in commercial fish feed; 25 – 30% corn is more typical (National Research Council, 1983). Secondly, the 'cold' pelleting used to prepare FFMIR604-0103 is likely to degrade less mCry3A protein than normal methods of pelleting fish feed.

C.3. Hazard and Exposure Assessments of mCry3A for Non-Target Organisms

C.3.a Bobwhite quail – Colinus virginianus

C.3.a.1. Hazard assessment

An avian acute oral toxicity study on mCry3A protein was conducted according to US EPA Guideline No. 71-1. The test substance used was MCRY3A-0102 (Section 2a. above). The test organisms were 25-week old Northern bobwhite quails (*Colinus virginianus*). The birds were healthy in appearance and individuals weighed between 177 and 216 grams at the beginning of the test.

The control and treatment groups both comprised five males and five females. Individuals of each sex were allocated to the groups at random. Males and females were kept in separate pens. Birds were acclimated to the test pens for three weeks before the start of the test. Water and a game bird feed were supplied *ad libitum* during acclimation and testing. Birds were fasted for 19 hours before dosing.

Each individual in the treatment group was weighed and given an oral dose of 722 mg MCRY3A-0102/kg body weight in a single gelatin capsule; this dose is equivalent to 652 mg mCry3A/kg body weight. Individuals in the control group each received a single empty gelatin capsule. From the initiation to the end of the test each bird was observed daily for mortality, abnormal behavior or other signs of toxicity. Body weights were measured individually on Days 3, 7 and 14 of the test. Average feed consumption for each group was determined for Days 0-3, 4-7 and 8-14.

No deaths occurred during the study and all birds were normal in appearance and behavior. There were no treatment-related reductions in body weight of either sex, nor was there a treatment-related reduction in feed consumption during any period of the test. The results indicate that the median lethal dose (LD_{50}) of test substance MCRY3A-0102 is greater than 722 mg/kg body weight (corresponding to 652 mg mCry3A protein/kg body weight). The No Observable Effect Level (NOEL) in the study was 652 mg mCry3A/kg body weight (the highest dose tested).

C.3.a.2. Margin of Exposure

A dose of 652 mg mCry3A/kg body weight represents at least 1400X the daily dietary dose (DDD) for seed-eating birds. The calculation of the DDD is as follows:

$$DDD = \frac{FIR}{bw} \times C$$ (Crocker *et al*., 2002)

where: FIR = food intake rate; bw = body weight; C = concentration of mCry3A in food.

Birds feed rarely on leaves of corn; however birds such as crows (*Corvus brachyrhynchos*), grackles (*Quiscalus quiscula*) and sandhill cranes (*Grus canadensis*) uproot sprouting corn to feed on the germinating kernels (*e.g*., Steffey *et al*., 1999; Blackwell *et al*., 2001; Sterner *et al*., 2003). Red-winged blackbirds (*Agelaius phoeniceus*) and grackles destroy over 360,000 metric tons per annum of ripening field corn in the USA and Canada. Blackbirds typically slit open husks with their bills and puncture kernels in the milk stage (Steffey *et al*., 1999). Blackbirds are also common in corn stubble where they forage for spilled corn kernels and weed seeds (Linz *et al*., 2003). Therefore, the concentration of mCry3A in MIR604 kernels and *FIR/bw* ratios for cereal seed-eating birds were used to calculate the margin of exposure in this study.

The average concentration of mCry3A in kernels of MIR604 hybrids was 0.9 µg/g fresh weight (Joseph and Hill, 2003). Corrected for extraction efficiency (69.7%) this represents an actual concentration of 1.3 µg mCry3A/g fresh weight.

FIR/bw ratios for cereal seed-eating birds consuming fresh food were estimated by Crocker *et al*. (2002). Among the seven species represented, values range from 0.11 for the pheasant (*Phasianus colchicus*) to 0.35 for the tree sparrow (*Passer montanus*); these species also represent the range of body weights, 22 g for the sparrow to 953 g for the pheasant (heavier species have lower *FIR/bw* ratios). The range of daily dietary dose estimates is therefore 0.14 – 0.46 µg mCry3A/g body weight (*i.e.,* mg mCry3A/kg body weight). The test dose of 652 mg mCry3A/kg body weight therefore gives a margin of exposure between 4657 and 1417X the estimated daily dietary dose of mCry3A for seed-eating birds, assuming a diet of 100% fresh kernels from MIR604 plants.

C.3.a.3. Conclusion

No harmful effects were detected when 25-week old bobwhite quail (*Colinus virginianus*) were given a single oral dose of mCry3A representing no less than 1417X the extreme worst-case daily dietary dose of mCry3A to birds feeding on MIR604 corn tissue.

C.3.b. Rainbow trout – *Oncorhynchus mykiss*

C.3.b.1. Hazard assessment

A 28-day study of the effects of fish feed prepared from MIR604 grain (FFMIR604-0103) was carried out according to OECD Guideline 215 and US EPA OPPTS Guideline 885.4200. The test substance was prepared with the maximum amount of corn grain (50% w/w) that would provide a nutritionally balanced feed; about 25% corn by weight is typical in fish feed (National Research Council, 1983). In addition, 'cold' pelleting was used to minimize degradation of mCry3A compared with normal preparation methods (see C.2.a. above). A control substance, FFMIR604-0103C, was prepared in the same fashion from grain of a corn line isogenic to MIR604 (see C.2.a. above).

The test organism was rainbow trout (*Oncorhynchus mykiss*). Fish were obtained from a commercial fish farm and were kept in 25% seawater for 7 days on arrival at the test laboratory. Fish were acclimatized to the test temperature ($15 \pm 1^{o}C$) for at least 7 days before the initiation of the test. During this time, the fish were fed appropriate amounts of a commercial fish feed, but food was withheld for at least 24 hours before the test.

Forty fish were selected randomly for both the control and treatment populations. Immediately before the test, fish were anaesthetized, weighed individually and measured and then transferred to aerated buckets to recover from the anaesthetic. Once all the fish had recovered, they were transferred to the test vessels.

The test apparatus was a dynamic, continuous flow through system; the renewal rate was 400 ml min^{-1}. The test vessels had a maximum capacity of 54 liters and a working volume of 45 litres. Water in the test apparatus was de-chlorinated tap water to which salts were added to maintain minimum hardness, and sterilized by passage through an ultraviolet sterilizer. The water was not aerated and the test apparatus was housed in a temperature-controlled room with a nominal temperature of $15 \pm 1^{o}C$. The photoperiod was 16 hours dark and 8 hours light, with 20-minute dawn and dusk transitions.

Fish in the control tank were fed FFMIR604-0103C and the fish in the treatment tank were fed FFMIR604-0103; both populations received feed at the rate of 4% of total body weight per day. For the first 14 days, feeding was based on the weight of the fish at the beginning of the test. The quantity of feed was recalculated following weighing of the fish on Day 14 of the test. Uneaten food and feces were removed daily.

Fourteen days after the start of the test, the fish were starved for 24 hours, anaesthetized, weighed and measured. Fish were returned to the test vessels for a further 14 days exposure. On Day 28, fish were again anaesthetized, weighed and measured.

Fish were observed daily for mortality and symptoms of toxicity. Any dead fish were removed, but not replaced, and the amount of feed supplied was adjusted accordingly. Detailed observations of symptoms and feeding responses were made on Days 4, 7, 15

and 22. Physical and chemical parameters of the water were determined periodically and remained within guidelines throughout the test.

One fish (2.5%) in the treatment tank was observed to be dead on Day 21. Transient dark coloration, sounding and surfacing were recorded in one to three fish (2.5 – 7.5%) in the treatment population on Day 15. The levels of mortality and symptoms are below the levels that would invalidate the test (10%).

No significant difference was found between the weights of the treatment and control groups at 0, 14 or 28 days. The results indicate that exposure to mCry3A in fish feed FFMIR604-0103 for 28 days had no significant effects on the growth and mortality of juvenile rainbow trout.

C.3.b.2. Exposure assessment

Consumption of fish feed prepared from grain of hybrid corn derived from Event MIR604 is the main route of exposure of fish to mCry3A. As described above, the test substance in the trout growth study, FFMIR604-0103, was formulated and manufactured to maximize the amount of mCry3A protein in the finished feed, giving a margin of exposure over any conceivable mCry3A concentration in fish feed prepared in the usual way. However, even though FFMIR604-0103 was prepared to maximize the concentration of mCry3A, it was not known at the time of the trout growth study whether FFMIR604-0103 contained any mCry3A. Therefore, a study to estimate the concentration of mCry3A in FFMIR604-0103 was carried out following completion of the trout growth study. Two outcomes were possible: mCry3A would be detected in the feed indicating that the trout growth study was a valid assessment of the toxicity of mCry3A to fish; or no mCry3A would be detected, indicating no risk of mCry3A to fish because of no exposure under worst-case conditions, but rendering the trout growth study superfluous.

C.3.b.3. Quantification of mCry3A in the Feed Used in the Rainbow Trout Growth Study

Four substances were analysed in this study: FFMIR604-0103 and FFMIR604-0103C, the test and control substances in the trout growth study; and KMIR604-0103 and KMIR604-0103C, samples of ground corn kernels from which FFMIR604-0103 and FFMIR604-0103C, respectively, were prepared. Protein was extracted from these substances and the extracts were analysed for the presence of mCry3A using ELISA. ELISA was carried out using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies. Methods followed standard operating procedures and were the same for each substance.

In KMIR604-0103, the grain sample from which FFMIR604-0103 was prepared, mCry3A was detected at a concentration of 0.30 µg/g fw weight grain. Modified Cry3A was detected in FFMIR604-0103 at an average concentration of 0.09 µg/g fw weight feed; three random samples of FFMIR604-0103 gave very similar results (standard

deviation = 0.005) indicating that the mCry3A was distributed uniformly throughout the feed. No mCry3A was detected in the control fish feed, FFMIR604-0103C, or in the grain sample from which it was prepared, KMIR604-0103C.

The results confirm that mCry3A and/or immunoreactive polypeptides derived from mCry3A were present in the test substance FFMIR604-0103 used in the rainbow trout growth study. These data indicate that the trout growth study can be considered a test of the hazard of mCry3A to fish.

C.3.b.4. Margin of Exposure

We have no estimate of the degree to which cold pelleting reduces the degradation of mCry3A, but a worst-case assumption is that it does not reduce degradation of mCry3A compared with normal pelleting. Even if cold pelleting does not conserve integrity of mCry3A, test substance FFMIR604-0103 still provides a margin of exposure. First, FFMIR604-0103 contains about twice the usual proportion of corn grain in fish feed (National Research Council, 1983). Also, feed is very unlikely to be prepared from 100% grain of MIR604 hybrids. A reasonable assumption is that the adoption rate of MIR604 hybrids will be no more than *ca*. 5% of field corn grown in the USA. These conservative assumptions give a margin of exposure of 1/(0.5 x 0.054) = 37.0X EEC.

Another way of looking at the margin of exposure is to consider the maximum possible exposure of fish to mCry3A *via* fish feed. Ground grain of corn hybrids derived from MIR604, test substance KMIR604-0103, was found to contain 0.30 µg/g fw weight grain. At most, fish feed could contain 35% MIR604 corn grain, assuming that the corn source is 100% MIR604 grain. If it were possible to prepare fish feed with no degradation of mCry3A, the feed would contain 0.11 µg/g fw weight feed. Hence test substance FFMIR604-0103 exposed fish to 0.09/0.11 x 100 = 82% of the theoretical maximum environmental concentration of mCry3A.

C.3.b.5. Conclusion

No harmful effects were detected when juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed for 28 days to a diet containing mCry3A. The concentration of mCry3A in the diet was no less than 37.0X the EEC of mCry3A for fish consuming feed prepared from bulk corn grain containing grain from MIR604 hybrids.

C.3.c. Insidious flower bug – *Orius insidiosus*

C.3.c.1. Hazard assessment

A 21-day study of the effects of mCry3A on *Orius insidiosus* was carried out according to EPA OPPTS Guideline 885.4340. The test substance was MCRY3A-0102 incorporated into an artificial diet to give a nominal concentration of 50 µg mCry3A/g diet. The design of the test is based on the protocol of Bakker *et al*. (2000). This protocol is one of a group of standard testing methods (Candolfi *et al*., 2000) that are widely adopted in the European Union for assessing the effects of plant protection

products on non-target arthropods. As such, the method of Bakker *et al*. has been ring tested for repeatability and rigorous validity criteria have been set.

The test organisms were a discrete cohort of nymphs of *O. insidiosus*. Nymphs were 2-3 days old at the initiation of the test. Forty bugs were used in each treatment.

At the start of the test, MCRY3A-0102 was mixed with an artificial meat-based diet at a rate equivalent to 50 μg mCry3A/g diet. The diet had been cooked previously to denature proteases that might digest mCry3A. Diet was treated with deionised water to provide a control substance and with the orally active insect growth regulator teflubenzuron (10 μg/g diet) as a toxic reference treatment.

Immediately after preparation, aliquots of treated diet were placed in small pots and covered with a thin film (Parafilm®). The diet was presented in this manner so that bugs could feed by piercing the film, simulating their method of feeding by piercing prey with their mouthparts and removing the contents. A single bug was placed in a test arena with a pot of diet and a ball of cotton wool moistened with tap water to provide drinking water.

Additional pots of food were frozen to preserve the freshness of the diet mixture and to prevent degradation of the mCry3A. Aliquots of food were thawed daily and used to replace the food in each area. Drinking water was supplied constantly throughout the test. The study was carried out in a controlled environment room maintained at 25-26°C and 61-87% relative humidity (RH), with a 16-hour photoperiod. The environmental conditions were monitored throughout the test.

The condition of the nymphs was assessed daily until they became adults (usually 10 – 17 days at 25°C) or until 21 days after test initiation, whichever was sooner. After 21 days, all nymphs in the control and treatment groups had become adults or had died. In the control there was 23% pre-imaginal mortality, compared with 18% in the MCRY3A-0102 treatment. The 21 day corrected mortality for the MCRY3A-0102 treatment was therefore 0%. Mortality in the teflubenzuron treatment was 98%.

The test met the validity criteria of lower than 25% pre-imaginal mortality in the control group and higher than 50% mortality in the toxic reference group. Environmental conditions remained within guidelines throughout the test. Although the control mortality met the validity criterion, 77% survival might be regarded as low compared with other toxicity studies. It should be remembered that the study ran for 3 weeks, and it not easy to keep individual insects alive that long on an artifical diet. The US EPA regards 20% control mortality as acceptable; this was exceeded on day 19 in this study, at which time there was 18% mortality in the mCry3A treated group. Therefore, the insects received prolonged exposure to mCry3A before the test would have been terminated under the US EPA's control mortality criterion.

Under these laboratory conditions, mCry3A was not harmful to *Orius insidiosus*. The nominal median lethal concentration (LC_{50}) was >50 μg mCry3A/g diet and the nominal

No Observable Effect Concentration (NOEC) was 50 μg mCry3A/g diet (the highest concentration tested).

C.3.c.2. Exposure assessment

Orius bugs were exposed to a nominal concentration of 50 μg mCry3A/g diet during the toxicity study. A subsequent study was carried out to determine whether the bugs were exposed to this intended concentration of mCry3A.

C.3.c.3. Characterization and quantification of mCry3A in the treatment diet in the *Orius insidiosus* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Orius* toxicity study was carried out to determine the exposure of the bugs to mCry3A in that study. Diets treated with MCRY3A-0102 and untreated diets were prepared at Mambo-Tox, Southampton, UK as described in the summary of the toxicity study. Samples of these diets were stored frozen at Mambo-Tox during the *Orius* toxicity study and then shipped on dry ice to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA for analysis.

Protein extracts were prepared from the treated and untreated *Orius* diets according to standard operating procedures. The concentrations of mCry3A in the extracts were determined by quantitative ELISA using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies according to standard operating procedures.

The intactness of the mCry3A in the treated diet was determined by Western blot analysis. Aliquots of the treated diet extract, a solution of MCRY3A-0102 and the untreated diet extract were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

Bioactivity of mCry3A in the treated diet was measured in bioassays against first-instar Colorado potato beetles (CPB). CPB larvae were fed a standard CPB diet supplemented with 10 or 20% treated diet from the *Orius* study, or 10 or 20% untreated diet. Other treatments were: untreated CPB diet (negative control), CPB diet treated with a solution of MCRY3A-0102 (positive control) and CPB diet treated with water (control for the positive control). Each treatment comprised 3 replicates of 10 larvae. Each replicate was housed in a covered 47 mm diameter Petri dish kept at room temperature. In all treatments, diets were replaced *ca.* 24 and 48 h after the start of the assay. Mortality was measured daily for 4 days.

ELISA showed that 95.6% of the mCry3A was recovered from the treated diet; no mCry3A was detected in the extract from the untreated diet. A Western blot of treated

diet extract revealed a single band of *ca*. 67.7 kDa, the predicted molecular weight of mCry3A. In the bioassay, 83 and 90% of CPB larvae were dead after 4 days in the 10 and 20% treated diet groups, respectively (nominal concentrations of mCry3A were 5 and 10 µg mCry3A/g complete diet). In the groups fed diet supplemented with MCRY3A-0102 to 12.5 and 50 µg mCry3A/g complete diet, 73 and 80% of CPB larvae were dead after 4 days. In the groups fed 10% and 20% untreated diet, CPB mortality was 13% after 4 days in each case. There was 10% CPB mortality in both negative control groups.

The results of this study confirm that intact, bioactive mCry3A was present in the treated diet from the *Orius* study at the intended concentration of 50 µg/g diet.

C.3.c.4. Margin of Exposure

Orius insidiosus is a common beneficial insect in cornfields in the USA. Nymphs and adults prey on thrips, mites, insect eggs and caterpillars (Fauvel, 1999; Steffey *et al*., 1999). *Orius* is a particularly useful beneficial insect as it can maintain high population densities during periods when prey is scarce; it does this by feeding on corn silks and pollen, without apparent adverse effects on pollination (Steffey *et al*., 1999).

Orius could be exposed to mCry3A in MIR604 *via* several routes: through feeding on pollen, by eating sap from leaf tissue (*e.g.,* Coll and Guershon, 2001), by eating silks, or by eating prey that have eaten MIR604 tissue. The absence of detectable mCry3A protein in pollen of MIR604 plants (Joseph and Hill, 2003) indicates that exposure via this route is unlikely. *Orius* has been observed to feed on leaf tissue from which it extracts sap; detailed analysis by Armer *et al*. (1996) using $^{14}CO_2$ radiolabelled photosynthate showed that *Orius* feeding on soybean leaves accumulated little if any radiolabel. Based on their own observations and a review of similar experiments, Armer *et al*. concluded that *Orius* feeds principally from xylem vessels and hence plants provide the insect with water rather than nutrients. Therefore it is very unlikely that the main route of exposure of *Orius* to mCry3A will be through sap. If exposure were possible *via* sap, worst case EEC would be 6.6 µg mCry3A/g diet (based on corrected protein concentrations in leaves of MIR604 hybrids, see Chapter 7, Part C.1.a. above); this represents a margin of exposure of 50/6.6 = 7.6X EEC.

Silks, however, do represent a realistic potential route of exposure to mCry3A. At seed maturity in MIR604 hybrids, silks were estimated to contain on average 1.2 µg mCry3A/g fresh weight before correction for extraction efficiency (Joseph and Hill, 2003). The efficiency of extraction of mCry3A from silks has not been determined, however 70% efficiency seems a reasonable conservative assumption based on the measurements of other plant tissues (Joseph and Hill, 2003). The corrected concentration is therefore 1.7 µg mCry3A/g fresh weight, and hence the margin of exposure based on consumption of silks is 29.4X EEC.

The final potential route of exposure of *Orius* to mCry3A is via consumption of prey that has eaten MIR604 leaf tissue. No studies have estimated the concentration of mCry3A in herbivores that have fed on leaves of MIR604; nor to our knowledge are there studies on the concentration of native Cry3A in herbivores that have eaten plants

containing this protein. However, various studies have estimated the concentration of Cry1Ab, in herbivores that had fed on *Bt* corn or artificial diets containing the protein. Data on Cry1Ab in corn probably represent the best available data to estimate the concentration of mCry3A in herbivores feeding on MIR604: the herbivores tested are found in corn fields, both Cry1Ab and mCry3A are Cry proteins and the proteins are present in corn tissue.

Experiments by Head *et al*. (2001) found the concentration of Cry1Ab in corn leaf aphids (*Rhopalosiphum maidis*) to be 250-500 times lower than that in the artificial diet solution they were fed; no protein was detected in aphids feeding on Bt corn plants. Trace amounts of Bt protein have been detected in grain aphids (*Rhopalosiphum padi*) feeding on corn expressing Cry1Ab (Raps *et al*., 2001; Dutton *et al*., 2002). Head *et al*. (2001) found that concentrations of Cry1Ab in lepidopteran pests of corn (*Ostrinia nubilalis* – European corn borer, *Helicoverpa zea* – corn earworm, and *Agrotis ipsilon* – black cutworm) were between 10 and 140 times lower than the concentration of protein in artificial diets. *Spodoptera littoralis* (Egyptian cotton leafworm) larvae on Bt corn plants contained concentrations of Cry1Ab between 4.8 and 8.5 times lower than the leaves on which they were feeding (Raps *et al*., 2001; Dutton *et al*., 2002). The highest concentration of Cry1Ab relative to the dietary concentration was found in spider mites (*Tetranychus urticae*) feeding on Bt corn, which had a concentration of Cry1Ab 1.4 times lower than leaf tissue (Dutton *et al*., 2002).

Although *Orius* is most effective at controlling Lepidoptera such as corn earworm, it will prey on spider mites (Steffey *et al*., 1999). As spider mites have the highest concentration of Cry1Ab, their rate of incorporation can be used to calculate a worst-case margin of exposure to mCry3A for *Orius*. Assuming that mCry3A in leaves of MIR604 is incorporated into herbivores in the same fashion as Cry1Ab in the study of Dutton *et al*., spider mites are expected to contain 6.6/1.4 = 4.7 µg mCry3A/g fresh weight (based on corrected protein concentrations in leaves of MIR604 hybrids, see Chapter 7, Part C.1.a. above). Therefore in this study the worst-case margin of exposure for *Orius* eating spider mites that have fed on MIR604 leaves was 10.6X EEC. The omnivorous diet of *Orius* means that the margin of exposure is likely to be many times higher than this realistic worst-case value.

C.3.c.5. Conclusion

No harmful effects were detected when *Orius insidiosus* nymphs were exposed for 21 days to an artificial diet containing mCry3A at no less than 10.6X the worst-case average EEC of mCry3A in the diet of *Orius* species exposed to MIR604 hybrid corn plants.

C.3.d. Seven Spot Ladybird Beetle – *Coccinella septempunctata*

C.3.d.1. Hazard assessment

A 24-day study of the effects of mCry3A on the ladybird beetle *Coccinella septempunctata* was carried out according to EPA OPPTS Guideline 885.4340. The design of the test is based on the protocol of Schmuck *et al*. (2000). This protocol is one

of a group of standard testing methods (Candolfi *et al*., 2000) for assessing the effects of plant protection products on non-target arthropods that are widely accepted in the European Union. As such, the method of Schmuck *et al*. has been ring tested for repeatability and rigorous validity criteria have been set.

Artificial diets can be used to rear *C. septempunctata* larvae, but they require occasional supplementation with live aphids; a diet of aphids is sufficient for *C. septempunctata* to complete its life cycle (Schmuck *et al*., 2000). For simplicity of test design, and closest agreement with existing test protocols, pea aphids (*Acyrthosiphon pisum*) were used as the diet in this study. The test substance was presented by dipping aphids into a weak solution of Agral 90 (a wetting agent) containing MCRY3A-0102 at a concentration equivalent to 50 μg mCry3A/mL. This system was taken to simulate the possible exudation of honeydew containing mCry3A and any uptake of mCry3A into the bodies of the aphids.

The test was initiated with 4-day old (2nd instar) ladybird beetle larvae. The larvae were obtained from a discrete cohort of eggs laid by a culture of field-collected insects maintained at the test facility. The eggs and young larvae were maintained in batches of 10-25 in Petri dishes kept at 25°C in a 16-hour photoperiod. Larvae were fed untreated pea aphids until the start of the test.

The test substance was MCRY3A-0102 dissolved in a 40 μL/L solution of Agral 90; the toxic reference substance was 150 g/L teflubenzuron in a 40 μL/L solution of Agral 90; and the control treatment was a 40 μL/L solution of Agral 90. Each treatment population comprised 40 larvae, individually confined in test arenas. The treatments were applied via pea aphids that had been immersed in the appropriate solution and then allowed to dry for about 30 minutes. The number of aphids given was adjusted during the test as the individuals grew. Freshly treated aphids were supplied daily to larvae and any aphids remaining from the previous day were removed. This procedure was followed until pupation.

Pupae were left in the test arenas and feeding was halted. When adults emerged, their sex was determined and, where numbers allowed, a male and a female were placed in a test arena. Unpaired beetles were kept on their own. All adults were used in the assessments. The test was carried out in a controlled environment room maintained at 21-27°C and 35-92% RH with a 16 hour photoperiod.

Observations of larvae were made at the time of feeding. The number of dead, moribund and live larvae and the number of pupae were recorded. Observations of adults were also made at the time of feeding; the number of dead and live adults was recorded.

Pre-imaginal mortality was zero in both the control and treatment populations; mortality was 100% in the toxic reference treatment population. Adult mortality was 7.5% in the control population and 15% in the mCry3A-treated population, giving a corrected mortality of 8.1%. This was not statistically significantly different from the control mortality at the 5% level. The development time from larva to pupa was not significantly different between the control and treatment populations. The time from

larva to adult was slightly shorter in the treatment population (9.48 compared with 9.80 days in the control group); in other words pupae developed into adults faster in the treatment population. This difference was statistically significant at 5%. The difference in development times did not result in a statistically significant difference in adult survival.

The validity criteria of no greater than 20% control mortality and greater than 40% mortality in the toxic reference population were met. Temperature and RH deviated from established test protocols for short periods, but were not considered to have affected the integrity of the test as the control mortality was acceptable. Under these laboratory conditions, mCry3A was not harmful to *Coccinella septempunctata*. The LC_{50} of mCry3A dissolved in an Agral solution supplied via dipped aphids was >50 µg/mL; the NOEC of the mCryA solution was 50 µg/mL (the highest concentration tested).

C.3.d.2. Exposure Assessment

Ladybird beetles were exposed to a nominal concentration of 50 µg mCry3A/mL solution on dipped aphids during the toxicity study. A subsequent study was carried out to quantify the exposure in terms of µg mCry3A/g diet.

C.3.d.3. Characterization and quantification of mCry3A in the treatment diet in the *Coccinella septempunctata* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Coccinella* toxicity study was carried out to determine the exposure of the ladybird beetles in that study. Pea aphids treated with a solution of MCRY3A-0102 were prepared at Mambo-tox in the same manner and in parallel with the treated pea aphids used in the *Coccinella* toxicity study. A sample of the treated aphids and a sample of untreated aphids were frozen immediately after preparation and were shipped on dry ice to Syngenta Biotechnology, Inc. for analysis.

Protein extracts from treated and untreated aphids were prepared according to standard operating procedures. The concentration of mCry3A in the extracts was measured by quantitative ELISA, according to standard operating procedures, using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

The intactness of mCry3A in the extracts was assessed by Western blot. Aliquots of the extract of treated aphids, two aliquots of a solution of MCRY3A-0102 and an aliquot of the extract of untreated aphids were subjected to SDS-PAGE according to standard operating procedures. The gel was electroblotted and the membrane probed with immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

ELISA showed a concentration of 9.0 µg mCry3A/g aphids. The Western blot revealed a single immunoreactive band corresponding to the predicted molecular weight of mCry3A (*ca.* 67.7 kDa.).

C.3.d.4. Margin of Exposure

Until the late 1980s, three species, *Coleomegilla maculata*, *Hippodamia convergens* and *H. tredecimpunctata* comprised the majority of the ladybird beetle fauna of corn in the USA. Surveys summarized by Hodek and Honek (1993), found that these species formed 100% of a sample of ladybird beetles collected from field corn in Minnesota, 94.2% of a sample from sweet corn in North Dakota and 97.9% of a sample from field corn in South Dakota. Since the late 1980s, *Coccinella septempunctata*, a species introduced for biological control of cereal aphids, has increased rapidly and is now a 'predominant' ladybird beetle in corn in the eastern United States (Steffey *et al*., 1999).

Hodek and Honek (1993) estimated that the diet of ladybird beetles in the tribe Coccinellini contains *ca.* 85% aphids, the remainder being other sucking insects such as psyllids. *Hippodamia* and *Coccinella* are members of this tribe. *Coleomegilla maculata* has a somewhat different diet; it is an important predator of aphids and will also prey on mites and insect eggs and plant pollen can constitute up to 50% of its diet.

Aphids that have fed on corn plants expressing Cry1Ab contain very low concentrations of Cry1Ab relative to the plants (Head *et al*., 2001; Raps *et al*., 2001; Dutton *et al*., 2002). The highest estimate is that aphids contain Cry1Ab at a concentration 250 times less than the corn on which they are feeding (Head *et al*., 2001). On the other hand, the concentration of Cry1Ab in mites was only 1.4 times lower than the corn. As discussed above, the Cry1Ab corn data provide the best data to estimate the concentrations of mCry3A in herbivores feeding on MIR604. If the Cry1Ab in *Bt* corn conversion rates apply to incorporation of mCry3A into insects feeding on MIR604 corn hybrids, aphids are expected to contain 6.6/250 = 0.026 µg mCry3A /g fresh weight and mites 6.6/1.4 = 4.7 µg mCry3A /g fresh weight[4]. Pollen of MIR604 hybrids contains no detectable mCry3A (Joseph and Hill, 2003) and eggs of insects that have fed on MIR604 hybrids are unlikely to contain any mCry3A as proteins tend not to bioaccumulate.

Based on these considerations, a realistic worst-case exposure to mCry3A for ladybird beetles in corn is a diet of 85% aphids and 15% spider mites. This gives an EEC of:

$$(0.15 \times 4.7) + (0.85 \times 0.026) = 0.73 \text{ µg mCry3A /g fresh weight diet}$$

Therefore the margin of exposure to mCry3A in the *Coccinella* toxicity study is 9.0/0.73 = 12.3X EEC.

C.3.d.1. Conclusion

[4] 6.6 µg /g fresh weight is the average concentration of mCry3A in leaves of MIR604 hybrids – corrected for extraction efficiency – see Section C.1.a above.

No harmful effects were detected when *Coccinella septempunctata* larvae and adults were exposed for 24 days to an artificial diet containing mCry3A at no less than 12.3X the average worst-case EEC of mCry3A in the diet of ladybird beetles exposed to MIR604 hybrid corn plants.

C.3.e. Rove beetle – *Aleochara bilineata*

C.3.e.1. Hazard assessment

A study of the effects of mCry3A on the rove beetle *Aleochara bilineata* was carried out according to EPA OPPTS Guideline 885.4340. The design of the test is based on the protocol of Grimm *et al*. (2000). This protocol is one of a group of standard testing methods (Candolfi *et al*., 2000) for assessing the effects of plant protection products on non-target arthropods that are widely accepted in the European Union. As such, the method of Grimm *et al*. has been ring tested for repeatability and rigorous validity criteria have been set.

For the test, adult beetles that emerged over 24 hours were confined individually in wells of polystyrene tissue culture plates. The wells were lined with moistened filter paper and the beetles were supplied with a pellet of raw minced beef for food. The plates were placed in a dark cold room (0-8°C) for six days to delay physiological development. Three days before the start of the test the beetles were moved back to a controlled environment room maintained at 23-25°C, 64-79% RH and a 16-hour photoperiod.

On the day that the test started, the beetles (physiologically 4 days old) were brought together in small groups in 9-cm-diameter plastic Petri dishes, lined with damp filter paper. Groups comprising 10 males and 10 females were impartially selected and placed together in 5-cm-diameter plastic Petri dishes, ready for transfer into the freshly-treated test arenas.

The food supplied to the treatment population was puréed beef supplemented with MCRY3A-0102 at a rate equivalent to 50 μg mCry3A protein/g diet. The diet was cooked prior to addition of MCRY3A-0102 to denature proteases that might degrade mCry3A. The control diet was prepared in the same way, with deionised water as the reference item; the positive control diet was cooked beef with teflubenzuron at 10 μg/g diet.

Aliquots of the treated diet were placed in small receptacles. These were supplied to adult *A. bilineata* (10 males and 10 females per replicate, 4 replicates per treatment) for 35 days. Additional packages of treated food were frozen, to maintain their freshness and the stability of mCry3A in the treatment diet. Each day, aliquots of treated food were de-frosted and used to replace the food in each arena. At the end of the test, remaining aliquots were kept frozen prior to conducting tests to determine the stability of mCry3A in the diet.

The beetles were initially confined in small, ventilated pots for one week to maximize their exposure to the treated diet. They were then transferred to boxes containing damp

sand for a further four weeks. Treated diet was supplied daily and fresh water was added to the sand every 1-3 days as required to maintain the starting weight of the test arenas.

The fecundity of the beetles was assessed by the provision of pupae of the onion fly (*Delia antiqua*). The pupae were maintained under the conditions used for the initial cold treatment of the *Aleochara* beetles and were used within three weeks of their arrival at the test facility. The fly pupae were added to each arena on three occasions: 14, 21 and 28 days after test initiation (DAT). The survival of the adult beetles originally introduced was assessed at 35 DAT and they were then removed from the arenas. The number of F_1 adult *A. bilineata* that developed from the parasitised fly pupae was recorded over the subsequent 6 weeks.

After 35 days, mortality in the various treatments was 34% in the control population, 31% in the MCRY3A-0102 treatment population and 35% in the teflubenzuron treatment (2% corrected mortality). The mean number of progeny was 647 in the control population and 663 in the treatment population. This represents 2.5% greater fecundity in the treatment population, although this difference is not statistically significant. The mean number of offspring in the teflubenzuron treatment was 3, a 99.5% reduction compared with the control.

For the test to be considered valid, the mean number of beetles emerging from parasitized fly pupae in the control treatment should be >400 per replicate. Also, the mean number of beetles emerging in the toxic reference treatment should be reduced by >50%, relative to the control. Both of these criteria were met. Because the beetles are kept for 5 weeks on an artificial diet, adult survival is often low and no validity criterion is set for this endpoint. During the study, physical conditions in the controlled environment room deviated from established protocols for short periods. These fluctuations were not considered to have invalidated the test as the control and toxic reference populations exceed the validity criteria.

Under these laboratory conditions, mCry3A was not harmful to the fecundity of *Aleochara bilineata*. The NOEC was 50 µg mCry3A/g diet (the highest concentration tested).

C.3.e.2. Exposure assessment

The *Aleochara* beetles were exposed to a nominal concentration of 50 µg mCry3A/g diet during the toxicity study. A subsequent study was carried out to determine whether the beetles were exposed to this intended concentration of mCry3A.

C.3.e.3. Characterization and quantification of mCry3A in the treatment diet in the *Aleochara bilineata* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Aleochara* toxicity study was carried out to assess the exposure of rove beetles to mCry3A in that study. Diet treated with MCRY3A-0102 and an untreated diet were prepared at Mambo-Tox, Southampton, UK as described in the summary of the toxicity study. Samples of

these diets were stored frozen at Mambo-Tox during the *Aleochara* toxicity study and then shipped on dry ice to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA for analysis.

Protein extracts of treated and untreated diets were prepared according to standard operating procedures. The concentrations of mCry3A in the extracts were determined by quantitative ELISA according to standard operating procedures using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

The intactness of the mCry3A in the treated diet was determined by Western blot analysis. Aliquots of the treated diet extract, a solution of MCRY3A-0102 and the untreated diet extract were subjected to SDS-PAGE using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

Bioactivity of mCry3A in the treated diet was measured in bioassays against first-instar Colorado potato beetles (CPB). CPB larvae were fed a standard CPB diet supplemented with 10 or 20% treated diet, or 10 or 20% untreated diet. Other treatments were: untreated CPB diet (negative control), CPB diet treated with a solution of MCRY3A-0102 (positive control) and CPB diet treated with water (control for the positive control). Each treatment comprised 3 replicates of 10 larvae. Each replicate was housed in a covered 47 mm diameter Petri dish kept at room temperature. In all treatments, diets were replaced *ca.* 24 and 48 h after the start of the assay. Mortality was measured daily for 4 days.

ELISA showed that 91.7% of the mCry3A was recovered from the treated diet; no mCry3A was detected in the extract from the untreated diet. A Western blot of treated diet extract revealed a single intense band of *ca*. 67.7 kDa, the predicted molecular weight of mCry3A. In the bioassay, 57 and 77% of CPB larvae were dead after 4 days in the 10 and 20% treated diet groups respectively (nominal concentrations of mCry3A were 5 and 10 µg mCry3A/g complete diet). In the groups fed diet supplemented with MCRY3A-0102 to 12.5 and 50 µg mCry3A/g complete diet, 73 and 80% of CPB larvae were dead after 4 days. In the groups fed 10% and 20% untreated diet, CPB mortality was 10% after 4 days in each case. There was also 10% CPB mortality in both negative control groups.

The results of this study confirm that intact, bioactive mCry3A was present in the treated *Aleochara* diet at the intended concentration (50 µg/g diet).

C.3.e.4. Margin of Exposure

Rove beetles (Staphylinidae) are common in cornfields. There is little information on their diets in corn, but in general rove beetles feed mainly on other invertebrates although

some species will feed on decaying plant material (Steffey *et al.*, 1999). Species of *Aleochara* are parasitoids of pupae of cyclorraphous Diptera (Grimm *et al*., 2000).

A worst-case EEC based on consumption of soil invertebrates can be calculated from the concentration of mCry3A in roots of MIR604 hybrids: 2.8 µg/g fresh weight averaged over all growth stages, which is equivalent to 3.7 µg/g fresh weight when corrected for extraction efficiency (75.1%) (Joseph and Hill, 2003). Assuming a worst-case rate of incorporation into prey of 1.4X lower than the plant concentration (Dutton *et al*., 2002; see *Orius* study summary above, Chapter 7, Part C.1.b.3.), rove beetles could be exposed to an average concentration 2.6 µg mCry3A/g fresh weight. A diet of 50 µg mCry3A/g fresh weight therefore provides a margin of exposure of 19.2X EEC for exposure *via* invertebrate prey.

The concentration of mCry3A in 'decaying' plant material has not been determined; in any case it is difficult to judge exactly what degree of decay renders plant material acceptable to rove beetles. A reasonable estimate could be the concentration of mCry3A in 15-Day silage: 2.73 µg mCry3A/g fresh weight, which is equivalent to 3.2 µg mCry3A/g fresh weight when corrected for extraction efficiency (84.5%) (Joseph and Hill, 2003). A diet of 50 µg mCry3A/g fresh weight therefore provides a margin of exposure of 15.6X EEC for exposure through decaying plant material.

C.3.e.5. Conclusion

No harmful effects were detected when rove beetles (*Aleochara bilineata*) were exposed to an artificial diet containing mCry3A at no less than 15.6X the average worst-case EEC of mCry3A in the diet of rove beetle species exposed to MIR604 hybrid corn plants.

C.3.f. Ground beetle – *Poecilus cupreus*

C.3.f.1. Hazard Assessment

A study of the effects of mCry3A on the ground beetle *Poecilus cupreus* was carried out according to EPA OPPTS Guideline 885.4340. The design of the test is based on the protocol of Heimbach (1998). A test of toxicity to adult *Poecilus* beetles is one of a group of standard tests (Candolfi *et al*., 2000) for assessing the effects of plant protection products on non-target arthropods that are widely accepted in the European Union. We chose to test the toxicity of mCry3A to larvae because adult *Poecilus* is recognized as being relatively insensitive to pesticides (e.g. Candolfi *et al*., 1999).

Larvae of *P. cupreus* were obtained from a commercial supplier. They were shipped on the day they began to hatch and arrived at the test facility the following day. The test started the day after the larvae were received, when they were *ca.* 2 days old. In the period before the test, individual larvae were kept separately in the wells of polystyrene tissue culture plates filled with moist peat. The larvae were stored in the dark at 20°C and

7-73% RH; they were fed pupae of the blowfly *Calliphora vomitoria* that were cut in half to allow the *Poecilus* access to the contents.

The test arenas were small glass tubes capped with a ventilated polythene stopper. The tubes were filled with a standard sandy soil (LUFA) moistened with tap water to 35% of its water holding capacity. During the test, the soil-filled tubes were stored upright in plastic boxes (*ca.* 28 x 16 x 9 cm, with one box per treatment). The floors of the boxes were lined with moist capillary matting. The boxes had ventilated lids. The study was carried out in constant darkness in a controlled environment cabinet maintained at 19-22°C and 64-90% RH.

Poecilus larvae were exposed to mCry3A by being fed blowfly pupae injected with 1 µl of a solution of MCRY3A-0102. The concentration of mCry3A in the treatment diet was 50 µg/g fly pupa. The control diet was prepared in the same way, with deionised water as the reference item. The positive control diet was fly pupae injected with 1 µl of a solution of 'Nemolt', giving 0.664 ng teflubenzuron/g fly pupa. Diets were prepared in a single batch sufficient to provide each larva with one pupa per day. Larvae were provided with a fresh pupa on day 1 of the test and the remaining pupae were stored at –20 to –30°C.

Each treatment comprised 40 larvae confined individually in the soil-filled tubes. Larvae were transferred from the tissue culture plates using a soft brush and allocated randomly to the treatments. Each larva was provided with a freshly treated pupa that had been cut in half and place cut side up on the surface of the soil. The ventilated stoppers were then fitted.

Each larva was given a freshly defrosted pupa daily. The remains of the previous day's pupa were removed, taking care not to remove soil or very young larvae. Feeding continued until the *Poecilus* became pre-pupae.

The larvae were assessed 3 times per week for the first 2 weeks of the test. Thereafter assessments were made twice weekly. At each assessment, the larvae were categorized as alive, dead, missing or pupated. From day-21, if a larva had not been observed for three successive assessments, a thorough search was made by tipping the soil out of the tubes onto a white tray and sifting through it. Missing larvae were assumed to have died and decomposed. From day-32, the tubes were checked daily and the number of emerging adults recorded. Once the color of the adult skin had changed from white to its normal green-black, the beetles were weighed and sexed.

Mortality in the mCry3A treatment was 10% compared with 20% in the control treatment. The mean weight of the emerging beetles was very similar (81.5 mg in the control, 82.9 in the mCry3A treatment). All beetles died in the toxic reference (teflubenzuron) treatment.

For the test to be considered valid, pre-imaginal mortality should not exceed 20% in the control treatment and should exceed 50% in the toxic reference treatment. These criteria were met.

Under these laboratory conditions, mCry3A was not harmful to larvae of *P. cupreus*. The nominal median lethal concentration (LC_{50}) was >50 µg mCry3A/g blowfly pupa and the nominal No Observable Effect Concentration (NOEC) was 50 µg mCry3A/g blowfly pupa (the highest concentration tested).

C.3.f.2. Exposure assessment

The *Poecilus* larvae were exposed to a nominal concentration of 50 µg mCry3A/g diet during the toxicity study. A subsequent study was carried out to determine whether the bugs were exposed to this intended concentration of mCry3A.

C.3.f.3. Characterization and quantification of mCry3A in the treatment diet in the *Poecilus cupreus* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Poecilus* toxicity study was carried out to assess the exposure of the beetles to mCry3A in that study. Blowfly pupae treated with MCRY3A-0102 were prepared at Mambo-Tox, Southampton, UK as described in the summary of the toxicity study. A batch of pupae prepared in parallel with those in the toxicity study was stored at –20 to –30°C at the test facility. On completion of the toxicity study, the pupae were shipped on dry ice to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA for analysis.

Extracts of treated and untreated blowfly pupae were prepared according to standard operating procedures. The concentrations of mCry3A in the extracts were determined by quantitative ELISA according to standard operating procedures using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

The intactness of the mCry3A in the treated diet was determined by Western blot analysis. Aliquots of the extracts from treated and untreated pupae, and a solution of MCRY3A-0102 were subjected to SDS-PAGE using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

ELISA determined the concentration of mCry3A in the blowfly pupae to be *ca.* 12 µg mCry3A/g pupae, equivalent to an average concentration of 0.82 µg mCry3A/pupa, and 24% of the mCry3A injected into the pupae. No mCry3A was detected in the extracts from untreated pupae. A Western blot revealed a single immunoreactive band at the predicted molecular weight of intact mCry3A (*ca.* 67.7. kDa) in the extracts from pupae treated with mCry3A; no immunoreactive material was detected in extracts from untreated pupae.

The results show that intact mCry3A was present at *ca.* 12 µg /g treated blowfly pupae used to expose *Poecilus* larvae to mCry3A in the toxicity study at Mambo-Tox. Other studies (Graser, 2004c, d, f) have shown that an immunoreactive band of 67.7 kDa on a Western blot probed with anti-Btt polyclonal antibodies correlates strongly with bioactivity against CPB larvae; it is highly likely, therefore, that *Poecilus* larvae were exposed to bioactive mCry3A.

C.3.f.4. Margin of Exposure

Nine species of carabid beetles are reported commonly from cornfields (Steffey *et al*., 1999), although mainly others have been recorded (e.g. Varchola and Dunn, 2001). Most are non-target (beneficial) organisms, but some species are pests that eat germinating corn seeds (Steffey *et al*., 1999).

The main route of exposure of non-target carabids to mCry3A will be through eating prey that has eaten tissue of MIR604 hybrids. Larvae of the black cutworm (*Agrotis ipsilon*) are common prey for carabid beetles in cornfields (Steffey *et al*., 1999). Cutworms cause severe damage in young corn when alternative food plants have been removed by cultivation of herbicides. Cutworm larvae sever young corn plants and eat them in burrows in the soil; larvae also occasionally burrow into stems of older plants (Steffey *et al*., 1999).

The average concentration of mCry3A in plants of MIR604 hybrids at the whorl stage is 1.5 µg/g fresh weight, after correcting for extraction efficiency (Joseph and Hill, 2003). Head *et al*. (2001) showed that the concentration of Cry1Ab in black cutworm larvae was *ca*. 10 times lower than the concentration of Cry1Ab in their diet. If mCry3A from MIR604 hybrids is incorporated into black cutworms at the same rate as Cry1Ab, the best estimate based on limited data (see above), then carabid beetles will be exposed to 0.15 µg mCry3A/g fresh weight *via* black cutworm larvae. Taking this as the EEC, the margin of exposure in the *Poecilus* toxicity study is 80X EEC.

Cry1Ab is toxic to some Lepidoptera, and therefore it is possible that black cutworm larvae could accumulate a higher concentration of mCry3A than Cry1Ab relative to the respective concentrations of these proteins in their diet. However, the LC_{50} of Cry1Ab to black cutworm larvae is >80,000 ppm (MacIntosh *et al*., 1990), whereas Head *et al*. (2001) used only 20 ppm. Nevertheless, it is possible that the concentration of mCry3A in cutworm larvae relative to their food is underestimated using the conversion rate of Cry1Ab from the study of Head *et al*. (2001).

An extreme worst-case is to assume that the concentration of mCry3A in the cutworms is 1.4 times lower than in MIR604 hybrids. This conversion factor is derived from the concentration of Cry1Ab in spider mites relative to their diet (Dutton *et al*., 2002, see Chapter 7, Part C.3.d.4. above); Cry1Ab is not toxic to spider mites and therefore should not affect their feeding rates. The extreme worst-case exposure of non-target carabids to mCry3A on this basis is 1.07 µg mCry3A/g fresh weight prey, giving a margin of exposure of 11.2X EEC in the *Poecilus* toxicity study.

The adults of two species of carabid, *Stenolophus lecontei* (seedcorn beetle) and *Clivina impressifrons* (slender seedcorn beetle), are sporadic pests in corn when they eat germinating corn seed (Steffey *et al*., 1999). If seedcorn beetles were to eat only kernels of MIR604 corn hybrids they would be exposed to 1.3 µg mCry3A/g fresh weight diet (the average concentration of mCry3A in corn kernels when corrected for extraction efficiency [Joseph and Hill, 2003]); this is 9.2X lower than the concentration of mCry3A in the *Poecilus* toxicity study. However, seedcorn beetles tend to feed on other arthropods and eat corn seeds only when other food is in short supply (e.g. Pausch, 1979). Therefore seedcorn beetles will be exposed to concentrations of mCry3A far below 10 times that in the *Poecilus* toxicity study. Furthermore, the *Poecilus* study exposed carabid larvae to mCry3A, whereas only adult seedcorn beetles will be exposed to mCry3A *via* kernels of MIR604 hybrids; adult carabids are far less sensitive to toxins than are larvae. Seedcorn beetles are therefore very unlikely to be harmed by mCry3A expressed in MIR604.

C.3.f.5. Conclusion

No harmful effects were detected when carabid beetle larvae (*Poecilus cupreus*) were exposed to an artificial diet containing mCry3A at no less than 11.2X the average worst-case EEC of mCry3A in the diet of non-target carabid species exposed to MIR604 hybrid corn plants.

C.3.g. Honeybee – *Apis mellifera*

C.3.g.1. Hazard Assessment

A 26-day (5 days treatment followed by 21 days observation) study of the effects of mCry3A on honeybee brood development was carried out according to EPA OPPTS Guideline 885.4380. The method is adapted from that of Oomen *et al*. (1992) for testing the effects of insect growth-regulating insecticides on honeybees. *A. mellifera* is a standard test organism for testing plant protection chemicals and this study fulfils the requirement to test the effect of mCry3A on a pollinator.

The test substance in this study was MCRY3A-0102 dissolved in a 50% w/v solution of sucrose; the concentration of mCry3A was 50 µg/g solution. The toxic reference substance was Dimilin Flo, a formulation of the insect growth regulator diflubenzuron, dissolved in a 50% sucrose solution. Diflubenzuron is known to affect the development of honeybee brood (based on the experience of staff at the test facility), but has low toxicity to adult honeybees. The concentration of diflubenzuron was 0.375 mg/mL sucrose solution. The control reference substance was 50% sucrose solution.

On the morning of treatment application, a sufficient frames were removed from Honeybee hives (n = 4 per treatment) to record the position of 100 cells containing eggs and 100 cells containing 1- to 3-day old larvae. The position of these cells was recorded by laying a transparent acetate sheet over the relevant frame and tracing the cells using a fine-tipped indelible marker pen. Each hive received a fresh batch of 200 mL of the appropriate test solution daily for 5 days. The solutions were quickly removed from the

containers by the bees and none of the previous test solution remained at the time of each application. Over a 21-day period following the initiation of treatments, the frames containing the “marked” cells were removed and the developmental success of the brood was recorded.

During the pre-treatment identification of the egg and larvae cells, the relative proportions of brood and food (in the form of pollen and nectar) on the frames within each hive were recorded. At the end of the study, this assessment was repeated in order to assess whether the test treatments had affected the relative proportions of brood cells within the hives.

Dead bee traps were fitted to the hives to record the number of dead adult bees, larvae and pupae being removed from the treated hives by worker bees over a 21-day period following the initiation of the 5-day treatments.

In the cells containing eggs at the start of the test, mortality was 28.5% in the control hives and 27.3% in the hives treated with MCRY3A-0102. There was 100% mortality in hives treated with Dimilin Flo. In the cells containing larvae at the beginning of the test, there was 6.0% mortality in the control hives and 6.8% mortality in the hives treated with MCRY3A-0102; this equated to a corrected mortality of 0.9%, which was not statistically significantly different from zero. Again, 100% mortality was recorded from cells in hives treated with Dimilin Flo. Prior to treatment, the proportion of cells occupied by brood was not significantly different among treatments. However, at the end of the test there were fewer cells occupied by brood in the MCRY3A-0102 and Dimilin Flo treatments (40.7% and 27.7% respectively) than in the control hives (49.6%). The difference was statistically significant in the case of the Dimilin Flo, but not in the case of MCRY3A-0102.

The number of dead bees and brood deposited in the dead bee traps during the treatment and observation periods was too low for statistical analysis. The low numbers of dead bees and brood in the MCRY3A-0102 and control treatments showed that the hives were apparently healthy and had high survival. Dimilin Flo was not expected to affect worker bees as it has low contact and oral toxicity in laboratory tests. Although Dimilin Flo had a dramatic effect on brood development, this was not reflected by an increase in the number of dead brood deposited in the bee traps.

There were slight deviations from the protocol relating to the number and age of brood identified at the start of the test; however they were not considered to have affected the validity of the test. Under conditions of the test, exposure of the hives to sucrose solution containing 50 µg mCry3A/g had no adverse effects on the condition and survivorship of honeybee larvae developing in brood cells within the hives. The test substance also had no apparent effect on the survival of adult honeybees.

C.3.g.2. Exposure Assessment

Hives were exposed to a nominal concentration of 50 µg mCry3A/g diet. The honeybees were supplied daily with freshly prepared diet to maximise the probability that

hives were exposed to the nominal concentration of mCry3A. As recommended by Oomen *et* al. (1992) the test was carried out while there were few natural nectar sources so that the bees did not store the test substances rather than feed them to the larvae. At each application of fresh diet, none of the previous test solutions remained in the diet containers, and there was a highly significant effect of the toxic reference treatment. These observations provide a weight of evidence that the developing brood were exposed to the nominal concentration of mCry3A.

C.3.g.3. Margin of Exposure

Honeybees are unlikely to be exposed to mCry3A via MIR604; corn does not produce nectar and MIR604 pollen does not contain detectable amounts of mCry3A (Joseph and Hill, 2003). The rationale for this test is that the honeybee is a representative of the order Hymenoptera. Many parasitoids of corn pests are wasps in the families Braconidae, Ichneumonidae and Trichogrammatidae of the Hymenoptera (Steffey *et al*., 1999). These groups mainly parasitise eggs and larvae of Lepidoptera. Larvae of these parasitoids could be exposed to mCry3A in the bodies of hosts that have eaten tissue of MIR604.

At present there is no validated test to expose larvae of parasitic Hymenoptera to high doses of protein (although it is possible to expose adults). It is possible, however, to expose larvae of honeybees. Hymenoptera are almost certainly more sensitive to toxins as larvae than as adults, therefore a study of larval honeybees is likely to be more representative than a study of adult parasitoids to assess the effects of mCry3A on parasitoid larvae.

As discussed above, Head *et al*. (2001), Raps *et al*. (2001) and Dutton *et al*. (2002) have studied the incorporation of Cry1Ab into the bodies of the larvae of Lepidoptera feeding on transgenic corn plants; these data represent the best data for predicting the incorporation of mCry3A into the bodies of herbivores feeding on MIR604. The highest incorporation was found in *Spodoptera littoralis*, which contained 4.8 times less Cry1Ab by weight than the leaves on which they were feeding. If mCry3A is incorporated into Lepidoptera at the same rate relative to leaf tissue, then the highest concentration of mCry3A in Lepidoptera will be 4.8X lower than in MIR604 leaf tissue. The average concentration of mCry3A in leaves of MIR604 hybrids is 6.6 µg/g fresh weight (see Chapter 7, Part B.1. above). Therefore the average concentration in the larvae of Lepidoptera is expected to be 1.4 µg/g fresh weight[5]. A test concentration of 50 µg mCry3A/g diet therefore gives a margin of exposure of about 35X EEC.

C.3.g.4. Conclusion

No harmful effects were detected when honeybee (*Apis mellifera*) hives were exposed to an artificial diet containing mCry3A at no less than 35.7X the average worst-case EEC of mCry3A in the diet of parasitic Hymenoptera exposed to MIR604 hybrid corn plants. Pollinators are highly unlikely to be exposed to mCry3A *via* hybrid MIR604 corn plants.

[5] With the provisos noted in the discussion of *Poecilus* above

C.3.h. Earthworm – *Eisenia foetida*

C.3.h.1. Hazard Assessment

A 14-day study of the acute toxicity of MCRY3A-0102 to the earthworm *Eisenia foetida* was carried out according to OECD Guideline 207 for earthworm acute toxicity tests. *E. foetida* is a standard organism for testing the effects of plant protection products on soil invertebrates.

The test was conducted with an artificial soil comprising 10% sphagnum peat, 20% kaolin clay and 70% silver sand (quartz) with water added to 50% of the soil's water holding capacity. Calcium carbonate was added to adjust the soil pH to 6.0 ± 0.5. For the treatment population, MCRY3A-0102 was added to give a concentration of 250 µg mCry3A/g moistened soil. The control population was exposed to the same soil without MCRY3A-0102. In addition, the LC_{50} for a toxic reference substance (2-chloracetamide) was determined in soil with the same composition as the control and reference treatments.

Treatments were incorporated into the artificial soil substrate, which was then transferred to 1 L glass jars. Each jar contained 500 g (dry weight) of soil. Ten adult *E. foetida* (between 300 and 600 mg in weight) were then introduced into each replicate jar (n = 4 per treatment). The jars were kept in a controlled environment room maintained at 18-20°C and 57-89% relative humidity, with constant light at 485-569 lux. The mortality of the earthworms was assessed 7 and 14 days after treatment (DAT). Any dead earthworms were removed at the time of assessment. The body weight of the earthworms was recorded before and after treatment.

At 7 DAT, there was 0% mortality in the control population and 2.5% mortality in the treatment population. At 14 DAT, mortality in the control population was still 0% and in the treatment population it had risen to 5.0%. The difference between the control and treatment was not statistically significant at either time. During the study the weight of earthworms in the control population decreased by 11.4% and the weight of earthworms in the treatment population decreased by 5.8%; this difference was not significant. The LC_{50} of 2-chloracetamide was determined to be 18.0 mg/kg dry weight soil, showing the earthworms were sufficiently sensitive to meet Guideline requirements.

The test is considered valid if the mortality in the control population is below 10% and the decrease in weight of the control population is no greater than 20%. Both of these criteria were met. Under conditions of the test, MCRY3A-0102 was not harmful to the earthworm *Eisenia foetida*. The LC_{50} of mCry3A to *E. foetida* was >250 µg/g moistened soil and the NOEC was 250 µg/g soil (the highest concentration tested).

C.3.h.2. Exposure assessment

Earthworms were exposed to a nominal concentration of 250 µg mCry3A/g moistened soil during the toxicity study. A subsequent study was carried out to

determine whether the earthworms were exposed to this intended concentration of mCry3A.

C.3.h.3. Characterization and quantification of mCry3A in the artificial soil in the *Eisenia foetida* toxicity study

Characterization and quantification of mCry3A in the artificial soil in the *Eisenia foetida* toxicity study was carried out to assess the exposure of earthworms to mCry3A in that study. To avoid disturbance of the worms, soil samples were not taken from the toxicity study. As a surrogate for soil in the MCRY3A-0102 treatment, soil prepared in the same manner, but not containing worms, was maintained in parallel under the conditions of the toxicity study. Samples of the mCry3A-treated soil were taken immediately after preparation (0 days) and at 3, 7 and 14 days after treatment, and stored at *ca*. –20°C. On completion of the toxicity study, the samples of treated soil were shipped frozen to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA, where they were stored at –80°C until analysis. A sample of untreated artificial soil, from the same batch of soil used in the toxicity study, was shipped at the same time (and stored at –80°C).

The concentrations and intactness of mCry3A in the treated soil samples were measured by ELISA and Western blot analysis respectively. Extracts of treated and untreated soils were prepared according to standard operating procedures. ELISA was carried out according to standard operating procedures using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

Aliquots of the treated soil extract, a solution of MCRY3A-0102 and the untreated soil extract were subjected to SDS-PAGE using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

The bioactivity of mCry3A in the treated soil samples was also measured by bioassay of 1st instars of CPB. Standard CPB diets were mixed with 5 and 10% (w/w) of each treated soil sample, giving nominal concentrations of 12.5 and 25.0 µg mCry3A/g complete diet. Negative control diets were prepared in the same manner using samples of untreated soil to which deionized water had been added in the same proportion (w/v) used to prepare the control soil in the *Eisenia* toxicity study. A positive control diet was prepared by adding MCRY3A-0102 to give 12.5 and 50.0 µg mCry3A/g CPB diet. Assays using CPB diet treated with deionised water in the same proportion (w/v) as the MCRY3A-0102 solution in the positive control diet, and untreated CPB diet were also carried out. Mortality of CPB larvae was assessed daily for 5 days.

ELISA of the treated soil extracts detected 18.1% of the nominal concentration of mCry3A in the 0-day sample, 14.8% in the 3-day sample, 6.3% in the 7-day sample and 7.6% in the 14-day sample. No mCry3A was detected in extracts of the untreated soil. In all extracts of treated soil samples, a Western blot detected an immunoreactive band

corresponding to the predicted molecular weight of intact mCry3A (*ca*. 67.7 kDa). In the 3-day and subsequent samples, one or more additional bands of apparent mCry3A degradation products were detected; these were more prominent in the 7 and 14-day samples. The main degradation products appeared as a doublet at *ca.* 57 kDa.

Treated soil showed high bioactivity against 1st-instar CPB at all sampling times. After 5 d, mortality on the treated soil diets was between 60 and 83%, with little difference apparent among sampling times and between the different soil incorporation rates in the CPB diet. Mortality on the positive control diet was similar to that on the treated soil diets after 5 days (87-97%). Mortality on the water-treated soil diet was low (13-20%) after 5 days and similar to the water-treated and untreated CPB diets (both 20% mortality).

This study shows that active mCry3A was present in the treated artificial soil throughout the 14-day exposure part of *Eisenia* toxicity study. Although only about 18% of the nominal concentration of mCry3A was recovered from the 0-day sample, 7.6% of the nominal concentration of mCry3A could still be detected after 14 days. Therefore, the low recovery was probably due to low extractability of the protein from the artificial soil, rather than rapid degradation of the protein immediately after addition to the soil; unlike the study of mCry3A in live soil reported in Chapter 9 below, rapid degradation is not expected in artificial soils due to low microbial activity. The Western blot showed that intact mCry3A was present 14 days after the soil was treated, although some degradation did occur. However, bioassays against first-instar CPB confirmed that this protein was bioactive. These results show that earthworms were exposed to intact, bioactive mCry3A throughout the 14-day toxicity study.

C.3.h.4. Margin of Exposure

Earthworms could be exposed to mCry3A *via* several routes (Raybould, 2004b), but the most likely route is ingestion of senescent plant material that is incorporated into the soil. The average concentration of mCry3A in senescent roots of MIR604 hybrids is 3.8 µg/g fresh weight, after correcting for extraction efficiency (75.1%); the average concentration of mCry3A in senescent leaves of MIR604 hybrids is 5.5 µg/g fresh weight, after correcting for extraction efficiency (77.1%) (Tables 1 and B-1 in Joseph and Hill, 2003). An extreme worst-case exposure would occur if earthworms ate a diet comprising 100% senescing leaves of MIR604 hybrids; on this basis, 250 µg mCry3A/g moistened soil gives a margin of exposure of 46X EEC. In reality, senescing plant material will be greatly diluted in a large volume of soil and therefore 46X EEC is a very conservative estimate.

C.3.h.5. Conclusion

No harmful effects were detected when earthworms (*Eisenia foetida*) were exposed to an artificial soil containing mCry3A at no less than 46X the average worst-case EEC of mCry3A in the diet of earthworms exposed to MIR604 hybrid corn plants.

C.3.i. Wild mammals

C.3.i.1. Hazard Assessment

No ecotoxicology studies were carried out solely for the assessment of safety of mCry3A to wild mammals. However, the study of acute oral toxicity of mCry3A to mice (Johnson, 2003) can be used for this purpose. Five male and five female mice were each exposed to a single dose of MCRY3A-0102 at 2632 mg/kg body weight, representing approximately 2377 mg/kg body weight of mCry3A. The test substance was dissolved in an aqueous suspension of 1% methylcellulose. A control group received the same volume of 1% methylcellulose used to deliver the test substance. Fourteen days after treatment there were no signs of toxicity in the mice given MCRY3A-0102; all endpoints were comparable in the control and test groups. The LD_{50} value for male and female mice is therefore greater than 2377 mg mCry3A/kg body weight (the single dose tested).

C.3.i.2. Margin of Exposure

Mammals can be serious pests of corn. Rodents such as thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), deer mice (*Peromysus maniculatus*), house mice (*Mus domesticus*), and prairie and meadow voles (*Microtus* spp.) will feed on germinating corn seeds. Frequently these species remove so many seeds that the field needs to be replanted. Woodchucks (*Marmota monax*) also feed on sprouting corn seed, but because they feed along the edges of fields, they usually cause less serious damage than other rodents. Larger mammals such as white-tailed deer (*Odocoilus virginianus*) and raccoons (*Procyon lotor*) cause injury to ripening ears. Deer typically nip off ear tips, whereas raccoons chew through husks. In some areas these species are hunted specifically to reduce damage to cornfields (Steffey *et al*., 1999).

The daily dietary dose (DDD) of mCry3A for mammals can be calculated using the method described for birds in Section 3.a.1. above, *i.e.*:

$$DDD = \frac{FIR}{bw} \times C$$

Crocker *et al.* (2002) estimated the ratio of food intake rate and body weight (*FIR/bw*) for several rodent species. The values for the harvest mouse (*Micromys minutus*) and the wood mouse (*Apodemus sylvaticus*) consuming cereal seeds are 0.33 and 0.28, respectively. Kernels of MIR604 hybrids contain an average of 0.9 µg mCry3A/g fresh weight, which represents 1.3 µg mCry3A/g fresh weight after correcting for extraction efficiency (69.7%); therefore a worst-case DDD for rodents eating a diet comprising 100% kernels of MIR604 is approximately 0.43 mg mCry3A/kg body weight. This represents over 5500X the dose of mCry3A in the mouse study.

Deer eating the tips of corn ears might be exposed to leaf and kernel tissue. Leaf tissue of MIR604 has a much higher concentration of mCry3A than do kernels (10.1 µg

mCry3A/g fresh weight compared with 1.6 µg mCry3A/g fresh weight at seed maturity)[6] and therefore represents a worst case diet, provided the calorific value of leaf tissue is not so high that deer eat much less of it than kernels to attain their energy needs. Grasses have a slightly higher calorific value than cereal seeds (Table 4 in Crocker *et al*., 2002;), but the difference is small compared with the difference in concentration of mCry3A between leaves and kernels of MIR604. Therefore a diet of 100% leaves of MIR604 is taken to be the worst case exposure for deer and other large mammals to mCry3A.

The *FIR*/*bw* for fallow deer eating grass is 0.09 (Crocker *et al*., 2002). Taking the concentration of mCry3A in the diet to be 10.1 µg mCry3A/g fresh weight, the value for leaf tissue at seed maturity, the worst-case DDD for a large mammal eating a diet comprising 100% ears of MIR604 corn is 0.91 mg mCry3A/kg body weight. This represents over 2600X the dose in the mouse study.

C.3.i.3. Conclusion

No harmful effects were detected when mice were given a single oral dose of mCry3A representing no less than 2600X the extreme worst-case daily dietary dose of mCry3A to mammals feeding on MIR604 hybrid corn.

D. RISK ASSESSMENT FOR NON-TARGET ORGANISMS

A comparison of native and mCry3A showed that these proteins have the same spectrum of activity against various insect pest species, apart from the intended increase in toxicity of mCry3A to certain species of *Diabrotica* (Table 14). From a review of the literature (Tables 15, 16 and 17) it is concluded that the toxicity of native Cry3A is limited to certain species in 3 families of Coleoptera: the Chrysomelidae, the Curculionidae and the Tenebrionidae. If native and mCry3A differ only in their activity against *Diabrotica* species, it follows that mCry3A should pose negligible hazard (show low toxicity) to species outside those 3 families; the ecotoxicology studies summarised in Section C tested this hypothesis.

Nine species of non-target organism were exposed to concentrations or doses of mCry3A in excess of expected environmental concentrations of mCry3A that might result from the proposed cultivation of MIR604 (Table 18). No harmful effect of mCry3A was detected in any study. Taken together, the studies give a robust test of the hypothesis that the hazard of mCry3A is limited to the Chrysomelidae, the Curculionidae and the Tenebrionidae. Several features provide high predictive power to extrapolate the absence of detectable effects to species that were not tested: high margins of exposure; long exposure times in many studies; sensitive endpoints in many studies; and the testing of species closely related to the target organism (see Chapman *et al*., 1998, for a discussion of uncertainty in predictions from laboratory studies).

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[6] The average concentration of mCry3A leaves of MIR604 hybrids at seed maturity is 7.8 µg/g and the extraction efficiency is 77.1%; the average concentration of mCry3A in kernels of MIR604 hybrids at seed maturity is 1.1 µg/g and the extraction efficiency is 69.7% (Tables 1 and B-1 from Joseph and Hill, 2003).

The conclusion that mCry3A has no detectable toxicity at no less than 10X EEC, except to species in the Chrysomelidae, the Curculionidae and the Tenebrionidae, means that risk to the diversity or abundance of NTOs outside these families from the proposed cultivation of MIR604 is minimal. In addition to the lack of hazard of mCry3A, the risk to certain NTOs from MIR604 is minimal because of negligible exposure; this applies to pollinators in particular, because mCry3A was not detected in MIR604 pollen.

E. POTENTIAL FOR EXPOSURE OF NON-TARGET CHRYSOMELIDAE, CURCULIONIDAE AND TENEBRIONIDAE (COLEOPTERA) TO MCRY3A PROTEIN FROM MIR604 PLANTS

The data presented above and in the previously submitted volume by Garcia-Alonso and Vlachos (2003) demonstrate that activity of mCry3A is expected to be limited to beetles, and in particular to certain members of the Chrysomelidae (leaf beetles, flea beetles and rootworms), the Curculionidae (weevils and snout beetles) and Tenebrionidae (darkling beetles) families. The Chrysomelidae and Curculionidae are herbivores and some species are serious crop pests, and by definition these species are not non-target organisms. It is possible that some species in these families could be beneficial if they feed on and control weed species. Such species could potentially be exposed to MIR604 pollen, but not to mCry3A because the protein is not expressed in pollen at detectable levels (Joseph and Hill, 2003). Therefore no non-target species of the Chrysomelidae or Curculionidae are likely to be harmed by mCry3A expressed in MIR604.

The Tenebrionidae are scavengers of plant material as both larvae and adults. Some species, known as mealworms, are pests of stored grain. Species known as darkling beetles or false wireworms can be pests of crops such as cotton and cucurbits. The most serious damage caused by darkling beetles is the cutting of stems of young seedlings just below the soil surface. Further feeding can also occur on flowers or the undersides of leaves, but this is rarely a cause of economic loss.

Darkling beetles are unlikely to be exposed to mCry3A through eating fresh tissue of MIR604 because they are not recorded as a pest species in corn (*e.g.*, Steffey *et al*., 1999). From what is known of the ecology of darkling beetles, we cannot eliminate the possibility that some species may be exposed to mCry3A by feeding on senescent tissue of MIR604 within cornfields.

Wu and Dean (1996) measured the LD_{50} of a native Cry3A to *Tenebrio molitor* (yellow mealworm), which we can regard as a representative species of the Tenebrionidae, and *Leptinotarsa decemlineata* (Colorado potato beetle), a species of Chrysomelidae commonly used in bioassays for native Cry3A because of its sensitivity to this protein (*e.g.*, Wu *et al*., 2000). The LD_{50} for *T. molitor* was 11.4 µg Cry3A/larva and that for *L. decemlineata* was 91 ng Cry3A/larva (*i.e.,* each *T. molitor* larva is about 125-fold less sensitive than each Colorado potato beetle larva). The concentration of mCry3A in senescing leaves of MIR604 hybrids is 4.2 µg/g fresh weight; corrected for 77.1% extraction efficiency for leaves, this amounts to 5.5 µg/g fresh weight (Joseph and Hill,

2003). Assuming the activity of mCry3A to these species is roughly the same as the native Cry3A used by Wu and Dean (1996), *T. molitor* larvae would need to ingest roughly 2.1 g of senescing leaf tissue of MIR604 to receive the median lethal dose of mCry3A. This represents about 3500X the body weight of a first instar and about 15X the body weight of a final instar of *T. molitor* (body weight data for *T. molitor* obtained from National University of Singapore [1998]). Given the omnivorous diet of the Tenebrionidae and the likely rapid degradation of mCry3A in soil (Raybould, 2004b), any darkling beetle species in cornfields are extremely unlikely to be exposed to sufficient mCry3A to result in a significant impact, even if they were sensitive to mCry3A.

F. ENDANGERED SPECIES CONSIDERATIONS

F.1. Endangered Coleoptera

Studies of non-target organisms and pest species have revealed no unexpected effects of mCry3A to Coleoptera. Therefore the expectation is that the activity of mCry3A will be limited to certain species within the Chrysomelidae, Curculionidae and Tenebrionidae families of beetles (Garcia-Alonso and Vlachos, 2003). There are currently no endangered or threatened species in any of these families (U.S. Fish and Wildlife Service Endangered Species Program, http://endangered.fws.gov/); this includes the salt creek tiger beetle (*Cicindela nevadica lincolniana*; family Cicindelidae), which is a new addition to the federal Endangered Species list with effect from 7th November 2005. In addition, there are no endangered or threatened beetle species in habitats in which corn is grown (US EPA, 2003). Pollen of MIR604 has no detectable mCry3A and corn pollen is not known to disperse into the habitats or breeding grounds of endangered beetles (US EPA, 2003). Therefore no endangered or threatened beetles are expected to be harmed by mCry3A expressed in MIR604 hybrids.

F.2. Other endangered species

Extensive testing of mCry3A, native Cry3A and other Cry3 proteins (Section D above; Garcia-Alonso and Vlachos, 2003; US EPA, 2001; US EPA, 2003) has shown no harmful effects to taxa outside the Coleoptera; groups tested include mammals, birds, fish, earthworms and several orders of insects. The lack of detectable expression of mCry3A in pollen of MIR604 inbred lines and hybrids, the lack of weediness of corn, the absence of sexually compatible wild relatives of corn in the USA and the very low probability that mCry3A will show significant dispersal in soil (Raybould, 2003) means that endangered and threatened species could only be exposed to mCry3A by eating MIR604 plants or organisms that had fed on MIR604 tissue.

EPA reviewed the likelihood of exposure of endangered bird and bat species to Cry3Bb1 (a coleopteran-active protein) expressed in corn Event MON863 (US EPA, 2003). EPA concluded that endangered birds and bats rarely forage in agricultural fields, and that insectivorous species tend to take flying insects rather than larvae (the life stage most likely to contain significant amounts of Cry protein). Indirect effects of MON863 could

occur through changes in prey abundance, however such changes would most likely be beneficial to rare species as their prey will increase due to reductions in pesticide use (US EPA, 2003). Similar changes are expected for MIR604 and therefore cultivation of MIR604 is extremely unlikely to have harmful effects on any endangered or threatened species.

F.3. Risks to the Monarch Butterfly

Although the monarch butterfly is not an endangered species, there are well-known reports that transgenic corn may harm populations of this insect (e.g. Losey *et al*., 1999). Laboratory studies have shown that first instar monarch larvae could be exposed to toxic concentrations of Cry1Ab through eating milkweed leaves (their primary plant food source) dusted with pollen from transgenic corn events expressing Cry1Ab (Hellmich *et al.*, 2001). However, a risk assessment using estimates of the proportion of the monarch population exposed to potentially harmful amounts of pollen showed that Cry1Ab posed no significant risk to monarchs (Sears *et al.*, 2001). Exposure of the first and second instars to corn pollen is low because the vast majority of monarch larvae do not develop on milkweed in or adjacent to cornfields during anthesis (pollen shed) of corn.

Event MIR604-derived hybrids pose no detectable risk to monarchs. First, mCry3A as expressed in MIR604 hybrids is extremely unlikely to be hazardous to monarchs. Extensive safety testing data indicate that activity of mCry3A is limited to species in 3 families of Coleoptera; monarchs are Lepidoptera. Secondly, monarchs are unlikely to be exposed to mCry3A; in addition to the low exposure of monarch larvae to corn pollen, mCry3A has not been detected in MIR604 pollen (see Chapter 6. **Quantification of mCry3A and PMI Proteins in Event MIR604**). Therefore, there is neither detectable hazard nor exposure of mCry3A to monarch larvae and Event MIR604-derived hybrids will pose negligible risk to this species.

Chapter 8. Food and Feed Safety

A. INTRODUCTION

This chapter represents a summary of data and information relevant to the food and feed safety of a modified Cry3A (mCry3A) insect control protein and the genetic material required for its production (*via* pZM26) in transgenic maize (corn) plants derived from Syngenta Seeds' transformation Event MIR604. Event MIR604 also expresses phosphomannose isomerase (PMI) as a selectable marker. A permanent exemption from the requirement of tolerances has been established for the PMI protein in all crops (US EPA, 2004a). This tolerance exemption was granted in response to a petition (EPA File Symbol PP 3E6748) submitted to the US Environmental Protection Agency by Syngenta Seeds. A large body of PMI protein characterization and mammalian safety data accompanied the PMI tolerance petition. Accordingly, the experimental details and results of this safety assessment, which included an evaluation of the potential for PMI to become a food allergen, are not presented in the present petition for deregulation. However, pertinent summary information can be found in Vlachos and Joseph, 2003, and US EPA, 2004a.

In connection with a December 15, 2003 application for an Experimental Use Permit for MIR604 corn (granted in May 2005), Syngenta Seeds petitioned the US EPA to grant a temporary exemption from tolerances (EPA File Symbol 4G6808; US EPA, 2004b) for the mCry3A protein. Syngenta Seeds has also petitioned the US EPA (EPA File Symbol PP 4F6838; US EPA, 2004b) to grant a permanent exemption from tolerances for mCry3A in all corn. That petition was submitted on April 30, 2004, concurrently with a FIFRA Section 3 registration application for MIR604 corn (US EPA, 2004c). These EPA applications are currently pending.

The scientific studies listed below (Table 20) contain data and detailed information that are relevant to the characterization and mammalian safety of Event MIR604 plants and/or the transgenic proteins produced therein. Those studies with EPA MRID #s were submitted to the US EPA concurrently with previous regulatory applications for MIR604 corn and the tolerance exemptions for mCry3A protein, or the tolerance exemption for PMI.

Table 20. List of Data Volumes Supporting the Food and Feed Safety of Event MIR604 Corn

Study Name	EPA MRID #	Reference
Characterization of Modified Cry3A Protein Produced in Event MIR604-Derived Maize (Corn) and Comparison with Modified Cry3A Protein Expressed in Recombinant *Escherichia coli*	46155603	Joseph and Graser, 2003a
Quantification of Modified Cry3A and PMI Proteins in Transgenic Maize (Corn) Tissues, Whole Plants, and Silage Derived from Transformation Even MIR604	46155604	Joseph and Hill, 2003
Characterization of Modified Cry3A Test Substance (MCRY3A-0102) and Certificate of Analysis	46155605	Joseph and Graser, 2003b
Further Characterization of Modified Cry3A Test Substance MCRY3A-0102	46155606	Moffatt, 2003
In vitro Digestibility of Modified Cry3A Protein (MCRY3A-0102 and IAPMIR604-0103) Under Simulated Mammalian Gastric Conditions	46155607	Joseph and Graser, 2003c
Effect of Temperature on the Stability of Modified Cry3A Protein (MCRY3A-0102)	46155608	Joseph, 2003
Analysis for the Presence of Modified Cry3A Protein in Wet and Dry Milled Fractions, Corn Oil and Corn Chips from Corn (Maize) Event MIR604	46155609	Joseph, R. and Kramer, C, 2003
Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Mouse	46155610	Johnson, 2003
Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Known Toxins[2]	NA[1]	Hart and Rabe, 2004a
Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Allergens[2]	NA	Hart and Rabe, 2004b
Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Known Toxins[2]	NA	Hart and Rabe, 2004c
Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Allergens[2]	NA	Hart and Rabe, 2004d
Characterization of Phosphomannose Isomerase (PMI) Produced in Maize (Corn) Plants Derived from Event MIR604 and Comparison to PMI as Contained in Test Substance PMI-0198	NA	Hill, 2004

[1]NA = not assigned.

[2]Previous versions of the four reports of amino acid sequence homology searches were submitted to the US EPA in 2003 in support of an Experimental Use Permit application for Event MIR604 corn (67979-EUP-U) or a petition for permanent exemption from tolerances for the PMI protein in all crops (Petition File Symbol PP 3E6748). The present reports represent the results of more recent bioinformatics searches of updated databases, however, the conclusions of the initial assessments (Zawodny, 2003a, 2003b, 2003c, 2003d, 2003e and 2003f) remain essentially unchanged.

B. HUMAN EXPOSURE CONSIDERATIONS: MODIFIED CRY3A PROTEIN

B.1. Dietary Exposure to Nucleic Acids

The nucleic acids (DNA and the RNA encoded by it) present in Event MIR604-derived corn plants as a result of transformation will not present a dietary safety concern. Based on the ubiquitous occurrence and known safety of nucleic acids in the food supply, the World Health Organization (WHO; FAO/WHO, 1991), and the US FDA (US FDA, 1992) have stated that the consumption of DNA from all sources, including genetically modified crops, poses no safety issue. In addition, a tolerance exemption under the Federal Food Drug and Cosmetic Act regulations has been established for residues of nucleic acids that are part of plant-incorporated protectants or associated inert ingredients (US EPA, 2001b).

B.2. Prior Human Exposure to Modified Cry3A Protein

Prior dietary exposure to the mCry3A protein has not occurred. However, it is conceivable that a low level of dietary exposure to the native Cry3A protein has occurred, as it is registered for use in the U.S. in NewLeaf® Bt potatoes and has been registered as a component of various *Bacillus thuringiensis*-based microbial insecticides (see Table 21), and is permanently exempt from food and feed tolerances (US EPA 2004b).

B.3. Expression Levels of mCry3A Protein in Event MIR604 Plants

Quantifiable levels of mCry3A protein were detected in all Event MIR604-derived plant tissues analyzed except pollen (Joseph and Hill, 2003). Across all growth stages, mean mCry3A levels measured in leaves, roots and whole plants ranged from *ca.* 3 - 23 µg/g fresh wt. (4 - 94 µg/g dry wt.), *ca.* 2 - 14 µg/g fresh wt. (7 - 62 µg/g dry wt.), and *ca.* 0.9 - 11 µg/g fresh wt. (3 - 28 µg/g dry wt.), respectively. Mean mCry3A levels measured in kernels at seed maturity and senescence ranged from *ca*. 0.6 – 1.4 µg/g fresh wt. (0.8 – 2.0 µg/g dry wt.). Mean mCry3A levels measured in silk tissue at anthesis were below the lower limit of quantification (LOQ), <0.1 µg/g fresh wt. (<1.0 µg/g dry wt.). Mean mCry3A levels measured in silk tissue at seed maturity ranged from *ca*. 0.6 – 1.9 µg/g fresh wt. (1 – 3 µg/g dry wt.). No mCry3A protein was detectable in pollen from either the inbred MIR604-A or the hybrids MIR604-B and MIR604-C [limit of detection (LOD) = 0.07 µg/g fresh wt., 0.15 µg/g dry wt.].

The stability of mCry3A protein expression over multiple generations was evaluated. Overall, levels were similar across the four generations analyzed and there was no evidence of any significant trend either up or down, indicating that the expression of mCry3A protein is stable.

A more detailed summary of the Event MIR604 expression data can be found in Chapter 6, **Quantification of mCry3A and PMI Proteins in Event MIR604.**

Table 21. List of Commercial Products Containing *Bacillus thuringiensis* subsp. *tenebrionis* or Cry3A delta endotoxin.

Product Name	Company Name	Strain or Host	Percent AI	EPA ID#	Date EPA Approved	Pests	US States Approved
Trident	Certis USA, LLC	Bacillus thuringiensis subsp. tenebrionis	14.32	70051-64	12/12/1988	Colorado Potato Beetle (larvae)	No data available
Trident II Biological Insecticide	Sandoz Agro, Inc.	Bacillus thuringiensis subsp. tenebrionis	0.64	55947-138	3/1/1990	Colorado Potato Beetle (larvae); Elm Leaf Beetle	No data available
Foil Bfc Oil Flowable Bioinsecticide	Ecogen, Inc.	Bacillus thuringiensis subsp. kurstaki strain EG2424	10	55638-10	2/5/1991	Colorado Potato Beetle (larvae)	NC, RI (Discontinued, all states)
Ditera Technical Powder	Abbott Laboratories	Bacillus thuringiensis subsp. tenebrionis	99.9999	275-79	2/7/1991	No data available	No data available
Ditera Wettable Powder	Abbott Laboratories	Bacillus thuringiensis subsp. tenebrionis	3.65	275-80	2/7/1991	Colorado Potato Beetle (larvae); Elm Leaf Beetle; Mexican Bean Beetle	No data available
M-Trak Bioinsecticide	Ecogen, Inc.	Delta endotoxin of Bacillus thuringiensis subsp. san diego encapsulated in killed Pseudomonas fluorescens	10	55638-44	6/27/1991	Colorado Potato Beetle (larvae); Elm Leaf Beetle; Elm Caligrapha (larvae); Cottonwood Leaf Beetle; Imported Willow Leaf Beetle; Mealworms (larvae)	No data available
Raven	Certis USA, LLC	Bacillus thuringiensis sub. kurstaki strain EG7673 Lepidopteran active toxin	25	70051-82	1/18/1995	Colorado Potato Beetle (larvae); Elm Leaf Beetle; Cottonwood Leaf Beetle; Imported Willow Leaf Beetle; Mexican Bean Beetle	DE, NC, OK (Cancelled or Dropped, all states)

Plant Pesticide B.t. Subsp. Tenebrionis Colorado Potato Beetle Protein	Monsanto Company	Bacillus thuringiensis subspecies tenebrionis delta endotoxin as produced in potato by Cry IIIA gene and its controlling sequences and found in the following constructs:PV-STBT02, PV-STBT04, and PV-STMT01, IR-22,	0.2	524-474	5/2/1995	Colorado Potato Beetle	DE, IA, NC, OK, RI (Cancelled, Inactive or Dropped, all states)
New Leaf Plus Potatoes	Monsanto Company	Bacillus thuringiensis subspecies tenebrionis delta endotoxin as produced in potato by Cry IIIA gene and its controlling sequences and found in the following constructs:PV-STBT02, PV-STBT04, and PV-STMT01, IR-22; Potato leafroll virus (PLRV) replicase protein as produced in potato plants.	CryIIIA - 0.2; PLRV - 0.03	524-498	11/18/1998	Colorado Potato Beetle; Potato Leaf Roll Virus	No data available
Novodor Technical	Valent Biosciences Corporation	Bacillus thuringiensis subspecies tenebrionis, strain NB-176	92.6	73049-47	4/29/2000	No data available	No data available
Novodor Flowable Concentrate	Valent Biosciences Corporation	Bacillus thuringiensis subspecies tenebrionis, strain NB-176	10	73049-48	4/29/2000	Colorado Potato Beetle (larvae); Elm Leaf Beetle (larvae)	DE, ID, MD, MN, NC, NV, NY, RI, SD, VA, WA, WI
Bacillus Thuringiensis Subsp Tenebrionis Slurry	Valent Biosciences Corporation	Bacillus Thuringiensis Subsp Tenebrionis, strain NB-176	17	73049-64	3/3/2006	No data available	No data available

Source: Kelly Registration Systems, Inc., 10115 Highway 142 N., Covington, Georgia 30014; http://www.krsnetwork.com/home.asp

B.4. Fate of Modified Cry3A Protein in Processed Corn Products

Modified Cry3A protein (mCry3A) was quantitatively analyzed in wet and dry milled fractions generated from standard food processing procedures carried out on maize (corn) grain derived from Event MIR604, together with a corresponding non-transgenic control (Joseph and Kramer, 2003). In addition, flaking grits and flour produced during dry milling were further processed to oil and corn chips, respectively. All milling fractions, oil, and corn chips were analyzed for mCry3A by enzyme linked immunosorbent assay (ELISA).

The mCry3A level measured in a sample of the starting grain material derived from Event MIR604 was *ca*. 1.1 µg mCry3A/g. Among the wet milled fractions, the medium fiber (0.46 µg mCry3A/g), fine fiber (0.26 µg mCry3A/g), and gluten meal fractions (0.24 µg mCry3A/g) yielded quantifiable amounts of mCry3A. The coarse fiber, germ, and starch fractions had detectable but not quantifiable levels of mCry3A (<0.06 µg mCry3A/g). The steep water fraction showed no detectable mCry3A.

The analysis of different samples derived from the dry milling process resulted in quantifiable levels of mCry3A protein in all 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. Modified Cry3A levels measured 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 measured in the flour used in the preparation of corn chips was 0.32 µg mCry3A/g, no mCry3A was detected in the corn chips. Similarly, mCry3A was not detectable in oil, whereas the mCry3A concentration measured in the starting material, flaking grits, was 2.12 µg/g.

C. MAMMALIAN TOXICOLOGY ASSESSMENT OF MODIFIED CRY3A PROTEIN

C.1. Target Organism Specificity of Modified Cry3A Protein

The observed activity spectrum of the mCry3A protein (see Chapter 7. **Environmental Safety**), its mode-of-action and its structural similarity to Bt proteins for which human safety has previously been established (see Chapter 1. **Syngenta Seeds Petition for the Determination of Non-Regulated Status of Corn Event MIR604**) support the prediction that no adverse health effects will result from exposure to mCry3A protein from Event MIR604 corn. Nevertheless, as described below, Syngenta has conducted an extensive characterization and safety assessment of the mCry3A protein.

C.1.a. Test Substance Used for Safety Studies

The mCry3A protein cannot be reasonably extracted in sufficient quantities from Event MIR604 corn plants to deliver the high doses typically employed in standard acute oral toxicity studies. Therefore, mCry3A was produced and purified from a recombinant microbe (Joseph and Graser, 2003b).

The mCry3A test substance was prepared by expressing the same modified *cry3A* gene that was used for plant transformations in an *E. coli* over-expression system. The modified *cry3A* gene was linked to the *Bacillus thuringiensis cry1Ac* native promoter in a Bluescript™ vector and transformed into *E. coli* strain DH5α. The *cry1Ac* promoter permits expression of the modified *cry3A* gene in the stationary phase of bacterial cell growth. The resulting lyophilized protein preparation, designated test substance MCRY3A-0102, was insecticidally active and had a 144-hour LC_{50} of 1.4 μg/ml (95% confidence interval: 0.7 - 2.2 μg/ml) against larvae of western corn rootworm (WCRW). The purity of mCry3A in the test substance was determined by densitometric analysis of a Coomassie blue stained gel. The results of this analysis showed that test substance MCRY3A-0102 contained *ca.* 90.3% mCry3A by weight. Western blot analysis of the test substance revealed a single immunoreactive band corresponding to the predicted molecular weight of *ca*. 67,700 daltons. Re-analysis of test substance MCRY3A-0102 *ca*. nine months after its initial characterization, demonstrated that it was substantially stable under the conditions of storage (-20°C).

In a separate study (Moffatt, 2003), mass spectral analysis revealed that test substance MCRY3A-0102 contained two closely related proteins in a ratio of 2:3. The lesser of these two components (with the lower molecular weight) corresponded to the intended mCry3A protein (598 amino acids). The other component also contained these same 598 amino acids but was shown to contain an additional 16 amino acids at the N-terminus. As part of the test substance characterization study, small amounts of each of these two proteins, designated mCry3A-SF and mCry3A-LF, respectively, were prepared and their bioactivity determined against WCRW. The results of the bioassay demonstrated that, as expected, both proteins were of similar activity. On this basis, and also taking account of their high degree of structural homology (97.4 % amino acid identity), the two forms of mCry3A in test substance MCRY3A-0102 can be considered to be equivalent. It was demonstrated that these two forms of the protein in sample MCRY3A-0102 differed by 16 amino acid residues due to translation of the protein occurring from two different initiation codons in the vector sequence used for production of MCRY3A-0102 in *E. coli*. It is important to note that this only occurred in production of this test substance and could not have occurred in transformed plants. The extra initiation codon in the vector sequence used for the test substance production was present in the *cry1Ac* gene promoter used to express the *mcry3A* gene in *E. coli*. There is no additional initiation codon present in the gene promoter in the vector used for plant transformation.

C.1.b. Equivalence of mCry3A Protein in Test Substance MCRY3A-0102 and mCry3A Protein as Produced in Event MIR604 Plants

To justify the use of *E. coli*-expressed mCry3A protein as a surrogate for mCry3A as produced in Event MIR604 corn, an extensive "bridging" study was conducted (Joseph and Graser, 2003a). *E. coli*-produced test substance MCRY3A-0102 was compared by analysis of various functional and biochemical parameters to mCry3A protein produced in transgenic corn event MIR604. The mCry3A proteins from both sources were demonstrated to have the predicted molecular weight of *ca*. 67,700 Da and immunologically cross-reacted with the same anti-mCry3A antibody. No evidence of any post-translational glycosylation of mCry3A protein from either source was observed. Comparisons of the biological activity of *E. coli*-expressed and corn-expressed mCry3A

protein in a larval diet bioassay using western corn rootworm showed very similar activities (LC_{50} values). Based on these results, it can be concluded that mCry3A proteins from recombinant *E. coli* and MIR604-derived corn are substantially equivalent and that the microbial test substance MCRY3A-0102 is a suitable surrogate for mCry3A protein produced in transgenic corn Event MIR604.

Additional evidence supporting the equivalence of mCry3A in test substance MCRY3A-0102 and Event MIR604 plants is contained in Chapter 3 of this submission, **Molecular Analysis of Event MIR604**, and in the report, "Further Characterization of Modified Cry3A Test Substance MCRY3A-0102" (Moffatt, 2003).

C.2. Evaluation of mCry3A Amino Acid Sequence Homology with Known Toxins

Although the report of a previous toxin homology search (Zawodny, 2003a) was submitted to the U.S. EPA in support of mCry3A safety, due to the continuing deposition of new sequence entries into the database upon which this analysis relies, a new updated report of the toxin homology search is provided with this petition. However, this new report does not substantially alter the conclusions of the previous report submitted to EPA.)

To determine whether the mCry3A protein has any significant amino acid homology with protein sequences identified as toxins, the amino acid sequence was systematically compared to the latest posting of the National Center for Biotechnology Information (NCBI) Database (NCBI, 2004) containing all publicly available protein sequences (Hart and Rabe, 2004a). The procedure used allowed a determination of (1) whether any proteins in the database showed significant homology to the mCry3A protein, indicating they may be closely related to modified Cry3A, and (2) whether any sequences with significant homology to mCry3A were known to be toxins. The mCry3A query sequence showed no significant amino acid homology to any non-Bt proteins identified as, or known to be, toxins.

C.3. Evaluation of mCry3A Amino Acid Sequence Homology with Known or Putative Allergens

Although the report of a previous allergen homology search was submitted to the U.S. EPA in support of mCry3A safety (Zawodny, 2003b), due to the subsequent deposition of new protein sequence entries into the allergen database upon which this analysis relies, a new updated report of the *allergen homology search is provided with this petition. However, this new report has not substantially altered the conclusions of the previous report submitted to EPA.*

An extensive bioinformatics search was performed to determine whether the amino acid sequence of the mCry3A protein shows homology with proteins known or suspected to be allergens (Hart and Rabe, 2004b). Two different similarity searches were performed comparing the mCry3A protein to the entries in the Syngenta Biotechnology Incorporated (SBI) Allergen Database. This database was compiled from entries identified as allergens or putative allergens in public protein databases, and was

supplemented with additional amino acid sequences identified from the scientific literature.

First, overall homology was examined by comparing sequential 80-amino acid peptides of the mCry3A protein sequence to the allergen sequences using the FASTA search algorithm. Each successive 'window' of 80 amino acids was offset from the previous window by one residue, such that each peptide overlapped the previous peptide by 79 amino acids. Any 80-amino acid peptide of the query sequence having greater than 35% amino acid identity to an allergen sequence was defined as having significant homology to the allergen sequence. Second, the mCry3A protein sequence was screened for matches of eight or more contiguous amino acids using a program that compared every possible peptide of eight contiguous amino acids between the mCry3A sequence and the allergen sequences. The purpose of this analysis was to screen for short, local regions of amino acid identity that might indicate the presence of common IgE-binding epitopes.

The results of these analyses revealed that there was no significant similarity between any of the sequential mCry3A 80-amino acid peptides and any entries in the SBI Allergen Database. Additionally, there were no alignments of eight or more contiguous identical amino acids between the mCry3A protein and any of the proteins in the allergen database. Thus, the mCry3A protein shows no significant amino acid homology to known or putative allergenic proteins.

C.4. Acute Oral Mouse Toxicity Study of Modified Cry3A Protein

An acute mouse oral toxicity study was conducted at the Syngenta Central Toxicology Laboratory (Alderley Park, Macclesfield, Cheshire, UK) according to US EPA Test Guideline OPPTS 870.1100 (Johnson, 2003). Test substance MCRY3A-0102 (see description and analyses of test substance in Section C.1.a., above) was administered to 5 male and 5 female mice [strain Alderley Park albino mouse (AP_fCD-1); 8 - 9 weeks old] *via* a gavage dose of 2632 mg/kg body weight. The test substance contained *ca.* 90.3% mCry3A protein by weight. Therefore, the mice received *ca.* 2377 mg mCry3A/kg body weight. A negative control group (5 mice/sex) concurrently received the dosing vehicle alone, a suspension of 1% methylcellulose, at the same dosing volume as used for the test material mixture. Food was provided *ad libitum*, except during the *ca.* 3.5 hours prior to dosing, when the animals were fasted. Water was provided *ad libitum* throughout the study. Observations for mortality and clinical/behavioural signs of toxicity were made frequently on the day of dosing and daily[7] thereafter for 14 days. Detailed clinical observations were made for each animal at each observation time. Body weights were recorded daily[1] and food consumption was recorded, in most cases, at daily intervals throughout the study. Surviving animals were euthanized 14 days post dosing and subjected to gross necropsy. Organ weights (brain, liver with gall bladder, kidneys and spleen) were recorded and principal tissues (brain, cecum, colon, duodenum, ileum, jejunum, rectum, stomach, heart, kidney, liver with gall bladder, lung and spleen) were processed for microscopic examination.

[1] Except for day 13 of the study when, in error, observations were not made

No test substance-related mortalities occurred during the study, and no clinical signs attributable to the test substance were observed. There were no treatment-related effects on body weight, food consumption, or organ weights, nor were any treatment-related effects observed following macroscopic or microscopic examination of tissues. One female mouse in the test substance group was euthanized on day 2 of the study due to clinical signs consistent with a dosing injury, which was confirmed upon post-mortem examination.

MCRY3A-0102 was not acutely toxic to mice. There was no evidence of toxicity of the test substance at 2632 mg MCRY3A-0102/kg body weight, representing *ca*. 2377 mg mCry3A protein/kg body weight. The estimated LD_{50} value for pure mCry3A protein in male and female mice is >2377 mg/kg body weight, the single dose tested.

C.5. *In vitro* Digestibility Study of Modified Cry3A Protein

The susceptibility of mCry3A protein to proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin (Joseph and Graser, 2003c). mCry3A from two sources, transgenic maize (corn) and recombinant *E. coli* (test substance MCRY3A-0102), was readily degraded in SGF. No intact mCry3A (*ca.* 67,700 molecular weight) or immunoreactive fragments were detected following digestion in SGF for 2 minutes, as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis. These data support a conclusion that mCry3A expressed in transgenic plants will be readily digested as conventional dietary protein under typical mammalian gastric conditions.

The observation that some food allergens exhibit proteolytic stability has led to the common belief that proteins that are resistant to gastric digestion are more likely to become food allergens. However, some researchers (Veiths *et al.,* 1999; Kenna and Evans, 2000; Fu, 2002; reviewed by Fu *et al.,* 2002) have questioned the validity of digestion stability as a criterion for protein allergenicity assessment. However, at the present time, information on gastric digestibility may be utilized in a comprehensive weight-of-evidence approach to assessing allergenic potential.

C.6. Effect of Heat on the Stability of Modified Cry3A Protein

The effect of temperature on mCry3A protein was determined by incubating test substance MCRY3A-0102 for 30 minutes at a range of temperatures (4ºC, 25ºC, 37ºC, 65ºC and 95ºC) followed by bioassay against WCRW larvae (Joseph, 2003). At 95ºC mCry3A was completely inactivated. At 4ºC, 25ºC, and 37ºC there was little or no effect on mCry3A bioactivity, whereas at 65ºC there was some reduction in the bioactivity.

D. EVIDENCE THAT MODIFIED CRY3A PROTEIN IS UNLIKELY TO BECOME A FOOD ALLERGEN

While virtually all allergens are proteins, only a few of the many proteins found in foods are allergenic. Although the probability that any specific novel protein will become a food allergen is, therefore, small, the potential allergenicity of the mCry3A

protein was evaluated using an extensive weight-of-evidence approach. The methods employed generally followed a decision tree for allergenicity assessments, as recommended and described by several international organizations[8] and other experts (Metcalfe *et al*., 1996; FAO/WHO, 2001; Taylor, 2002).

Four general concerns regarding potential allergenicity arise in the context of producing novel proteins in food plants:

- Is the novel protein derived from a source known to produce allergenic proteins and, therefore, might individuals previously sensitized to one or more of these allergens be inadvertently exposed *via* food from the modified crop?
- Is the novel protein sufficiently similar to known protein allergens such that it might elicit an allergic cross-reaction in sensitized individuals?
- Does the novel protein have particular physio-chemical characteristics that would make it more likely to sensitize some individuals, if sufficient dietary exposure occurred?
- Would the novel protein be present in sufficiently high concentrations in food to promote sensitization in the minority of individuals who might be predisposed to sensitization?

The following discussion presents specific test results and information regarding the mCry3A protein to address each of these concerns. Based on the weight of evidence from diverse sources, it can be concluded that mCry3A is very unlikely to represent a potential allergen in food. Additionally, no reports of food allergenicity have been reported to be associated with the use of Bt Cry3A-based insecticidal products in the several years that they have been used on food crops or as a plant-incorporated protectant.

D.1. Low Potential for Exposure to Modified Cry3A *via* Food

Many allergenic proteins, especially those in commonly allergenic foods, are abundant in the offending food, and are present at concentrations typically ranging between 1% and 80% of total protein (Metcalfe *et al.*, 1996). The average mCry3A concentration measured in unprocessed corn grain from Event MIR604 represents less than 0.0001% of the total protein (this calculation is based on corn grain containing 10% total protein by weight, and assumes less than 1 ppm (1 µg mCry3A/g) in the grain; see Chapter 6. **Quantification of mCry3A and PMI Proteins in Event MIR604**)

D.2. Cry3A is Not Derived from a Known Source of Oral Allergens

The source of native Cry3A protein is *Bacillus thuringiensis*. Bacteria have no history of allergenicity (Taylor and Hefle, 2001; FAO/WHO, 2001). Additionally, despite decades of widespread use of Bt insecticides on food crops, there have been no reports of oral allergies to these preparations, and the US EPA has stated that laboratory

[8] The International Food Biotechnology Council (IFBC), the Allergy and Immunology Institute of the ILSI, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO)

animal studies submitted to the Agency have not indicated any potential for allergic reactions to Bt or its components (US EPA, 2001a).

D.3. Modified Cry3A Does Not Have Amino Acid Sequence Homology to Known Allergens

As described above (Chapter 8, Part C.3.), an extensive bioinformatics search determined that the mCry3A protein shows no significant amino acid homology to known or putative allergenic protein sequences.

D.4. Modified Cry3A is Unstable to Heat and Food Processing

Many food allergens are stable to heat and processing. However, the mCry3A protein has been demonstrated to lose all bioactivity upon heating at 95°C (see Chapter 8, Part C.6., above), and is substantially degraded or eliminated by standard corn processing methods that result in protein degradation, *e.g.* wet milling and the alkaline process used in making corn chips (see Chapter 8, Part B.4., above).

Disulfide bonds between cysteine residues can stabilize some proteins and appear to contribute to their allergenicity, as evidenced by the mitigating effect of the reducing agent thioredoxin on wheat and milk allergenicity (Buchanan *et al.,* 1997; del Val *et al*., 1999). The three-dimensional structure of native Cry3A protein does not include disulfide bonds (Li *et al.,* 1991), therefore it is highly unlikely that the mCry3A protein is stabilized by disulfide bonds. The amino acid sequence of the mCry3A protein contains only three cysteine residues, the same number as the native Cry3A protein. The very low likelihood of disulfide bonds within the mCry3A molecule, in addition to its lability to heat and processing, suggests that the mCry3A protein does not have features that contribute to high stability and, presumably, higher allergenic potential.

D.5. Modified Cry3A is Susceptible to Gastric Digestion

As previously described above (Chapter 8, Part C.5.) the mCry3A protein is readily degraded by pepsin under simulated gastric conditions. No intact mCry3A or immunoreactive fragments were detected following digestion in SGF for 2 minutes.

D.6. Modified Cry3A is Not Glycosylated

As previously described above (Chapter 8, Part C.1.b.), analysis of the mCry3A protein revealed no evidence of post-translational glycosylation.

E. MAMMALIAN SAFETY ASSESSMENT OF PMI MARKER PROTEIN

E.1. Permanent Exemption from Tolerances for PMI in All Crops

A permanent exemption from tolerances for the PMI protein as an inert ingredient in all plants has been granted by the U.S. EPA (U.S. EPA, 2004a) and details of the

mammalian safety assessment for PMI can be found elsewhere (Vlachos and Joseph, 2003).

E.2. Identification of Changes in PMI Protein as Expressed in Event MIR604

The maize transformant that gave rise to the MIR604 transgenic line was transformed using *Agrobacterium* and the plasmid pZM26, which contains the *E. coli*-derived *manA* gene that encodes PMI. As described in Chapter 3. **Molecular Analysis of Event MIR604**, post-transformation analysis of the DNA sequence encoding PMI in Event MIR604 plants determined that two unintended amino acid changes had occurred in the PMI protein as expressed in this event. Valine-61 has been substituted by alanine, and glutamine-210 has been substituted by histidine. To establish whether PMI from Event MIR604 plants was substantially equivalent to PMI as contained in test substance PMI-0198 (*E. coli*-produced PMI), several biochemical and functional parameters were evaluated; PMI proteins from both sources were compared by their apparent size, immunoreactivity with the same anti-PMI antibody, enzymatic activity and the presence or absence of glycosylation (Hill, 2004).

Western blot analysis determined that as expected, PMI extracted from Event MIR604 maize leaf tissue is of similar size and immunoreactivity to *E. coli*-produced PMI. The enzymatic activity of PMI from both sources was comparable, confirming that the two unintended amino acid changes in PMI protein as expressed in Event MIR604 had no functional effect on the PMI protein. Additionally, there was no evidence of post-translational glycosylation of PMI from either source. On the basis of these comparisons, it can be concluded that the PMI proteins from these two sources are substantially equivalent (Hill, 2004).

E.3. PMI as Expressed in Event MIR604 Plants: Evaluation of Amino Acid Homology with Known Toxins

Although the reports of previous toxin homology searches were submitted to the U.S. EPA in support of the safety of PMI (Zawodny, 2003e) and PMI as expressed in MIR604 plants (Zawodny, 2003c), due to the continuing deposition of new protein sequence entries into the databases upon which these analyses rely, a new updated report of the toxin homology search is provided with this petition.

To determine whether the PMI protein as expressed in Event MIR604 corn ("MIR604 PMI") has any significant amino acid homology with protein sequences identified as toxins, its amino acid sequence was systematically compared to the latest posting of the National Center for Biotechnology Information (NCBI) protein database (NCBI, 2004) containing all publicly available protein sequences (Hart and Rabe, 2004c). The procedure used allowed a determination of (1) whether any proteins in the database showed significant homology to the MIR604 PMI protein, indicating they may be closely related to MIR604 PMI, and (2) whether any sequences with significant homology to MIR604 PMI were known to be toxins. The MIR604 PMI query sequence showed no significant amino acid homology to any proteins identified as, or known to be, toxins.

E.4. PMI as Expressed in Event MIR604 Plants: Evaluation of Amino Acid Homology with Known or Putative Allergens

Although the reports of previous allergen homology searches were submitted to U.S. EPA in support of the safety of PMI (Zawodny, 2003f) and PMI as expressed in MIR604 plants (Zawodny, 2003d), due to the subsequent deposition of new protein sequence entries into the allergen database upon which these analyses rely, a new updated report of the allergen homology search is provided with this petition.

Two different similarity searches were performed comparing the MIR604 PMI protein to the entries in the Syngenta Biotechnology Incorporated (SBI) Allergen Database (Hart and Rabe, 2004d). This database was compiled from entries identified as allergens or putative allergens in public protein databases, and was supplemented with additional amino acid sequences identified from the scientific literature. The SBI Allergen Database is updated annually.

First, overall homology was examined by comparing sequential 80-amino acid peptides of the MIR604 PMI protein sequence to the allergen sequences using the FASTA search algorithm. Each successive 'window' of 80 amino acids was offset from the previous window by one residue, such that each peptide overlapped the previous peptide by 79 amino acids. Any 80-amino acid peptide of the query sequence having greater than 35% amino acid identity to an allergen sequence was defined as having significant homology to the allergen sequence. Second, the MIR604 PMI protein sequence was screened for matches of eight or more contiguous amino acids using a program that compared every possible peptide of eight contiguous amino acids between the MIR604 PMI sequence and the allergen sequences. The purpose of this analysis was to screen for short, local regions of amino acid identity that might indicate the presence of common IgE-binding epitopes.

The results of these analyses revealed that there was no significant similarity between any of the sequential MIR604 PMI 80-amino acid peptides and any entries in the SBI Allergen Database. As indicated above, the SBI Allergen Database is updated annually and subsequent to these searches, results from a recent allergen homology search (Hart and Rabe, 2004d) detected one region of sequence homology of eight contiguous identical amino acids between MIR604 PMI and a recently described allergen, α-parvalbumin (110 amino acids; Accession Number CAC83047) from *Rana species* (frog) CH2001. This allergen was identified and reported by Hilger *et al.,* 2002. The specific amino acid sequence in common was "DLSDKETT", which occurs at positions 327 – 334 of MIR604 PMI and positions 77 – 84 of the allergen sequence (Hart and Rabe, 2004d). It is of note that the two amino acid substitutions, described in Section E.2. above, that were identified in the Event MIR604-derived PMI protein do not occur within the above identified common sequence and thus are not fundamentally related to the results of the sequence homology search.

Hilger *et al.* (2002) described a severe case of food-induced anaphylaxis in a single individual who consumed frog legs of Indonesian origin. Using the patient's serum, Hilger *et al*. proceeded to identify the causative agent of this anaphylactic response as α-parvalbumin from an unidentified frog (Rana) described as *Rana species* CH2001. The

response of this patient appeared to be quite specific to the frog leg sample of Indonesian origin, in that the patient's serum showed no cross-reactivity to related parvalbumins from *Rana esculenta*, the common edible frog. To determine if IgE antibodies present in this patient's serum recognized PMI, Syngenta sent two protein samples to the Hilger group for analysis of cross-reactivity. The first protein, designated "POI1" (Protein Of Interest 1), was a PMI test substance, PMI-0198, produced from an *E. coli* over-expression system and used in previous safety testing as a surrogate for PMI as expressed in transgenic plants. As discussed above, the two amino acid changes that occurred in Event MIR604 PMI do not lay within the common sequence indicated above and thus the PMI test substance PMI-0198, which does not contain these substitutions, is appropriate for these cross-reactivity studies. As an internal check, an unrelated protein designated "POI2" (bovine serum albumin) and a positive control extract from *Rana species* CH2001 were also included in the experiments performed in Dr. Hilger's laboratory for cross-reactivity analysis.

The results of the serum screening analysis demonstrated no cross-reactivity between the human serum IgE and PMI (POI1). Negative control serum screening analysis demonstrated no cross-reactivity with BSA (POI2). The positive control extract from *Rana species* CH2001 demonstrated cross-reactivity as expected. This indicates that the allergic patient's serum IgE does not recognize any portion of the PMI protein as an allergenic epitope. Therefore, the observed low degree of sequence identity between MIR604 PMI and α-parvalbumin from *Rana species* CH2001 is not biologically relevant.

F. NON-HUMAN EXPOSURE AND SAFETY CONSIDERATIONS

Field corn (maize) is readily consumed by livestock in the United States, with roughly 80% of the crop fed to livestock. Livestock that feed on maize include cattle, pigs, poultry, sheep and goats. The safety of Event MIR604 maize as livestock feed is supported by its compositional and nutritional equivalence to non-transformed control and commercially available maize lines, the absence of any unintended or toxic effects associated with Event MIR604 maize grain when incorporated into the diets and fed to broiler chickens, the rapid digestibility in mammalian gastric fluid and lack of toxicity of the introduced mCry3A insecticidal protein.

F.1. Prior Livestock Exposure to Modified Cry3A Protein

Prior dietary exposure of livestock to mCry3A via feed has not occurred. However, it is conceivable that a low level of exposure to the native Cry3A protein has occurred, as it is registered for use in the U.S. in NewLeaf Bt potatoes and as a component of encapsulated *Bacillus thuringiensis*-based microbial insecticides (see Table 17), and is permanently exempt from food and feed tolerances (US EPA 2004a). Livestock consuming feed containing Event MIR604 maize may receive low exposure to the mCry3A protein, as quantifiable levels of mCry3A were detected in all Event MIR604-derived plant tissues except pollen (see part B.3. above). However, the rapid degradation of mCry3A observed under *in vitro* conditions simulating the gastric environment of mammals (see section C.5. above) indicates that any protein consumed by livestock will be rapidly digested and metabolised as conventional dietary protein.

F.2. Livestock Exposure to Nucleic Acids

All whole foods and feedstuffs contain DNA and humans and animals, including livestock, routinely consume significant quantities of DNA from a variety of sources. The nucleic acids (DNA and the RNA encoded by it) present in Event MIR604-derived corn plants as a result of transformation will not present a dietary safety concern. Based on the ubiquitous occurrence and known safety of nucleic acids in the food supply, the World Health Organization (WHO; FAO/WHO, 1991), and the US FDA (US FDA, 1992) have stated that the consumption of DNA from all sources, including genetically modified crops, poses no safety issue. In addition, a tolerance exemption under the Federal Food Drug and Cosmetic Act regulations has been established for residues of nucleic acids that are part of plant-incorporated protectants or associated inert ingredients (US EPA, 2001b).

F.3. Nutritional Data

The nutritional composition of MIR604-derived maize plants and isogenic non-transgenic control plants was assessed (See Chapter 5, **Compositional Analysis of Event MIR604 Corn** for details and data). Key nutritional components in maize grain and whole plants (forage) derived from Event MIR604 and near isogenic non-transgenic control plants were compared (Kramer, 2004). The whole plants and grain analyzed were from hybrid pairs grown at 12 locations in the USA over two growing seasons (2002 and 2003). As would be expected from an analysis of this size, sporadic statistically significant differences were observed for some parameters between the MIR604 transgenic and near isogenic controls. All components evaluated in this study were within the range of reported literature values for maize with the exception of potassium in forage and phytosterols in grain.

At the time the forage potassium data were generated, Syngenta was unable to identify and provide a control range for concentrations of potassium in conventional corn forage. Syngenta subsequently conducted a study to measure potassium concentrations in non-transgenic forage using the same methodology that was employed in the original analysis of the MIR604 forage (de Fontes and Kramer, 2006). In addition, further literature investigations revealed historical data that was previously overlooked. Forage potassium concentrations for MIR604 and its controls were compared and found to be within both the newly measured and literature ranges.

Average phytosterol levels in both control and transgenic grain samples were below the average concentration reported in the literature. Syngenta subsequently conducted a study to measure campesterol and stigmasterol concentrations in non-transgenic grain using the same methodology that was employed in the original analysis of the MIR604 grain (de Fontes and Kramer, 2005). Grain campesterol and stigmasterol concentrations for MIR604 and its controls were compared and found to be within the newly measured ranges.

For all other analytes, no consistent patterns emerged to suggest that biologically significant changes in composition or nutritive value of the grain or forage had occurred as an unintended result of the transformation process or expression of the transgene. The

conclusion based on these data is that there is strong evidence that the genetically modified MIR604 hybrids are substantially equivalent in composition to the isogenic controls, and other commercial hybrids.

The nutritional quality of Event MIR604 maize grain was assessed in a 49-day feeding trial in broiler chickens (Brake, 2004). Broiler chickens are highly sensitive to small nutrient changes within their diets because of their extremely rapid growth. Male and female broiler chickens were fed diets containing Event MIR604 maize grain or non-transformed control or commercially available ("NC 2003") maize lines. The diets were formulated based on the individual nutrient analyses for each of the grains to meet standard nutritional recommendations for poultry.

The transgenic MIR604 hybrid diets supported rapid broiler chicken growth at low mortality rates and excellent feed conversion ratios without significant impact on overall carcass yield or quality. The absence of biologically relevant differences in growth and feed conversion parameters confirms the nutritional equivalence of Event MIR604 corn to its isogenic control, the absence of any unintended effects and the absence of toxicity and supports the conclusion that consumption of MIR604 maize in animal feed does not pose a safety concern.

F.4. Potential Toxicity of Introduced Protein

As described above, the lack of observed toxicity in rodents acutely exposed to high oral doses of the mCry3A protein indicates that any residues of mCry3A in MIR604 used in animal feeds will not pose a safety concern (section C.4.). In addition, as described in Section C.2. above, the potential toxicity of the mCry3A and PMI proteins was also assessed by comparing the amino acid sequences against the latest posting of the National Center for Biotechnology Information (NCBI) Entrez Protein Database (NCBI, 2004) containing all publicly available protein sequences identified as toxins. The mCry3A query sequence showed no significant amino acid homology to any non-delta endotoxin proteins identified as, or known to be, toxins. The absence of toxicity of the protein introduced into Event MIR604 maize supports the safety of Event MIR604 maize as livestock feed.

G. CONCLUSIONS

The large body of data and information described herein support the conclusion that the modified Cry3A protein as expressed in Event MIR604 corn will pose no hazard to humans or domestic animals upon commercial approval of the use of Event MIR604 corn. Additionally, the PMI protein has been granted a permanent exemption from tolerance in all crops from the U.S. EPA based on an extensive body of data that demonstrate this selectable marker presents no risk to humans, animals or the environment.

Chapter 9. Environmental Consequences of Introduction

A. INTRODUCTION

As described in Chapters 1 and 6, mCry3A has demonstrated very specific, targeted and enhanced pesticidal activity toward certain maize coleopteran pest species: northern, western and Mexican corn rootworm. Corn plants transformed with the modified *cry3A* gene display resistance to these pests (Chen and Stacy, 2003). Expression of mCry3A in Event MIR604-derived hybrids has been shown to occur in root and other tissues, and during the course of normal agricultural practice, the active principle mCry3A could theoretically enter neighboring environments through establishment of weedy populations of MIR604 plants outside fields, through transfer and expression of the mCry3A gene in other organisms via sexual hybridization or horizontal gene transfer, through off-crop movement of MIR604 pollen or via degradation of plant tissue and subsequent movement of mCry3A through soil. Below are arguments and reviews of studies that indicate that introduction of Event MIR604-derived hybrids will lead to minimal exposure of the environment to mCry3A. In addition, when coupled with the data described in Chapter 7 (**Environmental Safety**) demonstrating no detectable hazard of mCry3A to non-target organisms that might feed on MIR604 growing in cornfields, it is clear that Event MIR604-derived hybrids present very little environmental risk and may provide substantial benefits over current traditional corn rootworm control practices.

B. EXPRESSION LEVELS OF MCRY3A IN HYBRIDS DERIVED FROM MIR604

Details of the study that determined expression levels of mCry3A in MIR604 tissues are provided in the Chapter 6. **Quantification of mCry3A and PMI Proteins in Event MIR604.** A summary of the study report (Joseph and Hill, 2003) is provided here.

Corn tissues derived from three genotypes were sampled at four growth stages: whorl stage, 6 weeks after planting; anthesis, 10-11 weeks after planting; seed maturity, 18-20 weeks after planting; and senescence, about 24 weeks after planting. The concentrations of mCry3A in leaves, roots, kernels, silks and pollen were determined by ELISA. Measurements of mCry3A concentrations in whole plants and in silage were also made.

With one exception, all tissues at all sampling times contained detectable amounts of mCry3A. The only tissue in which mCry3A was not detected was pollen (limit of detection = 0.07 µg/g fresh weight). The finding of effective absence of mCry3A in pollen from MIR604 hybrids was supported by the lack of detectable mCry3A protein in pollen from the inbred line; all pollen grains are expected to contain the *mcry3A* gene in the MIR604 inbred, whereas only 50% of pollen grains of MIR604 hybrids are expected to contain the gene.

The stability of expression profiles over multiple generations was investigated in four successive backcross generations under greenhouse conditions. Leaf tissue was sampled at anthesis and the amount of mCry3A was measured using ELISA. The concentrations of mCry3A in all generations were similar and no trend towards increased or decreased

expression was observed. An independently validated detection method for mCry3A as expressed in Event MIR604 hybrids has been developed and submitted to the US EPA (Steiner and Larkin, 2004).

C. DEGRADATION OF mCRY3A IN SOIL

C.1. Background

The EPA has recently reviewed data concerning the degradation in the soil of various Cry proteins registered as plant-incorporated protectants (US EPA, 2001a). Laboratory soil degradation studies on the lepidopteran-active proteins Cry1Ab, Cry1Ac or Cry1F in field-collected soils, as either pure protein or in plant material, indicate that these proteins are degraded rapidly. The period for the Cry protein concentration or bioactivity to fall to half its initial value (the DT_{50}) is typically between 2 and 22 days for Cry1A proteins. The DT_{50} for Cry1F is just over 3 days (US EPA, 2001a).

Subsequent to the EPA review, a further plant-incorporated protectant, Cry3Bb1, which is active against certain Coleoptera, has been registered (US EPA, 2003). The DT_{50} of this protein was determined in the laboratory in a range of soil types and found to be between less than 1 day and up to 9 days.

The weight of evidence is that Cry proteins, like virtually all proteins, are inherently degradable in live soils *via* enzymatic degradation by ubiquitous soil proteases. Therefore the expectation is that mCry3A is also inherently degradable in live soil; a laboratory study was carried out to test this hypothesis.

C.2. Laboratory study of the degradation of mCry3A in soil

To test the hypothesis that mCry3A will degrade rapidly in live soil, mCry3A was incorporated into a sample of field-collected soil and its degradation was measured by bioassay against larvae of the Colorado potato beetle (CPB), which are known to be sensitive to mCry3A (Kramer and Joseph, 2004).

Soil was obtained from a farm in Grundy County, Iowa, USA in October 2003. Agvise Laboratories (Northwood, North Dakota, USA) identified the soil as silty clay loam with a moisture holding capacity at 1/3 bar of 27.7%. The soil was sieved and acclimated at 25ºC ± 1ºC under constant moisture levels for 10 days prior to the start of the test. Biomass determinations indicated that microbial activity was maintained during the period of the study and that the soil was suitable for use in this study.

The source of mCry3A for the study was the test substance MCRY3A-0102, a 90.3% pure sample of microbially expressed mCry3A. The preparation and characterization of MCRY3A-0102 have been described (Joseph and Graser, 2003b). In a separate study, test substance MCRY3A-0102 has been shown to be a suitable surrogate for mCry3A protein produced in transgenic corn Event MIR604, as assessed by various biochemical and functional parameters (Joseph and Graser, 2003a).

Soil was weighed and transferred to flasks such that 50 g dry weight equivalent of soil at 18.25% moisture was added to each flask. Soil samples were treated with 5 ml of a solution of MCRY3A-0102 to give a concentration of 230 µg mCry3A/g dry weight soil; this concentration is about 18X the highest concentration of mCry3A in whole plants of MIR604. This is a considerably higher concentration of mCry3A than would result from incorporation of residues of MIR604 hybrids in the field as the residues will be diluted in a large volume of soil (US EPA, 2001a).

Treated soil samples were capped, shaken and then weighed to determine actual moisture levels in the sample flasks. Moisture levels were adjusted and maintained throughout incubation at 75% ± 12% of field moisture capacity (FMC) at 1/3 bar, to simulate average field conditions. The samples were kept in a constant temperature room at 25°C ± 1 ^{o}C. Duplicate samples were removed from the constant temperature room at 1, 3, 7, 12 and 30 days after treatment and, along with two samples taken immediately after treatment, were frozen and stored at –20°C before analysis.

The DT_{50} for the bioactivity of mCry3A in the treated soil was measured using a bioassay. For each incubation period, a sample of treated soil was incorporated into a standard CPB diet at 10% w/w. Triplicate aliquots of each duplicate soil sample were taken to prepare 6 treatment diets; 2 bioassay plates, comprising 10 first instar CPB each, were prepared from each diet, giving 12 bioassay plates for each incubation period. Bioassay plates were kept at room temperature and mortality of the CPB larvae was measured after 72 hours.

For soil samples collected up to and including day 7 post soil treatment, mortality in the bioassays of mCry3A-treated soil was high (48 - 54%; 13-27% std. dev.). A positive control, consisting of CPB diet treated with MCRY3A-0102 to a concentration of 23 µg mCry3A/g diet (*i.e.* the nominal concentration of mCry3A in the treated soil diet), gave 53% (12% std. dev.) mortality. Mortality in the 12- and 30-day incubation treatments was 11% (13% std. dev.) and 9% (9% std. dev.), respectively. Mortality was 18% (15% std. dev.) in a water-treated soil control.

The reductions in mortality of CPB in the bioassay were modeled using simple first-order kinetics, and indicated that the DT_{50} of mCry3A in soil was 7.6 days. The results of this study confirm that mCry3A will be rapidly degraded in soil.

D. PERSISTENCE AND SPREAD OF THE *mcry3a* GENE

A full assessment of the environmental fate of mCry3A requires not only consideration of the expression and degradation of the protein in fields during and immediately after cultivation of MIR604 corn, but also the theoretical possibility that mCry3A could persist or spread more widely because of gene flow or the establishment of weedy populations of MIR604 (Raybould, 2004a). Additional details can be found in Chapter 2. **The Corn (Maize) Family**.

D.1. Gene Flow

Corn (*Zea mays* ssp. *mays*) will hybridize with a group of taxa collectively called teosinte. Several types of teosinte are classified as subspecies of *Zea mays*, whereas others are regarded as separate species of *Zea*. Teosinte species are natives of Central America and have co-existed with cultivated corn for several thousand years. They have remained genetically distinct from cultivated varieties despite occasional introgression (US EPA, 2001a). Teosinte species are not natives of the USA, but isolated populations have been recorded in Florida and Texas, the former a possible remnant of the use of annual teosinte as a forage grass. These populations are apparently now extinct in both states (US EPA, 2001a). Teosinte species are grown in botanical gardens, but fertilization of these plants with pollen from MIR604 is extremely unlikely.

Species of the genus *Tripsacum* are considered close relatives of corn. There are sixteen species of *Tripsacum* worldwide, of which three occur in the USA: *T. dactyloides*, a widespread forage grass; *T. floridanum*, known from southern Florida; and *T. lanceolatum*, which is present in Arizona and possibly New Mexico (US EPA, 2001a).

Corn breeders view *Tripsacum* as a potential source of useful genes for traits including apomixis, pest and disease resistance and drought tolerance (OECD, 2003) and therefore substantial effort has been made to obtain and characterize corn X *Tripsacum* hybrids. Hybrids between corn and *Tripsacum* species are difficult to obtain outside the laboratory or greenhouse and are often sterile. Only one record exists of an open-pollinated hybrid between *Zea* and *Tripsacum*, which involved species native to Guatemala. After consultation with experts on improvement of forage grasses, US EPA (2001a) concluded that the chance of natural introgression of genes from corn to *Tripsacum* was 'extremely remote' and that no other species in the continental USA would interbreed with commercial corn.

The data reviewed above indicate the very low probability of transfer of the *mcry3A* gene from MIR604 to wild relatives of corn. Species of *Zea* other than corn are not recorded outside botanical gardens in the USA. *Tripsacum dactyloides* is widespread, but does not hybridize readily with corn, and the probability of backcross or F_2 progeny of *Tripsacum* X *Zea* hybrids being produced in the field is negligible. Therefore, mCry3A protein is unlikely to spread from cultivated MIR604 corn and persist in the environment as the result of gene flow.

D.2. Potential for Horizontal Gene Transfer

In its recent reassessment of the environmental safety of Bt plant-incorporated protectants, EPA conducted an extensive review of information relevant to the theoretical risks of horizontal gene transfer (HGT). Studies reviewed by EPA showed no evidence for HGT under field conditions, and only equivocal evidence for HGT under laboratory conditions designed to maximize the recovery of transformants (US EPA, 2001a). Conner *et al*. (2003) also reviewed the literature and found very few examples where HGT had been demonstrated convincingly, and these cases relied on artificially high sequence homology between the transgene and the potential recipient (*e.g*., de Vries *et al*., 2001). Conner *et al*. (2003) also reported that new data comparing full genomic

sequences of various prokaryotes and eukaryotes have identified putative HGT events, however alternative interpretations of these data are possible (see references in Conner *et al*., 2003). There is no reason to suppose that corn derived from Event MIR604 is likely to transfer genes by HGT at a higher rate than any other plant, and therefore the likelihood of exposure to mCry3A through microorganisms expressing the *mcry3A* gene from MIR604 is minimal.

EPA also concluded that there were no significant hazards should a *cry* gene be transferred from a transgenic plant to a microorganism. *Bacillus thuringiensis* is common in soil and *cry* genes have been available for HGT to other species for long periods and no harmful events appear to have resulted from this prolonged exposure.

Soil microorganisms have not previously been exposed to the *mcry3A* gene contained in MIR604. While native and modified Cry3A proteins have a high degree of sequence homology, the DNA sequence of *mcry3A* was altered substantially from native *cry3A* to optimize codon use for expression in plants (Rabe, 2004). The change to the plant-preferred pattern of codon usage means that the *mcry3A* gene likely has lower homology to potential recombination sites in soil microorganisms than has the native *cry3A* gene.

Should *mcry3A* be integrated into a plasmid or chromosome of a bacterium, mCry3A protein is extremely unlikely to be produced because the maize metallothionein promoter linked to *mcry3A* in MIR604 (Rabe, 2004) is unlikely to function in bacteria and codon use in *mcry3A* is optimized for expression in corn, not bacteria (*e.g.,* Baneyx, 1999; Chen and Stacy, 2003). Laboratory testing of the effects of mCry3A protein on a variety of non-target organisms revealed no adverse effects (Chapter 7. **Environmental Safety**). Therefore in the extremely unlikely event that *mcry3A* is stably integrated and expressed in a soil microorganism, no harmful effects are expected.

D.3. Weediness

Corn has lost the ability to survive outside cultivation (OECD, 2003). It can over-winter and germinate in a subsequent crop as a volunteer weed. For example, corn is a common volunteer in soybeans. However, several features of corn make it unlikely to form self-sustaining weedy populations in agriculture: it is easily controlled in subsequent crops with selective herbicides; seed dispersal is limited because seeds are held inside the husks of the cob; and the seeds lack dormancy so that young plants are exposed to harsh winter conditions. Corn does not persist in habitats outside agriculture because, in addition to the features listed above, it requires disturbed ground to germinate and it is very uncompetitive against perennial vegetation.

Volunteers of MIR604 in following crops can be controlled in the same way as volunteers of any other corn variety; enhanced resistance to corn rootworm is highly unlikely to increase the frequency of volunteers. Furthermore, enhanced resistance to corn rootworm is unlikely to increase the invasiveness of corn in uncultivated land because ease of weed control, lack of dormancy and low competitiveness are the main reasons why corn is not a persistent weed. The lack of invasiveness of corn, particularly in non-agricultural habitats, means that mCry3A will not spread from sites of cultivation and persist in the environment as weedy populations of MIR604.

E. ENVIRONMENTAL FATE AND EXPOSURE OF mCRY3A

The data reviewed above indicate that exposure to mCry3A protein will be limited to the fields in which MIR604 corn will be grown; mCry3A is unlikely to persist in fields for a long period after grain from MIR604 has been harvested.

E.1. Exposure During Cultivation

No mCry3A protein was detected in pollen of MIR604 hybrids or inbreds and therefore ecologically significant exposure to mCry3A outside cultivation is unlikely to occur through contact with pollen. Furthermore, the rapid degradation of mCry3A in soil means that the protein is very unlikely to spread from cultivation into surface or ground water. These conclusions are particularly pertinent to the potential exposure of aquatic organisms to mCry3A (Section E.3 below). In short, exposure to mCry3A during cultivation will be limited to direct contact with living, senescent or dead MIR604 tissues in cornfields and possible short-term exposure to exuded proteins in soil.

E.2. Exposure Following Cultivation

Persistence of mCry3A protein following planting of MIR604 hybrids is likely to be for a limited period and be confined to sites of cultivation. After harvest, some protein will remain in MIR604 plant material (for example, roots and vegetative material plowed into the soil) until the plant tissues degrade. mCry3A protein is expected to degrade rapidly and not move off site. Control of volunteers will minimize the potential for prolonged exposure to mCry3A following cultivation of MIR604. Expression of mCry3A in soil microorganisms that have been transformed with the *mcry3A* gene by HGT is highly improbable and is also very unlikely to be hazardous.

Corn is not a weedy species and in the unlikely event that any feral plants of MIR604 corn result from accidental seed spillage, they are not expected to survive to flowering outside cultivation. There are no sexually compatible wild relatives of corn in the USA and therefore mCry3A protein will not spread and persist through introgression of the *mcry3A* gene into wild plants.

E.3. Organisms Likely to be Exposed to mCry3A

Aquatic organisms are unlikely to be exposed because of the low probability that mCry3A will enter watercourses through movement of soil particles, pollen dispersal or seed spillage (Vlachos, 2004); corn fields may become flooded during heavy rain, though degradation and dilution of mCry3A, and uncertainty about the immigration of aquatic organisms into flooded fields makes quantification difficult. Therefore other than humans and animals that eat corn grain, or animals that eat corn forage or silage, the only organisms likely to be exposed to mCry3A as a result of cultivation of MIR604 hybrids are pests and non-target organisms that feed on corn tissue, predators and parasitoids of these animals, and soil organisms. Environmental exposure will be limited spatially to areas where MIR604 is grown. Temporal exposure will be limited to the period of cultivation for non-target organisms feeding on the above ground parts of corn. Soil organisms may be exposed to mCry3A during the cultivation and *via* plant material

incorporated into the soil after harvest. The rapid degradation of mCry3A in the soil indicates that the duration of potential post-harvest exposure of soil organisms to significant amounts of mCry3A protein will be limited to a few weeks. A second period of exposure to all non-target organisms will occur if MIR604 volunteers arise, although volunteers should result in much lower exposure than planting of the crop. The precise routes of exposure and the safety of mCry3A to these non-target organisms are assessed in Chapter 7 (**Environmental Safety**).

F. CURRENT CORN ROOTWORM CONTROL PRACTICES

Insect pest management practices employed to reduce rootworm damage generally fall into two categories: (1) crop rotation and (2) application of chemical insecticides. As the preferred diet of rootworm larvae is corn roots and corn rootworm beetles lay their eggs in a diapause condition, farmers have adopted a crop rotation strategy that can significantly interrupt the corn rootworm life cycle. By following a first year corn crop with soybeans or other non-corn crop in the second season's planting, the population of emerging beetles is reduced as the primary food source (corn roots) for the hatching larvae is not available. With the pest population reduced, corn can then be planted again in the third season with little risk of economic impact. This crop rotation (primarily corn and soybeans) has been used since the mid-70s as an effective non-chemical control strategy for corn rootworm.

In areas where crop rotation has been used extensively, rootworm variants have evolved which have minimized the effectiveness of the crop rotation strategy. The first adaptation is referred to as the 'extended diapause' variant and was first documented in northwestern Iowa. In this instance, eggs laid by Northern corn rootworm (CRW) beetles remain in diapause two or more winters before hatching. In 2002, USDA-NASS (USDA, 2002) estimated that this extended diapause variant is present in 9.8 million acres of corn in the U.S.

The second adaptation, referred to as the 'soybean variant', was first documented in east central Illinois. In this case the Western CRW adult beetle deposit its eggs in neighboring soybean fields, which hatch in the following year's corn crop. According to USDA-NASS estimates, in 2002, 7.1 million acres of corn were planted in the heart of the soybean variant infestation zone that covers large portions of Illinois and Indiana. According to Payne *et al*. (2003) this new variant of CRW has also spread through most of northern Indiana, eastern Illinois, southern Michigan and western Ohio. Given historic movement patterns, the new variants may soon spread as far west as eastern Iowa (Onstad *et al*., 1999). This expanded area is equivalent to 16 million acres with the westward expansion being the greatest threat. Western CRW have also been detected in soybean fields located in counties extending to the Wisconsin border. As these new CRW variants spread throughout the U.S. corn belt, the effectiveness of this non-chemical control strategy will diminish.

In 2003, over 16 million acre treatments occurred using 6 organophosphates (OPs), 1 carbamate, 2 pyrethroids, and fipronil comprising the top ten chemical pesticides used to prevent corn rootworm damage (Steiner *et al*., 2004). Including all chemical alternatives

used in 2003, over 21 million acre treatments occurred (Table 22). On a percentage basis, OPs accounted for 72% of the pounds of active ingredient applied in 2003 (Table 23); OPs and OP-pyrethroid combinations accounted for 63% of the acre treatments. Pyrethroids accounted for only 12% of the pounds but 32% of the acre treatments for corn rootworm.

Table 22. Corn Rootworm Pesticides by Class

Chemical Class	Pounds of Active Ingredient	Acre Treatments	Grower Costs (US dollars)
OP	4,306,445	4,601,773	50,611,707
Pyrethroid	728,276	6,948,614	93,171,730
OP-Pyrethroid	554,693	8,864,582	55,919,425
Carbamate	216,175	266,934	3,745,043
Other	150,575	870,264	12,659,437
TOTALS	5,956,164	21,552,167	216,107,342

Table 23. Corn Rootworm Pesticides Percent of Use by Class

Chemical Class	Percent of Use Based on Pounds of AI	Percent of Use Based on Acre Treatments
OP	72	22
Pyrethroid	12	32
OP-Pyrethroid	9	41
Carbamate	4	1
Other	3	4
TOTALS	100	100

A number of products that comprise these classes of chemical pesticides have been classified for restricted use by the U.S. EPA (US EPA 2004d). EPA pointed out that phorate and terbufos (OPs) had been reviewed under the Agency's special review process because use of those pesticides may result in unreasonable adverse effects. In addition, EPA referenced its work on the avian risks presented by granular pesticides used on corn. Specifically, the Agency noted that phorate, terbufos, and chlorpyrifos are still used to combat corn rootworm. Also, EPA reported that, while carbofuran (a carbamate) was no longer used as a granular product, the non-granular formulation was still of concern. Finally, EPA noted that many of the corn rootworm pesticides were restricted use compounds because of human health and environmental concerns. These restrictions include extensive personal protective equipment that must be used by workers in order to use the pesticides, as well as significant personnel training to ensure compliance. Regardless of whether the restricted use pesticides are used in or out of compliance, many raise ecological concerns. Additionally, non-selective insecticides are expected to be toxic to many non-target arthropods, including beneficial arthropods, occurring in or near the treated fields.

Due to the wide spread use of chemical insecticides to control corn root worm infestations in the U.S. corn belt, both larval and adult CRW resistance has been reported (Scharf, *et al*., 2001; Zhou, *et al*., 2003). Control failures linked to corn rootworm resistance to carbaryl (Zhu, et al., 2001), methyl parathion (an organophosphate; Meinke, et al., 1998) and fipronil (Scharf, *et al*., 2000) have been reported. Continued extensive use of these and other chemical insecticides and spread of resistant phenotypes will most likely result in an increase of control failures and increase the demand for alternative methods of corn rootworm control.

G. BENEFITS OF INTRODUCTION OF EVENT MIR604-DERIVED HYBRIDS

Introduction of Event MIR604-derived hybrids could reduce many of the environmental risks associated with extensive use of conventional pesticides. mCry3A protein in Event MIR604 corn presents little if any risk to man and other non-target organisms. In contrast, the major chemical alternatives present numerous risk concerns. In addition, a potential 4.5 million acre treatments and about 1.25 million pounds of active ingredients could be eliminated during the first five years of sales of MIR604 corn (Steiner *et al*., 2004). From a comparative risk perspective, MIR604 corn presents a significant reduced risk profile compared to the chemical alternatives. Unlike chemical treatments, the plant-based delivery of active ingredient afforded by MIR604 hybrids offers consistent performance under varying environmental conditions. Unpredictable environmental factors can influence the efficacy of chemical treatments. In addition, with MIR604 corn, there will be reduced potential for crop injury resulting from herbicide/insecticide interactions and provide a viable alternative to crop rotation which has become less effective because of variant CRW strains. Finally, transgenic plant technology is easier to use than chemicals that require equipment calibration and application decisions concerning timing and whether to use the chemicals.

In addition to being safer to use, MIR604 hybrids deliver at a minimum comparable efficacy and yield performance compared to the chemical alternatives (see Chapter 4, **Agronomic Performance of Event MIR604**). Syngenta's efficacy data show that MIR604 corn performs significantly better than non-treated and chemically treated controls. Under environmental conditions of drought or heavy CRW pressure that can result in significantly reduced yield, the use of MIR604 can result in a positive yield impact. Under such conditions, grower monetary benefits from increased yield will be maximized. In less severe conditions, the benefits will be reduced.

While some benefits may be difficult to quantify and while environmental factors will influence efficacy and yield of any corn hybrid, the use of MIR604 corn will provide positive benefits for growers. Finally, as discussed above, the use of MIR604 corn provides substantial human health and environmental benefits that growers can achieve at the same time they accrue the economic advantages of MIR604 corn. Considering risks and benefits, including economic benefits, the deregulation of MIR604 corn will have significant public benefits, including direct benefits to US agriculture.

H. IRM AND STEWARDSHIP

Because of the clear agronomic, environmental and economic benefits of Event MIR604-derived hybrids outlined in this petition, it is essential that, once this technology is deployed, its continued utility is protected through the implementation of an appropriate and effective insect resistance management (IRM) program. Such a plan is founded on the best available understanding of the action of MIR604, the biology of the target CRW pests, the nature of the potential selection for resistance to mCry3A and a clear appreciation of resistance management options available to mitigate against any resistance development (FIFRA SAP, 1998). Moreover, an IRM plan for MIR604 must be compatible with existing IRM plans that include other CRW control such as YieldGard Rootworm® (Cry3Bb, Monsanto) and other corn pest (Lepidoptera) control (e.g., Bt11; Cry1A(b), Syngenta) technologies. Syngenta has developed an IRM plan based on the above principles and this plan will employ strategies that will include: a minimum structured refuge of non-CRW-control corn, complementary and harmonized IRM practices and an aggressive stewardship program that will maintain the long-term efficacy of MIR604 maize by reducing the potential for pests to develop resistance to the mCry3A protein (McCaffery, 2004).

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APPENDIX 1

EVENT MIR604 HYBRID 2002-2003 AGRONOMIC DATA

APPENDIX 1A

MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRID EFFICACY DATA

APPENDIX 1A (CONT'D)

TABLE 1A

CORN ROOTWORM ROOT DAMAGE RATING 0-3.9 SCALE[1]

0 - 3 Rating	Description of Rootworm Damage
0.01	No damage to 1-2 light surface scars on roots
0.02	3+ light surface scars ≤ 4 heavy scars
0.05	5+ heavy scars (long, deep scars), but **NO** root pruning
0.10	One root pruned to ≤ 2 inches accompanied with heavy scars, typically.
0.25	2+ roots pruned to ≤ 2 inches (up to 1/4 nodes, equivalent, pruned back)
0.50	Equivalent of 0.50 node of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
0.75	Equivalent of 0.75 node of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.00	Equivalent of 1.00 node of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.25	Equivalent of 1.25 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.50	Equivalent of 1.50 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.75	Equivalent of 1.75 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.00	Equivalent of 2.00 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.25	Equivalent of 2.25 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.50	Equivalent of 2.50 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.75	Equivalent of 2.75 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
3.00	Equivalent of 3.00 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
3.90	Total Root Crown Destruction - No roots left

[1]At the VT to R1 stage (approximately 7 weeks following infestation) plants were manually dug from the ground, washed free of soil and rated for rootworm damage using a damage rating derived from the Iowa State 0-3 node-injury rating scale (http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html). According to the upper range of this scale, a rating of 3.00 denotes '*three or more nodes eaten'*, a high degree of root damage, whereas a rating of 0.0 is no damage. In some instances of extreme corn rootworm pressure, total root crown destruction can occur such that no roots are left on the corn plant. Depending on the degree of root crown destruction, this level of root damage is assigned by Syngenta a rating as high as 3.9.

APPENDIX 1A (CONT'D)

TABLE 2A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2,] , 2002

LOCATION, YEAR	ENTRY[4]	MEAN CRW ROOT DAMAGE RATING[5]	# OF REPS
Stanton, MN, 2002 Trial SYN137[3]	MIR604 early hybrid A1	1.092[7]	3
	MIR604 early hybrid A2	1.087	3
	Control early hybrid 1A	2.988	3
	LSD (0.05) [6]	**1.080**	
Bloomington, IL, 2002 Trial SYN177[3]	MIR604 early hybrid A1	0.949	3
	Control early hybrid 1A	3.130	3
	LSD (0.05)	**0.367**	
Bloomington, IL, 2002 Trial SYN178[3]	MIR604 early hybrid A2	0.798[8]	2
	MIR604 early hybrid A1	1.142	3
	Control early hybrid 1A	3.555	3
	LSD (0.05)	**0.318**	

[1]Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm plus artificial infestation with western corn rootworm.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3]Individual corn plants were artificially infested with freshly deposited Western corn rootworm eggs at an estimated calculated density of 1182 eggs per plant for trial SYN137 and 1462 eggs per plant for trials SYN177 and SYN178. Additional details can be found in Chapter 4, Section B.

[4]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid A1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data

[5]CRW root damage rating scale 0-3 (see Table 1A).

[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. [7]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level.

[8]One replication was mistakenly treated with insecticide for this MIR604 hybrid, and was not included in the LSD calculation.

APPENDIX 1A (CONT'D)

TABLE 3A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2], 2003

LOCATION, YEAR	ENTRY[4]	MEAN CRW ROOT DAMAGE RATING[5,11]	# OF REPS
Stanton, MN, 2003 Trial SYN142[8]	MIR604 early hybrid G3	0.11	2
	MIR604 early hybrid G8	0.09	2
	Control early hybrid 1D[6]	2.11	2
	LSD (0.05) [7]	**1.07**	
Willmar, MN, 2003 Trial SYN143[9]	MIR604 early hybrid G3	0.05	4
	MIR604 early hybrid G8	0.05	4
	Control early hybrid 1D	1.41	4
	LSD (0.05)	**0.20**	
Bloomington, IL, 2003 Trial SYN180[3]	MIR604 early hybrid A1	0.10	3
	Control early hybrid 1A	2.54	3
	MIR604 early hybrid G3	0.26	3
	Control early hybrid 1D	2.62	3
	LSD (0.05)	**0.38**	
Bloomington, IL, 2003 Trial SYN181[3]	MIR604 early hybrid G3	0.05	4
	MIR604 early hybrid G5	0.10	4
	Control early hybrid 1D	1.73	4
	LSD (0.05)	**0.22**	
Bloomington, IL, 2003 Trial SYN182[8]	MIR604 late hybrid E1	0.27	4
	MIR604 late hybrid E4	0.29	4
	Control late hybrid 2E	2.46	4
	LSD (0.05)	**0.49**	
Bloomington, IL, 2003 Trial SYN183[8]	MIR604 late hybrid E1	0.20	4
	MIR604 late hybrid E4	0.17	4
	Control late hybrid 2E	1.86	4
	LSD (0.05)	**0.38**	
Champagne, IL, 2003 Trial SYN001[10]	MIR604 late hybrid E5	1.04	4
	MIR604 late hybrid E6	0.87	4
	Control late hybrid 2E	2.37	4
	LSD (0.05)	**0.49**	
York, NE, 2003 Trial SYN002[10]	MIR604 late hybrid E5	0.02	4
	MIR604 late hybrid E6	0.04	4
	Control late hybrid 2E	1.79	4
	LSD (0.05)	**0.38**	

APPENDIX 1A (CONT'D)

TABLE 3A (CONT'D)

Fortescue, MO, 2003 Trial SYN003[10]	MIR604 late hybrid E5	0.32	4
	MIR604 late hybrid E6	0.17	4
	Control late hybrid 2E	2.65	4
	LSD (0.05)	**0.40**	
Monteno, IL, 2003 Trial SYN004[10]	MIR604 late hybrid E5	0.28	4
	MIR604 late hybrid E6	0.24	4
	Control late hybrid 2E	1.51	4
	LSD (0.05)	**0.28**	
Janesville, WI, 2003 Trial SYN005[10]	MIR604 late hybrid E5	1.63	4
	MIR604 late hybrid E6	1.36	4
	Control late hybrid 2E	2.71	4
	LSD (0.05)	**0.63**	
Richland, IA, 2003 Trial SYN006[10]	MIR604 late hybrid E5	0.54	4
	MIR604 late hybrid E6	0.75	4
	Control late hybrid 2E	2.27	4
	LSD (0.05)	**0.33**	
Champagne, IL, 2003 Trial SYN007[10]	MIR604 late hybrid E5	0.88	4
	MIR604 late hybrid E6	1.01	4
	Control late hybrid 2E	2.64	4
	LSD (0.05)	**0.45**	
Norway, KS, 2003 Trial SYN008[10]	MIR604 late hybrid E5	0.36	4
	MIR604 late hybrid E6	0.38	4
	Control late hybrid 2E	0.29	4
	LSD (0.05)	**0.48**	
York, NE, 2003 Trial SYN009[10]	MIR604 late hybrid E5	0.02	4
	MIR604 late hybrid E6	0.04	4
	Control late hybrid 2E	1.88	4
	LSD (0.05)	**0.28**	
Minnehaha, SD, 2003 Trial SYN010[10]	MIR604 late hybrid E5	0.09	4
	MIR604 late hybrid E6	0.05	4
	Control late hybrid 2E	0.29	4
	LSD (0.05)	**0.22**	

APPENDIX 1A (CONT'D)

TABLE 3A (CONT'D)

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm plus artificial infestation with western corn rootworm unless noted otherwise in the table.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3]Individual corn plants were artificially infested with freshly deposited Western corn rootworm eggs at a density of 594 eggs per plant for trial SYN180 and 405 eggs per plant for trial SYN181. Additional details can be found in Chapter 4, Section B.

[4]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid D3) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data.

[5]CRW root damage rating scale 0-3 (see Table 1A).

[6]Only control hybrids 2E were negative segregants of their respective MIR604 hybrids. All other control hybrids were non-transgenic parental inbred lines.

[7]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[8]Trial was not artificially infested.

[9]Trial was infested primarily with naturally occurring northern corn rootworm.

[10]These trials were conducted in a randomized complete block design, 2 x 20 ft. rows, 4 replicates, 25 seed per row. 10 root masses per plot (5 randomly selected plants per row) were evaluated at the VT to R1 stage (approximately 7 weeks following infestation). Plants were manually dug from the ground, washed free of soil and rated for rootworm damage according to the Iowa State 0-3 node-injury rating scale (see Table 1A above). (http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html).

[11]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level except for trial SYN008, Norway, KS.

APPENDIX 1A (CONT'D)

TABLE 4A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS IN GLASSHOUSE TRIALS[1]

LOCATION, YEAR	ENTRY[2]	MEAN CRW ROOT DAMAGE RATING[3]	# OF REPS
Stanton, MN, 2003 Trial SYN160	MIR604 early hybrid D1	0.019[6]	20
	MIR604 early hybrid D2	0.022	20
	Control early hybrid 1D[4]	0.730	20
	LSD (0.05)[5]	**0.092**	
Stanton, MN, 2003 Trial SYN165	MIR604 early hybrid D1	0.024	12
	MIR604 early hybrid D2	0.015	12
	Control early hybrid 1D	0.638	12
	LSD (0.05)	**0.138**	

[1]Individual potted corn plants (V2 to V3 leaf stage) were artificially infested with freshly deposited Northern corn rootworm eggs at a density of 43 eggs per plant for trial SYN160 and 58 eggs per plant for trial SYN165. Additional details can be found in Chapter 4, Section B.

[2]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid D1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data

[3]CRW root damage rating scale 0-3 (see Table 1A).

[4]All control hybrids were negative segregants of their respective MIR604 hybrids.

[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[6]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level.

APPENDIX 1A (CONT'D)

TABLE 5A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

LOCATION, YEAR	ENTRY[2]	MEAN CRW ROOT DAMAGE RATING[3]	# OF REPS
Monoville, TX, 2003 Trial SYN011[1]	MIR604 late hybrid E5	1.37[7]	4
	MIR604 late hybrid E6	0.94	4
	Control late hybrid 1E[4]	2.71	4
	LSD (0.05) [5, 6]	**0.56 – 0.60**	
Abbott, TX, 2003 Trial SYN012[1]	MIR604 late hybrid E5	0.91	4
	MIR604 late hybrid E6	0.42	4
	Control late hybrid 1E	2.81	4
	LSD (0.05)[5]	**0.63**	

[1]Trials comprised single row plots using a randomised complete block design with natural populations of Mexican corn rootworm.

[2]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid E5) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2003 data

[3]CRW root damage rating scale 0-3.9 (see Table 1A).

[4]All control hybrids were negative segregants of their respective MIR604 hybrids.

[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[6]Missing plot value in replicate 3 of the Negative Control 1E, causes an unbalanced design; LSD is dependent upon n = no. of reps and is variable for unbalanced design.

[7]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level.

APPENDIX 1B

YIELD DATA FOR MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS

APPENDIX 1B (CONT'D)

TABLE 1B

YIELD COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2], 2002

LOCATION(S), YEAR	ENTRY[3]	YIELD (BUSHELS/ACRE)	# OF REPS
Stanton, MN, 2002 Trial SYN653[7]	MIR604 early hybrid B1 (Bt)[4]	145.8	5
	MIR604 early hybrid B2 (Bt)	133.2	5
	Control early hybrid 1B (Bt)[5]	140.7	5
	LSD (0.05)[6]	**13.90**	
Bloomington-1, Bloomington-2, Bondville, St. Joseph, IL 2002 Trial SYN673[7]	MIR604 late hybrid C1 (Bt)	**110.1**[8]	5
	MIR604 late hybrid C2 (Bt)	**102.2**	5
	Control late hybrid 1C (Bt)	45.6	5
	LSD (0.05)	**19.42**	

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid B1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data.

[4]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, both MIR604-derived and control hybrids expressing the Cry1Ab protein (Bt) were included in the trials.

[5]All control hybrids were negative segregants of their respective MIR604 hybrids.

[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[7] Randomized complete block design for trial SYN653 and Split plot design at all locations for trial SYN673.

[8]MIR604 hybrid mean values in **bold** are significantly different from the negative control at the 0.05% level.

TABLE 2B

YIELD COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

LOCATION (S), YEAR	ENTRY[2]	YIELD (BUSHELS/ ACRE)	# OF REPS
Faribault, LeRoy, Mankato, Owatonna, Stanton, MN, 2003 Trial SYN652	MIR604 early hybrid G3	114.6	5
	MIR604 early hybrid G4	115.8	5
	Control early hybrid 2G[3]	116.2	5
	MIR604 early hybrid H6[4] (Bt)	122.2	5
	MIR604 early hybrid H7 (Bt)	118.9	5
	Control early hybrid 3H (Bt)	116.6	5
	LSD (0.05) [5]	**8.4**	
Hampton, IA; Brookings, SD; Janesville, WI, Rochelle, IL 2003 Trial SYN656	MIR604 early hybrid G3	144.4	3
	MIR604 early hybrid G4	143.6	3
	Control early hybrid 2G	140.4	3
	LSD (0.05)	**16.5**	
Bondville, Chillicothe, Hudson, Leroy, Shirley, IL, 2003 Trial SYN672	MIR604 late hybrid E2	103.1	5
	MIR604 late hybrid E3	101.7	5
	Control late hybrid 1E	104.5	5
	MIR604 late hybrid F4 (Bt)	107.9	5
	MIR604 late hybrid F3 (Bt)	108.5	5
	Control late hybrid 2F (Bt)	98.3	5
	LSD (0.05)	**11.0**	
Glidden, Washington, IA; Leesburg, IN; Henderson, KY; Seward, NE, 2003 Trial SYN676	MIR604 late hybrid E2	102.6	3
	MIR604 late hybrid E3	97.2	3
	Control late hybrid 1E	109.1	3
	LSD (0.05)	**18.1**	
Hampton, IA; Stanton, MN, 2003 Trial SYN653[7]	MIR604 early hybrid H6 (Bt)	151.3	4
	Control early hybrid 3H (Bt)	150.2	4
	LSD (0.05)	17.4	4
Bondville, Chillicothe, Hudson, Leroy, IL; Seward, NE, 2003 Trial SYN673[7]	MIR604 late hybrid F4[4] (Bt)	**120.5**[6]	4
	MIR604 late hybrid F3 (Bt)	112.0	4
	Control late hybrid 2F (Bt)	106.4	4
	LSD (0.05)	**9.6**	

[1] Trials comprised single row plots using a randomised complete block design with natural populations of western and northern corn rootworm.

[2]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid D3) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data.

[3]All control hybrids were negative segregants of their respective MIR604 hybrids.

[4]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 hybrids H and F and the controls 3H and 2F expressing the Cry1A(b) protein (Bt) were included in the trials.

APPENDIX 1B (CONT'D)

TABLE 2B (CONT'D)

[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[6]Mean values in **bold** are significantly different from the negative control at the 0.05% level.

[7]These trials were conducted with trap crop populations of CRW. Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

APPENDIX 1C

AGRONOMIC DATA FOR MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS

APPENDIX 1C (CONT'D)

TABLE 1C
AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2], 2002

Location	Bloomington-1, Bloomington-2, Bondville, St. Joseph, IL 2002 Trial SYN673					Stanton, MN 2002 Trial SYN653				
Reps	5					5				
Agronomic Trait[3]	MIR604 late hybrid C1[4] (Bt)	MIR604 late hybrid C2 (Bt)	Control late hybrid 1C[5] (Bt)	**LSD (0.05)[6]**	Locations w/ data	MIR604 early hybrid B1 (Bt)	MIR604 early hybrid B2 (Bt)	Control early hybrid 1B (Bt)	**LSD (0.05)**	Locations w/ data
DROPP	0	0	0.4	NA[7]	4	-	-	-	**-**	**-**
EMRGP	**76.5**[8]	81.8	81.3	1.3	4	-	-	-	**-**	**-**
ERHTN	-[9]	-	-	-	**-**	109	97	95	15	1
GMSTP	19.9	**19.2**	19.9	0.5	4	**28.1**	**28.0**	31.2	1.4	1
HAVPN	**24523**	26205	26048	417	4	30492	30492	30492	450	1
HU5SN	-	-	-	-	**-**	**1298**	**1302**	1360	24	1
HU5PN	-	-	-	-	**-**	**1298**	**1292**	1334	24	1
INTLR	**8.1**	**8.3**	5.9	0.6	4	-	-	-	-	**-**
LRTLP	59	59	37	23	4	**26**	**24**	6	13	1
PLHTN	-	-	-	-	**-**	275	268	277	17	1
POL5N	-	-	-	-	**-**	**61**	**61**	63	1	1
PSTSP	74	92	64	30	4	-	-	-	-	**-**
SLK5N	-	-	-	-	**-**	**61**	62	63	1	1
STKLP	**5.9**	**6.3**	3.3	2.0	4	1	3	3	4	1
TWSMN	**61.7**	**62**	52.8	2.4	4	46.9	47.6	46.9	2.1	1

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3] See Chapter 4, Table 1 for list and description of agronomic parameters.

[4]All control hybrids were negative segregants of their respective MIR604 hybrids.

[5]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.

[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.

[7]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.

[8]Mean values in **bold** are significantly different from the negative control at the 0.05% level.

[9]No data collected for this trait at these trial sites.

APPENDIX 1C (CONT'D)

TABLE 2C

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

Location	Glidden, Washington, IA; Leesburg, IN; Henderson, KY; Seward, NE, 2003 Trial SYN676					Hampton, IA; Brookings, SD; Janesville, WI, Rochelle, IL 2003 Trial SYN656				
Reps	3					3				
Agronomic Trait[2]	MIR604 late hybrid E2	MIR604 late hybrid E3	Control late hybrid 1E[3]	**LSD (0.05)**[4]	Locations w/ data	MIR604 early hybrid G3	MIR604 early hybrid G4	Control early hybrid 2G	**LSD (0.05)**	Locations w/ data
DROPP	0	0	0	NA[5]	3	-[6]	-	-	-	-
EMRGP	86.6	87.1	87.0	NA	2	100.3	96.9	99.4	NA	2
ERHTN	80	72	85	17	1	113	113	113	10	1
GMSTP	**18.6**[7]	19.4	20.2	0.9	4	19.9	20.2	20.0	1.5	3
HAVPN	30126	30322	30479	NA	4	29756	29450	29705	NA	3
HU5SN	1291	1327	1321	33	1	958	1118	1129	NA	2
HU5PN	1200	1218	1226	48	1	1110	1104	1116	NA	2
INTLR	5.2	5.0	4.8	1.0	2	5.3	5.0	5.7	NA	1
LRTLP	0	4	0	NA	1	-	-	-	-	-
PLHTN	243	257	237	25	1	248	252	237	19	1
POL5N	57	58	58	2	1	-	-	-	-	-
PSTSP	62	45	48	36.2	2	-	-	-	-	-
SLK5N	61	63	62	NA	1	-	-	-	-	-
STKLP	8.1	2.4	1.9	NA	2	2.5	1.7	1.6	NA	3
TWSMN	58.5	58.8	59.3	NA	4	**57.2**	55.6	54.5	1.8	3

[1] Trials comprised single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm.
[2] See Chapter 4, Table 1 for a list and description of agronomic parameters.
[3] All control hybrids were negative segregants of their respective MIR604 hybrids.
[4] Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[5] NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[6] No data collected for this trait at these trial sites.
[7] Mean values in **bold** are significantly different from the negative control at the 0.05% level.

APPENDIX 1C (CONT'D)

TABLE 3C

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

Location	Faribault, LeRoy, Mankato, Owatonna, Stanton, MN, 2003 Trial SYN652							
Reps	5							
Agronomic Trait[2]	MIR604 early hybrid H7[3] (Bt)	MIR604 early hybrid H6 (Bt)	Control early hybrid 3H[4] (Bt)	MIR604 early hybrid G3	MIR604 early hybrid G4	Control early hybrid 2G	**LSD (0.05)[5]**	Locations w/ data
EMRGP	86.7	87.1	87.1	86.3	87.2	86.7	NA[6]	3
ERHTN	102	103	102	98	100	103	12.1	2
GMSTP	22.0	20.7	20.6	19.3	20.3	19.2	1.4	4
HAVPN	29379	29524	29524	29234	29532	29379	NA	4
HU5SN	1249	**1239**[7]	1264	1249	1234	1244	23	1
HU5PN	1244	1239	1249	1239	1234	1244	25	1
PLHTN	298	303	297	307	304	304	11.0	2
STKLP	11.0	12.7	11.8	14.0	20.1	10.8	10.4	4
TWSMN	38.4	**40.2**	38.2	**39.2**	37.3	37.6	1.7	4

[1] Trials comprised single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm.
[2] See Chapter 4, Table 1 for a list and description of agronomic parameters.
[3]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 hybrids D7 and D6 and control hybrid 3D expressing the Cry1A(b) protein (Bt) were included in the trials.
[4]All control hybrids were negative segregants of their respective MIR604 hybrids.
[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[6]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[7]Mean values in **bold** are significantly different from the negative control at the 0.05% level.

APPENDIX 1C (CONT'D)

TABLE 4C

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

Location	Bondville, Chillicothe, Hudson, Leroy, Shirley, IL, 2003 Trial SYN672							
Reps	5							
Agronomic Trait[2]	MIR604 late hybrid F4[3] (Bt)	MIR604 late hybrid F3 (Bt)	Control late hybrid 2F[4] (Bt)	MIR604 late hybrid E2	MIR604 late hybrid E3	Control late hybrid 1E	**LSD (0.05)[5]**	Loc. w/ data
EMRGP	1.2	1.2	1.2	1.0	1.2	1.0	1.9	5
ERHTN	106	110	107	106	106	110	7	5
GMSTP	21.7	**23.7**[7]	22.5	**21.0**	22.7	23.1	0.8	5
HAVPN	25783	25435	25618	25783	25252	25526	585	5
HU5SN	1514	1509	1509	1499	1534	1529	53	1
HU5PN	1509	1499	1504	1494	1534	1509	56	1
INTLR	7.2	6.3	7.0	7.6	7.2	6.8	0.9	5
LRTLP	7	6	2	2	6	5	NA[6]	1
PLHTN	220	232	224	217	226	219	8	5
STKLP	22.9	18.4	24.1	24.2	15.2	17.1	11.6	5
TWSMN	**58.7**	57.9	57.5	57.6	57.6	57.6	1.0	5

[1] Trials comprised single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm.
[2] See Chapter 4, Table 1 for a list and description of agronomic parameters.
[3]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 hybrids F4 and F3 and control hybrid 2F expressing the Cry1A(b) protein (Bt) were included in the trials.
[4]All control hybrids were negative segregants of their respective MIR604 hybrids.
[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[6]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[7]Mean values in **bold** are significantly different from the negative control at the 0.05% level.

APPENDIX 1C (CONT'D)

TABLE 5C

FOLIAR DISEASE RATING 1-9 SCALE USED BY SYNGENTA AGRONOMISTS

Rating	Description
1	No lesions or one or two restricted lesions on inoculated leaf. About One (1) percent of leaf surface area is affected.
2	A few scattered lesions on inoculated leaf. About two (2) - four (4) percent of leaf surface area.
3	Few lesions on inoculated leaf. 5-15% of leaf surface area affected. A few scattered lesions on leaves above and/or below inoculated leaf (less than 5% of leaf area affected).
4	Few lesions on inoculated leaf. Approximately 15-25 percent of leaf surface area affected. A few scattered lesions on leaves above and/or below inoculated leaf (less than 10% of leaf area affected).
5	Light-moderate number of lesions on inoculated leaf (25-35 percent of leaf surface area affected). Some lesions may be coalesced. Leaves above and/or below inoculated leaf with light lesion development. Less than 15 percent leaf surface area affected.
6	Moderate numbers of lesions on inoculated leaf (35-50 percent of leaf surface area affected). Some lesions may be coalesced. Leaves above and/or below inoculated leaf with light- moderate lesion number (15-30 percent of leaf surface area).
7	Lesions abundant on inoculated leaf (50-75 percent of leaf surface area affected). Some lesions coalesced. Lower and upper leaves with moderate lesion numbers (30-50 percent of leaf surface area). Some lesions may be coalesced
8	Lesions abundant on inoculated leaf (75-100 percent of leaf surface affected). Numerous lesions coalesced. Lower and upper leaves with moderate-heavy lesion number (50-75 percent of leaf surface affected). Numerous lesions may be coalesced.
9	Lesions highly abundant on all leaves. Lesions highly coalesced. Plants may be prematurely killed.

APPENDIX 1C (CONT'D)

TABLE 6C

COMPARISON OF DISEASE RATINGS[1] OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[2], 2002-2003

	Disease	Gray Leaf Spot	Northern Corn Leaf Blight	Southern Corn Leaf Blight	Eyespot
	Reps	4	4	4	4
Location	**Hybrid**				
Stanton, MN, 2002 SYN135 & SYN136	MIR604 Early Hybrid B1[3] (Bt)	4.25	7.5	-	-
	MIR604 Early Hybrid B2 (Bt)	3.75	6.5	-	-
	Early Control Hybrid 1B (Bt)	3.75	6.0	-	-
	LSD (0.05)	**1.15**	**1.5**	-	-
Bloomington, IL, 2002 SYN175 & SYN176	MIR604 Late Hybrid C2 (Bt)	7.0	-	7.3	-
	MIR604 Late Hybrid C1 (Bt)	7.0	-	7.5	-
	Late Control Hybrid 1C (Bt)	7.0	-	7.3	-
	LSD (0.05)	**0.3**	-	**0.6**	-
Stanton, MN, 2003 SYN136 & SYN137	MIR604 Early Hybrid H6 (Bt)	-	2.5	-	5.25
	MIR604 Early Hybrid H7 (Bt)	-	2.5	-	6.00
	Early Control Hybrid 3H (Bt)	-	2.5	-	5.50
	LSD (0.05)	-	**1.4**	-	**NA**
Bloomington, IL, 2003 SYN177	MIR604 Late Hybrid F1 (Bt)	6.2		-	-
	MIR604 Late Hybrid F2 (Bt)	7.2		-	-
	Late Control Hybrid 1F (Bt)	8.0		-	-
	LSD (0.05)	0.9		-	-

[1] Disease ratings (see table 5C above) were assigned according to a standard 1-9 foliar disease rating scale (Ullstrup et al., 1945).

[2]Trials were comprised of single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm. Plots relied upon natural disease pressure and were rated multiple times during the growing season on an individual plant basis.

[3]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.

APPENDIX 1D

EFFICACY, YIELD AND AGRONOMIC DATA FOR MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS WITH INSECTICIDE TREATMENT

APPENDIX 1D (CONT'D)

TABLE 1D

EFFICACY COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1, 2], 2002

LOCATION, YEAR	ENTRY[4]	INSECTICIDE TREATMENT	MEAN CRW ROOT DAMAGE RATING[5]	# OF REPS
Stanton, MN, 2002 Trial SYN137	MIR604 early hybrid A1	Force 3G[6]	0.083	3
	MIR604 early hybrid A2	Force 3G	0.133	3
	Control early hybrid 1A[7]	Force 3G	0.193	3
	LSD (0.05) [8]		**0.185**	
Bloomington, IL, 2002 Trial SYN179	MIR604 early hybrid A1	Force 3G	0.045	3
	MIR604 early hybrid A2	Force 3G	0.077	3
	Control early hybrid 1A	Force 3G	0.335	3
	LSD (0.05)		**0.105**	

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm plus artificial infestation with western corn rootworm.
[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.
[3]Individual corn plants were artificially infested with freshly deposited Western corn rootworm eggs at a density of 1182 eggs per plant for trial SYN137 and 1462 eggs per plant for trials SYN179. Additional details can be found in Chapter 4, Section B.
[4]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid A1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data
[5]CRW root damage rating scale 0-3 (see Appendix 1A, Table 1A above).
[6]Force 3G applied at 1.12 g ai/100 row meters
[7]All control hybrids were non-transgenic parental inbreds.
[8]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.

APPENDIX 1D (CONT'D)

TABLE 2D

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1], 2002

Location	Hudson, Newman, St. Joseph, Shirley IL, 2002 Trial SYN672					Delavan, Faribault, LeRoy, Owatonna, MN 2002 Trial SYN652				
Treatment[2]	Force 3G					Force 3G				
Reps	4					4				
Agronomic Trait[3]	MIR604 late hybrid C2[4] (Bt)	MIR604 late hybrid C1 (Bt)	Control late hybrid 1C[5] (Bt)	**LSD (0.05)**[6]	Loc. w/ data	MIR604 early hybrid B1 (Bt)	MIR604 early hybrid B2 (Bt)	Control early hybrid 1B (Bt)	**LSD (0.05)**	Loc. w/ data
YGSMP	**106.8**[7]	**110.8**	119.6	8.6	4	118.1	125.4	125.7	16.6	4
DROPP	0	0	0	NA[8]	3	0	0	0	NA	1
EMRGP	81.6	**78.0**	81.5	2.7	4	94.7	92.4	93.4	3	4
ERHTN	82	72	78	6	4	100	103	103	NA	1
GMSTP	**18.2**	**19.1**	19.6	0.4	4	**24.0**	**25.3**	26.9	1.4	4
HAVPN	26140	**24995**	26117	859	4	30952	30395	30686	611	4
HU9SN	**1530**	**1541**	1558	14	1	-[9]	-	-	-	-
HU9PN	**1452**	1490	1496	25	1	-	-	-	-	-
INTLR	7.4	7.4	7.0	1.1	4	-	-	-	-	-
LRTLP	74	80	60	20	1	5	7	10	7	4
PLHTN	185	181	182	7	4	258	259	260	NA	1
PSTSP	70	56	56	20	1	-	-	-	-	-
STKLP	7	4	3	5	4	4.4	**9.9**	3.7	5.9	4
TWSMN	**61**	**61.4**	62.5	0.8	4	**45.2**	46.7	47.4	1.4	4

[1] Trials comprised single row plots using a randomised complete block design with natural populations of western and northern corn rootworm.
[2]Force 3G applied at 1.12 g ai/100 row meters.
[3] See Chapter 4, Table 1 for a list and description of agronomic parameters.
[4]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.
[5]All control hybrids were negative segregants of their respective MIR604 hybrids.
[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[7]Mean values in **bold** are significantly different from the negative control at the 0.05% level.
[8]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[9]No data collected for this trait at these trial sites.

APPENDIX 1D (CONT'D)

TABLE 3D

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1, 2], 2002

Location	Bloomington 1, Bloomington 2, Bondville, St. Joseph, IL 2002 Trial SYN673					Stanton, MN 2002 Trial SYN653				
Reps	5									
Treatment[3]	Force 3G					Force 3G				
Agronomic Trait[4]	MIR604 late hybrid C1[5] (Bt)	MIR604 late hybrid C2 (Bt)	MIR604 late hybrid 1C[6] (Bt)	**LSD (0.05)**[7]	Locations w/ data	MIR604 early hybrid B1 (Bt)	MIR604 early hybrid B2 (Bt)	Control early hybrid 1B (Bt)	**LSD (0.05)**	Locations w/ data
YGSMP	**127.1**[8]	**125.5**	93.6	19.42	4	152.9	150.2	162.7	13.9	1
DROPP	0	0.3	0.4	NA[9]	4	-	-	-	-	-
EMRGP	77.1	81.7	83.5	1.3	4	-	-	-	-	-
ERHTN	-[10]	-	-	-	-	96	107	110	15	1
GMSTP	**20.0**	**19.4**	20.9	0.6	4	**27.4**	29.8	30.1	1.4	1
HAVPN	24698	26186	26758	417	4	30492	30492	30492	450	1
HU5SN	-	-	-	-	-	**1304**	1320	1334	24	1
HU5PN	-	-	-	-	-	**1293**	1316	1330	24	1
INTLR	7.9	**8.4**	7.3	0.6	4	-	-	-	-	-
LRTLP	24	22	3	23	4	15	**27**	11	13	1
PLHTN	-	-	-	-	-	**277**	**271**	295	17	1
POL5N	-	-	-	-	-	61	62	62	1	1
PSTSP	50	**86**	46	30	4	-	-	-	-	-
SLK5N	-	-	-	-	-	**61**	62	63	1	1
STKLP	**2.9**	5.8	5.3	2.0	4	1	3	3	4	1
TWSMN	61.9	61.7	62.2	2.4	4	46.9	47.6	46.9	2.1	1

[1]Trials comprised single row trap crop plots using a split plot design with natural populations of western and northern corn rootworm.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3]Force 3G applied at 1.12 g ai/100 row meters.

[4] See Table 2D for a list and description of agronomic parameters.

[5]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.

[6]All control hybrids were negative segregants of their respective MIR604 hybrids.

[7]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.

[8]Mean values in **bold** are significantly different from the negative control at the 0.05% level.

[9]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.

[10]No data collected for this trait at these trial sites.

APPENDIX 1D (CONT'D)

TABLE 4D

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1, 2], 2003

Location	Bondville, Chillicothe, Hudson, Leroy, IL; Seward, NE, 2003 Trial SYN674					Hampton, IA; Stanton, MN, 2003 Trial SYN654				
Treatment[3]	Force 3G					Force 3G				
Reps	4					4				
Agronomic Trait[4]	MIR604 late hybrid F4[5] (Bt)	MIR604 late hybrid F3 (Bt)	Control late hybrid 2F[6] (Bt)	**LSD (0.05)[7]**	Loc. w/ data	MIR604 early hybrid H7 (Bt)	MIR604 early hybrid H6 (Bt)	Control early hybrid 3H (Bt)	**LSD (0.05)**	Loc. w/ data
YGSMN	119.6	113.3	111.3	11.0	4	154.3	152.0	146.9	18.4	4
DROPP	0	0	0	NA[8]	1	-	-	-	-	-
EMRGP	79.3	78.8	77.5	NA	5	98.5	98.9	100	NA	2
ERHTN	100	103	103	8	5	109	110	115	15	1
GMSTP	**21.5**[9]	22.9	22.6	0.9	5	24.2	23.0	22.6	1.8	2
HAVPN	25557	25387	24980	NA	5	30558	30558	30558	NA	2
HU5SN	**1324**	1313	1291	29	1	-[10]	-	-	-	-
HU5PN	1219	1218	1225	30	1	-	-	-	-	-
INTLR	7.9	6.9	7.2	1.5	4	-	-	-	-	-
LRTLP	0	2	0	NA	4	-	-	-	-	-
PLHTN	230	240	231	9.1	5	299	290	306	19	1
POL5N	58	58	58	1	1	-	-	-	-	-
PSTSP	28	**8**	38	16	1	-	-	-	-	-
SLK5N	63	62	61	NA	1	-	-	-	-	-
STKLP	11.5	10.9	14.4	NA	4	3.0	0.8	1.5	NA	2
TWSMN	59.2	58.2	58.5	NA	5	54.8	55.5	53.7	NA	2

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm.
[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.
[3]Force 3G applied at 1.12 g ai/100 row meters.
[4] See Table 2D for a list and description of agronomic parameters.
[5]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.
[6]All control hybrids were negative segregants of their respective MIR604 hybrids.
[7]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[8]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[9]Mean values in **bold** are significantly different from the negative control at the 0.05% level.
[10]No data collected for this trait at these trial sites.

APPENDIX 2

UNPUBLISHED DATA REPORTS IN SUPPORT OF REGULATORY APPROVALS FOR EVENT MIR604 HYBRIDS

CBI DELETED COPY

ALL DATA AND INFORMATION IN THIS APPENDIX IS CONSIDERED CONFIDENTIAL BUSINESS INFORMATION

Appendix 2 – Section 1

[CBI-Deleted]

Molecular Characterization of Event MIR604 Maize (Corn) Expressing a Modified Cry3A *Bacillus thuringiensis* Protein for USDA Petition for Non-Regulated Status

Appendix 2 – Section 2

[CBI-Deleted]

Characterization and Safety of Modified Cry3A Protein and Maize (Corn) Plants Derived from Event MIR604.

Appendix 2 – Section 3

[CBI-Deleted]

Characterization of Phosphomannose Isomerase (PMI) Produced in Maize (Corn) Plants Derived from Event MIR604 and Comparison to PMI as Contained in Test Substance PMI-0198

Appendix 2 – Section 4

[CBI-Deleted]

Quantification of Modified Cry3A and PMI Proteins in Transgenic Maize (Corn) Tissues, Whole Plants, and Silage Derived from Transformation Event MIR604

Appendix 2 – Section 5

[CBI-Deleted]

Compositional Analysis of Grain and Whole Plants from Transgenic Maize (Corn) Event MIR604

Appendix 2 – Section 6

[CBI-Deleted]

Environmental Safety Assessment of Modified Cry3A Protein and Event MIR604 Corn to Non-Target Organisms

Appendix 2 – Section 7

[CBI-Deleted]

Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Mouse

Appendix 2 – Section 8

[CBI-Deleted]

Analysis for the Presence of Modified Cry3A Protein in Wet and Dry Milled Fractions, Corn Oil and Corn Chips from Processing of Event MIR604 Maize (Corn)

Appendix 2 – Section 9

[CBI-Deleted]

Phosphomannose Isomerase (Sample PMI-0198): Acute Oral Toxicity Study in Mice

Appendix 2 – Section 10

[CBI-Deleted]

Effect of Temperature on the Stability of Modified Cry3A Protein (MCRY3A-0102)

Appendix 2 – Section 11

[CBI-Deleted]

In vitro Digestibility of Modified Cry3A Protein (MCRY3A-0102 and IAMPMIR604-0103) Under Simulated Mammalian Gastric Conditions

Appendix 2 – Section 12

[CBI-Deleted]

In vitro Digestibility of PMI Protein Under Simulated Mammalian Gastric and Intestinal Conditions

Appendix 2 – Section 13

[CBI-Deleted]

Characterization and Safety of Phosphomannose Isomerase (PMI), a Selectable Marker Expressed in Event 3243M-Derived Maize (Corn) Plants

Appendix 2 – Section 14

[CBI-Deleted]

Summary of Data Demonstrating the Environmental Safety of Modified Cry3A *Bacillus thuringiensis* Insect Control Protein and Event MIR604-Derived Corn (Maize) to Non-Target organisms

Appendix 2 – Section 15

[CBI-Deleted]

An Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Northern Bobwhite

Appendix 2 – Section 16

[CBI-Deleted]

A 28-Day Laboratory Study to Evaluate the Effects of Modified Cry3A Maize Fish Feed (FFMIR604-0103) on the Growth of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

Appendix 2 – Section 17

[CBI-Deleted]

A Semi-Field Test to Evaluate the Effects of the Modified Cry3A Protein (MCRY3A-0102) on Brood Development of the Honybee, *Apis mellifera* (Hymenoptera: Apidae)

Appendix 2 – Section 18

[CBI-Deleted]

Characterization of Fish Feed Test Substance (FFMIR604-0103) Prepared from Event MIR604-Derived Maize Grain

Appendix 2 – Section 19

[CBI-Deleted]

A Laboratory Test of the Toxicity of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae)

Appendix 2 – Section 20

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Coccinella septempunctata* to Modified Cry3A Protein: Supplement to Report Titled 'A Laboratory Test of the Toxicity of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle *Coccinella septempunctata* (Coleoptera: Coccinellidae)'

Appendix 2 – Section 21

[CBI-Deleted]

A Laboratory Toxicity Test of the Modified Cry3A Protein (MCRY3A-0102) to Larvae of the Ground-Dwelling Beetle, *Poecilus cupreus* (Coleoptera: Carabidae)

Appendix 2 – Section 22

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Poecilus cupreus* to Modified Cry3A Protein: Supplement to Report Titled 'A Laboratory Toxicity Test of the Modified Cry3A Protein (MCRY3A-0102) to Larvae of the Ground-Dwelling Beetle *Poecilus cupreus* (Coleoptera: Carabidae)'

Appendix 2 – Section 23

[CBI-Deleted]

A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylinidae)

Appendix 2 – Section 24

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Aleochara bilineata* to Modified Cry3A Protein: Supplement to Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylinidae)'

Appendix 2 – Section 25

[CBI-Deleted]

A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)

Appendix 2 – Section 26

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Orius insidiosus* to Modified Cry3A Protein: Supplement to Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)'

Appendix 2 – Section 27

[CBI-Deleted]

Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the Earthworm *Eisenia foetida* in an Artificial Soil Substrate

Appendix 2 – Section 28

[CBI-Deleted]

Analysis of Artificial Soil Used to Expose *Eisenia foetida* to Modified Cry3A Potein: Supplement to Report Titled ‘Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the Earthworm *Eisenia foetida* in an Artificial Soil Substrate’

Appendix 2 – Section 29

[CBI-Deleted]

Environmental Fate Assessment of Modified Cry3A Protein in Event MIR604 Corn

Appendix 2 – Section 30

[CBI-Deleted]

Laboratory Soil Degradation of Modified Cry3A Protein (MCRY3A-0102)

Appendix 2 – Section 31

[CBI-Deleted]

Waiver Request: Freshwater Aquatic Invertebrate Testing of Modified Cry3A Protein as Expressed in Event MIR604 Corn (Maize)

Appendix 2 – Section 32

[CBI-Deleted]

Characterization of Modified Cry3A Protein Produced in Event MIR604-Derived Maize (Corn) and Comparison with Modified Cry3A Protein Expressed in Recombinant *Escherichia coli*

Appendix 2 – Section 33

[CBI-Deleted]

Characterization of Modified Cry3A Test Substance (MCRY3A-0102) and Certificate of Analysis

Appendix 2 – Section 34

[CBI-Deleted]

Further Characterization of Modified Cry3A Test Substance MCRY3A-0102

Appendix 2 – Section 35

[CBI-Deleted]

Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Toxins

Appendix 2 – Section 36

[CBI-Deleted]

Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Allergens

Appendix 2 – Section 37

[CBI-Deleted]

Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Toxins

Appendix 2 – Section 38

[CBI-Deleted]

Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Allergens

Appendix 2 – Section 39

[CBI-Deleted]

Evaluation of Transgenic Corn (Maize) MIR604 in Broiler Chickens

Appendix 2 – Section 40

[CBI-Deleted]

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

Appendix 2 – Section 41

[CBI-Deleted]

Amended Public Interest Document in Support of Registration of the Plant-Incorporated Protectant Modified Cry3A Protein as Expressed in Event MIR604 Corn

Appendix 2 – Section 42

[CBI-Deleted]

Insect Resistance Management for Syngenta Event MIR604 Maize (Corn)

Appendix 2 – Section 43

[CBI-Deleted]

Effects of Temperature on the Stability of Phosphomannose Isomerase (PMI)

Appendix 2 – Section 44

[CBI-Deleted]

Natural Variation of Phytosterol Levels in Maize (Field Corn) Grain

Appendix 2 – Section 45

[CBI-Deleted]

Demonstration that the Potassium Levels in Forage Derived from MIR604 Maize (Field Corn) are within the Range of Potassium Levels in Conventional Maize Forage

United States Department of Agriculture

Animal and Plant Health Inspection Service

Marketing & Regulatory Programs Business Services

4700 River Road
Riverdale, MD 20737

Review for Completeness for Petition 04-362-01p
8/2/06

Dr. Henry York-Steiner
Syngenta Seeds, Inc.
Regulatory Affairs Manager

Subject: Draft Review for Technical Completeness of Syngenta's Petition for Determination of Non-regulated Status for the Insect Resistant Transformation Event of *Zea mays*, namely MIR 604, APHIS number 04-362-01p

Dear Dr. Steiner,

This letter is written in reference to the Revised Petition for Determination of Non-regulated Status Corn Rootworm Protected Transformation Event MIR604 submitted to the USDA, Animal and Plant Health Inspection Service, Biotechnology Regulatory Services (APHIS-BRS), dated May 17, 2006. APHIS-BRS determines that there is a need for additional information and clarification before this petition can be deemed technically complete. Please respond to each of the 14 points listed below. These points are listed under their respective Chapters of the submitted petition.

The additional information or clarification can be written as responses within the context of this letter or by submitting a revised petition containing the additional information. If a revised version is submitted, please provide an electronic submission of your petition for publication on the USDA-APHIS BRS website.

Please provide the following information:

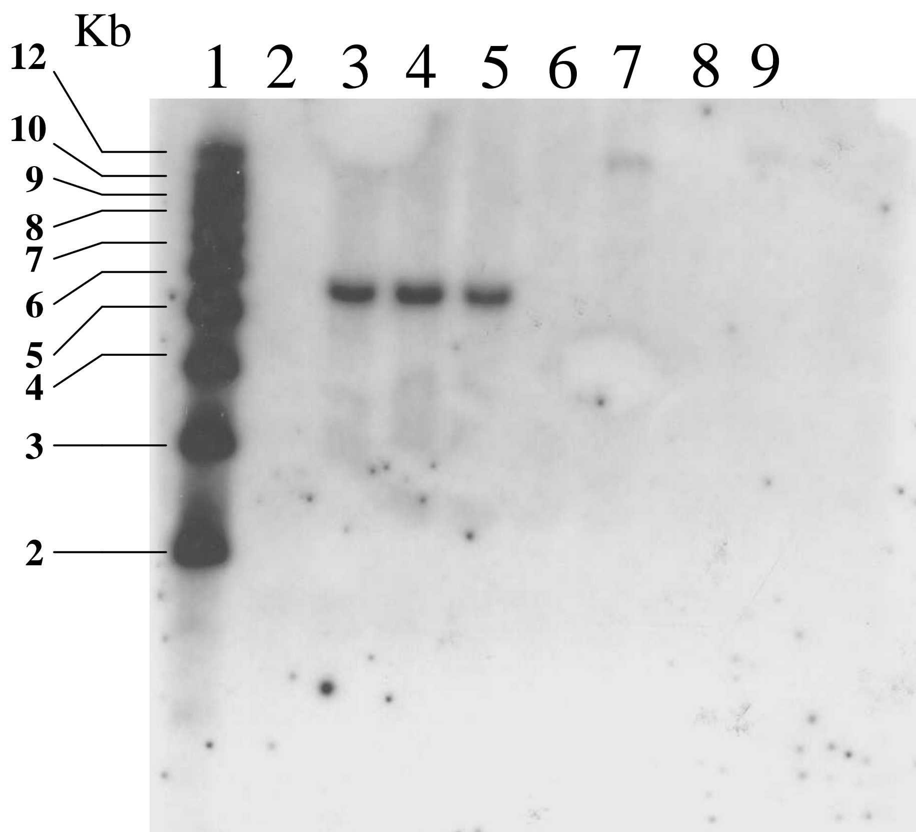
Chapter 3. Molecular Analysis of Event MIR604

1. Page 51. Figure 9. Explain why the intensities of the bands in lanes 5 and 7 are different.
   See Point #2
2. Page 52. Figure 11. Explain why the intensities of the bands in lanes 5 and 7 are different and why they appear to be of a different molecular weight. These results do not correlate with the presence of a single gene copy.
   There was a miscalculation in the copy number controls for this package (loaded less than a 1 copy equivalent for each of the Southerns). The mistake was realized after completion of this data set and it has since been corrected in all ongoing projects. Table 1 indicates the copy number equivalent loaded for each probe employed in Southern Analysis. Amounts of plasmid pZM26 present on each Southern represented less than a one copy equivalent of the element homologous to the probe employed in the various experiments. This indicates the respective experiments are sensitive enough to detect a less than

one copy equivalent, and the final conclusions are not impacted. Plasmid pZM26 is contains 13811 base pairs, and 19.40 pg would equal a one copy number equivalent.

The observed difference in molecular weight of the ZmUbiInt-hybridizing band in the lane containing *Kpn*I digested pZM26 (lane 7) and the ZmUbiInt-hybridizing band in the lane containing negative segregants spiked with *Kpn*I digested pZM26 DNA (lane 5) is due to the effect that the large amount (7.5 µg) of genomic DNA has on the migration of the small amount (2.80 pg) of plasmid in lane 5. We have previously observed that, relative to migration of purified plasmid DNA, the presence of a large amount of genomic DNA very often alters the migration of a small amount of plasmid during agarose gel electrophoresis. This observation does not affect the interpretation of the data.

3. Page 55. Figure 15. Lane 9 is blank. However, in Figure 5, page 49, a single band is present even though the same restriction enzyme and probe were used in Figure 15. Explain this discrepancy.
   As seen in figure 15 in the electronic version of the report, there is a faint band approximately 12kb in size in lane 9 which is similar to the band seen in lane 7. The band in lane 9 in figure 15 is similar to figure 5, as expected. Please refer to this electronic version of the Southern for figure 15.



Chapter 4. Agronomic Performance of Event MIR604 Hybrids

4. Page 62. Figure 18 does not have a caption or a reference. Provide both.
   There is no reference for this figure. This is an internal Syngenta document used to support NAFTA hybrid development and seed sales.

Chapter 7. Environmental Safety

5. Page 79. First paragraph bridges these petition data to that of Monsanto's Cry3A deregulated event. A comparison of the similarities and differences between the two proteins expressed would make your case more convincing. Provide a justification for using these data.
   The lack of hazard of mCry3A to NTOs is demonstrated by exposing representative indicator organisms to high doses of mCry3A in laboratory studies; the conclusion of lack of hazard stands independently of any data on native Cry3A. The native Cry3A data provide additional weight of evidence for species that we have not tested with mCry3A. Because there is no change in spectrum, apart from predicted intended effects on WCRW and NCRW, there is a high likelihood that NTOs insensitive to native Cry3A are also insensitive to mCry3A.

6. Page 79. Table 16. Collembola have routinely been used as an indicator of potential effects of Bt crops on beneficial decomposing organisms. The petition cites Sims and Martin (1997) as a Cry3A toxicity study for collembolans. However, additional information is needed to determine if the Sims and Martin (1997) study can be considered as a collembolan dietary toxicity test for event MIR604. Verification of functional equivalency of the Cry3A protein used by Sims and Martin to the Cry3A protein expressed in Syngenta's event MIR604 is needed. A description of the level of toxin tested by Sims and Martin (1997) relative to expression levels in event MIR604 plant tissue including roots and leaves also needs clarification to ensure the Collembola were exposed to at least 10 times the EEC.
   See Point #5. The native Cry3A data are simply being used to provide additional weight of evidence about lack of hazard.

7. Pages 102-103. Study design of *Aleochara bilineata* is unclear to us. Provide an explanation for the high mortality observed in the control group.
   The mortality is within the negative control validity criterion and 20% survival was exceeded on day 19. The general study design can be found in the IOBC Guidelines book. In this study, mortality is not an endpoint. We start with adult beetles and the test runs for 5 weeks. Note: no effect of IGR positive control on adults was expected or observed.

8. Page 108. Section C.3.g.1. Top of page. Nuclear hives of honey bees rather than individual larvae were exposed to mCry3A protein. Exposing individual larvae to Bt protein ensures exposure and allows for quantification of exposure. In the honey bee study, exposure to a concentration, not ingestion of a dose is claimed. It is unclear from the test methods described if the marked honey bee larvae ingested any Bt protein fed to the entire hive. The 100% mortality in cells in the positive control treatment confirms that the honey bees were ingesting the food source. If larvae were exposed, it is unclear what quantity of protein the larvae were exposed to and if the rate of exposure was uniform among larvae. Clarify methodology. The 100% mortality seen in the positive control treatment indicates that all the larvae were exposed. There is the possibility that the protein is deactivated by the nurse bees. In addition, the LC_{50} of mCry3A to larval honey bees was reported as >50 µg/g diet. Based on the method of exposure, it cannot be determined if this is the LC_{50} on per larvae basis. It is not possible to do a study using pollen as there is no expression of mCry3a in pollen. Clarify the method used to determine the LC_{50} on a per larvae basis. From the study described in Section C.3.g.1, the LC_{50} cannot be readily or accurately calculated on a per larvae basis. As discussed in the summary contained within the petition, the hive was exposed to the maximum concentration of test substance as the solution was entirely removed after every application. Further, 100% mortality was observed in hives treated with the toxic reference substance, indicating that the distribution of the test substances and controls by the adult bees was complete.

Chapter 8. Food and Feed Safety

9. Page 119. First paragraph, last sentence refers reader to 'pertinent summary information' that has been designated CBI (Vlachos and Joseph, 2003). Is there a published version of this information? Provide additional references. No published version available; the FR Notice for the tolerance exemption (EPA 2004a) is cited and as far as Syngenta is aware, is the only publicly available summary.

US EPA (2004a) Phosphomannose Isomerase and the Genetic Material Necessary for its Production in All Plants; Exemption from the Requirement of a Tolerance. 40 CFR Part 180. *Fed. Reg.* 69(94): 26770-26775, May 14, 2004. http://epa.gov/EPA-PEST/2004/May/Day-14/p10877.htm

Chapter 9. Environmental Consequences of Introduction

10. Pages 137-138. The examination of the degradation rate of mCry3A in soil was performed on a single soil sample. Therefore, the variation among the replicates taken from that sample should be reported as standard deviation, rather than standard error. Please correct. Also, additional literature

references should be provided to lend additional support to the argument that Cry proteins degrade rapidly within the soil environment.
The variation in the petition was mis-reported as standard error. The numbers reported in the revised petition are correct. The variation has been correctly reported as standard deviation in the revised petition.

Additional Literature:

Lab studies that show rapid degradation of Cry proteins

Hopkins, D. W., Gregorich, E. G. (2003) Detection and decay of the *Bt* endotoxin in soil from a field trial with genetically modified maize. *Europ. J. Soil Sci.* 54: 793-800.

Herman, R. A., Evans, S. L., Shanahan, D. M., Mihaliak, C. A., Bormett, G. A., Young, D. L., Buehrer, J. (2001) Rapid degradation of Cry1F delta-endotoxin in soil. Environ. Entomol. 30: 642-644.

These studies that show no accumulation of protein in the environment

Dubelman, S., Ayden, B. R., Bader, B. M., Brown, C. R., Jiang, C., Vlachos, D. (2005) Cry1Ab protein does not persist in soil after 3 years of sustained Bt corn use. Environ. Entomol. 34:915-921.

Head, G., Surber, J. B., Watson, J. A., Martin, J. W., Duan, J. J. (2002) No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use. Environ. Entomol. 31:30-36.

Ahmad, A., Wilde, G. E., Zhu, K. Y. (2005) Detectability of coleopteran-specific Cry3Bb1 protein in soil and its effect on nontarget surface and below-ground arthropods. Environ. Entomol. 34: 385-394.

A peer reviewed version of the EPA's BRAD on soil degradation

Sayre, P., Seidler, R. J. (2005) Application of GMOs in the U.S.: EPA research & regulatory considerations related to soil systems. Plant and Soil 275: 77-91.

Typographical errors noted:

11. Page 25. Third paragraph. Line 12. There is an extra "." following literature.
    The revised petition has been corrected.

12. Page 37. Second Vector backbone component, the reference should be Itoh and not Ioth.
    The revised petition has been corrected.

13. Page 61. Second paragraph, Line 6, data is a plural word and should be followed by "were" rather than "was".
    The revised petition has been corrected.

14. Page 79. First paragraph, Line 2, "e" is missing from "includ"e.
    The revised petition has been corrected.

Staff Contact
If you have any questions about the statements and information requested in this letter, please contact Catherine Preston at (301)734-5874 or facsimile number (301) 734-8669, or by e-mail, Catherine.A.Preston@aphis.usda.gov. Please refer to petition application number **04-362-01p** in your correspondence. APHIS looks forward to your reply.

Sincerely,

Neil Hoffman, Ph.D.
Director, Environmental Risk Analysis Division
Biotechnology Regulatory Services

Cc:
C. Preston
R. Alrefai
S. Koehler

Petition for the Determination of Non-Regulated Status

Corn Rootworm Protected Transformation Event MIR604

Revised

The undersigned submits this petition under 7 CFR 340.6 to Request that the Administrator, Animal and Plant Health Inspection Service, make a determination that the article should not be regulated under 7 CFR part 340.

Submitted by:
Henry-York Steiner
Regulatory Affairs Manager
Syngenta Seeds, Inc.
3054 East Cornwallis Road
Research Triangle Park, NC 27709-2257
Phone: (919) 541-8652
Fax: (919) 541-8535

Contributing Authors:

Robert Joseph[1], Linda Meyer[1], Alan Raybould[2], John Steffens[1], Jeff Stein[1] and Demetra Vlachos[1]

[1]Syngenta Seeds, Inc., 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257, USA; [2]Syngenta, Jealotts Hill International Research Center, Bracknell, Berkshire, RG42 6EY, UK

Date: May 17, 2006

CLAIM OF DATA CONFIDENTIALITY AND JUSTIFICATION FOR TREATMENT AS CBI

The information contained in Appendix 2 to this submission (hereinafter referred to as the "claimed information") is entitled to treatment as confidential business information ("CBI"), and should be protected against public disclosure. As discussed more fully below, the claimed information consists of valuable trade secret and confidential commercial information, the release of which would cause Syngenta substantial competitive harm. Consequently, this information should be protected against disclosure, pursuant to the Freedom of Information Act, 5 U.S.C. §§ 551 et seq. ("FOIA").

Nature of the Claimed Information

The claimed information consists of studies and data that have been developed by Syngenta in support of the commercialization of its new modified Cry3A (mCry3A) maize product. These data have been developed through extensive research, testing and analysis conducted and sponsored by Syngenta over several years, at a cost of several million dollars. These data provide essential product characterization information, key genetic sequence information, as well as information regarding the product's toxicological profile, its chemical profile, and its environmental fate and safety. Furthermore, these data provide an indispensable "road map" of the studies that are required in order to obtain the regulatory approvals necessary to market the company's new mCry3A maize product. Most of these studies have already been, or will be, submitted to the U.S. Environmental Protection Agency (EPA) under a claim of confidentiality, in order to obtain regulatory approvals for Syngenta's Cry 3A product under the Federal Insecticide, Fungicide, and Rodenticide Act, 7 U.S.C. §§ 136 et seq. ("FIFRA") and the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 321, et seq. ("FFDCA").

Basis for Confidentiality Claim

The Freedom of Information Act, in Exemption 4, specifically shields from public disclosure the following types of information:

> trade secrets and commercial or financial information obtained from a person and privileged or confidential.

See 5 U.S.C. § 552(b)(4). For purposes of this provision, the courts have defined "trade secret" information to mean a:

> secret, commercially valuable plan, formula, process, or device that is used for making, preparing, compounding, or processing of trade commodities and that can be said to be the end product of either innovation or substantial effort.

Public Citizen Health Research Group v. FDA, 704 F.2d 1280 (D.C.Cir. 1983). *See also, Sokolow v. FDA,* No. 1:97-CV-252, slip op at 7 (E.D. Tex. Feb 19, 1998), *aff'd* 162 F.3d 1160 (5th Cir. 1998) (information regarding the manner in which a drug is manufactured,

including "analytical methods employed to assure quality and consistency" and the "results of stability testing" are trade secret information for purposes of Exemption 4).

Similarly, APHIS, in its Policy Statement on the Protection of Privileged or Confidential Business Information, defines "trade secret" to include:

> production data, formulas, and processes, and quality control tests and data, as well as research methodology and data generated in the development of the production process.

50 Fed. Reg. 38561 (Sept. 23, 1985) (hereinafter referred to as APHIS's "Policy Statement on CBI").

The claimed information includes essential data pertaining to the formulation, characterization and stability of Syngenta's new mCry3A maize product. In addition, when examined as a whole, these studies provide a valuable road map of the studies needed to satisfy the data requirements for regulatory approval of the product under FIFRA and the FFDCA, which is an essential aspect of commercialization of the product. All of this information was developed at considerable expense and effort on the part of Syngenta. Accordingly, based on the standards enumerated by the courts and elaborated upon in APHIS's Policy Statement on CBI, the claimed information is eligible for treatment as "trade secret" information, and should be protected from disclosure under FOIA Exemption 4.

In addition, as discussed above, Exemption 4 shields from disclosure "commercial or financial information obtained from a person" that is "privileged or confidential." For purposes of this exemption, the courts have interpreted the term "commercial information" broadly, to include any information in which an entity has a commercial interest. *See Public Citizen Health Research Group v. FDA*, 704 F.2d 1280 (D.C. Cir. 1983). Moreover, the courts have found that such commercial information will be deemed "confidential" if its disclosure is "likely to cause substantial harm to the competitive position of the person from whom the information was obtained." *National Parks & Conservation Ass'n v. Morton*, 498 F.2d 765 (D.C. Cir. 1974). Similarly, APHIS has explained in its Policy Statement on CBI that:

> Documents containing commercial or financial information will be deemed confidential if review establishes that substantial competitive harm would result from disclosure.

50 Fed. Reg. 38561. According to the agency, confidential commercial information can include "safety data, efficacy or potency data, and environmental data." *Id.*

The courts have held that in order to demonstrate that information is confidential commercial information within the scope of Exemption 4, it is sufficient to show that there is actual competition and a "likelihood of substantial competitive injury" if the information is disclosed. *See CNA Financial Corp. v. Donovan*, 830 F.2d 1132 (D.C. Cir. 1987). Similarly, APHIS has explained in its interpretive guidance that a person seeking to protect confidential commercial information from disclosure must demonstrate that (i) the person faces active competition in the area to which the information pertains,

and (ii) release of the information would cause substantial competitive harm. 50 Fed. Reg. 38561.

There is no question that Syngenta faces active competition in its field of agricultural biotechnology, including competition from large multinational corporations such as Monsanto, Dow AgroSciences, Dupont, and others. Many of these competitors are developing or attempting to develop similar products that express insecticidal proteins. Thus, Syngenta satisfies the first criterion for protection of confidential commercial information under Exemption 4.

In addition, disclosure of the claimed information would likely result in substantial competitive harm to Syngenta. As alluded to previously, the claimed information consists of data that provide essential product characterization and composition information, as well as information regarding the product's toxicological profile, its chemical profile, and its environmental fate and safety. These data have been developed through extensive research, testing and analysis, at substantial cost to Syngenta. Access to this product information (including information pertaining to manufacturing and analytical protocols, methods and techniques) could provide our competitors with knowledge that would assist those competitors in developing similar products to compete with Syngenta's mCry3A product. Moreover, competitors would reap the benefits of Syngenta's research, thereby accelerating their ability to bring competitive products to market, without having to incur any of the costs of developing those data. In addition, access to the claimed information would provide competitors with commercially valuable insights into the product lines that Syngenta is intending to develop and bring to market, as well as the likely timetable for Syngenta's commercialization of those product lines.

Similarly, the claimed information, taken as a whole, provides a template of the studies that are needed to satisfy the data requirements necessary for regulatory approval of Syngenta's product under FIFRA and the FFDCA. In addition, the studies themselves have intrinsic value under these statutes, as reflected in the mandatory data compensation provisions in FIFRA Section 3(c)(1)(F) and FFDCA Section 408(i). Specifically, FIFRA Section 3(c)(1)(F) provides for a ten-year period of "exclusive use" of studies submitted to support the registration of new active ingredients. During this ten-year period, other manufacturers can rely on these "exclusive use" studies to support their own regulatory approvals under FIFRA only with the express permission of the original data submitter. 7 U.S.C. § 136a(c)(1)(F)(i). Following this period of exclusive use, other manufacturers are required to compensate the original data submitter if they wish to rely on those data to support their own (competitor product) registrations under FIFRA. *Id.* Similar data compensation protections are provided for studies submitted under Section 408(i) of the FFDCA. 21 U.S.C. § 346a(i). Consequently, if APHIS were to release these studies to the public, Syngenta would be deprived of potentially millions of dollars of compensation as a result of competitors being able to rely upon Syngenta's studies in order to obtain regulatory approvals their own products under FIFRA and the FFDCA, without having to compensate Syngenta for access to those studies.

Based on the foregoing, the claimed information is eligible for protection under Exemption 4 of FOIA.

Finally, Exemption 3 of FOIA protects from public disclosure information that is specifically exempted from disclosure by another statute. *See* 5 U.S.C. § 552(b)(3). Section 10 of FIFRA contains several provisions that restrict or prohibit the release of information submitted under the Act. Among other things, Section 10 of FIFRA strictly prohibits the release of such information to foreign or multinational companies. *See* 7 U.S.C. § 136h(g). Section 408 of the FFDCA provides similar protections against disclosure of data submitted under that statute. The restrictions on disclosure set forth in FIFRA and the FFDCA provide an independent basis for requiring APHIS to protect Syngenta's claimed information against disclosure, pursuant to Exemption 3 of FOIA.

Summary

Corn rootworm (*Diabrotica* spp) is a Coleopteran pest that costs farmers an estimated 1 billion dollars in crop damage and control costs each year. Rootworm larvae feed upon the root systems of corn plants causing damage that can lead to plant stress and/or lodging, resulting in yield loss. Crop rotation and insecticide application have been employed to reduce rootworm damage. However, corn rootworm variants and resistant phenotypes have reduced the effectiveness of these pest management strategies. Syngenta has developed a corn event, MIR604, that expresses a modified Cry3A (mCry3A) insect control protein which provides for excellent protection from rootworm larval damage during field trials conducted annually since 2001. Hybrids derived from Event MIR604 provide economic control of three major corn rootworm pest species in the U.S., western corn rootworm (*Diabrotica virgifera virgifera* Le Conte; WCRW), northern corn rootworm (*D. longicornis barberi* Smith and Lawrence; NCRW) and Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith; MCRW).

The amino acid sequence of the mCry3A protein corresponds to that of the native Cry3Aa2 protein from *B. thuringiensis* subsp. *tenebrionis (B.t.t.)*, except that (1) its N-terminus corresponds to methionine-48 of the native protein and (2) a consensus cathepsin G protease recognition site has been introduced. This cathepsin G recognition site is recognized by a chymotrypsin-like serine protease that is found in the midguts of CRW larvae providing greater toxicity towards certain species of CRW. mCry3A exhibits no toxicity toward either other corn pests (including the various Lepidoptera that attack corn) or against non-pest insect species.

Corn hybrids derived from Syngenta's transformation Event MIR604 contain two transgenes: (1) the modified *cry3A (mcry3A)* gene encoding the mCry3A insect control protein and (2) the *pmi* (*manA*) gene from *Escherichia coli,* which encodes the enzyme phosphomannose isomerase (PMI) as a selectable marker. Expression of the *mcry3A* gene in Event MIR604 is driven by the maize promoter from a metallothionein-like gene and *pmi* is driven by the maize polyubiquitin promoter. Data from Southern analysis and DNA sequencing demonstrate that MIR604 hybrids (1) contain a single copy of both the *mcry3A* and *pmi* genes, (2) do not contain any of the backbone sequences from the transformation plasmid pZM26, (3) the overall integrity of the intended insert and contiguousness of the functional elements have been maintained and (4) both *mcry3A* and *pmi* are inherited in the expected Mendelian ratio for a single locus.

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604. The *mcry3A* gene was transformed into a heterogenous genetic background selected for its suitability to transformation by *Agrobacterium*. Event MIR604 was crossed into a number of elite inbred lines and material generated from these crosses was used in molecular, expression, agronomic and other studies. Control germplasm was either near isogenic hybrids or negative segregants identified in field trials. To reduce the effect of insect damage to corn ears by lepidopteran pests, some agronomic experiments used MIR604 in a breeding stack with Bt11 which expresses the Cry1Ab protein (Bt). In these cases controls consisted of the corresponding Bt11-only hybrids.

MIR604 hybrids have been field tested for three years (2001-2003) at more than 25 locations in several states across the US cornbelt and have demonstrated statistically significant reductions in root damage relative to non-transgenic controls, equivalent or greater yields compared to near isogenic controls and no consistent differences in a number of agronomic traits examined. In addition, MIR604 hybrids are nutritionally equivalent compared to the near-isogenic controls, and to other commercial hybrids.

A comprehensive environmental safety assessment was conducted using bacterial and plant produced mCry3A test substances that showed mCry3A has a limited spectrum of activity within the Chrysomelidae family of coleopteran species, specifically to the major corn pest species WCRW, NCRW and MCRW. No pesticidal activity against other species tested was observed. Hazard and exposure assessments were performed on representative non-target species (bobwhite quail, rainbow trout, insidious flower bug, seven spot ladybird, rove beetle, ground beetle, honeybee, earthworm) and no harmful effects were detected in any of these tests.

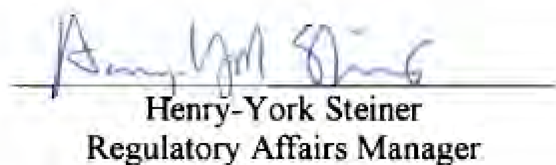
A thorough mammalian safety assessment was conducted for both mCry3A and PMI proteins as expressed in Event MIR604 hybrids. No adverse effects were observed for either protein. The large body of data and information described herein support the conclusion that the modified Cry3A protein as expressed in Event MIR604 corn will pose no hazard to humans or domestic animals upon commercial approval of the use of Event MIR604 corn. Additionally, the PMI protein has been granted a permanent exemption from tolerance in all crops from the U.S. EPA based on an extensive body of data that demonstrates this selectable marker presents no risk to humans, animals or the environment.

Event MIR604 hybrids have been field tested by Syngenta Seeds, under Notifications granted by USDA APHIS since 2001 in the primary corn growing regions of the midwestern US as well as in Hawaii and Puerto Rico. Information and data collected during these trials indicates that Event MIR604-derived hybrids exhibit no plant pathogenic properties, have no impact on biodiversity and are unlikely to harm other insects that are beneficial to agriculture. As corn does not establish weedy populations and has lost the ability to survive outside cultivation, MIR604 hybrids are no more likely to be a weed than non-transgenic maize. In addition, mCry3A is not expressed in pollen and thus MIR604 hybrids are unlikely to have an adverse ecological effect due to pollen exposure. Event MIR604-derived hybrids will lead to minimal exposure of the environment to mCry3A, will not promote gene flow increase the weediness or plant pest potential of any other cultivated plant or wild species or have an impact on non-target species, including humans.

Based on the information and data contained in this petition, Syngenta requests that USDA-APHIS/BRS make a determination of non-regulated status for Corn Rootworm Protected Transformation Event MIR604, any progeny derived from crosses between MIR604 and other corn varieties, and progeny derived from crosses of MIR604 with other transgenic corn varieties that have received non-regulated status under 7 CFR Part 340.

Statement of Grounds Unfavorable

The undersigned certifies, that to the best of their knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner that are unfavorable to the petition.



Syngenta Seeds, Inc.
3054 East Cornwallis Road
Research Triangle Park, NC 27709-2257
Phone: (919) 541-8652
Fax: (919) 541-8535

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Abbreviations Used in this Petition

aadA	Adenylytransferase gene
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service
Bp	Base pair
Bt	*Bacillus thuringiensis*
Bwt	Body weight
C	Concentration
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
ColE1	*E.coli* origin of replication
Cry	Crystal protein delta endotoxins
DDD	Daily dietary dose
DNA	Deoxyribonucleic acid
DT_{50}	Time to dissipation of 50% of the initial bioactivity
EEC	Estimated exposure concentration
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FFDCA	Federal Food Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FIR	Food Intake Rate
HPLC	High Performance Liquid Chromatography
IFBC	International Food Biotechnology Council
ILSI	International Life Sciences Institute
IRM	Insect resistance management
Kb	Kilobase
kDa	Kilodalton
Kg	Kilogram
LB	Left border
LC_{50}	50% lethal concentration
LC_{90}	90% lethal concentration
LD_{50}	50% lethal dose
LOQ	Level of quantitation
manA	Phosphomannose isomerase gene from *Escherichia coli*
mcry3A	gene in event MIR604 encoding the modified Cry3A insecticidal protein
mCry3A	the modified Cry3A insecticidal protein expressed in Event MIR604
µg	Microgram
mg	Milligram
MOE	Margins of exposure
MRID No.	Master Record Identification Number
MTL	maize promoter from a metallothionein-like gene
NOEC	No observable effect concentration

NOEL	No observable effect level
nos	Nopaline synthase terminator
OECD	Organization for Economic Cooperation and Development
PBN	FDA Pre-market Biotechnology Notification
PCR	Polymerase chain reaction
PMI	Selectable marker protein phosphomannose isomerase
pmi	Gene in event MIR604 encoding the selectable marker protein
PPQ	Plant Protection and Quarantine
pZM26	Plasmid employed to create event MIR604
RB	Right border
RepA	Bacterial replication protein
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SOP	Standard operating procedure
spec	Spectinomycin resistance gene
T0	First generation transgenic
T1	Second generation transgenic
T-DNA	Transfer DNA
Ti-plasmid	Tumor inducing plasmid
ubq3	Ubiquitin-3 gene isolated from *Arabidopsis*.
Ubq3int	Promoter plus the first intron isolated from *Arabidopsis* ubiquitin-3 gene.
USDA	United States Department of Agriculture
US EPA	United States Environmental Protection Agency
VS1	*Agrobacterium* origin of replication
WHO	World Health Organization

Chapter 1. Syngenta Seeds Petition for the Determination of Non-Regulated Status of Corn Event MIR604

A. RATIONALE FOR THE DEVELOPMENT OF EVENT MIR604 CORN

Corn rootworm (*Diabrotica* spp), a Coleopteran pest ubiquitous to corn growing areas of the United States, costs farmers an estimated 1 billion dollars in crop damage and control costs each year (Ostlie, 2001; Payne *et al*., 2003). Rootworm larvae feed upon the root systems of young, developing corn seedlings. Moderate pruning of roots by rootworms can lead to plant stress during periods of inadequate moisture due to the inability of the corn plant to adequately translocate water and minerals from the roots to the rest of the plant. Corn plants suffering from moderate to severe root pruning are also susceptible to lodging during rain and wind storms (Wedberg, 1996). Corn ears on lodged plants may under develop or not be harvestable due to inaccessibility to harvest equipment.

The rootworm life cycle plays an integral role in the extent and severity of damage caused by this pest. Rootworm eggs over-winter in the top six inches of soil and begin hatching from May to mid-June depending upon the environmental conditions. The larvae go through three larval stages (instars) all of which feed on corn roots. The 1st instar larvae feed on the smaller, branching roots while the later instars feed on the inner root tissues and invade the brace roots (Wedberg, 1996). As the rootworm eggs will hatch over a long period of time, different larval stages will be present at any given time. After approximately 3 weeks of feeding the larvae enter the pupal stage whereupon the adult beetle will emerge after 6 to 10 days. Females reach sexual maturity slowly and do not begin laying eggs until 2 weeks after mating. A female corn rootworm beetle can lay up to 1000 eggs over a several week period. The eggs are in a diapause condition and must go through a cold incubation before the larvae will hatch the following spring.

Management practices employed to reduce rootworm damage generally fall into two categories: (1) crop rotation and (2) application of chemical insecticides. As the preferred diet of rootworm larvae is corn roots and corn rootworms beetles lay their eggs in a diapause condition, farmers have adopted a crop rotation strategy that can significantly interrupt the corn rootworm life cycle. By following a first year corn crop with soybeans or other non-corn crop in the second season's planting, the population of emerging beetles is reduced as the primary food source (corn roots) for the hatching larvae is not available. With the pest population reduced, corn can then be planted again in the third season with reduced risk of economic impact. This crop rotation (primarily corn and soybeans) has been used since the mid-70s as a very effective non-chemical control strategy for corn rootworm.

In areas where crop rotation has been used extensively, rootworm variants have evolved which have minimized the effectiveness of the crop rotation strategy. The first adaptation is referred to as the 'extended diapause' variant and was first documented in Northwestern Iowa. In this instance, eggs laid by Northern CRW beetles remain in diapause 2 or more winters before hatching. In 2002, USDA-NASS (USDA, 2002)

estimated that this extended diapause variant is present in 9.8 million acres of corn in the U.S.

The second adaptation, referred to as the ‘soybean variant’, was first documented in east central Illinois. In this case, the Western CRW adult beetle migrates from the cornfield and deposits its eggs in neighboring soybean fields, which hatch in the following year’s corn crop. According to USDA-NASS estimates, in 2002, 7.1 million acres of corn were planted in the heart of the soybean variant infestation zone that covers large portions of Illinois and Indiana. According to Payne *et al*. (2003), this new variant of CRW has also spread through most of northern Indiana, eastern Illinois, southern Michigan and western Ohio. Given historic movement patterns, the new variants may soon spread as far west as eastern Iowa (Onstad *et al*., 1999). This expanded area is equivalent to 16 million acres with the westward expansion being the greatest threat. Western CRW have also been detected in soybean fields located in counties extending to the Wisconsin border.

The second common practice of corn rootworm control is soil applied insecticides and seed treatments. Most chemical control practices target the larval stage, applying insecticide into the soil to protect roots from larval feeding. It is important that the insecticide provides effective control for at least 7-10 weeks, as eggs hatch over a 3-6 week period and feeding continues for an additional 3-4 weeks. There are several factors that affect insecticide performance including planting date, insecticide placement, insecticide characteristics and environment. Seed treatments are another form of chemical control available to corn growers. However their effectiveness is generally poor in circumstances where CRW pressure is moderate to high. In 2003, farmers applied nearly 6 million pounds of insecticide over 21 million acres at a cost of $216,000,000.

Syngenta scientists have developed a corn event, designated Event MIR604, that expresses a novel proteinaceous active ingredient that provides for excellent protection from rootworm larval damage during field trials conducted since 2001. Event MIR604 expresses a modified Cry3A insect control protein that provides economic control of the three major corn rootworm species in the U.S., western corn rootworm (*Diabrotica virgifera virgifera* Le Conte), northern corn rootworm (*D. longicornis barberi* Smith and Lawrence) (Chen and Stacy, 2003) and Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith). Farmers who plant this product will realize significant benefits of consistent performance and protection against yield loss from rootworm damage.

Syngenta Seeds, Inc. requests the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, make a determination that the article Event MIR604, described herein, should not be regulated under 7 CFR part 340.

B. BACKGROUND INFORMATION

B.1. Modified Cry3A Plant-Incorporated Protectant

Syngenta Seeds has developed a genetically improved line of corn, Event MIR604, which produces a modified Cry3A (mCry3A) insect control protein. The pesticidal mCry3A protein is related to a class of insecticidal proteins that are produced in crystalline inclusions during sporulation of the gram-positive soil bacterium *B. thuringiensis*. The source of the native, unmodified Cry3A protein is *B. thuringiensis* subsp. *tenebrionis* (*B.t.t.*) (Sekar *et al.*, 1987). mCry3A has a similar spectrum of activity to the native Cry3A, but with additional very markedly enhanced, commercially exploitable toxicity towards the Western corn rootworm (WCRW; *Diabrotica virgifera virgifera* Le Conte), Northern corn rootworm (NCRW; *D. longicornis barberi* Smith and Lawrence) and Mexican corn rootworm (MCRW; *Diabrotica virgifera zeae* Krysan and Smith), all of which are major Coleopteran pests of corn in the U.S. Importantly, mCry3A has no activity either against other corn pests including the various Lepidoptera that attack corn or against non-pest insect species.

The amino acid sequence of the mCry3A protein corresponds to that of the native Cry3A protein from *B.t.t.*, except that (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 (Rabe, 2004). This cathepsin G recognition site has the sequence alanine-alanine-proline-phenylalanine (AAPF), and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein. Cathepsin G is a chymotrypsin-like serine protease.

Syngenta scientists recently discovered that the midguts of WCRW larvae contain cathepsin G-like activity (Chen and Stacy, 2003). The mCry3A protein as expressed in Event MIR604 plants was designed to have significantly greater toxicity to WCRW by inserting the cathepsin G site that could be recognized by this protease (Garcia-Alonso and Vlachos, 2003).

B.2. Molecular Analysis of DNA Insert in Event MIR604

Event MIR604 corn was created via *Agrobacterium*-mediated transformation of plasmid pZM26 into embryonic corn tissue suitable for transformation. A complete description of the donor genes, regulatory sequences, transformation vector and transformation method is provided in Chapter 3, **Molecular Analysis of Event MIR604** (Rabe, 2004).

Maize (corn) derived from Syngenta's transformation Event MIR604 contains two transgenes: (1) the modified *cry3A (mcry3A)* gene encoding the mCry3A insect control protein and (2) the *pmi* (*manA*) gene from *Escherichia coli,* which encodes the enzyme phosphomannose isomerase (PMI) as a selectable marker. PMI has no pesticidal properties and is considered an 'inert ingredient' by the US EPA. PMI allows transformed corn cells to utilize mannose as a sole carbon source, while corn cells lacking the *pmi* gene fail to grow (Negrotto *et al.*, 2000).

Expression of the *mcry3A* gene in Event MIR604 is driven by the maize promoter from a metallothionein-like gene (MTL promoter; de Framond, 1991); it confers root-preferential gene expression in corn. Expression of the *pmi* gene in Event MIR604 is driven by the maize polyubiquitin promoter (Christensen *et al.,* 1992), which confers constitutive expression in monocots.

Data from Southern hybridisation analysis and DNA sequencing demonstrate that single copies of both the *mcry3A* and *pmi* genes are present in Syngenta's corn Event MIR604. Additionally, Event MIR604 does not contain any of the backbone sequences from the transformation plasmid pZM26. Sequence analysis of the entire T-DNA insert present in Event MIR604 confirms that the overall integrity of the intended insert and contiguousness of the functional elements have been maintained. A 43 base-pair truncation at the right border junction of the T-DNA insert and 44 base-pair truncation at the left border junction of the T-DNA insert were identified. Three single nucleotide changes were also identified relative to the intended DNA sequence. One of these changes occurred within a promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and give rise to two amino acid changes. The nucleotide changes identified in the *pmi* gene in Event MIR604 resulted in the substitution of (1) alanine in place of valine-61 and (2) histidine in place of glutamine-210 in the amino acid sequence of the PMI protein. These substitutions have not resulted in any apparent functional change in PMI as expressed in Event MIR604 (Hill, 2004).

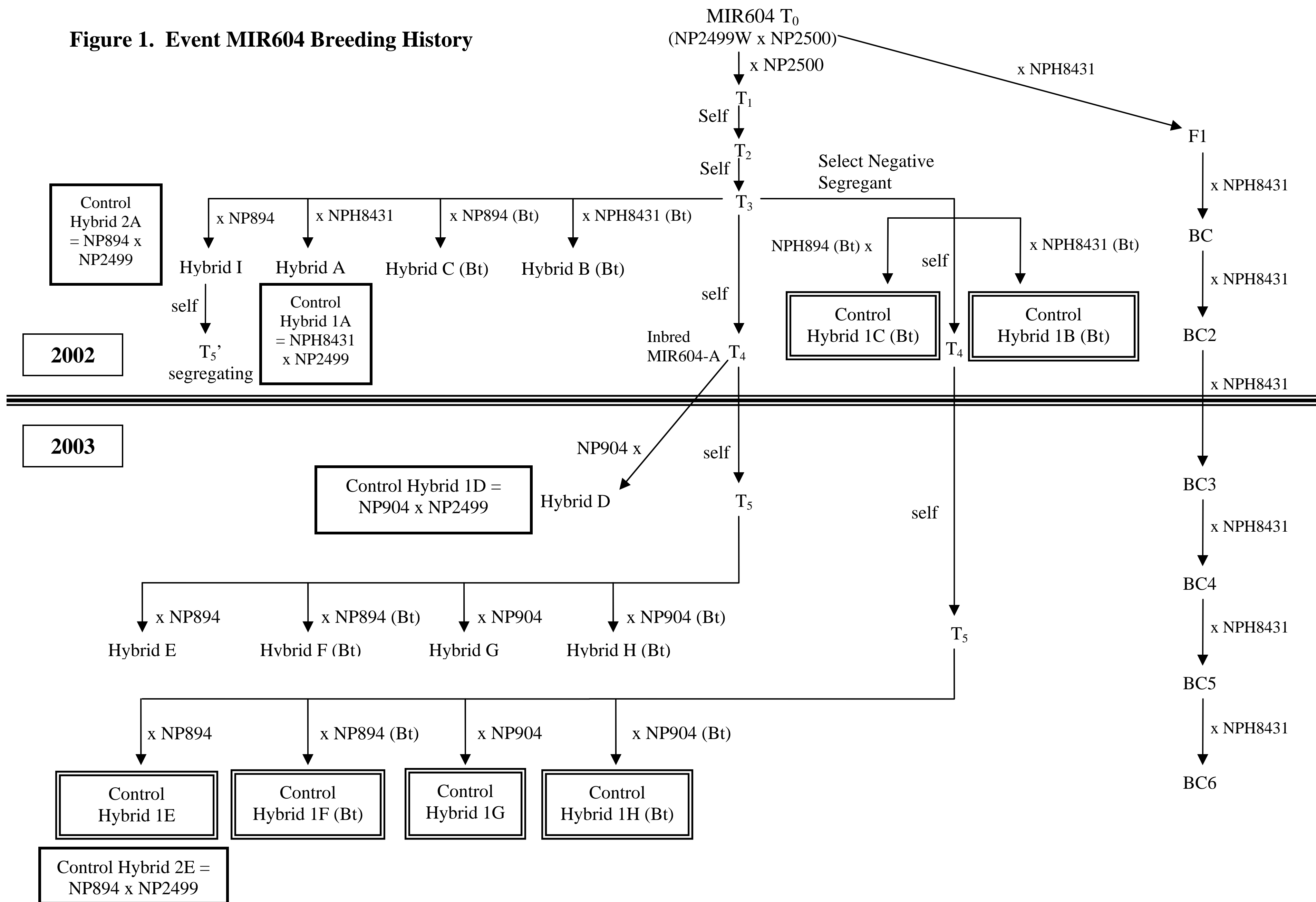
Statistical analysis confirmed the expected Mendelian inheritance ratio for both *mcry3A* and *pmi*. Additional information confirming the stability of expression of these genes in Event MIR604 is available in an accompanying data volume (Joseph and Hill, 2003). Levels of mCry3A and PMI proteins were determined to be stable in MIR604 plants over four successive backcross generations.

B.3. Breeding History of Event MIR604

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604 (see Figure 1). The pZM26 plasmid was transformed into a heterogenous genetic background (NP2499W x NP2500) derived from a cross between two dent corn inbred lines selected for its suitability to transformation by Agrobacterium. Once transformed, this line, designated Event MIR604, was crossed back into one of the parental inbred lines and then selfed 2 to 4 times to fix the transgene in the germplasm. A number of elite inbred lines were crossed into the Event MIR604 line and material generated from these crosses was used in agronomic and other studies (Figure 1). The early and late maturity MIR604 hybrids were made up by crossing different inbred corn lines which display varying days to maturity (maturity testers) into the fixed MIR604 germplasm.

Control germplasm was derived by one of two methods. In some experiments, the non-transgenic, near isogenic hybrids were constructed by using non-transformed germplasm closely matched to the genetic background of MIR604 for that particular stage of the breeding pedigree (indicated by single lined boxes in Figure 1). In other experiments, negative segregants were identified and used as breeding partners in crosses to elite inbred lines (indicated by double lined boxes in Figure 1).

Figure 1. Event MIR604 Breeding History



In order to reduce the confounding effect on yield of insect damage to the corn ears by lepidopteran pests, in some trials MIR604 was deployed in a breeding stack with Bt11 which expresses the Cry1Ab protein (indicated by '(Bt)' in Figure 1). In these cases controls consisted of the corresponding Bt11-only hybrids.

B.4. Agronomic Performance of Event MIR604

In 2002 and 2003, 22 different hybrids derived from traditional breeding of Event MIR604 were evaluated at 33 locations in the states of IA, IL, IN, KS, KY, MN, MO, NE, SD, TX, WI, for either insect efficacy evaluation or agronomic assessment, and yield (Chapter 4, **Agronomic Performance of Event MIR604 Hybrids**.). Event MIR604 hybrids consistently demonstrated statistically significant reductions in root damage relative to non-transgenic controls at all locations. Event MIR604 also provided statistically significant increases in harvestable yield compared to near isogenic controls under adverse growing conditions (e.g., drought), and was either equivalent or in some cases better in yield performance under non-adverse growing conditions. For all other agronomic traits, sporadic statistical differences were observed between Event MIR604 and its non-transgenic control but none were consistent over the two years of data collection or across locations.

B.5. Compositional Analysis of Event MIR604

Key nutritional components in maize grain and whole plants (forage) derived from Event MIR604 and near-isogenic control plants were compared. The whole plants and grain analyzed were from hybrid pairs (a hybrid pair consisting of transgenic and near-isogenic control plants) grown at 12 locations in the USA over two growing seasons (2002 and 2003). As would be expected from an analysis of this size, sporadic statistically significant differences were observed for some parameters between the MIR604 transgenic and near-isogenic controls. All components evaluated in this study were within the range of reported literature values for maize, with the exception of two components, potassium and phytosterols. At the time of analysis, a control range for concentrations of potassium in conventional corn forage was not available. Both control and transgenic values for phytosterols in grain were below the average concentration reported in the literature. . For all other analytes, no consistent patterns emerged to suggest that biologically significant changes in composition or nutritive value of the grain or forage had occurred as an unintended result of the transformation process or expression of the transgene. The conclusion based on these data is that grain and forage from genetically modified MIR604 hybrids are substantially equivalent in composition to the near-isogenic controls, and other commercial hybrids. Further details of this study can be found in Chapter 5, **Compositional Analysis of Event MIR604.**

B.6. The Mode of Action of Modified Cry3A Protein

Syngenta scientists have investigated the solubilization, proteolytic processing, receptor binding, and pore forming properties of the mCry3A protein in side-by-side comparisons with native Cry3A. The results showed that the solubility, and pore forming properties of mCry3A protein were similar compared to native Cry3A protein. The introduction of the cathepsin G recognition site into the Cry3A protein results in a much more rapid and complete processing of the 67 kDa mCry3A protein to a 55 kDa product

as compared to native Cry3A protein. Also, binding of the 67 kDa mCry3A protein to first instar WCRW membranes is similar to that of native Cry3A, but binding of the 55 kDa product is enhanced in the case of the mCry3A protein. Thus, mCry3A would be expected to behave, from a mode of action standpoint, as other pore-forming Cry proteins characterized to date. Further details of these studies can be found in Garcia-Alonso and Vlachos (2003) and in Chen and Stacy (2003) and Steiner et. al. (2005).

B.7. Environmental Safety Assessment

A comprehensive environmental safety assessment has been conducted on mCry3A as the active principle in Event MIR604. This assessment included a literature and data review of the specificity of both native Cry3A and mCry3A for vertebrate and non-vertebrate species. Laboratory assessments of toxicity to vertebrate and non-vertebrate organisms as well as carefully chosen non-target organisms were also performed. Further details of these studies are provided in Chapter 7, **Environmental Safety**.

The data show that the toxicity of native Cry3A proteins is specific to certain Coleoptera of the families Chrysomelidae, Tenebrionidae and Curculionidae. No toxic effects have been reported on non-target insect species or on other invertebrates or vertebrates. The modified Cry3A (mCry3A) toxin as expressed in Event MIR604 maize (corn) has an extended spectrum of activity within the Chrysomelidae family of coleopteran species, such that it has enhanced toxicity to the major corn pest species WCRW, NCRW and MCRW, but its activity against other species tested to date appears to be unchanged.

Several non-target species were selected for individual laboratory safety assessment based on their potential for exposure in an agricultural setting, taxonomic similarity to the target pest, feasibility of testing and to meet certain U.S. EPA requirements. The test species included: bobwhite quail (*Colinus virginianus*), rainbow trout (*Oncorhynchus mykiss*), insidious flower bug (*Orius insidiosus*), seven spot ladybird (*Coccinella septempunctata*), a rove beetle (*Aleochara bilineata*), ground beetle (*Poecilus cupreus*), honeybee (*Apis mellifera*), earthworm (*Eisenia foetida*). Hazard and exposure assessments were performed on each test species to determine minimum marginal exposure limits at pre-selected, appropriate endpoints for each species. No harmful effects were detected in any of the tests performed on the non-target species (Raybould, 2004b). The large body of ecological toxicity data support the conclusion that the modified Cry3A protein as expressed in Event MIR604 corn will pose little to no risk to the environment.

B.8. Food and Feed Safety Assessment

A thorough assessment of the safety, including the potential for exposure, mammalian toxicity and allergenicity, of both the mCry3A and PMI proteins as expressed in Event MIR604 has been conducted. Details of the safety assessment and supporting studies can be found in Chapter 8, **Food and Feed Safety.** The data support the conclusion that neither the mCry3A nor the PMI protein as expressed in Event MIR604-derived corn will pose health hazards to humans or domestic animals upon commercial approval of the use of Event MIR604-derived corn.

Although prior dietary exposure to the mCry3A protein has not occurred, it is conceivable that a low level of dietary exposure to the native Cry3A protein has occurred, as it is registered for use in the U.S. in various *B.t.*-based microbial insecticides (Trident, Foil, Ditera, M-Trak, Novodor, Raven) and NewLeaf® Bt potatoes, and is permanently exempt from food and feed tolerances.

Expression levels of mCry3A in Event MIR604 plants were assessed across all growth stages. Mean mCry3A levels measured in leaves, roots and whole plants ranged from *ca.* 3 - 23 μg/g fresh wt. (4 - 94 μg/g dry wt.), *ca.* 2 - 14 μg/g fresh wt. (7 - 62 μg/g dry wt.), and *ca.* 0.9 - 11 μg/g fresh wt. (3 - 28 μg/g dry wt.), respectively (Joseph and Hill, 2003). Mean mCry3A levels measured in kernels at seed maturity and senescence ranged from *ca*. 0.6 – 1.4 μg/g fresh wt. (0.8 – 2.0 μg/g dry wt.).

Modified Cry3A protein (mCry3A) was analyzed in wet- and dry milled fractions generated from standard food processing procedures carried out on maize (corn) grain derived from Event MIR604, together with a corresponding non-transgenic control (Joseph and Kramer, 2003). Quantifiable levels of mCry3A were detected in various wet- and dry-milled fractions ranging from 2.12 μg mCry3A/g in flaking grits to below detectable levels in coarse fiber, germ, and starch. Although the concentration of mCry3A measured in the flour used in the preparation of corn chips was 0.32 μg mCry3A/g, no mCry3A was detected in the corn chips. Similarly, mCry3A was not detectable in oil, whereas the starting material, flaking grits, contained the highest level of mCry3A.

Both mCry3A and PMI recombinant proteins expressed in *E. coli*, and demonstrated as equivalent to their respective proteins as expressed in Event MIR604-derived corn tissue, have been evaluated in acute oral mouse toxicity studies (Johnson, 2003; Kuhn, 1999). No adverse effects were observed for mCry3A at a dose of 2377 mg/kg body weight and no adverse effects were observed for PMI at 3080 mg/kg body weight. Both mCry3A and PMI protein sequences were evaluated for similarity to known toxin and allergen sequences. Neither mCry3A nor PMI showed significant amino acid homology to any proteins identified as or known to be toxins. In addition, mCry3A did not show any significant homology to known or putative allergens. The PMI protein sequence was found to have one region of sequence homology of eight contiguous amino acids with a recently identified frog allergen α–parvalbumin (Hilger *et al*., 2002). Upon subsequent specific serum testing with patient serum, it was established that PMI does not cross react with antibodies which recognize the frog α–parvalbumin allergen. This indicates that the allergic patient's serum IgE does not recognize any portion of the PMI protein as an allergenic epitope and, therefore, the low degree of amino acid sequence homology between the PMI protein and α–parvalbumin from edible frog is not biologically relevant or indicative of allergenic potential for PMI (Hart and Rabe, 2004d). Additionally, neither mCry3A nor PMI are derived from a known source of oral allergens, both are heat labile (Joseph, 2003; Hill, 2003), and both are readily digested in simulated mammalian gastic fluid (Joseph and Graser, 2003c; Privalle, 1999), further supporting the conclusion that neither mCry3A nor PMI are likely food allergens.

B.9. Environmental Consequences of Introduction of Event MIR604-derived Hybrids

Expression of mCry3A in Event MIR604-derived hybrids has been shown to occur in root and other tissues, and during the course of normal agricultural practice, the active principle mCry3A could *theoretically* enter neighboring environments through establishment of weedy populations of MIR604 plants outside fields, through transfer and expression of the mCry3A gene in other organisms via sexual hybridization or horizontal gene transfer, through off-crop movement of MIR604 pollen or via degradation of plant tissue and subsequent movement of mCry3A through soil.

The risk that mCry3A could persist or spread more widely because of gene flow or the establishment of weedy populations of MIR604 is very low given that species of *Zea* other than cultivated corn are not recorded outside botanical gardens in the USA and those related species which are widespread do not hybridize readily with cultivated corn. Corn has lost the ability to survive outside cultivation and is unlikely to form self-sustaining weedy populations in agriculture; it is easily controlled in subsequent crops with selective herbicides and seed dispersal is limited because seeds are held inside the husks of the cob. These properties indicate that exposure to mCry3A protein will be limited to the fields in which MIR604 corn will be grown and mCry3A is unlikely to persist in fields for a long period after grain from MIR604 hybrids have been harvested.

In addition to the low exposure risk, MIR604 is simpler to use and provides more consistent performance that results in superior efficacy and a positive yield impact compared to the chemical alternatives. In terms of reduction of root damage, the efficacy data show that MIR604 corn performs significantly better than non-treated and chemically treated controls. Yield data show an overall positive trend comparing MIR604 corn to both non-treated and chemically treated controls particularly in unfavorable growing conditions. To help insure the continued utility and long-term efficacy of MIR604 hybrids an appropriate insect resistance management (IRM) program has been proposed.

Introduction of Event MIR604-derived hybrids will lead to minimal exposure of the environment to mCry3A, will not promote gene flow or weediness in corn and provide yield and efficacy benefits, thus producing minimal consequence when introduced as a commercial product. Further detailed information can be found in Chapter 9, **Environmental Consequences of Introduction.**

C. REGULATORY PERMIT STATUS

An application for an Experimental Use Permit for mCry3A as a plant-incorporated protectant (Experimental Use Permit for Field Testing of the Modified Cry3A *Bacillus thuringiensis* Insect Control Protein as Expressed in Event MIR604-Derived Corn Plants (67979-EUP-U)) was submitted to the U.S. EPA on December 15, 2003 and approved on March 23, 2005. A permanent exemption from the requirement of tolerances for the inert marker protein, PMI, was recently granted for all crops (U.S. EPA, 2004a) on May 14, 2004. An amendment/extension to the existing Experimental Use Permit for MIR604 was submitted to the US EPA on August 16 2005 and approved by EPA on March 2 2006.

Syngenta Seeds, Inc. has also filed a U.S. EPA FIFRA Section 3 registration application on April 30, 2004 titled Plant-Incorporated Protectant Active Ingredient Modified Cry3A Protein as Expressed in Event MIR604-Derived Corn Plants (67979-L). The Section 3 application is expected to be approved by EPA in 2006. Accompanying this application was a request to the US EPA for a permanent exemption from the requirement of tolerances for mCry3A (US EPA, 2004b).

mCry3A corn event MIR604 also falls within the scope of the US Food and Drug Administration's (FDA) 1992 Statement of Policy: Foods Derived from New Plant Varieties, including genetically engineered varieties pursuant to 21 CFR Section 192.25 of the Federal Food, Drug, and Cosmetic Act. Syngenta has initiated a consultation with FDA and has filed a Pre-market Biotechnology Notification (PBN #0099) in 2005 and expects approval in 2006.

Syngenta is also pursuing field trial applications in the U.S. (see Table 1) to facilitate commercial development and regulatory approvals for Event MIR604 hybrids in the U.S. Event MIR604 hybrids have been planted in several states under USDA-APHIS comprehensive permit and notification since 2001.

Table 1. Summary of USDA-APHIS Notification/Permits and Field Trial Reports for Field Trials Planted with Event MIR604.

Year	USDA Notification or Permit No.	Planted Trial Sites by State[1]	Status of USDA field trial reports
2001	01-018-01n	HI	Submitted
	01-022-07 r/m	IL, PR	Submitted
2002	02-022-01 r/m	FL, IL, MN, TX	Submitted
	02-022-02 r/m	HI	Submitted
2003	03-021-01 r/m	FL,IA,IL,IN,KS,KY,MN,MO,MS,NE,PR,SD,TX,WI	Submitted
	03-021-02 r/m	HI	Submitted
	03-287-07n	PR	Submitted
	03-287-05n	FL, HI, IL	Submitted
	03-287-02n	HI	Submitted
	03-287-01n	HI	Submitted
	03-287-10n	HI	Submitted
	03-287-11n	HI	Submitted
	03-287-04n	IL	Submitted
	03-300-04n	PR	Submitted
2004	04-072-06n	AR,CA,CO,FL,HI,IA,ID,IL,IN,KS,KY,LA,MD,MN, MO,MS,NC,NE,NM,NY,OH,PA,PR,SD,TX,VA,WI	Submitted
	04-076-04n	FL,HI,IA,ID,IL,IN,KY,MN,MS,NE,PA,PR,SD,WI (IR/HT stacks)	Submitted
	04-085-08n	IA	Submitted
	04-086-03n	IL	Submitted
	04-140-01n	IA	Submitted
	04-203-09n	PR (IR/HT stacks)	Submitted

[1]If Status of USDA field trial reports listed as In Progress, then Trial sites may be approved, but not all trial sites planted.

Chapter 2. The Corn (Maize) Family

The following was excerpted, with minor edits from USDA APHIS Environmental Assessment 92-042-01 (authored by Dr. James Lackey), the Canadian Food and Inspection Authority (CFIA) Regulatory Directive Dir94-11 [http://www.inspection.gc.ca/english/plaveg/bio/dir/dir9411e.shtml] (CFIA, 1994) and the Organization for Economic Cooperation and Development (OECD) Consensus Document on the Biology of *Zea mays* subsp. mays (Maize) [http://www.oecd.org/document/9/0,2340,en_2649_201185_1889395_1_1_1_1,00.html] (OECD, 2003). Full descriptions and complete references can be obtained from these documents.

A. GENERAL DESCRIPTION OF *ZEA MAYS* L. (MAIZE/CORN)

Zea is a genus of the family Graminae (Poaceae), commonly known as the grass family. Maize (*Zea mays* L.) is a tall, monecious annual grass with overlapping sheaths and broad conspicuously distichous blades. Plants have staminate spikelets in long spike-like racemes that form large spreading terminal panicles (tassels) and pistillate inflorescences in the leaf axils, in which the spikelets occur in 8 to 16 rows, approximately 30 cm long, on a thickened, almost woody axis (cob). The whole structure (ear) is enclosed in numerous large foliaceous bracts and long styles (silks) protrude from the tip of the ear as a mass of silky threads (Hitchcock and Chase, 1971). Pollen is produced entirely in the staminate inflorescence and eggs, entirely in the pistillate inflorescence. Maize is wind pollinated and both self and cross-pollination are usually possible. Shed pollen usually remains viable for 10 to 30 minutes, but can remain viable for longer durations under favorable conditions (Coe *et al*., 1988). Cultivated maize is presumed to have been derived from teosinte (*Z. mexicana*) and is thought to have been introduced into the old world in the sixteenth century. Maize is cultivated worldwide and represents a staple food for a significant proportion of the world's population. No significant native toxins are reported to be associated with the genus Zea (International Food Biotechnology Council, 1990).

B. ORIGIN OF THE SPECIES *ZEA MAYS* L.

It is generally agreed that teosinte (*Z. mexicana*) is an ancestor of maize, although opinions vary as to whether maize is a domesticated version of teosinte (Galinat, 1988). Teosinte is an ancient wild grass found in Mexico and Guatemala. Because it has differentiated into various races, species and plant habits, taxonomic classification is still a matter of controversy. Doebley and Iltis (1980) and Iltis and Doebley (1980) classified the annual teosintes into two subspecies of *Z. mays*: ssp. *mexicana* (including races Chalco, Central Plateau and Nobogame) and ssp. *parviglumis*-var. *parviglumis* (race Balsas) and var. *huehuetenangensis* (race Huehuetenango) and the species *Z. luxurians* (race Guatemala). The perennial teosintes from Jalisco, Mexico are separated into two more species according to ploidy, *Z. perennis* and *Z. diploperennis*. The Meso-American region located within middle South Mexico and Central America is recognised as one of the main centres of origin and development of agriculture as well as centre of origin and diversification of more than one hundred crops (Vavilov, 1951; Smith, 1995; Harlan, 1992). At the present time, there is no agreement about where exactly maize was

domesticated and there are several proposals in this regard. Based on the findings of archaeological materials from the maize plant (pollen, cobs, husks, and other remnants) in the United States and Mexico, which are older than those found in South America, Randolph (1959) proposed that maize was domesticated, independently, in the southwestern United States, Mexico, and Central America. Mangelsdorf (1974) proposed that "corn had not one origin but several in both Mexico and South America", because the archaeological evidences are found in Mexico and several morphological characteristics of extant populations are found in the maize races of South America (Andes region) in comparison to those races of Meso-America.

C. CULTIVATION AND USE OF CORN

As discussed above, maize has been cultivated by the indigenous peoples of North America for thousands of years. The modern era of maize hybrid production began in the U.S. where research conducted in the early part of this century proved that hybrid maize could produce a yield superior to open-pollinated varieties (Sprague and Eberhart, 1977). Gradually, hybrid-derived varieties replaced the open-pollinated types in the 1930's and 1940's. Almost all corn grown in the United States now comes from hybrid seed that is obtained every planting season from private enterprises; the older open-pollinated varieties are virtually unknown in commerce (Hallauer et. al., 1988).

The production of hybrid seed requires the development and maintenance of inbred lines and subsequent controlled crosses to produce commercial seed. Self pollination is essential for inbred development while controlled cross pollination is mandatory for hybrid seed production. Mechanisms have been developed to ensure the correct form of pollination for each process and to prevent genetic contamination of seed stocks (Wych, 1988). Breeder or foundation seed is produced from self pollinated seed after the eighth or ninth generation of inbreeding. A high degree of self pollination is assured by planting in blocks that are isolated by a distance of at least 200 meters (~660 ft.) from any other contaminating source of pollen. Hybrid seed production is accomplished by interplanting rows of the male and female inbred parents (e.g., one row of male to four female rows). Hybrid seed production requires isolation similar to that for foundation seed. Self pollination of the female parent is prevented through detasseling prior to pollen shed or by the use of male sterile females. Genetic conformity of inbreds and hybrids is monitored and assured through grow-outs of representative seed lots and laboratory screening using such criteria as isozyme profiles.

Maize is planted when soil temperatures are warm (greater than or equal to 10°C) usually mid to late April through to mid-May in the US corn belt. Optimum yields occur when the appropriate hybrid maturity and population density are chosen. In addition, exogenous sources of nitrogen fertilizer are generally applied and weed and insect control measures are generally recommended. Choice of the appropriate hybrid for the intended growing area helps to ensure that the crop will mature before frost halts the growth of the plant at the end of the season; hybrids are categorized according to the amount of "heat units" that will be required for maturity. Therefore, a hybrid developed for a specific heat unit zone, will not mature in (cooler) areas that receive fewer "heat units". Traditional cultivation practices in maize often result in bare soil which is susceptible to erosion by wind or water; increasingly, "no till" maize is being grown in an effort to reduce this soil loss.

In 2003, there were more than 78 million acres planted to corn in the United States producing over 10 billion bushels of grain (USDA, 2004). Maize grown in the U.S. is predominantly of the yellow dent type, a commodity crop largely used to feed domestic animals, either as grain or silage. The remainder of the crop is exported or processed by wet- or dry-milling to yield products such as high fructose maize syrup and starch or oil, grits and flour. These processed products are used extensively in the food industry. For example, maize starch serves as a raw material for an array of processed foods, and in industrial manufacturing processes. Since the early 1980's a significant amount of grain has also been used for fuel ethanol production. The by-products from these processes are often used in animal feeds. For a full discussion of the uses of maize see Watson (1988).

D. POLLINATION OF CORN

Pollination, fertilization, and caryopsis development of corn follows the same pattern for chasmogamous wind-pollinated grasses, with the following exceptions:

1. Pollen is produced entirely in the staminate inflorescences. Eggs are produced entirely in the pistillate inflorescences.
2. Self-pollination and fertilization and cross-pollination and fertilization are usually possible and frequencies of each are usually determined by physical proximity and other environmental influences on pollen transfer. A number of complicating factors, such as genetic sterility factors and differential growth rates of pollen tubes may also influence the frequencies of self-fertilization versus cross-fertilization.
3. Corn styles and corn pollen tubes are the longest known in the plant kingdom.
4. Shed pollen typically remains viable for 10 to 30 minutes, but may remain viable of much longer under refrigerated conditions (Coe *et al*., 1988).
5. Pollen dispersal is limited due to its large size (0.1 mm diameter) and spherical nature. Numerous studies have shown that greater than 98% of pollen settles to the ground within 60 meters of its source (Raynor *et al*., 1972; Luna *et al*., 2001; Burris, 2002).
6. The staminate and pistillate inflorescences do not develop at the same time. The pistillate inflorescence is precocious. However, there is the appearance of slight protandry because the elongating styles (silks) are delayed for about seven days in emergence from the bracts of the pistillate inflorescence, while the later-developing staminate inflorescence is fully visible. The silks are receptive to pollen up to 10 days after emergence, but receptivity decreases rapidly thereafter (Walden and Everett, 1961).

Corn is primarily wind pollinated; insects are responsible for insignificant amounts of pollen dispersal (Russell and Hallauer, 1980).

E. INTER-SPECIES/GENUS HYBRIDIZATION

Maize and other species and subspecies of teosinte are sexually compatible and can produce fertile hybrids (Wilkes, 1977). Related *Zea* species are geographically restricted and occur only in Mexico and Guatemala. The closest known relative of *Zea* is

Tripsacum, a genus of eleven species, widely distributed between 42°N and 24°S latitude (de Wet *et al*., 1981). Three species occur in the United States, two of which, *Tripsacum floridanum* (Florida Gamagrass) and *Tripsacum lanceolatum* (Mexican Gamagrass), are confined to the southernmost states of the United States. Only one, *Tripsacum dactyloides* (Eastern Gamagrass), has a distribution that includes the northern (U.S.) maize belt (Gould, 1968).

F. POTENTIAL FOR INTROGRESSION FROM *Z. MAYS* INTO RELATIVES

An examination of the literature prior to 1980 would lead to the conclusion that there is constant gene flow between maize and teosinte, and that the weedy teosinte (*Z. mays* ssp. *mexicana*) is a hybrid of the two sub-species, and functions as a genetic bridge between the two (de Wet and Harlan, 1972; de Wet, 1975; Galinat, 1973). However, this premise has been re-evaluated using techniques of gene mapping, which failed to show any evidence of recent introgression between maize and teosinte (Smith *et al*., 1985). Moreover, *Z. mays* ssp. *mexicana* seems not to be a hybrid of the wild and cultivated forms of *Zea* and therefore probably does not serve as a genetic bridge as the physical similarities appear to be due to parallel adaptation to the same habitat (Doebley, 1984). There is evidence of highly restricted gene flow between *Zea* spp. that apparently occurs predominantly from teosinte into maize (Doebley *et al*, 1987). *Tripsacum* and *Zea* have different chromosome numbers (n = 9 versus n = 10). Crosses between *Z. mays* and *T. dactyloides* can be made, but only through human intervention and, even then, only with extreme difficulty. Moreover, the progeny are frequently sterile or genetically unstable (Manglesdorf, 1974). The process of transferring *Tripsacum* germplasm into maize is technically difficult. The transmission rate of the single extra *Tripsacum* chromosome added to the genome is so low and the rate of maize *Tripsacum* crossing over so reduced, as to practically exclude the general use of experimentally-introduced *Tripsacum* germplasm in maize improvement (Galinat, 1988).

G. VOLUNTEERS AND WEEDINESS IN CORN

Maize has lost the ability to survive in the wild due to its long process of domestication, and needs human intervention to disseminate its seed. Although corn from the previous crop year can over winter and germinate the following year, it cannot persist as a weed. The presence of corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with the hoe or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist during the following years. Volunteers are common in many agronomic systems, but they are easily controlled; however, maize is incapable of sustained reproduction outside of domestic cultivation. Maize plants are non-invasive in natural habitats (Gould, 1968). Some *Zea* species are successful wild plants in Central America, but they have no pronounced weedy tendencies (Galinat, 1988). In contrast to weedy plants, maize has a pistillate inflorescence (ear) with a cob enclosed with husks. Consequently seed dispersal of individual kernels does not occur naturally. Individual kernels of corn, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities (Hallauer, 2000).

Chapter 3. Molecular Analysis of Event MIR604

A. SUMMARY

Data from Southern analysis and DNA sequencing demonstrated that single copies of the modified *cry3A* (*mcry3A*) gene, phosphomannose isomerase (*pmi*) gene, MTL promoter and ZmUbiInt promoter were present in Syngenta's maize (corn) Event MIR604. Event MIR604 did not contain any of the backbone sequences from the transformation plasmid pZM26. Additionally, Southern analysis demonstrated that the T-DNA insert was stable over several generations of Event MIR604. Sequence analysis of the entire T-DNA insert present in Event MIR604 confirmed the overall integrity of the insert and that contiguousness of the functional elements had been maintained. A 43 bp truncation at the right border (RB) junction of the T-DNA insert and 44 bp truncation at the left border (LB) junction of the T-DNA insert were identified. Three single nucleotide changes were also identified in the T-DNA insert. One of these changes occurred within the MTL promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and give rise to two amino acid changes. These substitutions have not resulted in any apparent functional change in PMI as expressed in Event MIR604 (Hill, 2004). Statistical analysis confirmed the expected Mendelian inheritance ratio for both *mcry3A* and *pmi*.

B. INTRODUCTION

This chapter is being submitted to the USDA in support of a petition for non-regulated status for Syngenta Seeds' transformation Event MIR604. These plants express a modified Cry3A (mCry3A) protein that confers resistance to certain coleopteran pests. Additionally, the plants express phosphomannose isomerase (PMI), a selectable marker trait that is inert with regard to pesticidal properties (Negrotto, et. al., 2000; Reed, et. al., 2001; Wright, et. al., 2001). The present chapter presents a summary of data and information relevant to the molecular characterization of the T-DNA insert present in Syngenta's corn event MIR604. Included herein are data and information describing the genetic elements that have been introduced into Event MIR604, the process used for transformation, and a molecular and genetic characterization of Event MIR604-derived plants.

C. EVENT MIR604 MAIZE

Syngenta's maize Event MIR604 has been transformed with a synthetic, maize optimized, modified *cry3A* (m*cry3A*) gene whose expression produces a mCry3A insect control protein that is a member of a class of proteins which occur naturally in a gram-positive soil bacterium *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t*) (Sekar *et al.*, 1987). Additional changes in this maize-optimized gene were made, such that the mCry3A protein has enhanced activity against the western corn rootworm (WCRW; *Diabrotica virgifera virgifera*) and other related pests. The mCry3A amino acid sequence corresponds to that of the native Cry3A protein, except that (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-phenylalanine, and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein. The consensus recognition site for cathepsin-G was determined to be alanine-alanine-proline-phenylalanine by Blocker *et al.*, 1999 and Nakajima, *et al.*, 1979. Maize plants transformed with the synthetic modified *cry3A* gene from pZM26 (Figure 2) display resistance to these pests (Chen and Stacy, 2003).

Event MIR604 maize also contains the *pmi* gene, which was introduced *via* the same pZM26 transformation vector. This gene represents the *manA* gene from *Escherichia coli* and encodes the enzyme phosphomannose isomerase (PMI), which was employed as a selectable marker during the process of regenerating plant material following transformation (Negrotto *et al*., 2000). Maize cells expressing *pmi* can utilize mannose as a primary carbon source, whereas cells lacking *pmi* expression will fail to proliferate in a mannose-based culture medium. A complete description of the *pmi* gene and PMI protein is found in a separate report (Vlachos and Joseph, 2003).

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604. The *mcry3A* gene was transformed into a heterogenous genetic background selected for its suitability to transformation by *Agrobacterium*. Event MIR604 was crossed into a number of elite inbred lines and material generated from these crosses was used in the molecular and segregation analysis. The control germplasm in these experiments was negative segregants identified in the back cross populations.

D. DESCRIPTION OF THE TRANSFORMATION SYSTEM AND METHOD

Transformation of Syngenta's mCry3A-expressing maize Event MIR604 was conducted using immature maize embryos derived from a proprietary *Zea mays* line (Negrotto *et al*., 2000), via *Agrobacterium*-mediated transformation. By this method, genetic elements within the left and right border regions of the transformation vector are efficiently transferred and integrated into the genome of the plant cell, while genetic elements outside these border regions are generally not. Immature embryos were excised from 8 - 12 day old ears and rinsed with fresh medium in preparation for transformation. Embryos were mixed with the suspension of *Agrobacterium* cells harboring the transformation vector pZM26 (Figure 2), vortexed for 30 seconds, and allowed to incubate for an additional five minutes. Excess *Agrobacterium* solution was aspirated and embryos were then moved to plates containing a non-selective culture medium. Embryos were co-cultured with the remaining *Agrobacterium* at 22°C for 2-3 days in the dark. Embryos were transferred to culture medium supplemented with ticarcillin (100 mg/ml) and silver nitrate (1.6 mg/l) and incubated in the dark for ten days. The phosphomannose isomerase gene, *pmi*, was employed as a selectable marker during the transformation process (Negrotto *et al*., 2000). Embryos producing embryogenic callus were transferred to cell culture medium containing mannose. After initial incubation with *Agrobacterium*, transformed tissue was transferred to, and grown for four months on selective media containing 500 mg/L of the broad-spectrum antibiotic cefotaxime insuring that the *Agrobacterium* was cleared from the transformed tissue. Regenerated plantlets were tested for the presence of both the *pmi* and *mcry3A* genes, as well as for the

absence of the spectinomycin (spec) antibiotic resistance gene, by TaqMan® PCR analysis. Plants positive for both genes, and negative for spec, were transferred to the greenhouse for further propagation.

E. THE DONOR GENES AND REGULATORY SEQUENCES

E.1. Active ingredient cassette:

MTL promoter (2556 bp): Derived from the *Zea mays* metallothionein-like gene (GenBank Accession number S57628). Provides root-preferential expression in *Zea mays* (de Framond, 1991).

mcry3A **(1797 bp):** A modified version of a native Cry3A gene, which occurs in *Bacillus thuringiensis* subsp. *tenebrionis* (Sekar *et al.*, 1987). The gene was reengineered to incorporate a cathepsin-G serine protease recognition site within the expressed protein. The amino acid sequence of the expressed protein corresponds to that of the native Cry3A protein, except that (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 consensus recognition site for cathepsin-G was determined to be alanine-alanine-proline-phenylalanine by Blocker *et al.*, 1999 and Nakajima, *et al.*, 1979. This cathepsin-G recognition site has the sequence alanine-alanine-proline-phenylalanine, and has replaced the amino acids valine-155, serine-156, and serine-157 in the native protein. This modification increases the toxicity to target pests, particularly *Diabrotica virgifera virgifera* and *Diabrotica longicornis barberi* (Chen and Stacy, 2003). The entire coding region of the *mcry3A* gene was synthesized to accommodate the preferred codon usage for maize (Murray *et al.*, 1989).

NOS (253 bp): Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (GenBank Accession number V00087). Its function is to provide a polyadenylation site (Depicker *et al.*, 1982).

E.2. Selectable marker cassette:

ZmUbiInt (1993 bp): Promoter region from *Zea mays* polyubiquitin gene, contains the first intron comprising 1010 bp of the promoter (GenBank Accession number S94464). The entire promoter region provides constitutive expression in monocots (Christensen *et al.*, 1992).

pmi **(1176 bp):** *E.coli manA* gene encoding phosphomannose isomerase (GenBank Accession number M15380). Catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate (Negrotto *et al.*, 2000).

NOS (253 bp): Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (GenBank Accession number V00087). Its function is to provide a polyadenylation site (Depicker *et al.*, 1982).

E.3. Vector backbone components:

Spec (789 bp): Streptomycin adenylyltransferase, *aadA* gene from *E. coli* Tn7 (GenBank Accession Number X03043). Confers resistance to erythromycin, streptomycin, and spectinomycin; used as a bacterial selectable marker (Fling *et al*., 1985).

VS1ori (405 bp): Consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas* (similar to GenBank Accession Number U10487). Serves as origin of replication in *Agrobacterium tumefaciens* host (Ioth *et al*., 1984).

ColE1ori (807 bp): Origin of replication that permits replication of plasmid in *E. coli*. (similar to GenBank Accession Number V00268) (Itoh and Tomizawa, 1978).

LB (25 bp): Left border region of T-DNA from *Agrobacterium tumefaciens* nopaline ti-plasmid (GenBank Accession number J01825). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Zambryski *et al*., 1982).

RB (25 bp): Right border region of T-DNA from *Agrobacterium tumefaciens* nopaline ti-plasmid (GenBank Accession number J01826). Short direct repeat that flanks the T-DNA and is required for the transfer of the T-DNA into the plant cell (Wang *et al*., 1984).

***virG* (726 bp):** VirGN54D from pAD1289 (similar to GenBank Accession Number AF242881). The N54D substitution results in a constitutive *virG* phenotype. VirG is part of the two-component regulatory system for the vir regulon in *Agrobacterium* (Hansen *et al*., 1994).

***repA* (1074 bp):** pVS1 replication protein from *Pseudomonas*, which is a part of the minimal pVS1 replicon that is functional in gram-negative plant associated bacteria (GenBank Accession Number AF133831) (Heeb *et al*., 2000).

F. MOLECULAR ANALYSIS OF EVENT MIR604

F.1. Functional Element Copy Number Southern Analysis

Genomic DNA used for Southern analysis was isolated from pooled leaf tissue of ten plants representing the backcross six (BC6) generation (see Figure 3 for pedigree) of Event MIR604 using the method of Thomas *et al*., (1993). All plants used for DNA isolation were individually analyzed using TaqMan® PCR to confirm the presence of a single copy of the *mcry3A* gene and the *pmi* gene. For the negative segregant controls, DNA was isolated from pooled leaf tissue of plants representing either the backcross four (BC4) generation (used for the *mcry3A*, *pmi* and backbone Southern analysis) or the backcross six (BC6) generation (used for the MTL and ZmUbiInt Southern analysis) of Event MIR604. These plants were individually analyzed using TaqMan® PCR and the assays were negative for the *mcry3A* gene

and the *pmi* gene, but were as expected positive for the assay internal control, the endogenous maize *adh* gene.

Southern analysis was performed using state-of-the-art molecular biology techniques. Genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme, which has a single recognition site within the Event MIR604 T-DNA insert from plasmid pZM26 (Figure 2). This approach allows for determination of the number of copies of the elements, corresponding to the specific probe used for each Southern, which have been incorporated into Event MIR604. This results in one hybridization band per copy of the element present in Event MIR604. Following agarose gel electrophoresis and alkaline transfer to a Nytran® membrane, hybridizations were carried out using element specific full-length PCR generated probes (See Figures 4, 6, 8, 10, 12 and 14). The probes were labeled with ^{32}P *via* random priming using the Rediprime II™ system (Amersham Biosciences, Cat. No. RPN1633).

Included in each southern were three control samples:

1.) DNA from a negative (non-transformed) segregant used to identify any endogenous *Zea mays* sequences that may cross-hybridize with the element specific probe.
2.) DNA from a negative segregant into which is introduced an amount of digested pZM26 that is equal to one or less copy number based on probe length, to demonstrate the sensitivity of the experiment in detecting a single gene copy within the *Zea mays* genome.[1]
3.) Digested pZM26 plasmid that is equal to one copy number based on probe length, to demonstrate a positive control for hybridization as well as the sensitivity of the experiment.

These hybridization data provide confirmatory evidence to support the TaqMan® PCR analysis that Event MIR604 contains a single copy of the *mcry3A* gene and the *pmi* gene. Additionally, the data demonstrate that Event MIR604 contains a single copy of the MTL promoter and ZmUbiInt promoter and that Event MIR604 does not contain any of the vector backbone sequences present in pZM26. As expected for the *mcry3A* (Figure 5), *pmi* (Figure 7), MTL (Figure 9) and ZmUbiInt (Figure 11) probes the *Kpn*I digest resulted in a single hybridization band demonstrating that a single copy of each element is present in Event MIR604. Additionally for the full-length backbone probe (Figure 13) lack of hybridization demonstrates the absence of any pZM26 vector backbone sequences being incorporated into Event MIR604 during the transformation process.

F.2. Generational Stability Southern Analysis

[1] **Formula to determine one copy equivalent based on probe length**

(µg digested DNA loaded*1.00E+06 pg/µg*bp length of probe)/bp maize diploid genome =	pg for 1 copy
Example:	
Zea mays diploid genome size in bp:	5.34E+09
mcry3A probe length in bp:	1797
µg *Kpn*I-digested DNA loaded for Southern analysis:	7.5
Calculation for *mcry3A*: (7.5*1.00E+06*1797)/5.34E+09=	2.52 pg

Genomic DNA used for generational stability Southern analysis was isolated from pooled leaf tissue of ten plants per generation representing the backcross four (BC4), backcross five (BC5) and backcross six (BC6) generations of Event MIR604 using the method of Thomas *et al*., (1993). All plants used for DNA isolation were individually analyzed using TaqMan® PCR (data not shown) to confirm the presence of a single copy of the *mcry3A* gene and the *pmi* gene. For the negative segregant controls DNA was isolated from pooled leaf tissue of ten plants representing the BC6 generation of Event MIR604. These plants were individually analyzed using TaqMan® PCR (data not shown) and the assays were negative for the *mcry3A* gene and the *pmi* gene, but were as expected positive for the assay internal control, the endogenous maize *adh* gene.

As expected the hybridization pattern over several generations of Event MIR604 was identical using a *mcry3A* probe (Figure 15). The hybridization data demonstrates the T-DNA insert from pZM26 incorporated into Event MIR604 is stable over several generations.

F.3. T-DNA insert sequencing

The nucleotide sequence of the entire T-DNA insert present in Event MIR604 was determined to demonstrate overall integrity of the insert, contiguousness of the functional elements and to detect any individual basepair changes. The Event MIR604 insert was amplified from DNA derived from the BC5 generation as two individual overlapping fragments (Figure 16). Each fragment was amplified using one oligo homologous to plant genomic sequences flanking the Event MIR604 insert (data not shown) and one oligo homologous to the *mcry3A* gene. PCR amplification was carried out using the Expand High Fidelity PCR system (Roche, Cat. No. 1732650). Each sequencing fragment was individually cloned into the pCR® -XL-TOPO vector (Invitrogen, Cat. No. K4700-20) and three separate clones for each fragment were identified and sequenced. Sequencing was carried out using the ABI3730XL analyzer using ABI BigDye® 1.1 or Big Dye 3.1 dGTP (for GC rich templates) chemistry. The sequence analysis was done using the Phred, Phrap, and Consed package from the University of Washington and was carried out to an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998). The final consensus sequence was determined by combining the sequence data from the six individual clones (three for each sequencing fragment) to generate one consensus sequence of the Event MIR604 insert. To further validate any individual basepair discrepancies between the Event MIR604 insert and the pZM26 plasmid small (~300-500 bp) PCR products specific to any regions where a basepair discrepancy was seen in the initial consensus sequence were amplified using the same methodology above. For all putative basepair discrepancies in the Event MIR604 insert direct PCR product sequencing resulted in single clear peaks at all basepairs in question, indicating these discrepancies are likely present in the Event MIR604 insert (data not shown). Alignment was performed using the ClustalW program with the following parameters: scoring matrix blosum55, gap opening penalty 15, gap extension penalty 6.66 (Thompson *et al*, 1994).

The consensus sequence data for the Event MIR604 T-DNA insert demonstrate the overall integrity of the insert and that contiguousness of the functional elements within the insert as intended in pZM26 have been maintained (Rabe, 2004). Sequence analysis revealed that some truncation occurred at the RB and LB ends of the T-DNA insert

during the transformation process that resulted in Event MIR604. The RB portion of the T-DNA insert was truncated by 44bp and the LB end of the T-DNA insert was truncated by 43 bp (Rabe, 2004). These deletions have no effect on the efficacy of the T-DNA insert and this phenomenon has also been previously observed in *Agrobacterium* transformation (Tinland and Hohn, 1995). Additionally, three basepair changes were noted in the Event MIR604 T-DNA insert (Rabe, 2004). One change occurred within the MTL promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and did result in two amino acid changes; valine at position 61 has been substituted by alanine (V61A) and glutamine at position 210 has been substituted by histidine (Q210H) (Rabe, 2004). Alanine and valine are both aliphatic amino acids resulting in a conservative substitution. Replacement of glutamine with histidine results in the substitution of an acidic residue for a basic residue. These substitutions have not resulted in any apparent functional change in PMI as expressed in Event MIR604 (Hill, 2004).

G. MENDELIAN INHERITANCE OF TRANSGENE INSERT

The inheritance pattern of the T-DNA insert derived from pZM26 in Event MIR604 was investigated. The initial Event MIR604 plant (T_0 generation) was bred to a non-transgenic inbred isoline, creating the T_1 generation. A single plant from this T_1 generation, identified by immuno-detection strip to be positive for PMI, was selfed to yield the T_2 generation. A single plant, putatively homozygous for *mcry3A* by TaqMan® PCR, from the T_2 seed was selfed to yield the T_3 generation. The zygosity of the T_3 generation was confirmed by conducting a progeny test by planting a 20 kernel sample of seed and assaying the resulting plants for *mcry3A* by TaqMan® PCR (data not shown). Progeny plants from the T_3 generation were crossed to a non-transgenic inbred to yield the T_4 generation (designated Hybrid I in Figure 3.). These progeny plants were selfed in the field and seed was collected in bulk, creating the T_5 seed. Individual T_5 plants were assayed for the presence of the trait by immuno-detection (ELISA) of mCry3A, as well as by TaqMan® PCR (data not shown) for both the *pmi* and *mcry3A* genes. The expected Mendelian inheritance ratio of positive and negative plants for a hemizygous trait in these populations is 3:1.

Genotypic data (Tables 2 and 3) were used to assess the goodness-of-fit of the observed genotypic ratio to the expected genotypic ratio using Chi Square analysis with Yates correction factor (Strickberger, 1976).

$$X^2 = \sum[\text{Observed-expected- } 0.5]^2/\text{expected}$$

Table 2. Observed vs. Expected* Genotype for T_5 Generation as determined by ELISA for mCry3A.

	Observed	Expected
Trait positive	317	313.5
Trait negative	101	104.5
Total	418	418

* X^2= 0.1148

This analysis tested the hypothesis that the genetic trait is segregating in a Mendelian fashion. The critical value to reject the hypothesis at the 5% level is 3.84 (Strickberger 1976). Since the Chi squared value is less than 3.84 the hypothesis that the genetic trait is behaving in a Mendelian fashion is accepted.

Table 3. Observed vs. Expected* Genotype for T_5 Generation as determined by Taqman® PCR Analysis for *pmi* and *mcry3a* genes.

	Observed	Expected
Trait positive	315	313.5
Trait negative	103	104.5
Total	418	418

* X^2= 0.0128

The critical value to reject the hypothesis at the 5% level is 3.84 (Strickberger 1976). Since the Chi squared value is less than 3.84 the hypothesis that the genetic trait is behaving in a Mendelian fashion is accepted[2].

H. CONCLUSIONS

Data from Southern analysis and DNA sequencing demonstrate that single copies of the modified *cry3A* (*mcry3A*) gene, phosphomannose isomerase (*pmi*) gene, MTL promoter and ZmUbiInt promoter are present in Syngenta's maize (corn) Event MIR604. Event MIR604 does not contain any of the backbone sequences from the transformation plasmid pZM26. Additionally, the T-DNA insert is stable over several generations of Event MIR604. Sequence analysis of the entire T-DNA insert present in Event MIR604 confirms that the overall integrity of the insert and contiguousness of the functional elements has been maintained. A 43 bp truncation at the right border (RB) junction of the T-DNA insert and 44 bp truncation at the left border (LB) junction of the T-DNA insert was identified. Three single nucleotide changes were also identified. One of these changes occurred within the MTL promoter, a regulatory region that does not encode a protein. The remaining two changes occurred within the *pmi* coding sequence and give rise to two amino acid changes which have not resulted in any apparent functional change in PMI as expressed in Event MIR604. Statistical analysis confirmed the expected Mendelian inheritance ratio for both *mcry3A* and *pmi*.

[2] The same 418 samples were used in both the PCR and ELISA experiments. The difference in the number of observed scores was attributed to two plants which were scored negative in the Taqman assay while scoring positive by ELISA assay. It is not possible to determine whether this difference is due to false postives in the ELISA or false negatives in the Taqman PCR. Regardless, the data clearly demonstrate that the transgene is inherited in a Mendelian fashion.

Figure 2. Plasmid map of pZM26. Map identifies restriction sites used for Southern analysis.

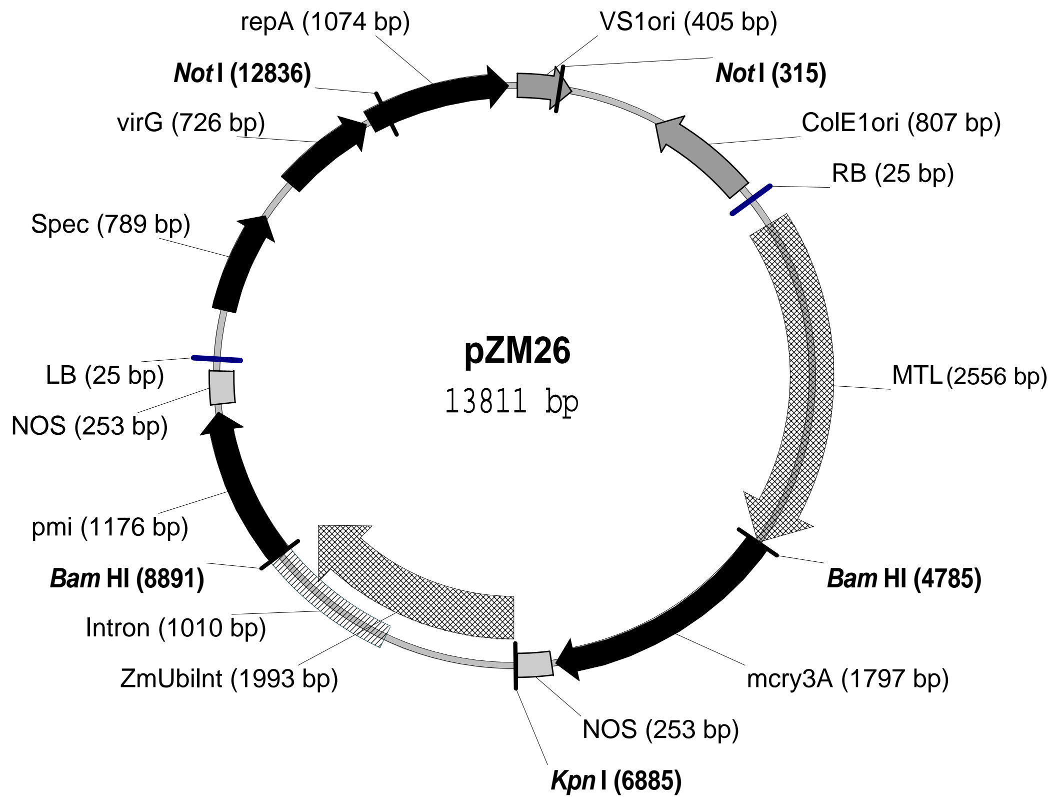


Figure 3. **MIR604 breeding history indicating generations used in the molecular analysis of Event MIR604.** The partial breeding history is extracted directly from Figure 1.

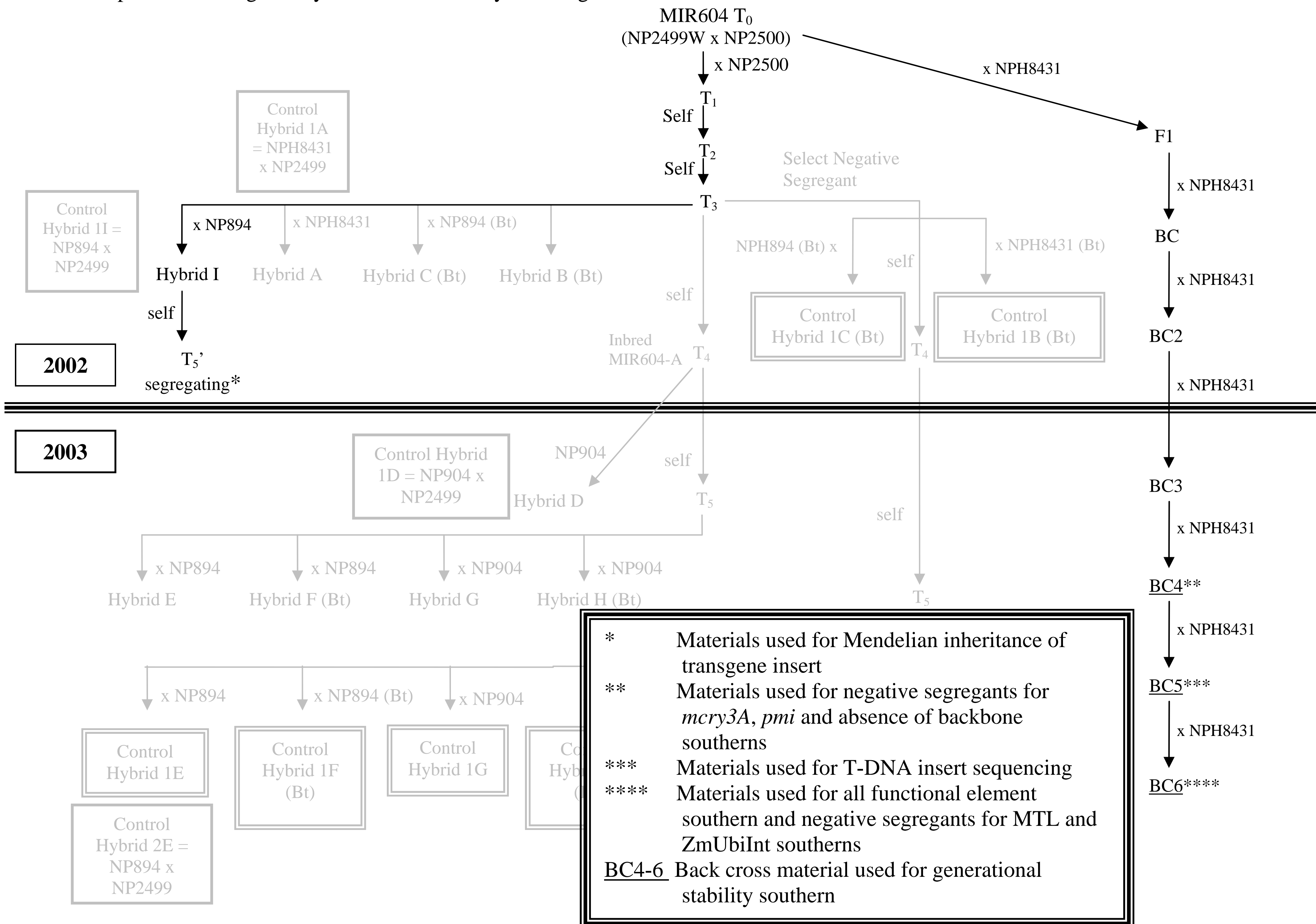


Figure 4. Location of *Kpn*I restriction site and position of *mcry3A* probe in the transformation vector pZM26 introduced into Event MIR604.

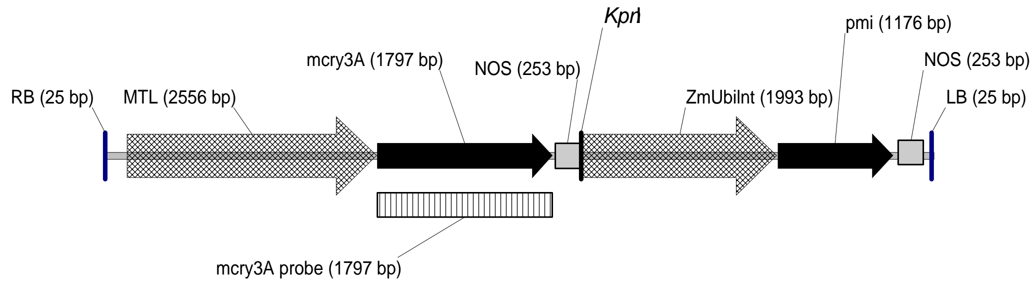


Figure 5. Southern analysis of Event MIR604 with *mcry3A* specific probe. Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *mcry3A* specific probe (1797 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC4 generation of Event MIR604; Lane 5: Negative segregants from BC4 generation of Event MIR604 spiked with 2.52 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 2.52 pg *Kpn*I digested pZM26 DNA.

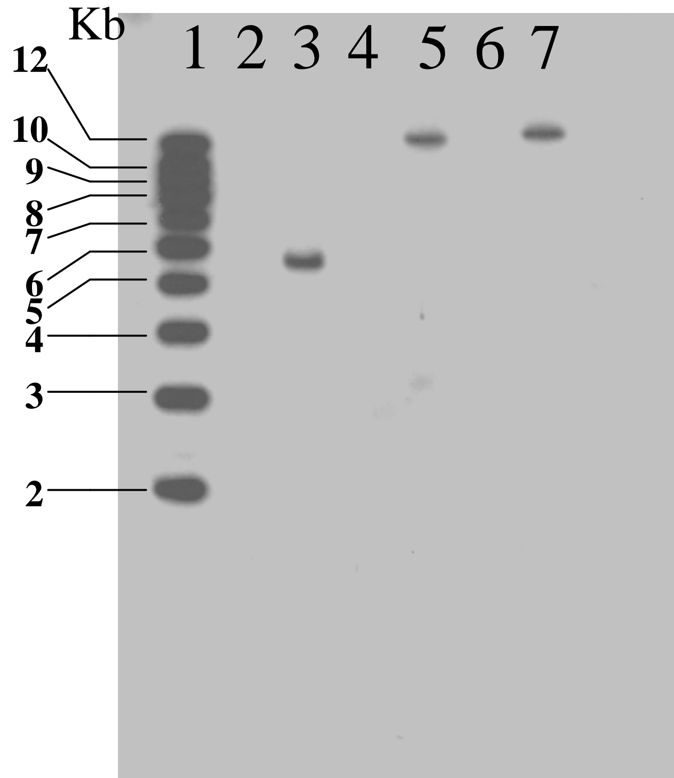


Figure 6. Location of *Kpn*I restriction site and position of *pmi* probe in the transformation vector pZM26 introduced into Event MIR604.

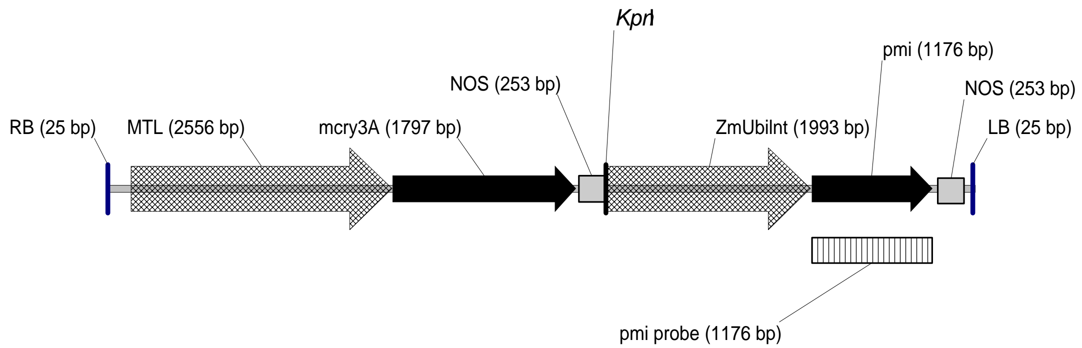


Figure 7. Southern analysis of Event MIR604 with *pmi* specific probe.
Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *pmi* specific probe (1176 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC4 generation of Event MIR604; Lane 5: Negative segregants from BC4 generation of Event MIR604 spiked with 1.65 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 1.65 pg *Kpn*I digested pZM26 DNA.

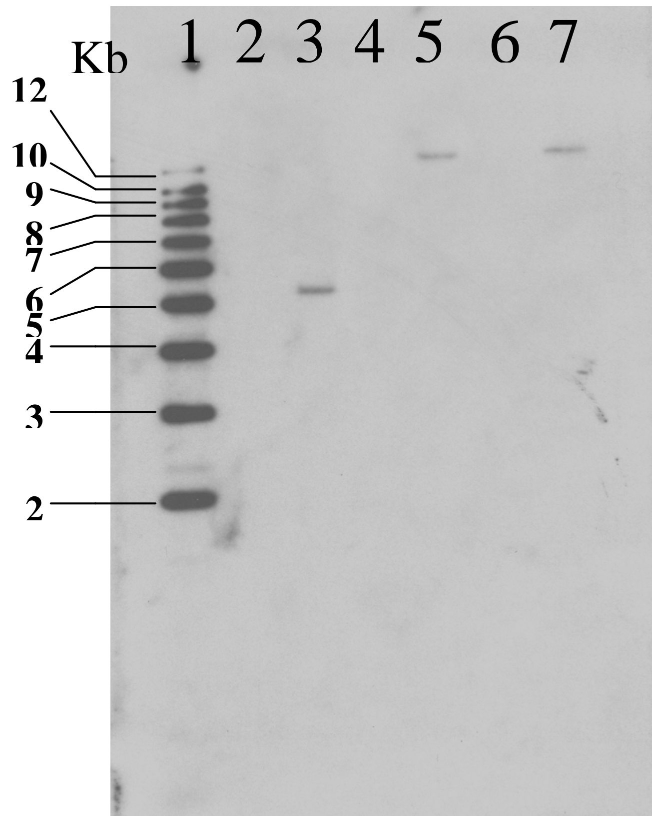


Figure 8. Location of *Kpn*I restriction site and position of MTL probe in the transformation vector pZM26 introduced into Event MIR604.

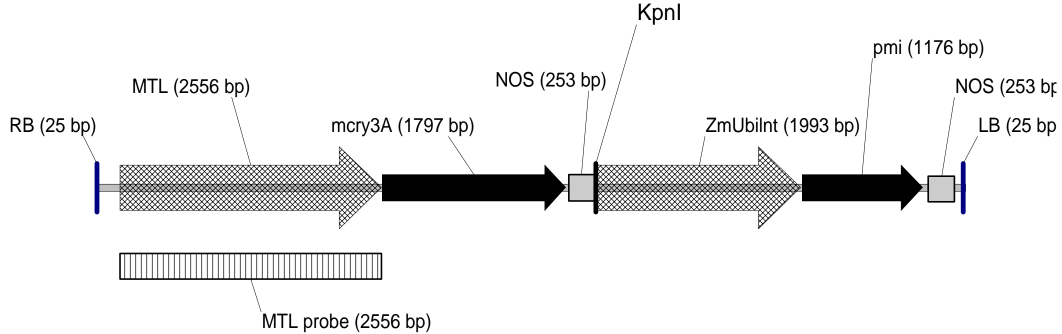


Figure 9. Southern analysis of Event MIR604 with MTL specific probe.
Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a MTL specific probe (2556 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC6 generation of Event MIR604; Lane 5: Negative segregants from BC6 generation of Event MIR604 spiked with 3.59 pg *Bam*HI/*Not*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 3.59 pg *Bam*HI/*Not*I digested pZM26 DNA. Note: *Bam*HI/*Not*I were used to digest pZM26 due to a preliminary experiment in which a *Kpn*I digest of pZM26 resulted in comigration with an endogenous *Zea mays* band that cross-hybridized with the MTL probe.

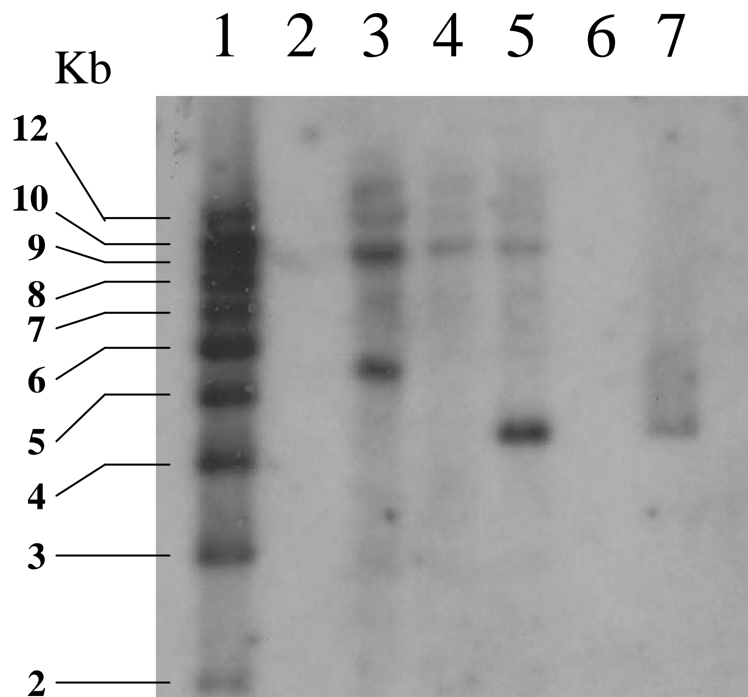


Figure 10. Location of *Kpn*I restriction site and position of ZmUbiInt probe in the transformation vector pZM26 introduced into Event MIR604.

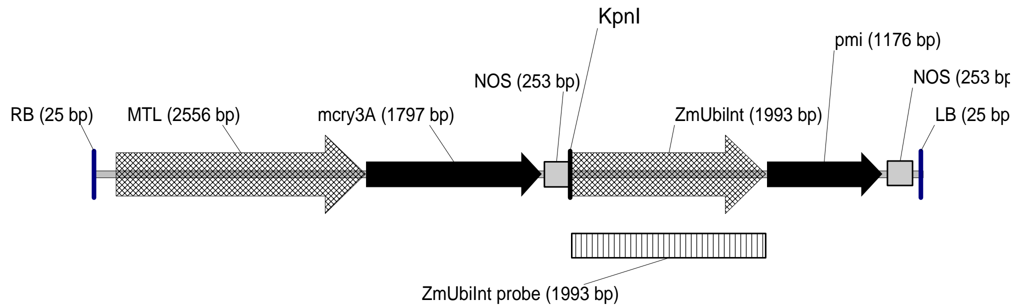


Figure 11. Southern analysis of Event MIR604 with ZmUbiInt specific probe. Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a ZmUbiInt specific probe (1993 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC6 generation of Event MIR604; Lane 5: Negative segregants from BC6 generation of Event MIR604 spiked with 2.80 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 2.80 pg *Kpn*I digested pZM26 DNA.

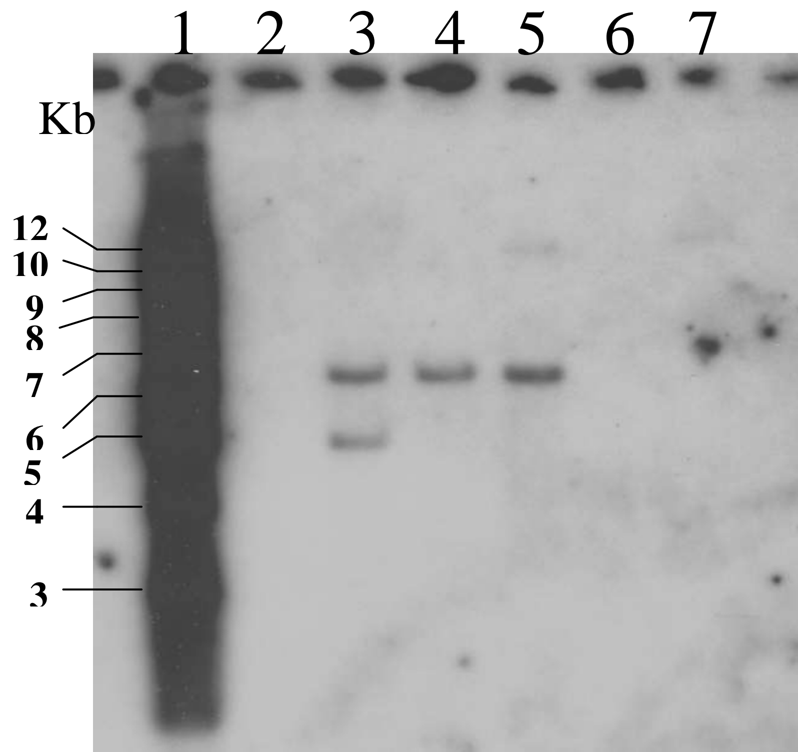


Figure 12. Location of *Kpn*I restriction site and position of backbone probe in the transformation vector pZM26.

Encompasses all basepairs outside of the LB and RB regions.

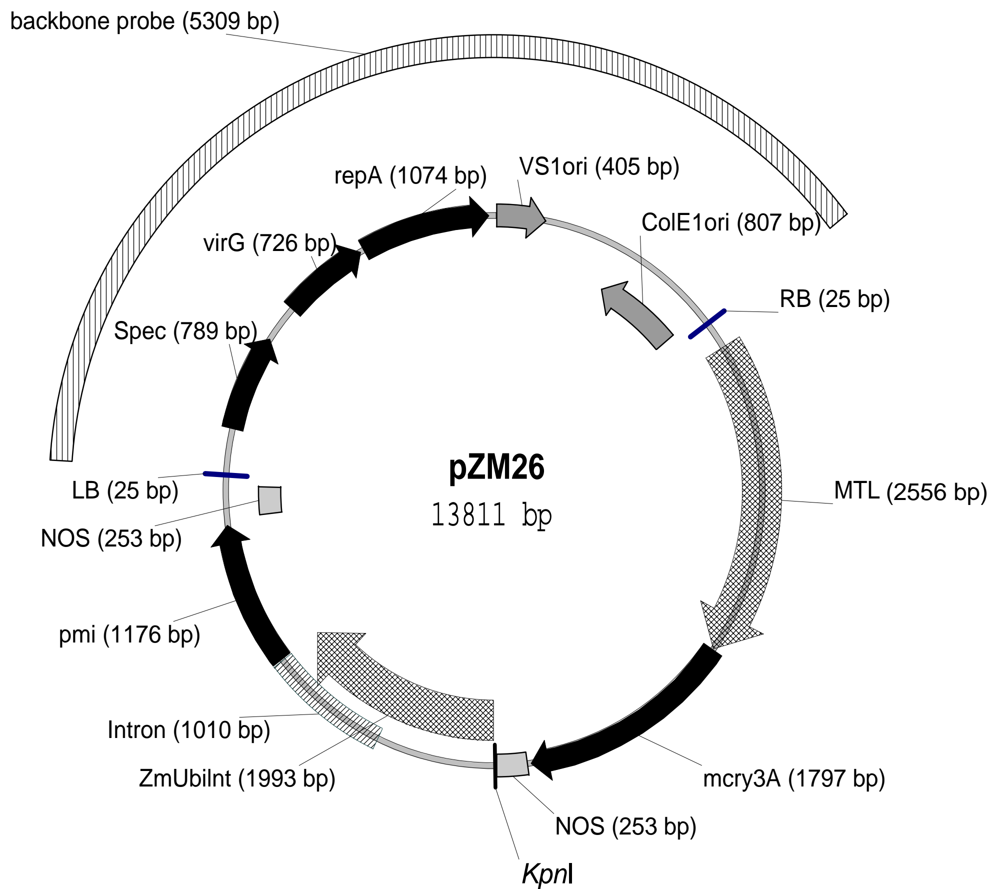


Figure 13. Southern analysis of Event MIR604 with backbone specific probe. Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a backbone specific probe (5309 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC6 generation Event MIR604; Lane 4: Negative segregants from BC4 generation of Event MIR604; Lane 5: Negative segregants from BC4 generation of Event MIR604 spiked with 7.46 pg *Kpn*I digested pZM26 DNA; Lane 6: Blank; Lane 7: 7.46 pg *Kpn*I digested pZM26 DNA.

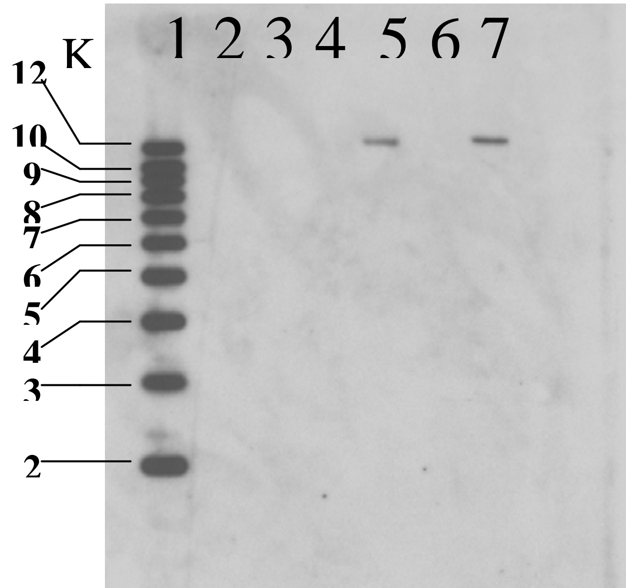


Figure 14. Location of *Kpn*I restriction site and position of *mcry3A* probe in the transformation vector pZM26 introduced into Event MIR604.

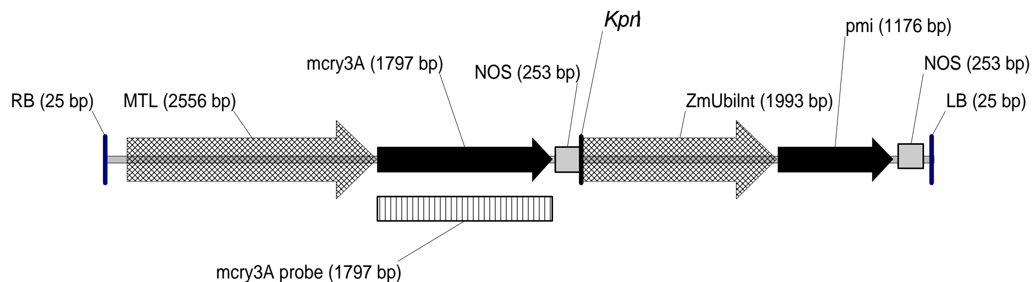


Figure 15. Generational stability southern analysis of Event MIR604 with *mcry3A* specific probe.

Maize genomic DNA (7.5 µg) was digested with *Kpn*I restriction enzyme and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *mcry3A* specific probe (1797 bp). Lane 1: Molecular weight markers (Kb DNA ladder, Stratagene Cat. No. 201115-81); Lane 2: Blank; Lane 3: BC4 generation Event MIR604; Lane 4: BC5 generation Event MIR604; Lane 5: BC6 generation Event MIR604; Lane 6: Negative segregants from BC6 generation of Event MIR604; Lane 7: Negative segregants from BC6 generation of Event MIR604 spiked with 2.52 pg *Kpn*I digested pZM26 DNA; Lane 8: Blank; Lane 9: 2.52 pg *Kpn*I digested pZM26 DNA.

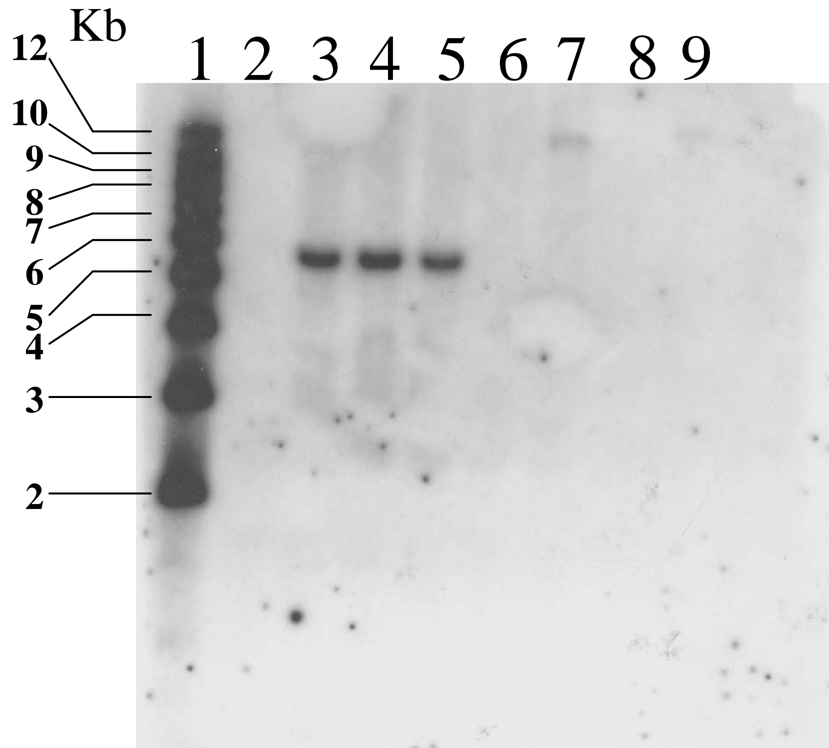
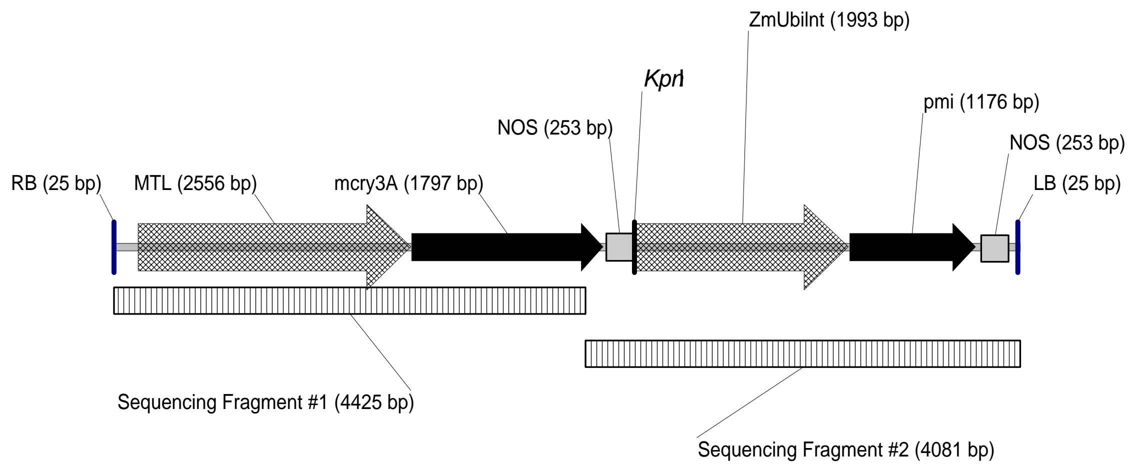


Figure 16. Location of fragments amplified from Event MIR604 to determine insert sequence.



Chapter 4. Agronomic Performance of Event MIR604 Hybrids

A. SUMMARY

Efficacy, yield, and agronomic performance of Event MIR604-derived field corn hybrids (MIR604 hybrids) were evaluated during 2002 and 2003 in 32 field trial locations across the U.S. corn belt (USDA Comprehensive Permit numbers 02-022-01 r/m, 02-022-02 r/m, 03-021-01 r/m, 03-021-02 r/m). Insect efficacy trials were conducted in field plots that were infested with naturally occurring populations of corn rootworm, with populations enhanced by use of trap crops the preceding season, or artificially infested with rootworm eggs. In addition to regular inspections for disease and insect pests, qualitative and quantitative comparisons for a number of morphological and agronomic traits were made between the transgenic and non-transgenic negative segregants or non-transgenic near isolines. Yield and agronomic trials were conducted both in the presence and absence of pest pressure, and in conjunction with an in-furrow insecticidal treatment. The traits chosen for agronomic comparison are those that are typically monitored by professional breeders and agronomists in the seed industry, and cover a broad range of characteristics that encompass the entire lifecycle of the maize plant.

The outcome of these studies, based upon data presented below, indicate that except for reduced damage to roots caused by corn rootworm the agronomic performance of MIR604-derived hybrids is similar, and for most traits, equivalent to their non-transformed near isogenic counterparts. In contrast to the similarity in most agronomic parameters, the yield performance of MIR604-derived hybrids in the presence of corn rootworm pressure is significantly increased relative to corn hybrids lacking this trait. Data referred to in this chapter are presented in Appendix 1, pp. 153-178.

B. AGRONOMIC METHODS

B.1. General Experimental Methods

The majority of experimental field trials were conducted as a simple random complete block design (RCBD). The experimental plots were divided into blocks of a size that would accomodate four rows 17 ft. long at a minimum. After blocking the plots, treatments were assigned at random within each block such that each treatment occurred once in every block. Within each block, control or test material was homogenously planted and agronomic, maintenance and havesting methods were applied as uniformly as possible. Comparisons of treatments and the computation of experimental error were performed within blocks. Any deviations from the basic trial design are footnoted in the trial data tables.

For efficacy trials, individual plants in field trials were artificially infested with an aqueous suspension of as few as 400 to as many as 1450 viable Western corn rootworm eggs (actual numbers of eggs per plant used in each trial are indicated as a footnote in the data tables). However, within an individual trial to maintain experimental integrity, the number of eggs per plant was standardized across plots. In artificially infested trials, as

plants reached the V2 to V3 leaf stage, eggs were mechanically placed 3 inches deep into the soil within 2-3 inches of the base of the stalk of each plant. Egg viability was assessed by immobilizing eggs onto a solid support, incubating in the laboratory and observing the proportion of emerged neonates versus unhatched eggs. Field infestations were performed with egg numbers normalized to account for differences in viability among egg populations. In cases where it was not possible or appropriate to artifically infest with Western corn rootworm eggs, the trial design included planting a trap crop with continuous corn or cucurbits the previous year to enhance the natural population of CRW. Egg densities within these plots were not assessed due to the technical complexities of such an analysis. However, root damage observed in the control corn plants within these plots was uniformly more severe compared to non-trap crop plots indicating that the CRW egg density was indeed enhanced.

To assess root damage by CRW feeding, corn plants at the VT to R1 stage (tasseling silking stage), were manually dug from the ground, soil washed from the roots and the roots examined and rated for corn rootworm damage according to the recent Iowa State 0-3 node-injury rating scale (http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html). Most trials included various chemical insecticide treatments as internal controls for comparison with the MIR604-derived hybrid entries. In some cases a chemical insecticide was applied in addition to the MIR604-derived hybrids to assess synergies between the two technologies and to serve as an experimental control. The chemical insecticide treatment included an in-furrow soil-applied granular treatment; Force 3G® (tefluthrin, Syngenta). This chemical formulation represents a highly popular and widely used CRW insecticide used on farms today. Application rates are indicated in the data tables.

B.2. MIR604 Pedigree

The introgression of the transgenes into the early and late maturing corn varieties utilized cross breeding from a single transformation event designated MIR604 (see Figure 17). Control germplasm consisted of near isogenic hybrids using non-transformed germplasm closely matched to the genetic background of MIR604 for that particular stage of the breeding pedigree (indicated by single lined boxes in Figure 17) or negative segregants used as breeding partners in crosses to elite inbred lines (indicated by double lined boxes in Figure 17). Further breeding details can be found in Chapter 1.

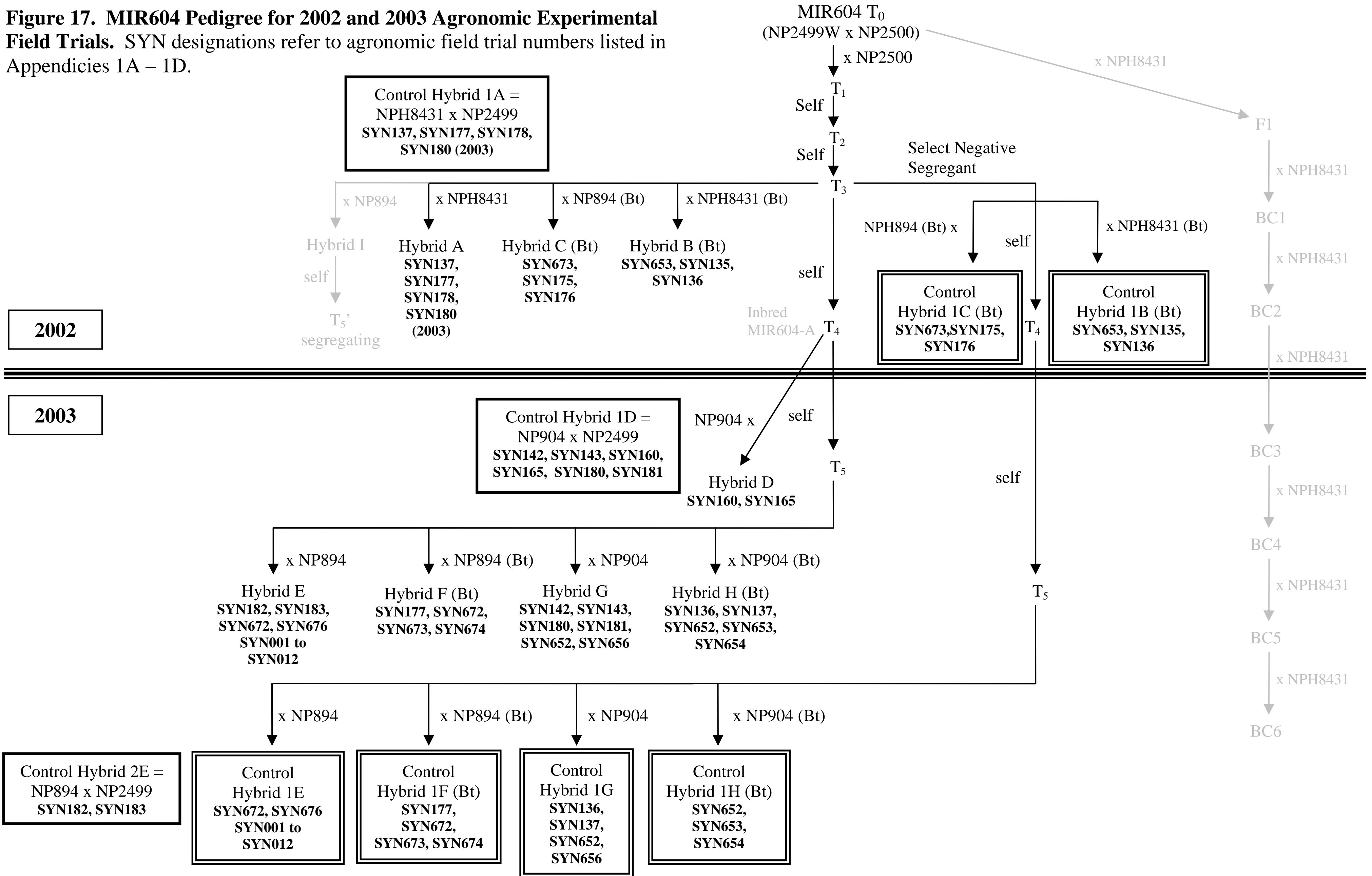


Figure 17. MIR604 Pedigree for 2002 and 2003 Agronomic Experimental Field Trials. SYN designations refer to agronomic field trial numbers listed in Appendicies 1A – 1D.

In order to reduce the confounding effect on yield of insect damage to the corn ears by lepidopteran pests, in some trials MIR604 was deployed in a breeding stack with Bt11 which expresses the Cry1Ab protein (indicated by '(Bt)' in the data tables and Figure 17). In these cases controls consisted of the corresponding Bt11-only hybrids.

C. CORN ROOTWORM CONTROL EFFICACY

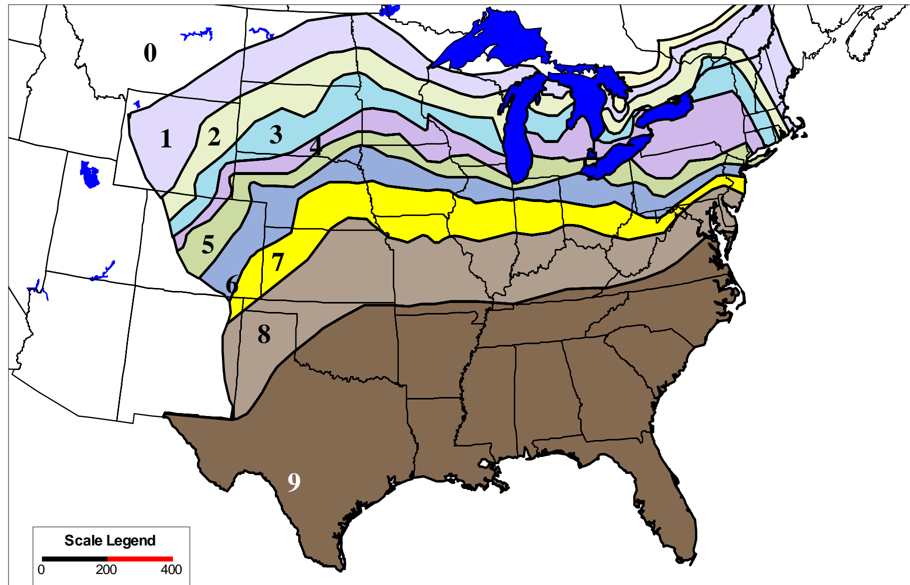
C.1. Western Corn Rootworm.

Western CRW is the most widespread and most damaging rootworm species affecting corn in the U.S. Root damage caused by CRW larval feeding is assessed by digging, examining and rating according to the Iowa State University 0-3 node-injury rating scale (Appendix 1A, Table 1A). According to the upper range of this scale, a rating of 3.00 denotes '*three or more nodes eaten'*, a high degree of root damage, whereas a rating of 0.0 is no damage. In some instances of extreme corn rootworm pressure, total root crown destruction can occur such that no roots are left on the corn plant. Depending on the degree of root crown destruction, this level of root damage is assigned by Syngenta a rating as high as 3.9.

In 2002, MIR604-derived hybrids were evaluated (see Appendix 1A, Table 1A) for insect efficacy at 3 locations in MN and IL (see Appendix 1A, Table 2A). MIR604-derived hybrids showed statistically significant reductions in root damage ratings relative to non-transgenic controls at all locations.

To assess MIR604 hybrids across hybrid maturity zones, a total of eight MIR604 hybrids were assessed for efficacy at 12 locations in 2003 (Appendix 1A, Table 3A). Mean root damage was significantly lower in the MIR604 hybrids compared to the non-transgenic negative isoline controls at all locations. MIR604 hybrids used in these studies included early and late maturity groups (Figure 18). Conventional corn hybrids are developed for optimal performance in specific maturity zones defined by the length of time from planting to harvesting grain. Early maturity hybrids are primarily adapted for growth in maturity zones 5 and lower while late maturity hybrids can be grown in maturity zones 5-8. In some cases efficacy was assessed in early maturity hybrids in trials outside their intended maturity zone. In these trials, the early maturity MIR604 hybrids demonstrated similar, significantly lower root damage as compared to controls when the same hybrid material was grown in their intended maturity zone (Appendix 1A, Table 3A, e.g., trial SYN180 conducted in Bloomington, IL). The same lower root damage ratings observed in the early maturity MIR604 hybrids were also seen in late-maturing MIR604 hybrids grown in the same area indicating that hybrid maturity group had little impact on the overall efficacy of MIR604 hybrids. Between different MIR604 hybrids, no statistical difference in root damage was seen at any of the locations, similar to data acquired in 2002. In two cases where corn rootworm pressure was very low, no statistically significant difference in root damage rating was seen between MIR604 derived hybrids and negative segregant controls (Appendix 1A, Table 3A, trials SYN008 and SYN010).

Figure 18. Designation of Syngenta Seeds Hybrid Corn Maturity Zones



B.2. Northern Corn Rootworm.

The Northern CRW is geographically not as widely distributed as the WCRW and is responsible for less damage to the U.S. corn crop. However, NCRW infestations can be severe in the northern maturity zones and often occur in the presence of WCRW. Due to the difficulties in rearing Northern corn rootworm, a limited number of Northern corn rootworm efficacy trials have been conducted on MIR604 hybrids. Mean root damage ratings for MIR604-derived and non-transgenic control hybrids in glasshouse trials artificially infested with northern corn rootworm show significantly greater efficacy compared to the negative control (Appendix 1A, Table 4A).

MIR604 hybrids were also exposed to natural populations of Northern corn rootworm at some of the locations listed in Appendix 1A, Tables 2A-3A. In particular, the Willmar, MN location (Trial SYN143, Appendix 1A, Table 3A) was populated primarily with Northern corn rootworm (NCRW) while the rest of the 2003 locations were a mixture of Western and Northern corn rootworms. Significantly less root damage was observed in MIR604 hybrids grown under natural NCRW pressure at Willmar, MN relative to near isogenic control hybrids. These results combined with those obtained in the artificially infested glasshouse trial indicate that MIR604 hybrid roots sustain significantly less NCRW feeding damage compared to the near isogenic controls.

C.3. Mexican Corn Rootworm.

Though not as prevalent as Western and Northern CRW, Mexican CRW can cause yield impacting root damage primarily in the southern maturity zones of 8 and 9 (Figure 16). MIR604 hybrids showed efficacy toward Mexican corn rootworm (MCRW) (Appendix 1A, Table 5A). MCRW trials were conducted at two Texas locations in 2003. Mean root damage ratings varied somewhat across the two locations. However, at both

locations, mean root damage in MIR604 hybrids was significantly lower than in the non-transgenic negative isoline controls.

D. YIELD EVALUATION

MIR604-derived hybrids were evaluated for yield (bushels per acre) at five field trials in MN and IL in 2002 (Appendix 1B, Table 1B). According to breeders and agronomists conducting the field trials, growing conditions were considered normal for MN in 2002 while IL field trial locations experienced drought and significant CRW feeding pressure. The impact on yield derived from MIR604 hybrids between the two growing conditions was significant. The average yield of MIR604 hybrids grown at the MN locations was statistically equivalent to the negative segregant controls. However, at the IL locations, the average yield increase of MIR604 hybrids was 61 bu/acre or a 233 % increase in yield over the negative controls (Appendix 1B, Table 1B).

Yield from MIR604 hybrids was also assessed in 2003 at 20 locations (Appendix 1B, Table 2B). In contrast to 2002, growing conditions for 2003 were typical for most of the trial sites. Yield loss associated with moderate corn rootworm damage largely depends upon environmental conditions which can lead to root lodging (high winds) or poor cob development and grain fill (drought stress). If the trial plots do not experience these conditions (as was the case in 2003), then yield losses can often be minimal despite corn rootworm feeding pressure. The average yield of MIR604-derived hybrids was statistically equivalent to or greater than the negative segregant controls at nearly all trial sites in 2003.

In the absence of high corn rootworm pressure and drought stress, MIR604-derived hybrids yield an equivalent amount of grain compared to their negative isoline controls over a wide geographical range. When MIR604-derived hybrids are grown under adverse conditions of heavy corn rootworm pressure and drought stress, a significant positive yield impact is observed which can provide significant yield preservation.

E. AGRONOMIC AND MORPHOLOGICAL CHARACTERISTICS

MIR604-derived hybrids and their non-transgenic controls were grown in 22 locations in 8 states during 2002 and 2003. Up to 18 separate agronomic traits were assessed at each trial; not all traits were recorded at each location (see Table 4). For the great majority of the agronomic traits compared in the two years of data, no statistically significant differences between MIR604-derived hybrids and their negative segregant control counterparts were observed. While some differences between transgenic and control plants were found to be significant, there were no consistent trends in the data across locations or across years that would indicate that any of these differences were due to the presence of the transgene. For example, agronomic traits HU5SN (heat units to 50% silking) and HUPSN (heat units to pollen shed) in the 2002 Stanton, MN trial (Appendix 1C, Table 1C) were significantly different (lower) for both MIR604-derived hybrids compared to their controls. However, these observations did not repeat themselves in the 2003 field trial held at Stanton, MN (Appendix 1C, Table 3C). These

differences also did not occur at other field trial sites in 2003, which were performed in the presence of natural Western and Northern CRW populations. Likewise, significant differences between agronomic traits (e.g. intactness rating, INTLR) observed between MIR604-derived hybrids and their negative isoline controls in 2002 field trials (Appendix 1C, Table 1C) were not observed in corresponding trials in 2003 (Appendix 1C, Table 2C and 4C).

Although instances of statistically different measurement between MIR604 and non-transgenic control hybrids were observed for some traits, the variation observed is within historical ranges derived from agronomic field trials conducted with transgenic events by Syngenta over the past decade. As MIR604 hybrids experience less root feeding damage than control hybrids, some significant agronomic and morphological differences are to be expected depending upon the trait being assessed and combination of pest pressure and environmental conditions experienced at each trial location.

TABLE 4. List and Definition of Traits Assessed in Agronomic Field Trials

Trait Code	Description
DROPP	Percent dropped ears just prior to harvest
EMRGP	Percent plants emerged. (Taken within approx. 14 days post-planting)
ERHTN	Ear height in cm. Taken at R2-R6 stage of corn development.
GMSTP	Grain moisture % measured at harvest time
HAVPN	Harvest population (plants per acre)
HU5PN	Heat units to 50% pollen shed
HU5SN	Heat units to 50% silking
HU9PN	Heat units to 90% pollen shed
HU9SN	Heat units to 90% silking
INTLR	Intactness rating (Late Season integrity of the plant above the ear; 1= completely intact, 9 = 100% of the plants are broken at the ear node)
LRTLP	Late root lodging % (recorded after anthesis)
PLHTN	Plant height in cm. Taken at R2 – R6 stage of corn development.
POL5N	Days to 50% plants pollen shedding
PSTSP	Push test for stalk/root quality on erect plants (data taken just prior to harvest)
SLK5N	Days to 50% plants silking
STKLP	Percent stalk lodging (data taken just prior to harvest)
TWSMN	Grain test weight in lbs/Bu at 15.5% moisture
YGSMP	Grain yield at standard 15.5% moisture

Specific disease trials were conducted in 2002 and 2003 where Syngenta Seeds agronomists, breeders and pathologists observed the disease susceptibility of MIR604-derived hybrids and negative segregant controls to a number of maize pathogens, including Northern corn leaf blight (*Helminthosporium turcicum*), Southern corn leaf blight (*Helminthosporium maydis*), Eyespot (*Kabatiella zeae*) and Gray leaf spot (*Cercospora zeae-maydis*). Disease susceptibility was measured by a 1-9 foliar disease

rating scale where 1 equals no lesions on a leaf and a score of 9 represents highly abundant lesions on all leaves (Appendix 1C, Table 5C). No indication of differential disease susceptibility was observed between negative segregant controls and MIR604-derived hybrids (Appendix 1C, Table 6C).

MIR604-derived hybrids have been grown under a number of USDA permits and notifications in 2002 and 2003 (see Chapter 1, Table 1). As part of the USDA field trial process, Syngenta is required to record any abnormal agronomic occurences observed during the course of the trial. For the 2002 and 2003 growing seasons, no adverse effects relating to non-target and pest insects were recorded. Similarly, there were no reports of enhanced susceptibility to insect pests in either 2002 or 2003.

F. PERFORMANCE OF MIR604 IN CONJUNCTION WITH INSECTICIDAL TREATMENT

A limited number of 2002 and 2003 field trials designed to (1) compare the insect control efficacy of MIR604-derived hybrids with currently practiced chemical insecticide treatments and (2) quantify any added efficacy, yield or agronomic performance afforded by the combination of applying low rates of chemical insecticides with MIR604 (Appendix 1D, Tables 1D-4D). MIR604 hybrids treated with Force 3G insecticide were assessed for efficacy in 2002 (Appendix 1D, Table 1D). During typical growing conditions experienced in Minnesota, root damage ratings for Force 3G treated MIR604 hybrids were not significantly different from similarly treated near isogenic control plants, though the values for root damage were numerically lower for both MIR604 hybrids (Appendix 1D, Table 1D). Under adverse growing conditions in Illinois for 2002, the mean root damage ratings for MIR604 hybrids were significantly lower compared to the negative controls. The difference between the two trials is most likely due to the inability of the insecticide treatment to protect the control roots during the drought conditions in Illinois.

Agronomic performance, including yield, of MIR604 hybrids compared to their near-isoline controls in the presence of Force 3G treatment was also assessed (Appendix 1D, Table 2D). Yield data across both 2002 and 2003 indicated no statistical difference between MIR604-derived hybrids and their negative segregant controls. The application of Force 3G on the negative isoline controls resulted in yields (in 2002) that were equivalent to those of Force 3G treated MIR604 hybrids despite the adverse growing conditions in IL. Clearly, the addition of an insecticide treatment helped protect against yield loss in the negative controls across the majority of locations in 2002 and 2003. One exception was trial (SYN673) in which the Force 3G treatment did not adequately protect the negative control hybrids due to adverse environmental conditions (wet early spring followed by drought) at both of the Bloomington locations in 2002. In this trial, the negative isoline control yield was significantly less than the MIR604 hybrid yield, a result which parallels 2002 yield data for a non-insecticide treated trial showing significant yield loss in the negative isoline control (see Appendix 1A, Table 2B). In 2003 trials, applying Force 3G insecticide to MIR604 hybrids does not appear to produce a synergistic effect on yield as the overall yield values are similar to the untreated non-transgenic controls (Appendix 1D, Tables 2D-4D).

Comparison of agronomic data clearly showed an absence of interaction between insecticidal treatment and performance of MIR604-derived hybrids (Appendix 1D, Tables 2D-4D). Some significant differences were observed between MIR604-derived hybrids and negative isoline controls (see Appendix 1D, Table 3D). However, these differences were not consistent between years, trials and hybrids.

G. CONCLUSIONS

Field trials conducted in 2002 and 2003 demonstrated that MIR604 hybrids provide consistent and statistically significant rootworm control (decreased root damage ratings) compared to non-transgenic controls. As a result, yield performance of MIR604-derived hybrids was statistically significantly greater compared to non-transgenic controls grown under adverse environmental conditions (e.g., drought) and equivalent to non-transgenic controls under normal growing conditions.

Agronomic data generated in 2002 and 2003 clearly show that MIR604-derived hybrids do not display statistically significant differences in agronomic characteristics that would render them phenotypically different than their near isogenic controls, except for traits (such as yield) that may be directly attributed to the root protection afforded by the inserted transgene.

MIR604-derived hybrids, in combination with an insecticide treatment, can provide significantly lower root damage than non-transgenic hybrids similarly treated during adverse growing conditions, and no adverse effects were observed in trials combining MIR604-derived hybrids and Force 3G insecticide.

Chapter 5. Compositional Analysis of Event MIR604

A. SUMMARY

Key nutritional components in maize grain and whole plants (forage) derived from Event MIR604 and near isogenic non-transgenic control plants were compared (Kramer, 2004; see Table 5 and 6 below). The whole plants and grain analyzed were from hybrid pairs (a hybrid pair consisting of transgenic and near isogenic control plants; see Figure 19) grown at 12 locations in the USA over two growing seasons (2002 and 2003). As would be expected from an analysis of this size, sporadic statistically significant differences were observed for some parameters between the MIR604 transgenic and near isogenic controls as represented in the summary of proximate analytes in Tables 7-10 below. All components evaluated in this study were within the range of reported literature values for maize with the exception of potassium in forage and phytosterols in grain.

At the time the forage potassium data were generated, Syngenta was unable to identify and provide a control range for concentrations of potassium in conventional corn forage. Syngenta subsequently conducted a study to measure potassium concentrations in non-transgenic forage using the same methodology that was employed in the original analysis of the MIR604 forage (de Fontes and Kramer, 2006; Table 11). In addition, further literature investigations revealed historical data that was previously overlooked. Forage potassium concentrations for MIR604 and its controls were compared and found to be within both the newly measured and literature ranges (Table 11).

Average phytosterol levels in both control and transgenic grain samples were below the average concentration reported in the literature (Table 12). Syngenta subsequently conducted a study to measure campesterol and stigmasterol concentrations in non-transgenic grain using the same methodology that was employed in the original analysis of the MIR604 grain (de Fontes and Kramer, 2005). Grain campesterol and stigmasterol concentrations for MIR604 and its controls were compared and found to be within the newly measured ranges (Table 12).

For all other analytes, no consistent patterns emerged to suggest that biologically significant changes in composition or nutritive value of the grain or forage had occurred as an unintended result of the transformation process or expression of the transgene. The conclusion based on these data is that there is strong evidence that the genetically modified MIR604 hybrids are substantially equivalent in composition to the isogenic controls, and other commercial hybrids.

Figure 19. **MIR604 Breeding history indicating generations used in the composition analysis of Event MIR604.** The partial breeding history is extracted directly from Figure 1.

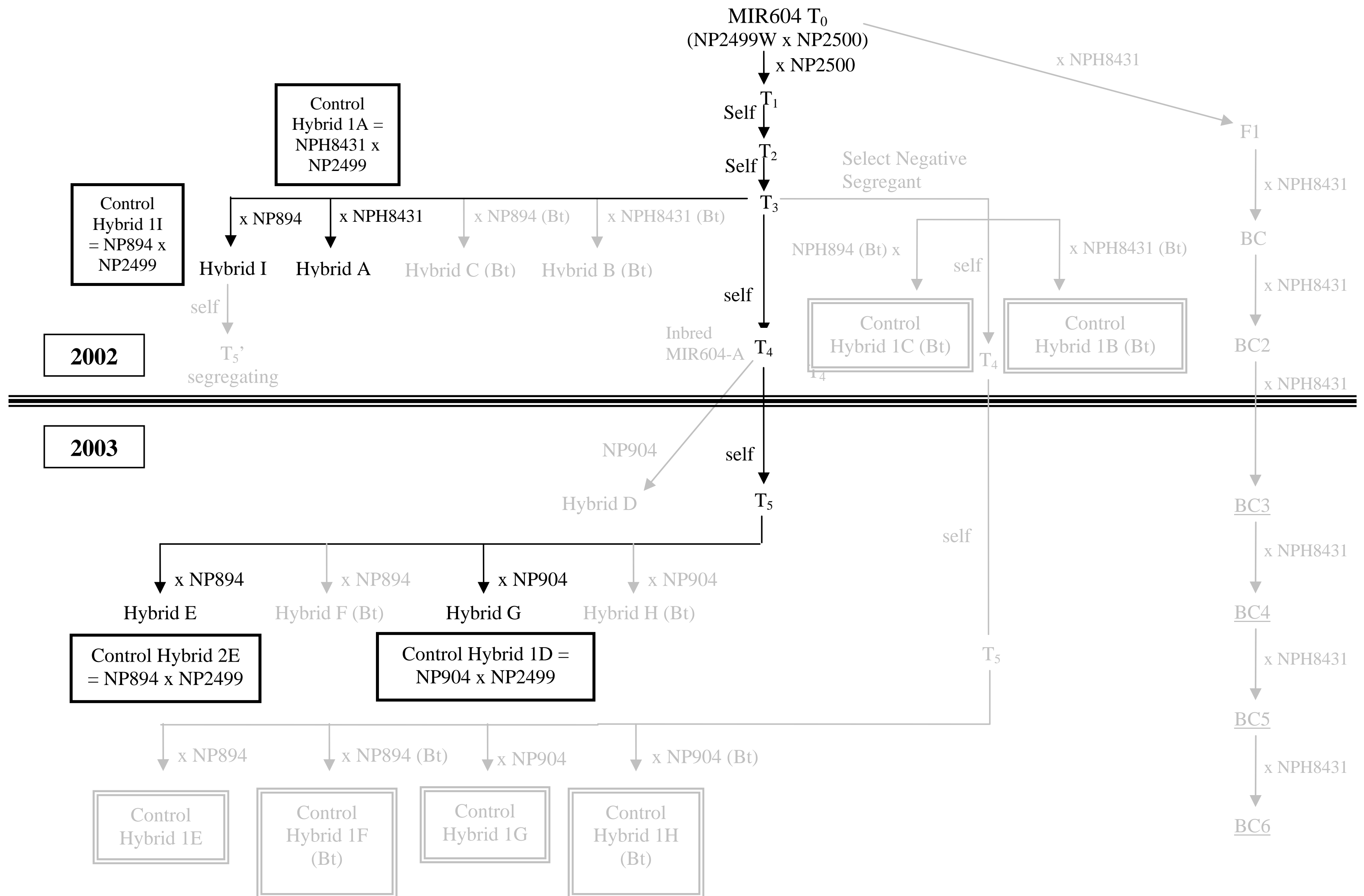


Table 5. Analytes Measured in Grain in 2002 and 2003 Growing Seasons[1]

Analyte	2002	2003
Proximates:		■
ash	■	■
fat	■	■
moisture	■	■
protein	■	■
carbohydrate		■
crude fiber	■	
Acid Detergent Fiber (ADF)		■
Neutral Detergent Fiber (NDF)		■
Total Dietary Fiber (TDF)		■
Minerals: Ca, Cu, Fe, Mg, Mn, P, K, Na, Zn, Cr or Se	■	■
Beta Carotene	■	■
Cryptoxanthin		■
Folic Acid	■	■
Vitamin B_1 (Thiamine)	■	■
Vitamin B_2 (Riboflavin)	■	■
Vitamin B_3 (Niacin)	■	■
Vitamin B_5 (Pantothenic Acid)		■
Vitamin B_6	■	■
Vitamin C		■
Vitamin E (Tocopherols)	■	■
Amino Acid Composition	■	■
Fatty Acid Profile (5 abundant)	■	■
Ferulic and p-Coumaric Acids		■
Furfural		■
Inositol		■
Phytic Acid		■
Raffinose		■
Trypsin Inhibitor		■
Phytosterols:		■
cholestrol		■
campesterol		■
stigmasterol		■
beta-sisterol		■

[1]shaded boxes indicate analytes that were measured in that year

Table 6. Analytes Measured in Forage in 2002 and 2003 Growing Seasons[1]

Analyte	2002	2003
Proximates:		■
ash	■	■
fat	■	■
moisture	■	■
protein	■	■
carbohydrate		■
crude fiber	■	
Acid Detergent Fiber (ADF)	■	■
Neutral Detergent Fiber (NDF)	■	■
Total Dietary Fiber (TDF)	■	■
Minerals: Ca, Cu, Fe, Mg, Mn, P, K, Na, Zn, Cr or Se	■	■

[1]shaded boxes indicate analytes that were measured in that year

Table 7. Summary of Proximate Analysis for 2002 MIR604 Grain. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids[1]. Hybrid designations correspond to those in Figure 19.

	2002						Literature Values				
Analyte[2]	MIR604 Hybrid A	Control Hybrid 1A	Std. Dev.	MIR604 Hybrid I	Control Hybrid 1I	Std. Dev.	OECD (2002)	ILSI (2004)	USDA (2004)	Watson (1987)	Souci (1994)
Moisture	9.18	8.90	0.33	8.93	9.37	0.43	7.0-23	6.1-26.2	10.37	7-23	12-13.2
Protein	10.44	10.02	0.49	11.21	11.41	0.39	6-12.7	6.15-15.01	9.42	6-12	7.61-9.84
Total Fat	2.65	2.63	0.08	3.09	2.89	0.24	3.1-5.8	1.742-5.564	4.74	3.1-5.7	3.20-4.30
Ash	1.56	1.55	0.10	1.59	1.62	0.07	1.1-3.9	0.616-6.282	1.2	1.1-3.9	N/A[4]
Starch	68.70	68.90	1.40	67.99	69.09	1.44	N/A	67.8-73.8	N/A	61-78	60.98-63.80
Crude Fiber[3]	3.49	3.39	0.21	3.49	3.46	0.25	N/A	N/A	N/A	N/A	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid A = Hybrid D, Control Hybrid 1A = Control Hybrid C, Hybrid I = Hybrid F, Control Hybrid 1I = Control Hybrid E.

[2]Percent dry weight except for Moisture (% fw).

[3]Literature values for crude fiber were determined by taking the cumulative low and high values from NDF, ADF and TDF (1.82 – 25.63 % dw for grain).

[4] Data not available.

Table 8. Summary of Proximate Analysis for 2003 MIR604 Grain. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids[1]. Hybrid designations correspond to those in Figure 19.

Analyte[2]	2003						Literature Values				
	MIR604 Hybrid G	Control Hybrid 1D	Std. Dev.	MIR604 Hybrid E	Control Hybrid 2E	Std. Dev.	OECD (2002)	ILSI (2004)	USDA (2004)	Watson (1987)	Souci (1994)
Moisture	9.84	9.84	0.40	9.90	10.32	0.35	7.0-23	6.1-26.2	10.37	7-23	12-13.2
Protein	10.88*	10.42	0.61	11.8*	11.00	0.70	6-12.7	6.15-15.01	9.42	6-12	7.61-9.84
Total Fat	3.53	3.38	0.37	3.88	3.46	0.32	3.1-5.8	1.742-5.564	4.74	3.1-5.7	3.20-4.30
Ash	1.55	1.51	0.15	1.50	1.48	0.11	1.1-3.9	0.616-6.282	1.2	1.1-3.9	N/A[4]
Carbohydrate[3]	84.0*	84.7	0.7	82.8*	84.0	0.6	82.2-82.9	77.4-89.5	74.26	N/A	N/A
Starch	55.2	56.7	2.5	54.5	55.7	2.0	N/A	67.8-73.8	N/A	61-78	60.98-63.80
NDF	13.4	12.9	1.2	12.9	13.2	2.0	8.3-11.9	5.59-22.64	N/A	8.3-11.9	N/A
ADF	5.5	4.9	0.8	5.2	4.9	0.9	3.0-4.3	1.82-11.34	N/A	3.3-4.3	N/A
TDF	13.4	14.1	0.9	13.1	13.5	0.8	11.1	11.8-25.63	N/A	N/A	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, Control Hybrid 1D = Control Hybrid E1, Hybrid E = Hybrid E4, Control Hybrid 2E = Control Hybrid E2.

[2]Percent dry weight except for Moisture (% fw)

[3]Carbohydrates are calculated as the percentage of dry weight = 100% - % protein - % fat - % ash.

[4]Data not available.

*An F-Test probability of <5% was observed indicating a significant difference between transgenic and control. However, all values are within historical ranges demonstrating that hybrids derived from Event MIR604 are not materially different in composition from near isogenic controls.

Table 9. Summary of Proximate Analysis for 2002 MIR604 Forage. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids. Hybrid designations correspond to those in Figure 19.

Analyte[1]	2002						Literature Values	
	MIR604 Hybrid A	Control Hybrid 1A	Std. Dev.	MIR604 Hybrid I	Control Hybrid 1I	Std. Dev.	OECD (2002)	ILSI (2004)
Moisture	64.21	64.63	2.59	71.62	71.94	2.05	62-72	55.3-80.4
Protein	7.27	7.46	0.27	8.02	8.24	0.55	4.7-9.2	3.14-11.56
Total Fat	1.54	1.92	0.38	1.37	1.68	0.37	1.5-3.2	0.373-4.570
Ash	3.46	3.76	0.15	4.01	3.82	0.29	2.9-5.7	1.997-9.638
NDF	37.41	38.20	3.82	39.43	37.63	1.76	40.0-48.2	20.29-63.71
ADF	20.84	21.78	1.63	21.77	21.30	1.23	25.6-34	16.13-41.92
Crude Fiber[3]	19.52	20.38	1.82	20.52	20.30	1.30	N/A[4]	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid A = Hybrid D, Control Hybrid 1A = Control Hybrid C, Hybrid I = Hybrid F, Control Hybrid 1I = Control Hybrid E.

[2]Percent dry weight except for Moisture (% fw)

[3]Literature values for crude fiber were determined by taking the cumulative low and high values from NDF, ADF and TDF (1.82 – 25.63 % dw for grain).

Table 10. Summary of Proximate Analysis for 2003 MIR604 Forage. Mean plus standard deviation of proximate compositional values from two MIR604 hybrids[1]. Hybrid designations correspond to those in Figure 19.

Analyte[2]	2003						Literature Values	
	MIR604 Hybrid G	Control Hybrid 1D	Std. Dev.	MIR604 Hybrid E	Control Hybrid 2E	Std. Dev.	OECD (2002)	ILSI (2004)
Moisture	66.89*	65.10	2.13	72.75*	70.61	2.32	62-72	55.3-80.4
Protein	9.03	8.31	0.96	8.46	8.60	0.85	4.7-9.2	3.14-11.56
Total Fat	2.15	2.12	0.48	1.46	1.64	0.46	1.5-3.2	0.373-4.570
Ash	4.43	4.24	0.55	4.23	4.08	0.56	2.9-5.7	1.997-9.638
Carbohydrate[3]	84.4	85.3	1.1	85.9	85.7	1.1	N/A[4]	76.4-91.5
NDF	41.2	42.4	3.4	44.3	45.2	5.3	40.0-48.2	20.29-63.71
ADF	28.1	28.8	4.0	27.7	29.4	3.4	25.6-34	16.13-41.92
TDF	48.0	47.8	3.2	54.1	53.0	3.7	N/A	N/A

[1]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, Control Hybrid 1D = Control Hybrid E1, Hybrid E = Hybrid E4, Control Hybrid 2E = Control Hybrid E2.

[2]Percent dry weight except for Moisture (% fw)

[3]Carbohydrates are calculated as the percentage of dry weight = 100% - % protein - % fat - % ash.

[4]Data not available.

[*]An F-Test probability of <5% was observed indicating a significant difference between transgenic and control. However, all values are within historical ranges demonstrating that hybrids derived from Event MIR604 are not materially different in composition from near isogenic controls.

Table 11. Potassium Levels Measured in Forage from Conventional and MIR604 Maize Hybrids. Hybrid designations correspond to those in Figure 19.

Analyte[1]	Conventional Corn		Transgenic Hybrids[2]		Literature Values					
	Mean	Range	MIR604 Hybrid G	MIR604 Hybrid E	Adams (1974)	Ballard *et al.* (2001)	Berger (1990)	Buchman-Smith *et al.* (1974)	Kappel *et al.* (1985)	Thomas *et al.* (2001)
Potassium	0.854	0.437-1.45	1.196	1.271	0.02-3.28	1.10-1.33	1.08	0.172-1.87	0.25-1.94	1.06-1.30

[1]Data for conventional corn are derived from 95 samples from 5 non-transgenic commercial hybrids. Data for transgenic corn are derived from 10 samples averaged across a miminum of 10 locations. All units are % dw.

[2]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, and Hybrid E = Hybrid E4.

Table 12. Summary of Phytosterol Analysis in Conventional Field Corn and MIR604 Grain. Hybrid designations correspond to those in Figure 19.

Analyte[1]	Conventional Corn		Transgenic Hybrids[2]		Literature Values[3]
	Mean	Range	MIR604 Hybrid G	MIR604 Hybrid E	
Campesterol	12.1	6.9-18.4	14.0	12.4	32
Stigmasterol	6.9	3.7-10.3	6.33	5.28	21
Beta-sitosterol	50.4	38.3-62.9	45.5	42.6	120

[1]Data for conventional corn are derived from 93 samples from 10 non-transgenic commercial hybrids. Data for transgenic corn are derived from 10 samples averaged across a miminum of 10 locations. All units are mg/100g dw.

[2]The hybrid designations in this table have been changed to maintain nomenclature consistency throughout this petition. The hybrids listed here are named differently in the Kramer 2004 report: Hybrid G = Hybrid E3, and Hybrid E = Hybrid E4.

[3]Souci (1994).

Chapter 6. Quantification of mCry3A and PMI Proteins in Event MIR604

A. SUMMARY

To characterize the range of expression of the mCry3A and PMI proteins in maize (corn) plants derived from Event MIR604, the concentrations of mCry3A protein (the active insecticidal principle) and PMI (the selectable marker) were determined by ELISA in several plant tissues and whole plants at four growth stages (whorl, anthesis, seed maturity and senescence; Joseph and Hill, 2003) in two field maize hybrids (Hybrid A and Hybrid I[3]) and one maize inbred (Inbred MIR604-A; see Figure 20). Hybrids A and I were derived from crossing the elite inbred MIR604-A into the T_3 generation of MIR604 (hemizygous for the transgenes) as depicted in the MIR604 breeding tree in Figure 20. Elite inbred MIR604-A was derived from recurrent selection followed by identification of a homozygous line. The quantity of mCry3A protein was also estimated on a per-acre and a per-hectare basis. Additionally, mCry3A and PMI levels in silage were measured 15, 29 and 75 days after ensiling the plant material from one maize hybrid (Hybrid A).

Quantifiable levels of mCry3A protein were detected in all Event MIR604-derived plant tissues analyzed except pollen. Across all growth stages, mean mCry3A levels measured in leaves, roots and whole plants ranged from *ca.* 3 - 23 µg/g fresh wt. (4 - 94 µg/g dry wt.), *ca.* 2 - 14 µg/g fresh wt. (7 - 62 µg/g dry wt.), and *ca.* 0.9 - 11 µg/g fresh wt. (3 - 28 µg/g dry wt.), respectively. Mean mCry3A levels measured in kernels at seed maturity and senescence ranged from *ca*. 0.6 – 1.4 µg/g fresh wt. (0.8 – 2.0 µg/g dry wt.). Mean mCry3A levels measured in kernels from the MIR604 hybrids at senescence (corresponding to the stage closest to grain harvest) were *ca*. 0.7 µg/g fresh wt. (0.9 µg/g dry wt.) across both hybrid genotypes. Mean mCry3A levels measured in silk tissue at anthesis were below the lower limit of quantification (LOQ), <0.1 µg/g fresh wt. (<1.0 µg/g dry wt.). Mean mCry3A levels measured in silk tissue at seed maturity ranged from *ca*. 0.6 – 1.9 µg/g fresh wt. (1 – 3 µg/g dry wt.). No mCry3A protein was detectable in pollen from neither the inbred MIR604-A nor the hybrids A and I (limit of detection (LOD) = 0.07 µg/g fresh wt., 0.15 µg/g dry wt.).

The levels of mCry3A were generally similar between hybrids for each tissue type at each time point. For the inbred line, mCry3A expression was generally higher than in the hybrids in leaves, roots and whole plants at whorl and anthesis stages and in roots at seed maturity. Over the growing season and across genotypes, estimates of mCry3A in MIR604-derived plants ranged from mean levels of *ca.* 8 g mCry3A/acre (21 g mCry3A/hectare) at senescence stage to *ca.* 240 g mCry3A/acre (592 g mCry3A/hectare) at seed maturity, assuming a planting density of 26,500 plants per acre (65,500

[3] The hybrids listed are designated differently in the Joseph and Hill 2003 report: Hybrid A = MIR604-B and Hybrid I = MIR604-C.

plants/hectare). In silage, the average mCry3A level measured was 2.5 µg/g fresh wt. (7.3 µg/g dry wt.) over 15, 29 and 75 days. By comparison, the level of mCry3A measured in the chopped plant material prior to ensiling was *ca*. 8 µg/g fresh wt. (20 µg/g dry wt).

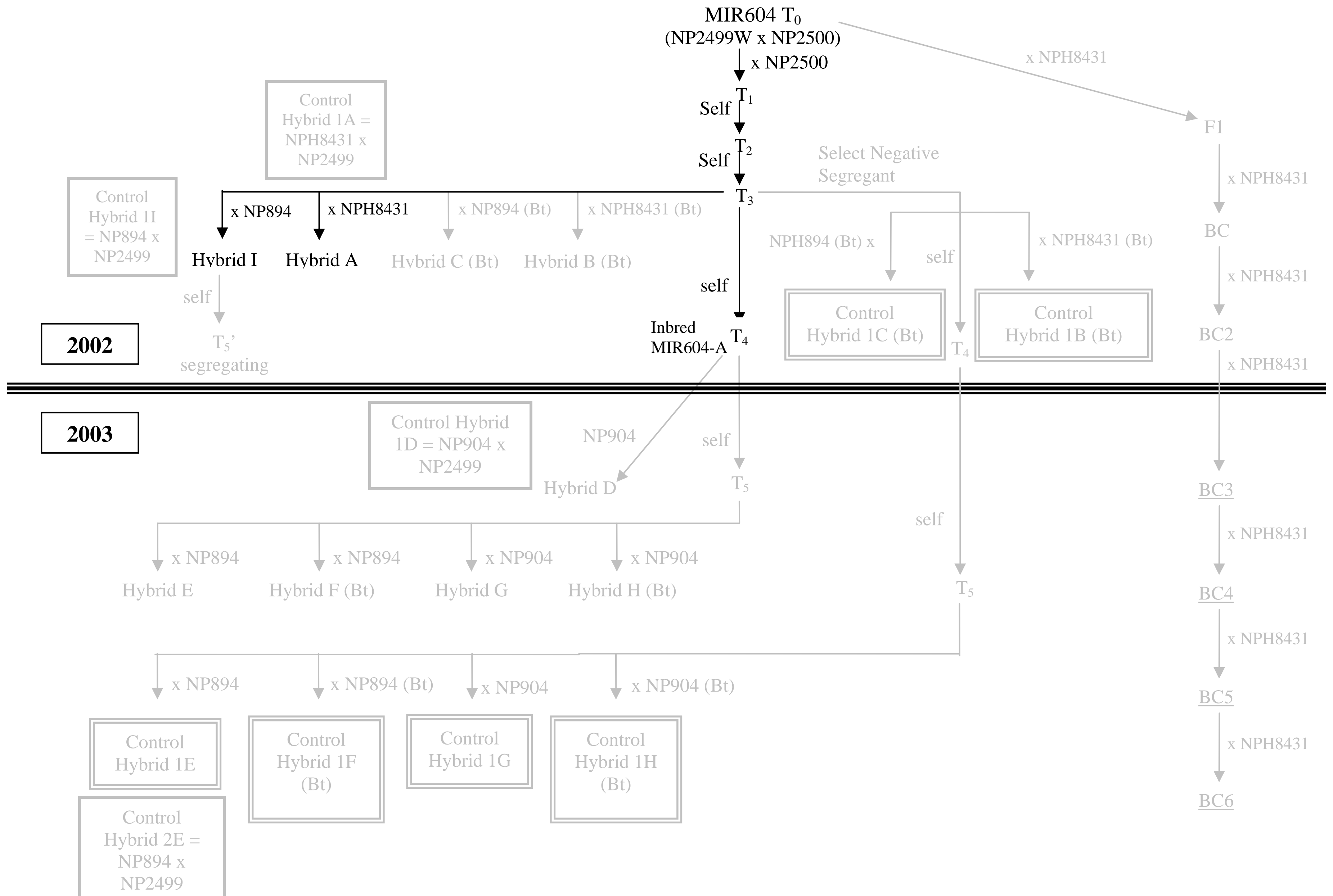
PMI protein was detected in most of the Event MIR604-derived plant tissues analyzed, albeit at low levels. Across all plant stages, mean PMI levels measured in leaves, roots and whole plants ranged from not detectable (ND) to *ca.* 0.4 µg/g fresh wt. (ND – 2.1 µg/g dry wt.), below the LOQ (<0.03 µg/g fresh wt.) to *ca*. 0.2 µg/g fresh wt. (<0.1 – 1.0 µg/g dry wt.), and below the LOQ (<0.02 µg/g fresh wt.) to *ca.* 0.3 µg/g fresh wt. (<0.04 – 2 µg/g dry wt.), respectively. Mean PMI levels measured in kernels at seed maturity and senescence ranged from below the LOQ (<0.06 µg/g fresh wt.) to *ca.* 0.4 µg/g fresh wt. (<0.07 – 0.5 µg/g dry wt.). Mean PMI levels measured in kernels from the MIR604 hybrids at senescence (corresponding to the stage closest to grain harvest) were <0.14 µg/g fresh wt. (<0.17 µg/g dry wt.) across both hybrid genotypes. Mean PMI levels measured in silk tissue at anthesis and seed maturity ranged from below the LOQ (<0.1 µg/g fresh wt.) to *ca.* 0.8 µg/g fresh wt. (<0.2 – 6.8 µg/g dry wt.). PMI in pollen ranged from *ca.* 1.9 – 2.6 µg/g fresh wt. (3.9 – 5.2 µg/g dry wt.).

The levels of PMI were generally similar among the inbred and hybrid genotypes for each tissue type at each time point. PMI was not detectable in silage at all three sampling times (day 15, 29 and 75). By comparison, the level of PMI measured in the chopped plant material prior to ensiling was *ca*. 0.3 µg/g fresh wt. (0.7 µg/g dry wt.).

The stability of mCry3A and PMI protein expression over multiple generations was evaluated. Seed from four successive backcross generations (representing genotypes that were hemizygous for the Event MIR604 transgenes) was grown under greenhouse conditions and leaf material was collected at anthesis for analysis of mCry3A and PMI protein levels. Mean mCry3A protein levels across the four generations were *ca*. 2.3 – 3.1 µg/g fresh wt. (*ca*. 11.8 – 15.5 µg/g dry wt.). Overall, levels were similar across the four generations analyzed and there was no evidence of any significant trend either up or down, indicating that the expression of mCry3A protein is stable.

A similar result was seen for the PMI protein. Mean PMI protein levels across the four generations were *ca*. 0.2 – 0.3 µg/g fresh wt. (*ca*. 1.1 – 1.3 µg/g dry wt.). Overall, levels were similar across the four generations analyzed and there was no evidence of any significant trend either up or down, indicating that the expression of PMI protein is stable. Therefore, both mCry3A and PMI appear to be stably expressed in Event MIR604 maize across multiple generations.

Figure 20. **MIR604 Breeding history indicating generations used in the quantification analysis of Event MIR604.** The partial breeding history is extracted directly from Figure 1.



Chapter 7. Environmental Safety of mCry3A

A. INTRODUCTION

Three sources of data are used to evaluate the environmental safety of the modified Cry3A (mCry3A) *Bacillus thuringiensis*-related insect control protein expressed in the transgenic corn Event MIR604. First, we present a summary of data collected by Syngenta to establish how the spectrum of activity of mCry3A differs from native Cry3A. These data suggest that the toxicity of mCry3A differs from native Cry3A only in the intended increase in activity against certain pest species in the genus *Diabrotica* (Coleoptera: Chrysomelidae). Secondly, we review data on the spectrum of activity of native Cry3A. These data are not used directly to evaluate the risks to non-target organisms from mCry3A in MIR604 because quantitative comparisons of the activity of native Cry3A in the studies and mCry3A in MIR604 are not possible. The purpose of citing these data is to provide a weight of evidence about the likely spectrum of activity (*i.e.*, the hazard) of mCry3A: as Syngenta's laboratory studies show that mCry3A and native Cry3A differ only in the intended increase of activity of mCry3A to certain *Diabrotica* species, the toxicity of native Cry3A is a good predictor of the hazard of mCry3A to non-target organisms (NTOs).

From the native Cry3A data we predict that mCry3A is unlikely to be hazardous except to certain species in 3 families of Coleoptera: the Chrysomelidae (leaf beetles, flea beetles and rootworms), the Curculionidae (weevils and snout beetles) and the Tenebrionidae (darkling beetles). The third source of data which was then used to test this hypothesis, was single-species laboratory studies exposing representative NTOs to concentrations of mCry3A in excess of the expected environmental concentrations (EECs) of mCry3A resulting from the proposed cultivation of MIR604 corn hybrids (hereafter MIR604). Test species were chosen to represent taxa that might be exposed to mCry3A *via* tissues of Event MIR604, or taxa related to the western corn rootworm (*Diabrotica virgifera virgifera*) and northern corn rootworm (*D. longicornis barberi*), the larvae of which are the main target pests of MIR604. In addition, test species were selected to include functional groups found in agricultural fields and other habitats into which mCry3A might spread: birds, freshwater fish, predators and parasitoids of crop pests, soil invertebrates and pollinators.

To ensure an adequate margin of exposure (*i.e.,* a "safety margin") under field conditions, studies on invertebrates were designed to expose test organisms to at least 10 times the EEC of mCry3A using conservative assumptions, yet using likely routes of exposure for the species in question. The margin of exposure for the bird study was calculated in terms of a dose rather than a concentration; bobwhite quail were exposed to about 1000X the worst-case daily dietary dose of mCry3A for a seed-eating bird.

The principal route of exposure to mCry3A for most non-target organisms will be through ingestion of plant material containing mCry3A, or through consumption of prey that has fed on plants containing mCry3A. Therefore to achieve a margin of exposure, most of the test species were exposed to mCry3A *via* a preparation of microbially

expressed mCry3A (Test Substance MCRY3A-0102; 90.3% pure) incorporated into artificial diets. For fish, the principal route of exposure to mCry3A will be consumption of fish feed formulated with corn grain; hence rainbow trout were given feed prepared from MIR604 grain. The safety margin was achieved by preparing the feed using the highest possible proportion of corn grain and the mildest pelleting to minimize heat degradation of mCry3A. No adverse effects of exposure to mCry3A were observed in any of the non-target organism studies.

For each of the tests, a summary of the study design and results is provided, together with a description of the test materials and, where relevant, studies to characterize mCry3A in the diet or test substrate. Final reports of the non-target organism studies (see Table 13) have been submitted to the US Environmental Protection Agency (US EPA) as part of US EPA Experimental Use Permit (67979-EUP-U) and Section 3 (67979-L) applications for Event MIR604 corn.

TABLE 13. List of Environmental Safety Reports Previously Submitted to U.S. EPA

Study Name	MRID #	Reference
Summary of Data Demonstrating the Environmental Safety of Modified Cry3A *Bacillus thuringiensis* Insect Control Protein and Event MIR604-Derived Corn (Maize) to Non-Target Organisms	46155615	Raybould, 2003
An Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Northern Bobwhite	46155616	Gallagher *et al*., 2003
A 28-Day Laboratory Study to Evaluate the Effects of Modified Cry3A Maize Fish Feed (FFMIR604-0103) on the Growth of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)	46155617	Hutchings and Caunter, 2003
A semi-field test to evaluate the effects of the modified Cry3A protein (MCRY3A-0102) on brood development of the honeybee, *Apis mellifera* (Hymenoptera: Apidae)	46155618	Halsall, 2003
Environmental Safety Assessment of Modified Cry3A Protein and Event MIR604 corn to Non-Target Organisms	46265601	Raybould, 2004b
Characterization of Fish Feed Test Substance (FFMIR604-0103) Prepared From Event MIR604-Derived Maize Grain	46265602	Graser, 2004a
A Laboratory Test of the Toxicity of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae)	46265603	Waterman, 2003
Analysis of Test Diet Used to Expose *Coccinella septempunctata* to Modified Cry3A Protein: Supplement to a Report Titled ‘A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle *Coccinella septempunctata* (Coleoptera: Coccinellidae)’	46265604	Graser, 2004c
A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Ground-Dwelling Beetle, *Poecilus cupreus* (Coleoptera: Staphylinidae)	46265605	Vinall, 2004a
Analysis of Test Diet Used to Expose *Poecilus cupreus* to Modified Cry3A Protein: Supplement to a Report Titled ‘A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) Larvae of the Ground-Dwelling Beetle, *Poecilus cupreus* (Coleoptera: Carabidae)’	46265606	Graser, 2004e
A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylinidae)	46265607	Vinall, 2003b
Analysis of Test Diet Used to Expose *Aleochara bilineata* to Modified Cry3A Protein: Supplement to a Report Titled ‘A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylindae)’	46265608	Graser, 2004d

Study Name	MRID #	Reference
A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)	46265609	Vinall, 2003a
Analysis of Test Diet Used to Expose *Orius insidiosus* to Modified Cry3A Protein: Supplement to a Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)'	46265610	Graser, 2004b
Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the Earthworm *Eisenia foetida* in an Artificial soil Substrate	46265611	Vinall, 2004b
Analysis of Soil Used to Expose *Eisenia foetida* to Modified Cry3A Protein: Supplement to a Report Titled 'Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the earthworm *Eisenia foetida* in an Artificial Soil Substrate'	46265612	Graser, 2004f
Environmental Fate Assessment of Modified Cry3A Protein in Event MIR604 Corn	46265613	Raybould, 2004a
Laboratory Soil Degradation of Modified Cry3A Protein (MCRY3A-0102)	46265614	Kramer and Joseph, 2004

B. HAZARD ASSESSMENT OF MODIFIED CRY3A

B.1. Syngenta studies comparing mCry3A and native Cry3A

Lepidopteran and coleopteran pests of corn, and a dipteran pest of fruit crops, were tested at Syngenta for sensitivity to mCry3A (see Table 14). In most cases, native Cry3A was also included in the tests for comparison. These laboratory bioassays were conducted as screening tests, using a concentration from 500-600 µg of either mCry3A or native Cry3A protein/ml diet. In most cases, first instars (10 larvae per replicate, 3 replicates) were fed artificial diet with an overlay of microbially expressed mCry3A or native Cry3A protein solution. The microbially expressed mCry3A protein was produced using the same modified *cry3A* gene that was used in the maize transformation that resulted in event MIR604. Due to problems with quarantine and commercial availability of Mexican corn rootworm (*Diabrotica virgifera zeae*; MCRW), this species was not tested in laboratory bioassays. However, Event MIR604 was tested for efficacy against this species in the field. Field trial data indicate that Event MIR604 derived corn plants have significant resistance against root damage caused by MCRW larval feeding when compared to negative controls (see **Agronomic Performance of Event MIR604 Hybrids**, Chapter 4).

Table 14. Susceptibility of insect pest species to native Cry3A and mCry3A proteins via direct exposure in laboratory studies conducted at Syngenta (unpublished data)

Order/ Species	Common name	Family	Cry3A	mCry3A
Coleoptera				
Leptinotarsa decemlineata	Colorado potato beetle	Chrysomelidae	Active	Active
Diabrotica virgifera virgifera	Western corn rootworm	Chrysomelidae	Not Active	Active
Diabrotica longicornis barberi	Northern corn rootworm	Chrysomelidae	Inconsistent Activity	Active
Diabrotica undecimpunctata	Southern corn rootworm Spotted cucumber beetle	Chrysomelidae	Not Active	Not Active
Diabrotica balteata	Banded cucumber beetle	Chrysomelidae	LowActivity[1]	Active
Anthonomus grandis	Cotton boll weevil	Curculionidae	Not Active	Not Active
Lepidoptera				
Agrotis ipsilon	Black cutworm	Noctuidea	Not Active	Not Active
Helicoverpa zea	Corn earworm	Noctuidae	Not Active	Not Active
Ostrinia nubilalis	European corn borer	Pyralidae[2]	Not Active	Not Active
Spodoptera frugiperda	Fall armyworm	Noctuidea	Not Active	Not Active
Pectinophora gossypiella	Pink bollworm	Gelechiidae	Not Active	Not Active
Heliothis virescens	Tobacco budworm	Noctuidea	Not Active	Not Active
Diptera				
Drosophila melanogaster	Fruit fly	Drosophilidae	Not tested	Not Active

[1] From Herrnsdtadt *et al.,* 1987
[2] Alternatively classified as Crambidae

These results confirmed that native Cry3A is primarily active against CPB and has minimal activity against NCRW, both members of the Chrysomelidae family of beetles. The results also showed that 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 corn in the USA.

The mCry3A as expressed in Event MIR604 corn showed moderate activity against *Diabrotica balteata*, whereas a previous report indicated that native Cry3A had low activity against these chrysomelid beetles (Herrnstadt *et al*., 1987). However, native Cry3A was not included in this Syngenta test so a direct comparison is not available.

In summary, these data show that the mCry3A toxin expressed in Event MIR604 has enhanced toxicity towards some species of chrysomelid beetles (NCRW and WCRW) when compared with native Cry3A, but has no apparent altered activity to the other insect species tested, with the possible exception of *D. balteata*.

B.2 Published literature and data reports

This section collates studies on the toxicity of native Cry3A from the scientific literature and regulatory reports submitted to the US EPA. The purpose is to delimit the taxonomic range of sensitivity to native Cry3A. Because studies in Section B.1.a suggest

that mCry3A has the same spectrum as native Cry3A, apart from intended enhanced activity against particular *Diabrotica* species, the limit of toxicity of native Cry3A predicts the taxonomic limits of the toxicity of mCry3A. The data in this section are not intended for use directly in a quantitative risk assessment of MIR604; they are intended to contribute to the weight of evidence that the hazard of mCry3A is restricted to species in 3 families of Coleoptera.

The effects of native Cry3A protein on many insect pests have been reported in the literature (Table 15). Results from different studies are sometimes difficult to compare, as the levels of activity are often qualitative (low, high, or intermediate activity); there are no established guidelines that define these categories in reference to mortality observed in the tests. However, the results obtained to date show that only pest species from the order Coleoptera are susceptible to the Cry3A toxin. Among the coleopteran pest species tested, only some species belonging to the Chrysomelidae, Curculionidae and Tenebrionidae families were shown to be susceptible to Cry3A in laboratory assays.

Additionally, transgenic plants expressing Cry3A protein have demonstrated efficacy in controlling coleopteran pests in the Chrysomelidae family. These include potatoes (Perlak *et al*., 1993; Adang *et al*., 1993) and tomatoes (Rhim *et al.*, 1995) for control of CPB; poplar plants for control of *Chrysomela tremulae* (Génissel *et al*., 2003); and eucalyptus plants for control of *Chrysophtharta bimaculata, Chrysophtharta agricola* and *Chrysophtharta variicolis* (Harcourt *et al*., 2000).

Table 15. Susceptibility of insect pest species to native Cry3A proteins via direct exposure in laboratory studies (van Frankenhuyzen and Nystrom, 2002)

ORDER/Family *Species*	**Gene**	**Stage/Instar**[1]	**Activity**	**Reference**
COLEOPTERA **Chrysomelidae**				
Chrysomela scripta	cry03Aa1	L2	highly active	Federici and Bauer, 1998
Diabrotica balteata	cry03Aa1	larva	low activity	Herrnstadt *et al.,* 1987
Diabrotica balteata	cry03Aa1	adult	low activity	Herrnstadt *et al.,* 1987
Diabrotica undecimpuctata	cry03Aa1	adult, L1, L2	low activity	Herrnstadt *et al.,* 1986
Diabrotica undecimpunctata	cry03Aa4	neonate	not active	Macintosh *et al.,* 1990
Diabrotica undecimpunctata	cry03Aa5	adult	not active	Johnson *et al.,* 1993
Diabrotica undecimpunctata	cry03Aa5	L1	low activity	Johnson *et al.,* 1993
Haltica tombacina	cry03Aa1	adult,L2, L3	highly active	Herrnstadt *et al.,* 1986
Leptinotarsa decemlineata	cry03Aa1	L1, L2	highly active	Herrnstadt *et al.,* 1986
Leptinotarsa decemlineata	cry03Aa2	neonate	active	Sekar *et al.,* 1987
Leptinotarsa decemlineata	cry03Aa2	L2	active	Wu and Dean., 1996
Leptinotarsa decemlineata	cry03Aa4	L1	active	MacIntosh *et al.,* 1990
Leptinotarsa decemlineata	cry03Aa4	neonate	active	McPherson *et al.,* 1988
Leptinotarsa decemlineata	cry03Aa5	neonate	active	Donovan., 1988
Leptinotarsa decemlineata	cry03Aa5	adult	not active	Johnson *et al,* 1993
Leptinotarsa decemlineata	cry03Aa5	L1	active	Johnson *et al.,* 1993
Phaedon cochleariae	cry03Aa	L2	highly active	Carroll *et al.,* 1989
Phyllotreta armoraciae	cry03Aa4	L1	not active	MacIntosh *et al.,* 1990
Pyrrhalta luteola	cry03Aa1	not specified	highly active	Herrnstadt *et al.,* 1986

ORDER/Family *Species*	Gene	Stage/Instar[1]	Activity	Reference
<u>COLEOPTERA</u>				
Tenebrionidae				
Tenebrio molitor	cry03Aa	not specified	low activity	Carroll *et al*., 1989
Tenebrio molitor	cry03Aa1	L1, L2, L3	intermediate activity	Herrnstadt *et al.,* 1986
Tribolium cataneum	cry03Aa1	adult, L3	intermediate activity	Herrnstadt *et al.,* 1986
Tenebrio molitor	cry03Aa2	L7	low activity	Wu and Dean., 1996
Curculionidae				
Anthonomus grandis	cry03Aa1	adult, L2, L3	highly active	Herrnstadt *et al.,* 1986
Anthonomus grandis	cry03Aa4	neonate	not active	Macintosh *et al.,* 1990
Hypera brunneipennis	cry03Aa1	adult	not active	Herrnstadt *et al.,* 1987
Hypera brunneipennis	cry03Aa1	larva	highly active	Herrnstadt *et al.,* 1987
Hypera postica	cry03Aa4	L1	not active	Macintosh *et al.,* 1990
Otiorhynchus sulcatus	cry03Aa1	L2, L3	intermediate activity	Herrnstadt *et al.,* 1986
Premnotrypes vorax[2]	cry03Aa	L1	active	Gomez *et al.,* 2000
Dermestidae				
Attagenus unicolor	cry03Aa1	L3	not active	Herrnstadt *et al.,* 1986
Bruchidae				
Callosobruchus maculatus	cry03Aa1	adult	low activity	Herrnstadt *et al.,* 1987
Callosobruchus maculatus	cry03Aa1	larva	low activity	Herrnstadt *et al.,* 1987
Nitidulidae				
Carpophilus hempterus	cry03Aa1	larva	not active	Herrnstadt *et al.,* 1987
Ptinidae				
Gibbium psylloides	cry03Aa1	adult	not active	Herrnstadt *et al.,* 1986
Scarabeidae				
Popillia japonica	cry03Aa4	L1	not active	Macintosh *et al.,* 1990
<u>DIPTERA</u> **Cuclidae** *Aedes aegypti*	cry03Aa1	L1	not active	Herrnstadt *et al.,* 1986
Muscidae *Musca domestica*	cry03Aa1	larva	not active	Herrnstadt *et al.,* 1987
<u>LEPIDOPTERA</u> **Noctuidae**				
Spodoptera exigua	cry03Aa1	L1	not active	Herrnstadt *et al.,* 1986
Trichoplusia ni	cry03Aa1	L1	not active	Herrnstadt *et al.,* 1986

[1] L1, L2, L3, etc. refer to instars 1, 2, and 3, etc.

[2] Entry is from the literature and does not appear in van Frankenhuyzen and Nystrom database

In addition to these studies, data supporting the registration of a Cry3A plant-incorporated protectant in Monsanto's NewLeaf® Bt potato includ tests of the sensitivity of selected insect pest species to Cry3A. These included three rootworm species; four lepidopterans (European corn borer, tobacco hornworm, corn earworm, and tobacco budworm); one dipteran (yellow fever mosquito); one orthopteran (German cockroach); and one hemipteran (green peach aphid) (US EPA, 2001a). Among the tested species, only the Colorado potato beetle (*Leptinotarsa decemlineata*: Chrysomelidae) displayed significant mortality.

In addition to the tests conducted to establish the toxicity of Cry3A protein to insect pest species, there are a number of studies in the literature that show that native Cry3A does not have any detectable toxicity to non-target insect species and other invertebrates (see Table 16).

Table 16. Non-target invertebrate species shown to be insensitive to native Cry3A protein as expressed in Bt potato via direct exposure in laboratory studies

Order/ Test Species	Common name	Reference
Coleoptera		
Hippodamia convergens	convergent ladybeetle	US EPA, 2001a
Hymenoptera		
Apis mellifera	honeybee	US EPA, 2001a
Nasonia vitripennis	parasitic wasp	US EPA, 2001a
Isotomidae		
Folsomia candida	springtail (collembola)	Sims and Martin, 1997
Xenylla grisea	springtail (collembola)	Sims and Martin, 1997
Neuroptera		
Chrysoperla carnea	green lacewing	US EPA, 2001a
NON-INSECT SPECIES		
Phylum/Subphylum/ ***Genus + species***		
Annelida		
Eisenia foetida	earthworm	US EPA, 2001a

There are many reports in the literature demonstrating that Bt δ-endotoxins do not have adverse toxic effects on mammalian or avian species, or freshwater fish (US EPA, 2001a; Mendelsohn *et al.,* 2003). Studies supporting the registration of a native Cry3A plant-incorporated protectant in Bt potato showed that this protein is not toxic to mammals or birds (Table 17).

Table 17. Tests of native Cry3A on vertebrate species.

Species	Study type	Treatment	Results	References
bobwhite quail	Dietary toxicity	50,000 ppm in Cry3A potato tubers	No treatment related adverse effects	US EPA, 2001a
mouse	Acute oral toxicity	5220 mg Cry3A/kg body weight	No treatment related adverse effects	US EPA, 2001a

B.3 Conclusions from Studies of Cry3A and mCry3A Among Target and Non-Target Species

The data described in this summary show that the toxicity of native Cry3A proteins is limited to certain species of Coleoptera in the families Chrysomelidae, Tenebrionidae and Curculionidae. No toxic effects have been recorded on non-target insect species or on other invertebrates or vertebrates. As described herein (see Table 14), data generated by Syngenta demonstrate that the mCry3A protein as expressed in Event MIR604 corn has an extended spectrum of activity against certain species of *Diabrotica*, so it is now highly active towards the major pest species western corn rootworm and northern corn rootworm, but its activity against other species tested appears to be unchanged; therefore we predict from these studies that mCry3A is hazardous only to certain species of Chrysomelidae, Tenebrionidae and Curculionidae.

The next section describes studies that test the hypothesis that the hazard of mCry3A is limited to certain species of Chrysomelidae, Tenebrionidae and Curculionidae. Estimates of exposure in these toxicity studies are compared with estimated environmental concentrations (EECs) or daily dietary doses (DDDs) of mCry3A that will result from the proposed cultivation of MIR604. The ratio of the exposure in the study to the EEC or the DDD is the margin of exposure (“safety margin”). High safety margins (>10X EEC) provide confidence that the results of the hazard studies are predictive of the effects of mCry3A on species that have not been tested; they also reduce the likelihood of type II statistical errors, that is the study fails to detect an effect when mCry3A is hazardous at the EEC.

C. LABORATORY SAFETY ASSESSMENT OF MODIFIED CRY3A PROTEIN AND EVENT MIR604 CORN TO NON-TARGET ORGANISMS

C.1 Applicability of Non-Target Organism Studies with Purified mCry3A Protein to the Environmental Safety of Corn Plants Derived from Event MIR604

C.1.a. Test Substances Used for Safety Studies and Margins of Exposure

All non-target organism tests with mCry3A, apart from the fish study, were conducted with a test substance prepared from cells of recombinant *Escherichia coli* over-expressing the gene for mCry3A that is present in MIR604. Purification of the *E. coli* culture yielded a white powder, designated MCRY3A-0102, containing 90.3% w/w mCry3A. The mCry3A in MCRY3A-0102 was shown to be chemically and biologically equivalent to mCry3A produced in MIR604 (Joseph and Graser, 2003a). MCRY3A-0102 was presented to the test organisms in appropriate artificial diets as judged by low mortality in negative control treatments and high mortality in positive control treatments.

Microbially expressed protein was chosen in preference to plant material containing mCry3A so that non-target organisms could be exposed to at least ten times the estimated exposure concentration of mCry3A. Exposure to ≥10X EEC provides a margin of safety to allow for the possibility that the EEC may be exceeded in certain environments where corn derived from Event MIR604 is grown. The margin of safety also allows for the possibility of different sensitivity to mCry3A within the groups of non-target organisms represented by the chosen test species. In all non-target arthropod studies, the test substance was presented in diets or other media at 50 µg mCry3A/g. Preliminary measurement of mCry3A concentrations in leaves of hybrids derived from Event MIR604 suggested a value of about 4 µg/g fresh weight (other plant tissues contained less than this value). Assuming an extraction efficiency of approximately 80%, leaves of MIR604 hybrids were estimated to contain about 5 µg mCry3A/g averaged over the life of the plant. As non-target arthropods tend not to consume corn leaf tissue, 5 µg/g represented a preliminary extreme worst-case EEC of mCry3A, and therefore the test organisms were exposed to 50 µg mCry3A/g to ensure an exposure of at least 10X EEC.

Confirmed values of concentrations of mCry3A in MIR604 hybrids were higher than original estimates. Leaves contained about 5.1 µg/g fresh weight averaged over four time points; the highest concentration was at seed maturity (7.8 µg/g fresh weight) and the lowest at anthesis (3.8 µg/g fresh weight) (Joseph and Hill, 2003). The extraction efficiency for MIR604 leaves was determined to be 77.1% (Joseph and Hill, 2003), giving a corrected mean value of 6.6 µg mCry3A/g fresh weight.

Although the concentration of mCry3A in test diets represents a clear margin of exposure based on the concentration of mCry3A protein in the leaves of MIR604, leaf concentration is not a realistic EEC because (by definition) non-target organisms do not consume significant quantities of crop tissues. When realistic routes of exposure are considered, test materials containing 50 µg mCry3A/g represent at least 10X EEC for the non-target arthropods tested. The rationale for setting the EEC for each test species is given in the study summaries in Part C.3. below.

Studies of the toxicity of mCry3A to earthworms and birds also used MCRY3A-0102 as the test substance. The calculation of the EEC for earthworms was based on the concentration of mCry3A in senescent leaf tissue of MIR604 hybrids (Joseph and Hill, 2003). The amount of protein used in the bird study was calculated from concentrations

in kernels of MIR604 hybrids (Joseph and Hill, 2003), and in this case the margin of exposure relates to a dose rather than a concentration of mCry3A. The calculation of an EEC for earthworms and a measure of dose for birds are described in the respective study summaries in Part C.3. below.

The assessment of toxicity of mCry3A to freshwater fish was the single exception to the use of MCRY3A-0102 as the test substance in non-target organism studies. The main anticipated route of exposure to fish will be the consumption of feed prepared from grain of MIR604 (Raybould, 2004b). Therefore fish feed was prepared using MIR604 grain in a manner intended to provide a margin of exposure. It is possible that fresh water fish could be exposed during flooding of corn fields. However, the exposure to mCry3A *via* this route is likely to be minimal compared with exposure through feed because of degradation and dilution of the protein; also it is not certain whether fish migrate into flooded fields when rivers are in spate.
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C.1.b. Selection of test species

The rationale for species selection was based on the requirement to test the hypothesis that modification of native Cry3A to enhance toxicity to western and northern corn rootworm larvae has not changed its spectrum of activity to non-target organisms. Several criteria were employed to select test species:

- Potential for exposure: common in cornfields, preys upon corn pests, eats corn tissue or processed corn, etc.
- Taxonomy: similarity to target pest or to exposed species for which no test exists.
- Test practicalities: availability of artificial diet, laboratory culture, sensitive endpoint, etc.
- Testing of particular functional groups as required by EPA (US EPA, 1996).

The species chosen for testing, along with the main reasons for their selection, are summarized in Table 18. A more detailed summary is given below:

C.1.b.1. Bobwhite quail - *Colinus virginianus* (Galliformes: Phasianidae)

Birds may be exposed to mCry3A through eating grain of MIR604 plants. Bobwhite quail is a standard representative bird species for testing the environmental effects of crop protection products and PIPs.

C.1.b.2. Rainbow trout – *Oncorhynchus mykiss* (Salmoniformies: Salmonidae)

The main route of exposure of freshwater fish to mCry3A is likely to be through feed prepared from MIR604 grain (Raybould, 2004b). The Rainbow trout was chosen for testing because it is a common freshwater fish often raised in fish farms and is a standard organism for laboratory tests.

C.1.b.3. Insidious flower bug – *Orius insidiosus* (Hemiptera: Anthocoridae)

O. insidiosus is a very common beneficial insect in cornfields in the United States, where it is useful in controlling pests such as corn earworm, corn leaf aphid, European corn borer, southwestern corn borer, spider mites and thrips (Steffey *et al*., 1999). In addition to importance in its own right, *O. insidiosus* is also a representative of the Hemiptera, an order that contains beneficial insects such as damsel bugs, assassin bugs and stink bugs, and also plant pests and even ectoparasites of birds and mammals.

Of all the non-target species tested, *O. insidiosus* is most likely to be exposed to mCry3A through consumption of tissue of MIR604. *Orius* adults lay eggs in corn leaves and the nymphs burrow through leaf tissue to reach the leaf surface to begin feeding. In later life stages, *Orius* bugs are generalist predators; they also eat pollen and have been observed apparently feeding on leaves. A closely related species, *O. laevigatus*, is used widely as a representative non-target arthropod in toxicity tests of crop protection products in the European Union. Experience with testing *O. laevigatus* provides useful information on the robustness of endpoints and expected levels of control mortality for the purpose of setting validity criteria for the *O. insidiosus* study.

C.1.b.4. Seven spot ladybird beetle – *Coccinella septempunctata* (Coleoptera: Coccinellidae)

The seven spot ladybird beetle is not a native of the USA, but has been widely introduced to control corn leaf aphids. It is the 'predominant' ladybird in the eastern United States (Steffey *et al*., 1999). Like the target rootworm pests, ladybirds are Coleoptera and therefore provide a good test of the hypothesis that the effect of modifying Cry3A is limited to the intended enhancement of toxicity to western and northern corn rootworm. *C. septempunctata* is widely used in the European Union as a representative non-target arthropod for testing the effects of crop protection products. *C. septempunctata* was chosen in preference to *Coleomegilla maculata* because the latter species consumes corn pollen, which is not a realistic route of exposure to mCry3A because the protein is undetectable in MIR604 pollen (Joseph and Hill, 2003).

C.1.b.5. Rove beetle – *Aleochara bilineata* (Coleoptera: Staphylinidae)

Aleochara bilineata has been recorded from the USA, though it is not a native species, and it was selected as a representative rove (staphylinid) beetle. Like ground beetles, rove beetles are common soil invertebrates in cornfields (Steffey *et al*., 1999) and have been recorded as eating eggs and larvae of corn rootworm species. Rove beetles are generalist predators, although some species will eat decaying plant tissue; larvae of *Aleochara* are parasitoids of fly pupae. The most likely route of exposure of rove beetles to mCry3A is through consumption of insects that have fed on MIR604 or by eating decayed tissues of MIR604. It is also possible that developing larvae could be exposed inside their host if it had eaten MIR604 tissue prior to pupating. As with *Coccinella* and *Poecilus*, one of the main reasons for selecting *Aleochara* was to investigate further the

spectrum of activity of mCry3A within the Coleoptera. *Aleochara* is a commonly used species for testing the safety of crop protection products in the European Union, and therefore there is extensive information on test endpoints for studies involving this species.

C.1.b.6. Carabid (ground) beetle – *Poecilus cupreus* (Coleoptera: Carabidae)

Poecilus cupreus is not recorded from the USA, but *P. chalcites* is common in US cornfields (Steffey *et al*., 1999). *P. cupreus* was selected for testing because it is a representative ground (carabid) beetle. Ground beetles are common generalist predators in the soil of cornfields (Steffey *et al*., 1999) and could be exposed to mCry3A through eating pest species that have eaten MIR604 tissue. Ground beetles are Coleoptera and are therefore another important taxon for indicating the spectrum of activity of mCry3A to non-target species. Historically, *Poecilus cupreus* adults were used in safety assessments of crop protection chemicals in the European Union. More recently, they have been used less because of a perceived lack of sensitivity (e.g. Candolfi *et al*., 1999). To overcome this drawback, larvae were used as they are expected to be more sensitive than the adults.

C.1.b.7. Honeybee – *Apis mellifera* (Hymenoptera: Apidae)

In general, corn is not an important source of food for honeybees, mainly because corn does not produce nectar. Honeybees will forage for corn pollen, although it is not a favored source of food. However, mCry3A is not detectable in pollen of MIR604 plants (Joseph and Hill, 2003) and therefore exposure of honeybees to mCry3A will be negligible. Nevertheless, *A. mellifera* is a useful test organism because it is a species of Hymenoptera for which methods exist to expose both larval and adult stages to test substances. Larval exposure is particularly valuable because there is potential for larvae of parasitic Hymenoptera to be exposed to mCry3A while they are developing within the body of an organism that has fed on MIR604 plants. At present there is no validated method for exposing parasitoid larvae to high doses of PIPs, although there are methods for exposing adults (*e.g.*, Romeis *et al*., 2003). Insect larvae are usually more sensitive to toxins than are adults of the same species; therefore larval honeybee exposure was considered to be a better predictor than exposure of adult parasitoids to assess the toxicity of mCry3A to parasitic Hymenoptera.

C.1.b.8. Earthworm - *Eisenia foetida* (Haplotaxida: Lumbricidae)

The earthworm *Eisenia foetida* is a standard soil invertebrate for testing the effects of crop protection chemicals and PIPs. Earthworms may be exposed to mCry3A through ingestion of soil containing fragments of MIR604 plant tissue, most likely derived from roots or senescent leaf material.

Table 18. Ecotoxicology studies: species selection

Test species	Common name	Order: family	Functional group	Comments
Colinus virginianus	bobwhite quail	Galliformes: Phasianidae	Bird	Representative
Oncorhynchus mykiss	rainbow trout	Salmoniformies: Salmonidae	Freshwater fish	Possible exposure *via* grain in feed
Orius insidiosus	insidious flower bug	Hemiptera: Anthocoridae	Predator (foliar)	Common beneficial insect in corn
Coccinella septempunctata	seven spot ladybird	Coleoptera: Coccinellidae	Predator (foliar)	Common beneficial insect in corn
Aleochara bilineata	rove beetle	Coleoptera: Staphylinidae	Predator (soil)	Representative
Poecilus cupreus	ground beetle	Coleoptera: Carabidae	Predator (soil)	Representative
Apis mellifera	honeybee	Hymenoptera: Apidae	Pollinator	Representative (e.g. for parasitoids)
Eisenia foetida	earthworm	Haplotaxida: Lumbricidae	Soil invertebrate	Representative

C.1.b.9. Functional groups not tested

EPA testing requirements specify that the effects of PIPs on freshwater invertebrates should be assessed. Historically, this has involved two-day exposures of *Daphnia magna* to pollen from the Event expressing the PIP, because dispersal of pollen into ponds and other water bodies is the most likely route of exposure for freshwater invertebrates. There is no detectable expression of mCry3A in MIR604 pollen (Joseph and Hill, 2003) and therefore minimal exposure of freshwater invertebrates to mCry3A is expected *via* this route. Another possible route of exposure of mCry3A to freshwater invertebrates is during flooding of corn fields. However, exposure outside corn fields is expected to be negligible *via* this route due to the degradability of the protein and its likely large dilution. Accordingly, a request to waive this test was made to the US EPA (Vlachos, 2004) as part of a FIFRA Section registration application; this application has been deemed complete by the US EPA, indicating that the request for a waiver was successful.

EPA also requires that the risk of PIPs to wild mammals be assessed. No test solely for this purpose was carried out; however, a 14-day mouse acute oral toxicity study (Johnson, 2003), undertaken to assess the safety of mCry3A to humans and livestock, was used to estimate the toxicity of mCry3A to wild mammals. The estimate of toxicity and data on exposure allow the risk to wild mammals from the cultivation of MIR604 corn to be assessed.

C.1.c. Test designs and endpoints

To provide maximum predictive power, the non-target organism studies attempted to achieve the following results within the constraints of test design:

- Exposure to ≥ 10X EEC mCry3A for as long as practicable
- Exposure of the potentially most sensitive life stage of the test species
- Measurement of sensitive endpoints
- Minimal effects on negative control populations
- Verification of route of exposure by use of appropriate toxic reference items

These objectives were met using a variety of techniques (see Table 19 for a summary). Where possible, fresh batches of artificial diet prepared with the test substance were presented daily to minimize any degradation of mCry3A during the test. Meat-based diets were necessary for some test species, and these were cooked before addition of the test substance to denature proteases that might degrade mCry3A; analysis of mCry3A in the diet confirmed that bioactive mCry3A was present in these diets. For non-target insects apart from *Aleochara*, early instars were used to start the test and, where possible, insects were reared through to adult emergence rather than pupation. Finally, teflubenzuron, an insect growth regulator that is active orally, was used as the toxic reference substance in all insect tests apart from the honeybee.

Table 19. Ecotoxicology studies: exposure routes, margins of exposure and endpoints

Test species	Exposure route to mCry3A	Minimum MoE	Supply of fresh mCry3A	Endpoints
bobwhite quail	Microbial protein in gelatin capsule	1400X DDD	Single oral dose	14d mortality, body weight, feed consumption
rainbow trout	Fish feed prepared from MIR604 grain	37.0X EEC	Daily for duration of test	28d mortality and growth
flower bug	Microbial protein in artificial diet	10.6X EEC	Daily for duration of test	Pre-imaginal mortality
ladybird beetle	Aphids dipped in solution of protein	12.3X EEC	Daily until pupation and after adult emergence	Pre-imaginal mortality and development; adult mortality
rove beetle	Microbial protein in artificial diet	15.6X EEC	Daily for first 35d of test	Fecundity
carabid beetle	Blowfly pupae injected with protein sol'n	11.2X EEC	Daily until pupation	Pre-imaginal mortality
honeybee	Microbial protein in sugar solution	36X EEC	Daily for first 5d of test	Brood development; adult and larval mortality
earthworm	Microbial protein in artificial soil	46X EEC	Single application	14d mortality and body weight
mouse†	Microbial protein in methycellulose sol'n	2600X DDD	Single oral dose	14d mortality, body weight, organ weights, feed consumption

MoE, Margin of Exposure; EEC, Estimated Environmental Exposure; DDD, Daily Dietary Dose
†Mammalian toxicology study

C.2. mCry3A Test Materials Used in Studies on Non-Target Organisms

Two types of test material were used in the studies that evaluated the safety of mCry3A protein for non-target species. As explained in section B.1 above, most studies were carried out with the test substance MCRY3A-0102 to achieve exposures to mCry3A that were at least 10X EEC for the test species concerned. The study of toxicity of mCry3A to freshwater fish was not carried out with MCRY3A-0102 because mCry3A is extremely unlikely to enter rivers, streams, lakes or ponds (Raybould, 2004b). Therefore, rainbow trout were fed a specially formulated fish feed, test substance FFMIR604-0103, made from grain of MIR604 (designated Hybrid I and the corresponding negative control hybrid 1I in Figure 1, Chapter 1: Syngenta Seeds Petition for the Determination of Non-regulated Status of Corn Event MIR604. Agronomic Performance of Event MIR604 Hybrids).

C.2.a. mCry3A protein produced in a microbial expression system (Test Substance MCRY3A-0102)

Test substance MCRY3A-0102 was prepared by expressing the modified *cry3A* gene used to generate corn Event MIR604 in an *E. coli* over-expression system. The modified *cry3A* gene was linked to the *Bacillus thuringiensis cry1Ac* native promoter in a Bluescript™ (Strategene, La Jolla, CA, USA) vector and transformed into *E. coli* strain DH5α. The *cry1Ac* promoter permits expression of the modified *cry3A* gene in the stationary phase of bacterial cell growth.

MCRY3A-0102 was prepared in two batches, both by Apex Bioscience, Inc., Research Triangle Park, NC, USA. Briefly, mCry3A protein was purified from *E. coli* following cell disruption using a grinding mill containing glass beads. Insoluble material was removed by centrifugation and inclusion bodies containing mCry3A were isolated, washed and solubilized in 50mM sodium carbonate. The solubilized proteins were subjected to anion exchange chromatography. MCry3A bound to the matrix was eluted with increasing sodium chloride concentration and then dialyzed *vs*. 20 mM ammonium bicarbonate, pH 9.5. The dialyzed fractions were lyophilised at Syngenta. The resulting lyophilised protein was designated as test substance MCRY3A-0102, and stored at –20°C. Upon characterization, this test substance was demonstrated to be *ca*. 90.3% pure mCry3A protein and to retain insecticidal activity against coleopteran larvae known to be sensitive to mCry3A. The preparation and characterization of test substance MCRY3A-0102 are described in detail in a separate report (Joseph and Graser, 2003b).

C.2.b. Fish feed prepared from MIR604 grain (Test Substance FFMIR604-0103)

Fish feed test substance FFMIR604-0103 was prepared by Zeigler Brothers, Inc. (Gardners, Pennsylvania, USA). The feed was formulated to contain 50% w/w of corn grain from an Event MIR604 hybrid; this is the maximum proportion of corn grain that can be used in fish feed, while maintaining a nutritionally balanced diet for juvenile trout. To minimize degradation of mCry3A protein by exposure to the typical heat and pressure conditions used in diet preparation, 'cold' pelleting was used to formulate the

feed. Grain from hybrid corn plants isogenic to Event MIR604 plants and grown concurrently under the same conditions was used to formulate the control fish feed, FFMIR604-0103C, in the same manner. A detailed description of the formulation of the test and control fish diets, as well as an analysis of the presence of mCry3A protein in the test diet is provided in a separate report (Graser, 2004a).

Exposure to mCry3A *via* FFMIR604-0103 provides a margin of safety in two ways. First, 50% w/w is higher than the standard amount of corn grain in commercial fish feed; 25 – 30% corn is more typical (National Research Council, 1983). Secondly, the 'cold' pelleting used to prepare FFMIR604-0103 is likely to degrade less mCry3A protein than normal methods of pelleting fish feed.

C.3. Hazard and Exposure Assessments of mCry3A for Non-Target Organisms

C.3.a Bobwhite quail – Colinus virginianus

C.3.a.1. Hazard assessment

An avian acute oral toxicity study on mCry3A protein was conducted according to US EPA Guideline No. 71-1. The test substance used was MCRY3A-0102 (Section 2a. above). The test organisms were 25-week old Northern bobwhite quails (*Colinus virginianus*). The birds were healthy in appearance and individuals weighed between 177 and 216 grams at the beginning of the test.

The control and treatment groups both comprised five males and five females. Individuals of each sex were allocated to the groups at random. Males and females were kept in separate pens. Birds were acclimated to the test pens for three weeks before the start of the test. Water and a game bird feed were supplied *ad libitum* during acclimation and testing. Birds were fasted for 19 hours before dosing.

Each individual in the treatment group was weighed and given an oral dose of 722 mg MCRY3A-0102/kg body weight in a single gelatin capsule; this dose is equivalent to 652 mg mCry3A/kg body weight. Individuals in the control group each received a single empty gelatin capsule. From the initiation to the end of the test each bird was observed daily for mortality, abnormal behavior or other signs of toxicity. Body weights were measured individually on Days 3, 7 and 14 of the test. Average feed consumption for each group was determined for Days 0-3, 4-7 and 8-14.

No deaths occurred during the study and all birds were normal in appearance and behavior. There were no treatment-related reductions in body weight of either sex, nor was there a treatment-related reduction in feed consumption during any period of the test. The results indicate that the median lethal dose (LD_{50}) of test substance MCRY3A-0102 is greater than 722 mg/kg body weight (corresponding to 652 mg mCry3A protein/kg body weight). The No Observable Effect Level (NOEL) in the study was 652 mg mCry3A/kg body weight (the highest dose tested).

C.3.a.2. Margin of Exposure

A dose of 652 mg mCry3A/kg body weight represents at least 1400X the daily dietary dose (DDD) for seed-eating birds. The calculation of the DDD is as follows:

$$DDD = \frac{FIR}{bw} \times C$$ (Crocker *et al*., 2002)

where: FIR = food intake rate; bw = body weight; C = concentration of mCry3A in food.

Birds feed rarely on leaves of corn; however birds such as crows (*Corvus brachyrhynchos*), grackles (*Quiscalus quiscula*) and sandhill cranes (*Grus canadensis*) uproot sprouting corn to feed on the germinating kernels (*e.g*., Steffey *et al*., 1999; Blackwell *et al*., 2001; Sterner *et al*., 2003). Red-winged blackbirds (*Agelaius phoeniceus*) and grackles destroy over 360,000 metric tons per annum of ripening field corn in the USA and Canada. Blackbirds typically slit open husks with their bills and puncture kernels in the milk stage (Steffey *et al*., 1999). Blackbirds are also common in corn stubble where they forage for spilled corn kernels and weed seeds (Linz *et al*., 2003). Therefore, the concentration of mCry3A in MIR604 kernels and *FIR/bw* ratios for cereal seed-eating birds were used to calculate the margin of exposure in this study.

The average concentration of mCry3A in kernels of MIR604 hybrids was 0.9 µg/g fresh weight (Joseph and Hill, 2003). Corrected for extraction efficiency (69.7%) this represents an actual concentration of 1.3 µg mCry3A/g fresh weight.

FIR/bw ratios for cereal seed-eating birds consuming fresh food were estimated by Crocker *et al*. (2002). Among the seven species represented, values range from 0.11 for the pheasant (*Phasianus colchicus*) to 0.35 for the tree sparrow (*Passer montanus*); these species also represent the range of body weights, 22 g for the sparrow to 953 g for the pheasant (heavier species have lower *FIR/bw* ratios). The range of daily dietary dose estimates is therefore 0.14 – 0.46 µg mCry3A/g body weight (*i.e.,* mg mCry3A/kg body weight). The test dose of 652 mg mCry3A/kg body weight therefore gives a margin of exposure between 4657 and 1417X the estimated daily dietary dose of mCry3A for seed-eating birds, assuming a diet of 100% fresh kernels from MIR604 plants.

C.3.a.3. Conclusion

No harmful effects were detected when 25-week old bobwhite quail (*Colinus virginianus*) were given a single oral dose of mCry3A representing no less than 1417X the extreme worst-case daily dietary dose of mCry3A to birds feeding on MIR604 corn tissue.

C.3.b. Rainbow trout – *Oncorhynchus mykiss*

C.3.b.1. Hazard assessment

A 28-day study of the effects of fish feed prepared from MIR604 grain (FFMIR604-0103) was carried out according to OECD Guideline 215 and US EPA OPPTS Guideline 885.4200. The test substance was prepared with the maximum amount of corn grain (50% w/w) that would provide a nutritionally balanced feed; about 25% corn by weight is typical in fish feed (National Research Council, 1983). In addition, 'cold' pelleting was used to minimize degradation of mCry3A compared with normal preparation methods (see C.2.a. above). A control substance, FFMIR604-0103C, was prepared in the same fashion from grain of a corn line isogenic to MIR604 (see C.2.a. above).

The test organism was rainbow trout (*Oncorhynchus mykiss*). Fish were obtained from a commercial fish farm and were kept in 25% seawater for 7 days on arrival at the test laboratory. Fish were acclimatized to the test temperature ($15 \pm 1^{o}C$) for at least 7 days before the initiation of the test. During this time, the fish were fed appropriate amounts of a commercial fish feed, but food was withheld for at least 24 hours before the test.

Forty fish were selected randomly for both the control and treatment populations. Immediately before the test, fish were anaesthetized, weighed individually and measured and then transferred to aerated buckets to recover from the anaesthetic. Once all the fish had recovered, they were transferred to the test vessels.

The test apparatus was a dynamic, continuous flow through system; the renewal rate was 400 ml min^{-1}. The test vessels had a maximum capacity of 54 liters and a working volume of 45 litres. Water in the test apparatus was de-chlorinated tap water to which salts were added to maintain minimum hardness, and sterilized by passage through an ultraviolet sterilizer. The water was not aerated and the test apparatus was housed in a temperature-controlled room with a nominal temperature of $15 \pm 1^{o}C$. The photoperiod was 16 hours dark and 8 hours light, with 20-minute dawn and dusk transitions.

Fish in the control tank were fed FFMIR604-0103C and the fish in the treatment tank were fed FFMIR604-0103; both populations received feed at the rate of 4% of total body weight per day. For the first 14 days, feeding was based on the weight of the fish at the beginning of the test. The quantity of feed was recalculated following weighing of the fish on Day 14 of the test. Uneaten food and feces were removed daily.

Fourteen days after the start of the test, the fish were starved for 24 hours, anaesthetized, weighed and measured. Fish were returned to the test vessels for a further 14 days exposure. On Day 28, fish were again anaesthetized, weighed and measured.

Fish were observed daily for mortality and symptoms of toxicity. Any dead fish were removed, but not replaced, and the amount of feed supplied was adjusted accordingly. Detailed observations of symptoms and feeding responses were made on Days 4, 7, 15

and 22. Physical and chemical parameters of the water were determined periodically and remained within guidelines throughout the test.

One fish (2.5%) in the treatment tank was observed to be dead on Day 21. Transient dark coloration, sounding and surfacing were recorded in one to three fish (2.5 – 7.5%) in the treatment population on Day 15. The levels of mortality and symptoms are below the levels that would invalidate the test (10%).

No significant difference was found between the weights of the treatment and control groups at 0, 14 or 28 days. The results indicate that exposure to mCry3A in fish feed FFMIR604-0103 for 28 days had no significant effects on the growth and mortality of juvenile rainbow trout.

C.3.b.2. Exposure assessment

Consumption of fish feed prepared from grain of hybrid corn derived from Event MIR604 is the main route of exposure of fish to mCry3A. As described above, the test substance in the trout growth study, FFMIR604-0103, was formulated and manufactured to maximize the amount of mCry3A protein in the finished feed, giving a margin of exposure over any conceivable mCry3A concentration in fish feed prepared in the usual way. However, even though FFMIR604-0103 was prepared to maximize the concentration of mCry3A, it was not known at the time of the trout growth study whether FFMIR604-0103 contained any mCry3A. Therefore, a study to estimate the concentration of mCry3A in FFMIR604-0103 was carried out following completion of the trout growth study. Two outcomes were possible: mCry3A would be detected in the feed indicating that the trout growth study was a valid assessment of the toxicity of mCry3A to fish; or no mCry3A would be detected, indicating no risk of mCry3A to fish because of no exposure under worst-case conditions, but rendering the trout growth study superfluous.

C.3.b.3. Quantification of mCry3A in the Feed Used in the Rainbow Trout Growth Study

Four substances were analysed in this study: FFMIR604-0103 and FFMIR604-0103C, the test and control substances in the trout growth study; and KMIR604-0103 and KMIR604-0103C, samples of ground corn kernels from which FFMIR604-0103 and FFMIR604-0103C, respectively, were prepared. Protein was extracted from these substances and the extracts were analysed for the presence of mCry3A using ELISA. ELISA was carried out using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies. Methods followed standard operating procedures and were the same for each substance.

In KMIR604-0103, the grain sample from which FFMIR604-0103 was prepared, mCry3A was detected at a concentration of 0.30 μg/g fw weight grain. Modified Cry3A was detected in FFMIR604-0103 at an average concentration of 0.09 μg/g fw weight feed; three random samples of FFMIR604-0103 gave very similar results (standard

deviation = 0.005) indicating that the mCry3A was distributed uniformly throughout the feed. No mCry3A was detected in the control fish feed, FFMIR604-0103C, or in the grain sample from which it was prepared, KMIR604-0103C.

The results confirm that mCry3A and/or immunoreactive polypeptides derived from mCry3A were present in the test substance FFMIR604-0103 used in the rainbow trout growth study. These data indicate that the trout growth study can be considered a test of the hazard of mCry3A to fish.

C.3.b.4. Margin of Exposure

We have no estimate of the degree to which cold pelleting reduces the degradation of mCry3A, but a worst-case assumption is that it does not reduce degradation of mCry3A compared with normal pelleting. Even if cold pelleting does not conserve integrity of mCry3A, test substance FFMIR604-0103 still provides a margin of exposure. First, FFMIR604-0103 contains about twice the usual proportion of corn grain in fish feed (National Research Council, 1983). Also, feed is very unlikely to be prepared from 100% grain of MIR604 hybrids. A reasonable assumption is that the adoption rate of MIR604 hybrids will be no more than *ca*. 5% of field corn grown in the USA. These conservative assumptions give a margin of exposure of 1/(0.5 x 0.054) = 37.0X EEC.

Another way of looking at the margin of exposure is to consider the maximum possible exposure of fish to mCry3A *via* fish feed. Ground grain of corn hybrids derived from MIR604, test substance KMIR604-0103, was found to contain 0.30 μg/g fw weight grain. At most, fish feed could contain 35% MIR604 corn grain, assuming that the corn source is 100% MIR604 grain. If it were possible to prepare fish feed with no degradation of mCry3A, the feed would contain 0.11 μg/g fw weight feed. Hence test substance FFMIR604-0103 exposed fish to 0.09/0.11 x 100 = 82% of the theoretical maximum environmental concentration of mCry3A.

C.3.b.5. Conclusion

No harmful effects were detected when juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed for 28 days to a diet containing mCry3A. The concentration of mCry3A in the diet was no less than 37.0X the EEC of mCry3A for fish consuming feed prepared from bulk corn grain containing grain from MIR604 hybrids.

C.3.c. Insidious flower bug – *Orius insidiosus*

C.3.c.1. Hazard assessment

A 21-day study of the effects of mCry3A on *Orius insidiosus* was carried out according to EPA OPPTS Guideline 885.4340. The test substance was MCRY3A-0102 incorporated into an artificial diet to give a nominal concentration of 50 μg mCry3A/g diet. The design of the test is based on the protocol of Bakker *et al*. (2000). This protocol is one of a group of standard testing methods (Candolfi *et al*., 2000) that are widely adopted in the European Union for assessing the effects of plant protection

products on non-target arthropods. As such, the method of Bakker *et al*. has been ring tested for repeatability and rigorous validity criteria have been set.

The test organisms were a discrete cohort of nymphs of *O. insidiosus*. Nymphs were 2-3 days old at the initiation of the test. Forty bugs were used in each treatment.

At the start of the test, MCRY3A-0102 was mixed with an artificial meat-based diet at a rate equivalent to 50 μg mCry3A/g diet. The diet had been cooked previously to denature proteases that might digest mCry3A. Diet was treated with deionised water to provide a control substance and with the orally active insect growth regulator teflubenzuron (10 μg/g diet) as a toxic reference treatment.

Immediately after preparation, aliquots of treated diet were placed in small pots and covered with a thin film (Parafilm®). The diet was presented in this manner so that bugs could feed by piercing the film, simulating their method of feeding by piercing prey with their mouthparts and removing the contents. A single bug was placed in a test arena with a pot of diet and a ball of cotton wool moistened with tap water to provide drinking water.

Additional pots of food were frozen to preserve the freshness of the diet mixture and to prevent degradation of the mCry3A. Aliquots of food were thawed daily and used to replace the food in each area. Drinking water was supplied constantly throughout the test. The study was carried out in a controlled environment room maintained at 25-26°C and 61-87% relative humidity (RH), with a 16-hour photoperiod. The environmental conditions were monitored throughout the test.

The condition of the nymphs was assessed daily until they became adults (usually 10 – 17 days at 25°C) or until 21 days after test initiation, whichever was sooner. After 21 days, all nymphs in the control and treatment groups had become adults or had died. In the control there was 23% pre-imaginal mortality, compared with 18% in the MCRY3A-0102 treatment. The 21 day corrected mortality for the MCRY3A-0102 treatment was therefore 0%. Mortality in the teflubenzuron treatment was 98%.

The test met the validity criteria of lower than 25% pre-imaginal mortality in the control group and higher than 50% mortality in the toxic reference group. Environmental conditions remained within guidelines throughout the test. Although the control mortality met the validity criterion, 77% survival might be regarded as low compared with other toxicity studies. It should be remembered that the study ran for 3 weeks, and it not easy to keep individual insects alive that long on an artifical diet. The US EPA regards 20% control mortality as acceptable; this was exceeded on day 19 in this study, at which time there was 18% mortality in the mCry3A treated group. Therefore, the insects received prolonged exposure to mCry3A before the test would have been terminated under the US EPA's control mortality criterion.

Under these laboratory conditions, mCry3A was not harmful to *Orius insidiosus*. The nominal median lethal concentration (LC_{50}) was >50 μg mCry3A/g diet and the nominal

No Observable Effect Concentration (NOEC) was 50 μg mCry3A/g diet (the highest concentration tested).

C.3.c.2. Exposure assessment

Orius bugs were exposed to a nominal concentration of 50 μg mCry3A/g diet during the toxicity study. A subsequent study was carried out to determine whether the bugs were exposed to this intended concentration of mCry3A.

C.3.c.3. Characterization and quantification of mCry3A in the treatment diet in the *Orius insidiosus* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Orius* toxicity study was carried out to determine the exposure of the bugs to mCry3A in that study. Diets treated with MCRY3A-0102 and untreated diets were prepared at Mambo-Tox, Southampton, UK as described in the summary of the toxicity study. Samples of these diets were stored frozen at Mambo-Tox during the *Orius* toxicity study and then shipped on dry ice to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA for analysis.

Protein extracts were prepared from the treated and untreated *Orius* diets according to standard operating procedures. The concentrations of mCry3A in the extracts were determined by quantitative ELISA using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies according to standard operating procedures.

The intactness of the mCry3A in the treated diet was determined by Western blot analysis. Aliquots of the treated diet extract, a solution of MCRY3A-0102 and the untreated diet extract were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

Bioactivity of mCry3A in the treated diet was measured in bioassays against first-instar Colorado potato beetles (CPB). CPB larvae were fed a standard CPB diet supplemented with 10 or 20% treated diet from the *Orius* study, or 10 or 20% untreated diet. Other treatments were: untreated CPB diet (negative control), CPB diet treated with a solution of MCRY3A-0102 (positive control) and CPB diet treated with water (control for the positive control). Each treatment comprised 3 replicates of 10 larvae. Each replicate was housed in a covered 47 mm diameter Petri dish kept at room temperature. In all treatments, diets were replaced *ca.* 24 and 48 h after the start of the assay. Mortality was measured daily for 4 days.

ELISA showed that 95.6% of the mCry3A was recovered from the treated diet; no mCry3A was detected in the extract from the untreated diet. A Western blot of treated

diet extract revealed a single band of *ca*. 67.7 kDa, the predicted molecular weight of mCry3A. In the bioassay, 83 and 90% of CPB larvae were dead after 4 days in the 10 and 20% treated diet groups, respectively (nominal concentrations of mCry3A were 5 and 10 µg mCry3A/g complete diet). In the groups fed diet supplemented with MCRY3A-0102 to 12.5 and 50 µg mCry3A/g complete diet, 73 and 80% of CPB larvae were dead after 4 days. In the groups fed 10% and 20% untreated diet, CPB mortality was 13% after 4 days in each case. There was 10% CPB mortality in both negative control groups.

The results of this study confirm that intact, bioactive mCry3A was present in the treated diet from the *Orius* study at the intended concentration of 50 µg/g diet.

C.3.c.4. Margin of Exposure

Orius insidiosus is a common beneficial insect in cornfields in the USA. Nymphs and adults prey on thrips, mites, insect eggs and caterpillars (Fauvel, 1999; Steffey *et al*., 1999). *Orius* is a particularly useful beneficial insect as it can maintain high population densities during periods when prey is scarce; it does this by feeding on corn silks and pollen, without apparent adverse effects on pollination (Steffey *et al*., 1999).

Orius could be exposed to mCry3A in MIR604 *via* several routes: through feeding on pollen, by eating sap from leaf tissue (*e.g.,* Coll and Guershon, 2001), by eating silks, or by eating prey that have eaten MIR604 tissue. The absence of detectable mCry3A protein in pollen of MIR604 plants (Joseph and Hill, 2003) indicates that exposure via this route is unlikely. *Orius* has been observed to feed on leaf tissue from which it extracts sap; detailed analysis by Armer *et al*. (1996) using $^{14}CO_2$ radiolabelled photosynthate showed that *Orius* feeding on soybean leaves accumulated little if any radiolabel. Based on their own observations and a review of similar experiments, Armer *et al*. concluded that *Orius* feeds principally from xylem vessels and hence plants provide the insect with water rather than nutrients. Therefore it is very unlikely that the main route of exposure of *Orius* to mCry3A will be through sap. If exposure were possible *via* sap, worst case EEC would be 6.6 µg mCry3A/g diet (based on corrected protein concentrations in leaves of MIR604 hybrids, see Chapter 7, Part C.1.a. above); this represents a margin of exposure of 50/6.6 = 7.6X EEC.

Silks, however, do represent a realistic potential route of exposure to mCry3A. At seed maturity in MIR604 hybrids, silks were estimated to contain on average 1.2 µg mCry3A/g fresh weight before correction for extraction efficiency (Joseph and Hill, 2003). The efficiency of extraction of mCry3A from silks has not been determined, however 70% efficiency seems a reasonable conservative assumption based on the measurements of other plant tissues (Joseph and Hill, 2003). The corrected concentration is therefore 1.7 µg mCry3A/g fresh weight, and hence the margin of exposure based on consumption of silks is 29.4X EEC.

The final potential route of exposure of *Orius* to mCry3A is via consumption of prey that has eaten MIR604 leaf tissue. No studies have estimated the concentration of mCry3A in herbivores that have fed on leaves of MIR604; nor to our knowledge are there studies on the concentration of native Cry3A in herbivores that have eaten plants

containing this protein. However, various studies have estimated the concentration of Cry1Ab, in herbivores that had fed on *Bt* corn or artificial diets containing the protein. Data on Cry1Ab in corn probably represent the best available data to estimate the concentration of mCry3A in herbivores feeding on MIR604: the herbivores tested are found in corn fields, both Cry1Ab and mCry3A are Cry proteins and the proteins are present in corn tissue.

Experiments by Head *et al*. (2001) found the concentration of Cry1Ab in corn leaf aphids (*Rhopalosiphum maidis*) to be 250-500 times lower than that in the artificial diet solution they were fed; no protein was detected in aphids feeding on Bt corn plants. Trace amounts of Bt protein have been detected in grain aphids (*Rhopalosiphum padi*) feeding on corn expressing Cry1Ab (Raps *et al*., 2001; Dutton *et al*., 2002). Head *et al*. (2001) found that concentrations of Cry1Ab in lepidopteran pests of corn (*Ostrinia nubilalis* – European corn borer, *Helicoverpa zea* – corn earworm, and *Agrotis ipsilon* – black cutworm) were between 10 and 140 times lower than the concentration of protein in artificial diets. *Spodoptera littoralis* (Egyptian cotton leafworm) larvae on Bt corn plants contained concentrations of Cry1Ab between 4.8 and 8.5 times lower than the leaves on which they were feeding (Raps *et al*., 2001; Dutton *et al*., 2002). The highest concentration of Cry1Ab relative to the dietary concentration was found in spider mites (*Tetranychus urticae*) feeding on Bt corn, which had a concentration of Cry1Ab 1.4 times lower than leaf tissue (Dutton *et al*., 2002).

Although *Orius* is most effective at controlling Lepidoptera such as corn earworm, it will prey on spider mites (Steffey *et al*., 1999). As spider mites have the highest concentration of Cry1Ab, their rate of incorporation can be used to calculate a worst-case margin of exposure to mCry3A for *Orius*. Assuming that mCry3A in leaves of MIR604 is incorporated into herbivores in the same fashion as Cry1Ab in the study of Dutton *et al*., spider mites are expected to contain 6.6/1.4 = 4.7 µg mCry3A/g fresh weight (based on corrected protein concentrations in leaves of MIR604 hybrids, see Chapter 7, Part C.1.a. above). Therefore in this study the worst-case margin of exposure for *Orius* eating spider mites that have fed on MIR604 leaves was 10.6X EEC. The omnivorous diet of *Orius* means that the margin of exposure is likely to be many times higher than this realistic worst-case value.

C.3.c.5. Conclusion

No harmful effects were detected when *Orius insidiosus* nymphs were exposed for 21 days to an artificial diet containing mCry3A at no less than 10.6X the worst-case average EEC of mCry3A in the diet of *Orius* species exposed to MIR604 hybrid corn plants.

C.3.d. Seven Spot Ladybird Beetle – *Coccinella septempunctata*

C.3.d.1. Hazard assessment

A 24-day study of the effects of mCry3A on the ladybird beetle *Coccinella septempunctata* was carried out according to EPA OPPTS Guideline 885.4340. The design of the test is based on the protocol of Schmuck *et al*. (2000). This protocol is one

of a group of standard testing methods (Candolfi *et al*., 2000) for assessing the effects of plant protection products on non-target arthropods that are widely accepted in the European Union. As such, the method of Schmuck *et al*. has been ring tested for repeatability and rigorous validity criteria have been set.

Artificial diets can be used to rear *C. septempunctata* larvae, but they require occasional supplementation with live aphids; a diet of aphids is sufficient for *C. septempunctata* to complete its life cycle (Schmuck *et al*., 2000). For simplicity of test design, and closest agreement with existing test protocols, pea aphids (*Acyrthosiphon pisum*) were used as the diet in this study. The test substance was presented by dipping aphids into a weak solution of Agral 90 (a wetting agent) containing MCRY3A-0102 at a concentration equivalent to 50 μg mCry3A/mL. This system was taken to simulate the possible exudation of honeydew containing mCry3A and any uptake of mCry3A into the bodies of the aphids.

The test was initiated with 4-day old (2nd instar) ladybird beetle larvae. The larvae were obtained from a discrete cohort of eggs laid by a culture of field-collected insects maintained at the test facility. The eggs and young larvae were maintained in batches of 10-25 in Petri dishes kept at 25°C in a 16-hour photoperiod. Larvae were fed untreated pea aphids until the start of the test.

The test substance was MCRY3A-0102 dissolved in a 40 μL/L solution of Agral 90; the toxic reference substance was 150 g/L teflubenzuron in a 40 μL/L solution of Agral 90; and the control treatment was a 40 μL/L solution of Agral 90. Each treatment population comprised 40 larvae, individually confined in test arenas. The treatments were applied via pea aphids that had been immersed in the appropriate solution and then allowed to dry for about 30 minutes. The number of aphids given was adjusted during the test as the individuals grew. Freshly treated aphids were supplied daily to larvae and any aphids remaining from the previous day were removed. This procedure was followed until pupation.

Pupae were left in the test arenas and feeding was halted. When adults emerged, their sex was determined and, where numbers allowed, a male and a female were placed in a test arena. Unpaired beetles were kept on their own. All adults were used in the assessments. The test was carried out in a controlled environment room maintained at 21-27°C and 35-92% RH with a 16 hour photoperiod.

Observations of larvae were made at the time of feeding. The number of dead, moribund and live larvae and the number of pupae were recorded. Observations of adults were also made at the time of feeding; the number of dead and live adults was recorded.

Pre-imaginal mortality was zero in both the control and treatment populations; mortality was 100% in the toxic reference treatment population. Adult mortality was 7.5% in the control population and 15% in the mCry3A-treated population, giving a corrected mortality of 8.1%. This was not statistically significantly different from the control mortality at the 5% level. The development time from larva to pupa was not significantly different between the control and treatment populations. The time from

larva to adult was slightly shorter in the treatment population (9.48 compared with 9.80 days in the control group); in other words pupae developed into adults faster in the treatment population. This difference was statistically significant at 5%. The difference in development times did not result in a statistically significant difference in adult survival.

The validity criteria of no greater than 20% control mortality and greater than 40% mortality in the toxic reference population were met. Temperature and RH deviated from established test protocols for short periods, but were not considered to have affected the integrity of the test as the control mortality was acceptable. Under these laboratory conditions, mCry3A was not harmful to *Coccinella septempunctata*. The LC_{50} of mCry3A dissolved in an Agral solution supplied via dipped aphids was >50 µg/mL; the NOEC of the mCryA solution was 50 µg/mL (the highest concentration tested).

C.3.d.2. Exposure Assessment

Ladybird beetles were exposed to a nominal concentration of 50 µg mCry3A/mL solution on dipped aphids during the toxicity study. A subsequent study was carried out to quantify the exposure in terms of µg mCry3A/g diet.

C.3.d.3. Characterization and quantification of mCry3A in the treatment diet in the *Coccinella septempunctata* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Coccinella* toxicity study was carried out to determine the exposure of the ladybird beetles in that study. Pea aphids treated with a solution of MCRY3A-0102 were prepared at Mambo-tox in the same manner and in parallel with the treated pea aphids used in the *Coccinella* toxicity study. A sample of the treated aphids and a sample of untreated aphids were frozen immediately after preparation and were shipped on dry ice to Syngenta Biotechnology, Inc. for analysis.

Protein extracts from treated and untreated aphids were prepared according to standard operating procedures. The concentration of mCry3A in the extracts was measured by quantitative ELISA, according to standard operating procedures, using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

The intactness of mCry3A in the extracts was assessed by Western blot. Aliquots of the extract of treated aphids, two aliquots of a solution of MCRY3A-0102 and an aliquot of the extract of untreated aphids were subjected to SDS-PAGE according to standard operating procedures. The gel was electroblotted and the membrane probed with immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

ELISA showed a concentration of 9.0 µg mCry3A/g aphids. The Western blot revealed a single immunoreactive band corresponding to the predicted molecular weight of mCry3A (*ca.* 67.7 kDa.).

C.3.d.4. Margin of Exposure

Until the late 1980s, three species, *Coleomegilla maculata*, *Hippodamia convergens* and *H. tredecimpunctata* comprised the majority of the ladybird beetle fauna of corn in the USA. Surveys summarized by Hodek and Honek (1993), found that these species formed 100% of a sample of ladybird beetles collected from field corn in Minnesota, 94.2% of a sample from sweet corn in North Dakota and 97.9% of a sample from field corn in South Dakota. Since the late 1980s, *Coccinella septempunctata*, a species introduced for biological control of cereal aphids, has increased rapidly and is now a 'predominant' ladybird beetle in corn in the eastern United States (Steffey *et al*., 1999).

Hodek and Honek (1993) estimated that the diet of ladybird beetles in the tribe Coccinellini contains *ca.* 85% aphids, the remainder being other sucking insects such as psyllids. *Hippodamia* and *Coccinella* are members of this tribe. *Coleomegilla maculata* has a somewhat different diet; it is an important predator of aphids and will also prey on mites and insect eggs and plant pollen can constitute up to 50% of its diet.

Aphids that have fed on corn plants expressing Cry1Ab contain very low concentrations of Cry1Ab relative to the plants (Head *et al*., 2001; Raps *et al*., 2001; Dutton *et al*., 2002). The highest estimate is that aphids contain Cry1Ab at a concentration 250 times less than the corn on which they are feeding (Head *et al*., 2001). On the other hand, the concentration of Cry1Ab in mites was only 1.4 times lower than the corn. As discussed above, the Cry1Ab corn data provide the best data to estimate the concentrations of mCry3A in herbivores feeding on MIR604. If the Cry1Ab in *Bt* corn conversion rates apply to incorporation of mCry3A into insects feeding on MIR604 corn hybrids, aphids are expected to contain 6.6/250 = 0.026 µg mCry3A /g fresh weight and mites 6.6/1.4 = 4.7 µg mCry3A /g fresh weight[4]. Pollen of MIR604 hybrids contains no detectable mCry3A (Joseph and Hill, 2003) and eggs of insects that have fed on MIR604 hybrids are unlikely to contain any mCry3A as proteins tend not to bioaccumulate.

Based on these considerations, a realistic worst-case exposure to mCry3A for ladybird beetles in corn is a diet of 85% aphids and 15% spider mites. This gives an EEC of:

$$(0.15 \times 4.7) + (0.85 \times 0.026) = 0.73 \text{ µg mCry3A /g fresh weight diet}$$

Therefore the margin of exposure to mCry3A in the *Coccinella* toxicity study is 9.0/0.73 = 12.3X EEC.

C.3.d.1. Conclusion

[4] 6.6 µg /g fresh weight is the average concentration of mCry3A in leaves of MIR604 hybrids – corrected for extraction efficiency – see Section C.1.a above.

No harmful effects were detected when *Coccinella septempunctata* larvae and adults were exposed for 24 days to an artificial diet containing mCry3A at no less than 12.3X the average worst-case EEC of mCry3A in the diet of ladybird beetles exposed to MIR604 hybrid corn plants.

C.3.e. Rove beetle – *Aleochara bilineata*

C.3.e.1. Hazard assessment

A study of the effects of mCry3A on the rove beetle *Aleochara bilineata* was carried out according to EPA OPPTS Guideline 885.4340. The design of the test is based on the protocol of Grimm *et al*. (2000). This protocol is one of a group of standard testing methods (Candolfi *et al*., 2000) for assessing the effects of plant protection products on non-target arthropods that are widely accepted in the European Union. As such, the method of Grimm *et al*. has been ring tested for repeatability and rigorous validity criteria have been set.

For the test, adult beetles that emerged over 24 hours were confined individually in wells of polystyrene tissue culture plates. The wells were lined with moistened filter paper and the beetles were supplied with a pellet of raw minced beef for food. The plates were placed in a dark cold room (0-8°C) for six days to delay physiological development. Three days before the start of the test the beetles were moved back to a controlled environment room maintained at 23-25°C, 64-79% RH and a 16-hour photoperiod.

On the day that the test started, the beetles (physiologically 4 days old) were brought together in small groups in 9-cm-diameter plastic Petri dishes, lined with damp filter paper. Groups comprising 10 males and 10 females were impartially selected and placed together in 5-cm-diameter plastic Petri dishes, ready for transfer into the freshly-treated test arenas.

The food supplied to the treatment population was puréed beef supplemented with MCRY3A-0102 at a rate equivalent to 50 µg mCry3A protein/g diet. The diet was cooked prior to addition of MCRY3A-0102 to denature proteases that might degrade mCry3A. The control diet was prepared in the same way, with deionised water as the reference item; the positive control diet was cooked beef with teflubenzuron at 10 µg/g diet.

Aliquots of the treated diet were placed in small receptacles. These were supplied to adult *A. bilineata* (10 males and 10 females per replicate, 4 replicates per treatment) for 35 days. Additional packages of treated food were frozen, to maintain their freshness and the stability of mCry3A in the treatment diet. Each day, aliquots of treated food were de-frosted and used to replace the food in each arena. At the end of the test, remaining aliquots were kept frozen prior to conducting tests to determine the stability of mCry3A in the diet.

The beetles were initially confined in small, ventilated pots for one week to maximize their exposure to the treated diet. They were then transferred to boxes containing damp

sand for a further four weeks. Treated diet was supplied daily and fresh water was added to the sand every 1-3 days as required to maintain the starting weight of the test arenas.

The fecundity of the beetles was assessed by the provision of pupae of the onion fly (*Delia antiqua*). The pupae were maintained under the conditions used for the initial cold treatment of the *Aleochara* beetles and were used within three weeks of their arrival at the test facility. The fly pupae were added to each arena on three occasions: 14, 21 and 28 days after test initiation (DAT). The survival of the adult beetles originally introduced was assessed at 35 DAT and they were then removed from the arenas. The number of F_1 adult *A. bilineata* that developed from the parasitised fly pupae was recorded over the subsequent 6 weeks.

After 35 days, mortality in the various treatments was 34% in the control population, 31% in the MCRY3A-0102 treatment population and 35% in the teflubenzuron treatment (2% corrected mortality). The mean number of progeny was 647 in the control population and 663 in the treatment population. This represents 2.5% greater fecundity in the treatment population, although this difference is not statistically significant. The mean number of offspring in the teflubenzuron treatment was 3, a 99.5% reduction compared with the control.

For the test to be considered valid, the mean number of beetles emerging from parasitized fly pupae in the control treatment should be >400 per replicate. Also, the mean number of beetles emerging in the toxic reference treatment should be reduced by >50%, relative to the control. Both of these criteria were met. Because the beetles are kept for 5 weeks on an artificial diet, adult survival is often low and no validity criterion is set for this endpoint. During the study, physical conditions in the controlled environment room deviated from established protocols for short periods. These fluctuations were not considered to have invalidated the test as the control and toxic reference populations exceed the validity criteria.

Under these laboratory conditions, mCry3A was not harmful to the fecundity of *Aleochara bilineata*. The NOEC was 50 µg mCry3A/g diet (the highest concentration tested).

C.3.e.2. Exposure assessment

The *Aleochara* beetles were exposed to a nominal concentration of 50 µg mCry3A/g diet during the toxicity study. A subsequent study was carried out to determine whether the beetles were exposed to this intended concentration of mCry3A.

C.3.e.3. Characterization and quantification of mCry3A in the treatment diet in the *Aleochara bilineata* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Aleochara* toxicity study was carried out to assess the exposure of rove beetles to mCry3A in that study. Diet treated with MCRY3A-0102 and an untreated diet were prepared at Mambo-Tox, Southampton, UK as described in the summary of the toxicity study. Samples of

these diets were stored frozen at Mambo-Tox during the *Aleochara* toxicity study and then shipped on dry ice to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA for analysis.

Protein extracts of treated and untreated diets were prepared according to standard operating procedures. The concentrations of mCry3A in the extracts were determined by quantitative ELISA according to standard operating procedures using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

The intactness of the mCry3A in the treated diet was determined by Western blot analysis. Aliquots of the treated diet extract, a solution of MCRY3A-0102 and the untreated diet extract were subjected to SDS-PAGE using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

Bioactivity of mCry3A in the treated diet was measured in bioassays against first-instar Colorado potato beetles (CPB). CPB larvae were fed a standard CPB diet supplemented with 10 or 20% treated diet, or 10 or 20% untreated diet. Other treatments were: untreated CPB diet (negative control), CPB diet treated with a solution of MCRY3A-0102 (positive control) and CPB diet treated with water (control for the positive control). Each treatment comprised 3 replicates of 10 larvae. Each replicate was housed in a covered 47 mm diameter Petri dish kept at room temperature. In all treatments, diets were replaced *ca.* 24 and 48 h after the start of the assay. Mortality was measured daily for 4 days.

ELISA showed that 91.7% of the mCry3A was recovered from the treated diet; no mCry3A was detected in the extract from the untreated diet. A Western blot of treated diet extract revealed a single intense band of *ca*. 67.7 kDa, the predicted molecular weight of mCry3A. In the bioassay, 57 and 77% of CPB larvae were dead after 4 days in the 10 and 20% treated diet groups respectively (nominal concentrations of mCry3A were 5 and 10 µg mCry3A/g complete diet). In the groups fed diet supplemented with MCRY3A-0102 to 12.5 and 50 µg mCry3A/g complete diet, 73 and 80% of CPB larvae were dead after 4 days. In the groups fed 10% and 20% untreated diet, CPB mortality was 10% after 4 days in each case. There was also 10% CPB mortality in both negative control groups.

The results of this study confirm that intact, bioactive mCry3A was present in the treated *Aleochara* diet at the intended concentration (50 µg/g diet).

C.3.e.4. Margin of Exposure

Rove beetles (Staphylinidae) are common in cornfields. There is little information on their diets in corn, but in general rove beetles feed mainly on other invertebrates although

some species will feed on decaying plant material (Steffey *et al.*, 1999). Species of *Aleochara* are parasitoids of pupae of cyclorraphous Diptera (Grimm *et al*., 2000).

A worst-case EEC based on consumption of soil invertebrates can be calculated from the concentration of mCry3A in roots of MIR604 hybrids: 2.8 µg/g fresh weight averaged over all growth stages, which is equivalent to 3.7 µg/g fresh weight when corrected for extraction efficiency (75.1%) (Joseph and Hill, 2003). Assuming a worst-case rate of incorporation into prey of 1.4X lower than the plant concentration (Dutton *et al*., 2002; see *Orius* study summary above, Chapter 7, Part C.1.b.3.), rove beetles could be exposed to an average concentration 2.6 µg mCry3A/g fresh weight. A diet of 50 µg mCry3A/g fresh weight therefore provides a margin of exposure of 19.2X EEC for exposure *via* invertebrate prey.

The concentration of mCry3A in 'decaying' plant material has not been determined; in any case it is difficult to judge exactly what degree of decay renders plant material acceptable to rove beetles. A reasonable estimate could be the concentration of mCry3A in 15-Day silage: 2.73 µg mCry3A/g fresh weight, which is equivalent to 3.2 µg mCry3A/g fresh weight when corrected for extraction efficiency (84.5%) (Joseph and Hill, 2003). A diet of 50 µg mCry3A/g fresh weight therefore provides a margin of exposure of 15.6X EEC for exposure through decaying plant material.

C.3.e.5. Conclusion

No harmful effects were detected when rove beetles (*Aleochara bilineata*) were exposed to an artificial diet containing mCry3A at no less than 15.6X the average worst-case EEC of mCry3A in the diet of rove beetle species exposed to MIR604 hybrid corn plants.

C.3.f. Ground beetle – *Poecilus cupreus*

C.3.f.1. Hazard Assessment

A study of the effects of mCry3A on the ground beetle *Poecilus cupreus* was carried out according to EPA OPPTS Guideline 885.4340. The design of the test is based on the protocol of Heimbach (1998). A test of toxicity to adult *Poecilus* beetles is one of a group of standard tests (Candolfi *et al*., 2000) for assessing the effects of plant protection products on non-target arthropods that are widely accepted in the European Union. We chose to test the toxicity of mCry3A to larvae because adult *Poecilus* is recognized as being relatively insensitive to pesticides (e.g. Candolfi *et al*., 1999).

Larvae of *P. cupreus* were obtained from a commercial supplier. They were shipped on the day they began to hatch and arrived at the test facility the following day. The test started the day after the larvae were received, when they were *ca.* 2 days old. In the period before the test, individual larvae were kept separately in the wells of polystyrene tissue culture plates filled with moist peat. The larvae were stored in the dark at 20°C and

7-73% RH; they were fed pupae of the blowfly *Calliphora vomitoria* that were cut in half to allow the *Poecilus* access to the contents.

The test arenas were small glass tubes capped with a ventilated polythene stopper. The tubes were filled with a standard sandy soil (LUFA) moistened with tap water to 35% of its water holding capacity. During the test, the soil-filled tubes were stored upright in plastic boxes (*ca.* 28 x 16 x 9 cm, with one box per treatment). The floors of the boxes were lined with moist capillary matting. The boxes had ventilated lids. The study was carried out in constant darkness in a controlled environment cabinet maintained at 19-22°C and 64-90% RH.

Poecilus larvae were exposed to mCry3A by being fed blowfly pupae injected with 1 µl of a solution of MCRY3A-0102. The concentration of mCry3A in the treatment diet was 50 µg/g fly pupa. The control diet was prepared in the same way, with deionised water as the reference item. The positive control diet was fly pupae injected with 1 µl of a solution of 'Nemolt', giving 0.664 ng teflubenzuron/g fly pupa. Diets were prepared in a single batch sufficient to provide each larva with one pupa per day. Larvae were provided with a fresh pupa on day 1 of the test and the remaining pupae were stored at –20 to –30°C.

Each treatment comprised 40 larvae confined individually in the soil-filled tubes. Larvae were transferred from the tissue culture plates using a soft brush and allocated randomly to the treatments. Each larva was provided with a freshly treated pupa that had been cut in half and place cut side up on the surface of the soil. The ventilated stoppers were then fitted.

Each larva was given a freshly defrosted pupa daily. The remains of the previous day's pupa were removed, taking care not to remove soil or very young larvae. Feeding continued until the *Poecilus* became pre-pupae.

The larvae were assessed 3 times per week for the first 2 weeks of the test. Thereafter assessments were made twice weekly. At each assessment, the larvae were categorized as alive, dead, missing or pupated. From day-21, if a larva had not been observed for three successive assessments, a thorough search was made by tipping the soil out of the tubes onto a white tray and sifting through it. Missing larvae were assumed to have died and decomposed. From day-32, the tubes were checked daily and the number of emerging adults recorded. Once the color of the adult skin had changed from white to its normal green-black, the beetles were weighed and sexed.

Mortality in the mCry3A treatment was 10% compared with 20% in the control treatment. The mean weight of the emerging beetles was very similar (81.5 mg in the control, 82.9 in the mCry3A treatment). All beetles died in the toxic reference (teflubenzuron) treatment.

For the test to be considered valid, pre-imaginal mortality should not exceed 20% in the control treatment and should exceed 50% in the toxic reference treatment. These criteria were met.

Under these laboratory conditions, mCry3A was not harmful to larvae of *P. cupreus*. The nominal median lethal concentration (LC_{50}) was >50 μg mCry3A/g blowfly pupa and the nominal No Observable Effect Concentration (NOEC) was 50 μg mCry3A/g blowfly pupa (the highest concentration tested).

C.3.f.2. Exposure assessment

The *Poecilus* larvae were exposed to a nominal concentration of 50 μg mCry3A/g diet during the toxicity study. A subsequent study was carried out to determine whether the bugs were exposed to this intended concentration of mCry3A.

C.3.f.3. Characterization and quantification of mCry3A in the treatment diet in the *Poecilus cupreus* toxicity study

Quantification and characterization of mCry3A in the diet used in the *Poecilus* toxicity study was carried out to assess the exposure of the beetles to mCry3A in that study. Blowfly pupae treated with MCRY3A-0102 were prepared at Mambo-Tox, Southampton, UK as described in the summary of the toxicity study. A batch of pupae prepared in parallel with those in the toxicity study was stored at –20 to –30°C at the test facility. On completion of the toxicity study, the pupae were shipped on dry ice to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA for analysis.

Extracts of treated and untreated blowfly pupae were prepared according to standard operating procedures. The concentrations of mCry3A in the extracts were determined by quantitative ELISA according to standard operating procedures using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

The intactness of the mCry3A in the treated diet was determined by Western blot analysis. Aliquots of the extracts from treated and untreated pupae, and a solution of MCRY3A-0102 were subjected to SDS-PAGE using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

ELISA determined the concentration of mCry3A in the blowfly pupae to be *ca.* 12 μg mCry3A/g pupae, equivalent to an average concentration of 0.82 μg mCry3A/pupa, and 24% of the mCry3A injected into the pupae. No mCry3A was detected in the extracts from untreated pupae. A Western blot revealed a single immunoreactive band at the predicted molecular weight of intact mCry3A (*ca.* 67.7. kDa) in the extracts from pupae treated with mCry3A; no immunoreactive material was detected in extracts from untreated pupae.

The results show that intact mCry3A was present at *ca.* 12 μg /g treated blowfly pupae used to expose *Poecilus* larvae to mCry3A in the toxicity study at Mambo-Tox. Other studies (Graser, 2004c, d, f) have shown that an immunoreactive band of 67.7 kDa on a Western blot probed with anti-Btt polyclonal antibodies correlates strongly with bioactivity against CPB larvae; it is highly likely, therefore, that *Poecilus* larvae were exposed to bioactive mCry3A.

C.3.f.4. Margin of Exposure

Nine species of carabid beetles are reported commonly from cornfields (Steffey *et al*., 1999), although mainly others have been recorded (e.g. Varchola and Dunn, 2001). Most are non-target (beneficial) organisms, but some species are pests that eat germinating corn seeds (Steffey *et al*., 1999).

The main route of exposure of non-target carabids to mCry3A will be through eating prey that has eaten tissue of MIR604 hybrids. Larvae of the black cutworm (*Agrotis ipsilon*) are common prey for carabid beetles in cornfields (Steffey *et al*., 1999). Cutworms cause severe damage in young corn when alternative food plants have been removed by cultivation of herbicides. Cutworm larvae sever young corn plants and eat them in burrows in the soil; larvae also occasionally burrow into stems of older plants (Steffey *et al*., 1999).

The average concentration of mCry3A in plants of MIR604 hybrids at the whorl stage is 1.5 μg/g fresh weight, after correcting for extraction efficiency (Joseph and Hill, 2003). Head *et al*. (2001) showed that the concentration of Cry1Ab in black cutworm larvae was *ca*. 10 times lower than the concentration of Cry1Ab in their diet. If mCry3A from MIR604 hybrids is incorporated into black cutworms at the same rate as Cry1Ab, the best estimate based on limited data (see above), then carabid beetles will be exposed to 0.15 μg mCry3A/g fresh weight *via* black cutworm larvae. Taking this as the EEC, the margin of exposure in the *Poecilus* toxicity study is 80X EEC.

Cry1Ab is toxic to some Lepidoptera, and therefore it is possible that black cutworm larvae could accumulate a higher concentration of mCry3A than Cry1Ab relative to the respective concentrations of these proteins in their diet. However, the LC_{50} of Cry1Ab to black cutworm larvae is >80,000 ppm (MacIntosh *et al*., 1990), whereas Head *et al*. (2001) used only 20 ppm. Nevertheless, it is possible that the concentration of mCry3A in cutworm larvae relative to their food is underestimated using the conversion rate of Cry1Ab from the study of Head *et al*. (2001).

An extreme worst-case is to assume that the concentration of mCry3A in the cutworms is 1.4 times lower than in MIR604 hybrids. This conversion factor is derived from the concentration of Cry1Ab in spider mites relative to their diet (Dutton *et al*., 2002, see Chapter 7, Part C.3.d.4. above); Cry1Ab is not toxic to spider mites and therefore should not affect their feeding rates. The extreme worst-case exposure of non-target carabids to mCry3A on this basis is 1.07 μg mCry3A/g fresh weight prey, giving a margin of exposure of 11.2X EEC in the *Poecilus* toxicity study.

The adults of two species of carabid, *Stenolophus lecontei* (seedcorn beetle) and *Clivina impressifrons* (slender seedcorn beetle), are sporadic pests in corn when they eat germinating corn seed (Steffey *et al*., 1999). If seedcorn beetles were to eat only kernels of MIR604 corn hybrids they would be exposed to 1.3 µg mCry3A/g fresh weight diet (the average concentration of mCry3A in corn kernels when corrected for extraction efficiency [Joseph and Hill, 2003]); this is 9.2X lower than the concentration of mCry3A in the *Poecilus* toxicity study. However, seedcorn beetles tend to feed on other arthropods and eat corn seeds only when other food is in short supply (e.g. Pausch, 1979). Therefore seedcorn beetles will be exposed to concentrations of mCry3A far below 10 times that in the *Poecilus* toxicity study. Furthermore, the *Poecilus* study exposed carabid larvae to mCry3A, whereas only adult seedcorn beetles will be exposed to mCry3A *via* kernels of MIR604 hybrids; adult carabids are far less sensitive to toxins than are larvae. Seedcorn beetles are therefore very unlikely to be harmed by mCry3A expressed in MIR604.

C.3.f.5. Conclusion

No harmful effects were detected when carabid beetle larvae (*Poecilus cupreus*) were exposed to an artificial diet containing mCry3A at no less than 11.2X the average worst-case EEC of mCry3A in the diet of non-target carabid species exposed to MIR604 hybrid corn plants.

C.3.g. Honeybee – *Apis mellifera*

C.3.g.1. Hazard Assessment

A 26-day (5 days treatment followed by 21 days observation) study of the effects of mCry3A on honeybee brood development was carried out according to EPA OPPTS Guideline 885.4380. The method is adapted from that of Oomen *et al*. (1992) for testing the effects of insect growth-regulating insecticides on honeybees. *A. mellifera* is a standard test organism for testing plant protection chemicals and this study fulfils the requirement to test the effect of mCry3A on a pollinator.

The test substance in this study was MCRY3A-0102 dissolved in a 50% w/v solution of sucrose; the concentration of mCry3A was 50 µg/g solution. The toxic reference substance was Dimilin Flo, a formulation of the insect growth regulator diflubenzuron, dissolved in a 50% sucrose solution. Diflubenzuron is known to affect the development of honeybee brood (based on the experience of staff at the test facility), but has low toxicity to adult honeybees. The concentration of diflubenzuron was 0.375 mg/mL sucrose solution. The control reference substance was 50% sucrose solution.

On the morning of treatment application, a sufficient frames were removed from Honeybee hives (n = 4 per treatment) to record the position of 100 cells containing eggs and 100 cells containing 1- to 3-day old larvae. The position of these cells was recorded by laying a transparent acetate sheet over the relevant frame and tracing the cells using a fine-tipped indelible marker pen. Each hive received a fresh batch of 200 mL of the appropriate test solution daily for 5 days. The solutions were quickly removed from the

containers by the bees and none of the previous test solution remained at the time of each application. Over a 21-day period following the initiation of treatments, the frames containing the "marked" cells were removed and the developmental success of the brood was recorded.

During the pre-treatment identification of the egg and larvae cells, the relative proportions of brood and food (in the form of pollen and nectar) on the frames within each hive were recorded. At the end of the study, this assessment was repeated in order to assess whether the test treatments had affected the relative proportions of brood cells within the hives.

Dead bee traps were fitted to the hives to record the number of dead adult bees, larvae and pupae being removed from the treated hives by worker bees over a 21-day period following the initiation of the 5-day treatments.

In the cells containing eggs at the start of the test, mortality was 28.5% in the control hives and 27.3% in the hives treated with MCRY3A-0102. There was 100% mortality in hives treated with Dimilin Flo. In the cells containing larvae at the beginning of the test, there was 6.0% mortality in the control hives and 6.8% mortality in the hives treated with MCRY3A-0102; this equated to a corrected mortality of 0.9%, which was not statistically significantly different from zero. Again, 100% mortality was recorded from cells in hives treated with Dimilin Flo. Prior to treatment, the proportion of cells occupied by brood was not significantly different among treatments. However, at the end of the test there were fewer cells occupied by brood in the MCRY3A-0102 and Dimilin Flo treatments (40.7% and 27.7% respectively) than in the control hives (49.6%). The difference was statistically significant in the case of the Dimilin Flo, but not in the case of MCRY3A-0102.

The number of dead bees and brood deposited in the dead bee traps during the treatment and observation periods was too low for statistical analysis. The low numbers of dead bees and brood in the MCRY3A-0102 and control treatments showed that the hives were apparently healthy and had high survival. Dimilin Flo was not expected to affect worker bees as it has low contact and oral toxicity in laboratory tests. Although Dimilin Flo had a dramatic effect on brood development, this was not reflected by an increase in the number of dead brood deposited in the bee traps.

There were slight deviations from the protocol relating to the number and age of brood identified at the start of the test; however they were not considered to have affected the validity of the test. Under conditions of the test, exposure of the hives to sucrose solution containing 50 μg mCry3A/g had no adverse effects on the condition and survivorship of honeybee larvae developing in brood cells within the hives. The test substance also had no apparent effect on the survival of adult honeybees.

C.3.g.2. Exposure Assessment

Hives were exposed to a nominal concentration of 50 μg mCry3A/g diet. The honeybees were supplied daily with freshly prepared diet to maximise the probability that

hives were exposed to the nominal concentration of mCry3A. As recommended by Oomen *et* al. (1992) the test was carried out while there were few natural nectar sources so that the bees did not store the test substances rather than feed them to the larvae. At each application of fresh diet, none of the previous test solutions remained in the diet containers, and there was a highly significant effect of the toxic reference treatment. These observations provide a weight of evidence that the developing brood were exposed to the nominal concentration of mCry3A.

C.3.g.3. Margin of Exposure

Honeybees are unlikely to be exposed to mCry3A via MIR604; corn does not produce nectar and MIR604 pollen does not contain detectable amounts of mCry3A (Joseph and Hill, 2003). The rationale for this test is that the honeybee is a representative of the order Hymenoptera. Many parasitoids of corn pests are wasps in the families Braconidae, Ichneumonidae and Trichogrammatidae of the Hymenoptera (Steffey *et al*., 1999). These groups mainly parasitise eggs and larvae of Lepidoptera. Larvae of these parasitoids could be exposed to mCry3A in the bodies of hosts that have eaten tissue of MIR604.

At present there is no validated test to expose larvae of parasitic Hymenoptera to high doses of protein (although it is possible to expose adults). It is possible, however, to expose larvae of honeybees. Hymenoptera are almost certainly more sensitive to toxins as larvae than as adults, therefore a study of larval honeybees is likely to be more representative than a study of adult parasitoids to assess the effects of mCry3A on parasitoid larvae.

As discussed above, Head *et al*. (2001), Raps *et al*. (2001) and Dutton *et al*. (2002) have studied the incorporation of Cry1Ab into the bodies of the larvae of Lepidoptera feeding on transgenic corn plants; these data represent the best data for predicting the incorporation of mCry3A into the bodies of herbivores feeding on MIR604. The highest incorporation was found in *Spodoptera littoralis*, which contained 4.8 times less Cry1Ab by weight than the leaves on which they were feeding. If mCry3A is incorporated into Lepidoptera at the same rate relative to leaf tissue, then the highest concentration of mCry3A in Lepidoptera will be 4.8X lower than in MIR604 leaf tissue. The average concentration of mCry3A in leaves of MIR604 hybrids is 6.6 μg/g fresh weight (see Chapter 7, Part B.1. above). Therefore the average concentration in the larvae of Lepidoptera is expected to be 1.4 μg/g fresh weight[5]. A test concentration of 50 μg mCry3A/g diet therefore gives a margin of exposure of about 35X EEC.

C.3.g.4. Conclusion

No harmful effects were detected when honeybee (*Apis mellifera*) hives were exposed to an artificial diet containing mCry3A at no less than 35.7X the average worst-case EEC of mCry3A in the diet of parasitic Hymenoptera exposed to MIR604 hybrid corn plants. Pollinators are highly unlikely to be exposed to mCry3A *via* hybrid MIR604 corn plants.

[5] With the provisos noted in the discussion of *Poecilus* above

C.3.h. Earthworm – *Eisenia foetida*

C.3.h.1. Hazard Assessment

A 14-day study of the acute toxicity of MCRY3A-0102 to the earthworm *Eisenia foetida* was carried out according to OECD Guideline 207 for earthworm acute toxicity tests. *E. foetida* is a standard organism for testing the effects of plant protection products on soil invertebrates.

The test was conducted with an artificial soil comprising 10% sphagnum peat, 20% kaolin clay and 70% silver sand (quartz) with water added to 50% of the soil's water holding capacity. Calcium carbonate was added to adjust the soil pH to 6.0 ± 0.5. For the treatment population, MCRY3A-0102 was added to give a concentration of 250 µg mCry3A/g moistened soil. The control population was exposed to the same soil without MCRY3A-0102. In addition, the LC_{50} for a toxic reference substance (2-chloracetamide) was determined in soil with the same composition as the control and reference treatments.

Treatments were incorporated into the artificial soil substrate, which was then transferred to 1 L glass jars. Each jar contained 500 g (dry weight) of soil. Ten adult *E. foetida* (between 300 and 600 mg in weight) were then introduced into each replicate jar (n = 4 per treatment). The jars were kept in a controlled environment room maintained at 18-20°C and 57-89% relative humidity, with constant light at 485-569 lux. The mortality of the earthworms was assessed 7 and 14 days after treatment (DAT). Any dead earthworms were removed at the time of assessment. The body weight of the earthworms was recorded before and after treatment.

At 7 DAT, there was 0% mortality in the control population and 2.5% mortality in the treatment population. At 14 DAT, mortality in the control population was still 0% and in the treatment population it had risen to 5.0%. The difference between the control and treatment was not statistically significant at either time. During the study the weight of earthworms in the control population decreased by 11.4% and the weight of earthworms in the treatment population decreased by 5.8%; this difference was not significant. The LC_{50} of 2-chloracetamide was determined to be 18.0 mg/kg dry weight soil, showing the earthworms were sufficiently sensitive to meet Guideline requirements.

The test is considered valid if the mortality in the control population is below 10% and the decrease in weight of the control population is no greater than 20%. Both of these criteria were met. Under conditions of the test, MCRY3A-0102 was not harmful to the earthworm *Eisenia foetida*. The LC_{50} of mCry3A to *E. foetida* was >250 µg/g moistened soil and the NOEC was 250 µg/g soil (the highest concentration tested).

C.3.h.2. Exposure assessment

Earthworms were exposed to a nominal concentration of 250 µg mCry3A/g moistened soil during the toxicity study. A subsequent study was carried out to

determine whether the earthworms were exposed to this intended concentration of mCry3A.

C.3.h.3. Characterization and quantification of mCry3A in the artificial soil in the *Eisenia foetida* toxicity study

Characterization and quantification of mCry3A in the artificial soil in the *Eisenia foetida* toxicity study was carried out to assess the exposure of earthworms to mCry3A in that study. To avoid disturbance of the worms, soil samples were not taken from the toxicity study. As a surrogate for soil in the MCRY3A-0102 treatment, soil prepared in the same manner, but not containing worms, was maintained in parallel under the conditions of the toxicity study. Samples of the mCry3A-treated soil were taken immediately after preparation (0 days) and at 3, 7 and 14 days after treatment, and stored at *ca*. –20°C. On completion of the toxicity study, the samples of treated soil were shipped frozen to the Regulatory Science Laboratory, Syngenta Biotechnology, Inc., Research Triangle Park, NC, USA, where they were stored at –80°C until analysis. A sample of untreated artificial soil, from the same batch of soil used in the toxicity study, was shipped at the same time (and stored at –80°C).

The concentrations and intactness of mCry3A in the treated soil samples were measured by ELISA and Western blot analysis respectively. Extracts of treated and untreated soils were prepared according to standard operating procedures. ELISA was carried out according to standard operating procedures using immuno-affinity purified rabbit anti-mCry3A polyclonal antibodies and immuno-affinity purified goat anti-Btt (native Cry3A from *Bacillus thuringiensis* subsp. *tenebrionis*) polyclonal antibodies.

Aliquots of the treated soil extract, a solution of MCRY3A-0102 and the untreated soil extract were subjected to SDS-PAGE using standard operating procedures. Proteins were electroblotted on to a membrane, which was then probed with immuno-affinity purified goat anti-Btt polyclonal antibodies. Immunoreactive proteins were visualized by colorimetry.

The bioactivity of mCry3A in the treated soil samples was also measured by bioassay of 1st instars of CPB. Standard CPB diets were mixed with 5 and 10% (w/w) of each treated soil sample, giving nominal concentrations of 12.5 and 25.0 µg mCry3A/g complete diet. Negative control diets were prepared in the same manner using samples of untreated soil to which deionized water had been added in the same proportion (w/v) used to prepare the control soil in the *Eisenia* toxicity study. A positive control diet was prepared by adding MCRY3A-0102 to give 12.5 and 50.0 µg mCry3A/g CPB diet. Assays using CPB diet treated with deionised water in the same proportion (w/v) as the MCRY3A-0102 solution in the positive control diet, and untreated CPB diet were also carried out. Mortality of CPB larvae was assessed daily for 5 days.

ELISA of the treated soil extracts detected 18.1% of the nominal concentration of mCry3A in the 0-day sample, 14.8% in the 3-day sample, 6.3% in the 7-day sample and 7.6% in the 14-day sample. No mCry3A was detected in extracts of the untreated soil. In all extracts of treated soil samples, a Western blot detected an immunoreactive band

corresponding to the predicted molecular weight of intact mCry3A (*ca*. 67.7 kDa). In the 3-day and subsequent samples, one or more additional bands of apparent mCry3A degradation products were detected; these were more prominent in the 7 and 14-day samples. The main degradation products appeared as a doublet at *ca.* 57 kDa.

Treated soil showed high bioactivity against 1st-instar CPB at all sampling times. After 5 d, mortality on the treated soil diets was between 60 and 83%, with little difference apparent among sampling times and between the different soil incorporation rates in the CPB diet. Mortality on the positive control diet was similar to that on the treated soil diets after 5 days (87-97%). Mortality on the water-treated soil diet was low (13-20%) after 5 days and similar to the water-treated and untreated CPB diets (both 20% mortality).

This study shows that active mCry3A was present in the treated artificial soil throughout the 14-day exposure part of *Eisenia* toxicity study. Although only about 18% of the nominal concentration of mCry3A was recovered from the 0-day sample, 7.6% of the nominal concentration of mCry3A could still be detected after 14 days. Therefore, the low recovery was probably due to low extractability of the protein from the artificial soil, rather than rapid degradation of the protein immediately after addition to the soil; unlike the study of mCry3A in live soil reported in Chapter 9 below, rapid degradation is not expected in artificial soils due to low microbial activity. The Western blot showed that intact mCry3A was present 14 days after the soil was treated, although some degradation did occur. However, bioassays against first-instar CPB confirmed that this protein was bioactive. These results show that earthworms were exposed to intact, bioactive mCry3A throughout the 14-day toxicity study.

C.3.h.4. Margin of Exposure

Earthworms could be exposed to mCry3A *via* several routes (Raybould, 2004b), but the most likely route is ingestion of senescent plant material that is incorporated into the soil. The average concentration of mCry3A in senescent roots of MIR604 hybrids is 3.8 μg/g fresh weight, after correcting for extraction efficiency (75.1%); the average concentration of mCry3A in senescent leaves of MIR604 hybrids is 5.5 μg/g fresh weight, after correcting for extraction efficiency (77.1%) (Tables 1 and B-1 in Joseph and Hill, 2003). An extreme worst-case exposure would occur if earthworms ate a diet comprising 100% senescing leaves of MIR604 hybrids; on this basis, 250 μg mCry3A/g moistened soil gives a margin of exposure of 46X EEC. In reality, senescing plant material will be greatly diluted in a large volume of soil and therefore 46X EEC is a very conservative estimate.

C.3.h.5. Conclusion

No harmful effects were detected when earthworms (*Eisenia foetida*) were exposed to an artificial soil containing mCry3A at no less than 46X the average worst-case EEC of mCry3A in the diet of earthworms exposed to MIR604 hybrid corn plants.

C.3.i. Wild mammals

C.3.i.1. Hazard Assessment

No ecotoxicology studies were carried out solely for the assessment of safety of mCry3A to wild mammals. However, the study of acute oral toxicity of mCry3A to mice (Johnson, 2003) can be used for this purpose. Five male and five female mice were each exposed to a single dose of MCRY3A-0102 at 2632 mg/kg body weight, representing approximately 2377 mg/kg body weight of mCry3A. The test substance was dissolved in an aqueous suspension of 1% methylcellulose. A control group received the same volume of 1% methylcellulose used to deliver the test substance. Fourteen days after treatment there were no signs of toxicity in the mice given MCRY3A-0102; all endpoints were comparable in the control and test groups. The LD_{50} value for male and female mice is therefore greater than 2377 mg mCry3A/kg body weight (the single dose tested).

C.3.i.2. Margin of Exposure

Mammals can be serious pests of corn. Rodents such as thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), deer mice (*Peromysus maniculatus*), house mice (*Mus domesticus*), and prairie and meadow voles (*Microtus* spp.) will feed on germinating corn seeds. Frequently these species remove so many seeds that the field needs to be replanted. Woodchucks (*Marmota monax*) also feed on sprouting corn seed, but because they feed along the edges of fields, they usually cause less serious damage than other rodents. Larger mammals such as white-tailed deer (*Odocoilus virginianus*) and raccoons (*Procyon lotor*) cause injury to ripening ears. Deer typically nip off ear tips, whereas raccoons chew through husks. In some areas these species are hunted specifically to reduce damage to cornfields (Steffey *et al*., 1999).

The daily dietary dose (DDD) of mCry3A for mammals can be calculated using the method described for birds in Section 3.a.1. above, *i.e.*:

$$DDD = \frac{FIR}{bw} \times C$$

Crocker *et al.* (2002) estimated the ratio of food intake rate and body weight (*FIR/bw*) for several rodent species. The values for the harvest mouse (*Micromys minutus*) and the wood mouse (*Apodemus sylvaticus*) consuming cereal seeds are 0.33 and 0.28, respectively. Kernels of MIR604 hybrids contain an average of 0.9 µg mCry3A/g fresh weight, which represents 1.3 µg mCry3A/g fresh weight after correcting for extraction efficiency (69.7%); therefore a worst-case DDD for rodents eating a diet comprising 100% kernels of MIR604 is approximately 0.43 mg mCry3A/kg body weight. This represents over 5500X the dose of mCry3A in the mouse study.

Deer eating the tips of corn ears might be exposed to leaf and kernel tissue. Leaf tissue of MIR604 has a much higher concentration of mCry3A than do kernels (10.1 µg

mCry3A/g fresh weight compared with 1.6 μg mCry3A/g fresh weight at seed maturity)[6] and therefore represents a worst case diet, provided the calorific value of leaf tissue is not so high that deer eat much less of it than kernels to attain their energy needs. Grasses have a slightly higher calorific value than cereal seeds (Table 4 in Crocker *et al*., 2002;), but the difference is small compared with the difference in concentration of mCry3A between leaves and kernels of MIR604. Therefore a diet of 100% leaves of MIR604 is taken to be the worst case exposure for deer and other large mammals to mCry3A.

The *FIR*/*bw* for fallow deer eating grass is 0.09 (Crocker *et al*., 2002). Taking the concentration of mCry3A in the diet to be 10.1 μg mCry3A/g fresh weight, the value for leaf tissue at seed maturity, the worst-case DDD for a large mammal eating a diet comprising 100% ears of MIR604 corn is 0.91 mg mCry3A/kg body weight. This represents over 2600X the dose in the mouse study.

C.3.i.3. Conclusion

No harmful effects were detected when mice were given a single oral dose of mCry3A representing no less than 2600X the extreme worst-case daily dietary dose of mCry3A to mammals feeding on MIR604 hybrid corn.

D. RISK ASSESSMENT FOR NON-TARGET ORGANISMS

A comparison of native and mCry3A showed that these proteins have the same spectrum of activity against various insect pest species, apart from the intended increase in toxicity of mCry3A to certain species of *Diabrotica* (Table 14). From a review of the literature (Tables 15, 16 and 17) it is concluded that the toxicity of native Cry3A is limited to certain species in 3 families of Coleoptera: the Chrysomelidae, the Curculionidae and the Tenebrionidae. If native and mCry3A differ only in their activity against *Diabrotica* species, it follows that mCry3A should pose negligible hazard (show low toxicity) to species outside those 3 families; the ecotoxicology studies summarised in Section C tested this hypothesis.

Nine species of non-target organism were exposed to concentrations or doses of mCry3A in excess of expected environmental concentrations of mCry3A that might result from the proposed cultivation of MIR604 (Table 18). No harmful effect of mCry3A was detected in any study. Taken together, the studies give a robust test of the hypothesis that the hazard of mCry3A is limited to the Chrysomelidae, the Curculionidae and the Tenebrionidae. Several features provide high predictive power to extrapolate the absence of detectable effects to species that were not tested: high margins of exposure; long exposure times in many studies; sensitive endpoints in many studies; and the testing of species closely related to the target organism (see Chapman *et al*., 1998, for a discussion of uncertainty in predictions from laboratory studies).

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[6] The average concentration of mCry3A leaves of MIR604 hybrids at seed maturity is 7.8 μg/g and the extraction efficiency is 77.1%; the average concentration of mCry3A in kernels of MIR604 hybrids at seed maturity is 1.1 μg/g and the extraction efficiency is 69.7% (Tables 1 and B-1 from Joseph and Hill, 2003).

The conclusion that mCry3A has no detectable toxicity at no less than 10X EEC, except to species in the Chrysomelidae, the Curculionidae and the Tenebrionidae, means that risk to the diversity or abundance of NTOs outside these families from the proposed cultivation of MIR604 is minimal. In addition to the lack of hazard of mCry3A, the risk to certain NTOs from MIR604 is minimal because of negligible exposure; this applies to pollinators in particular, because mCry3A was not detected in MIR604 pollen.

E. POTENTIAL FOR EXPOSURE OF NON-TARGET CHRYSOMELIDAE, CURCULIONIDAE AND TENEBRIONIDAE (COLEOPTERA) TO MCRY3A PROTEIN FROM MIR604 PLANTS

The data presented above and in the previously submitted volume by Garcia-Alonso and Vlachos (2003) demonstrate that activity of mCry3A is expected to be limited to beetles, and in particular to certain members of the Chrysomelidae (leaf beetles, flea beetles and rootworms), the Curculionidae (weevils and snout beetles) and Tenebrionidae (darkling beetles) families. The Chrysomelidae and Curculionidae are herbivores and some species are serious crop pests, and by definition these species are not non-target organisms. It is possible that some species in these families could be beneficial if they feed on and control weed species. Such species could potentially be exposed to MIR604 pollen, but not to mCry3A because the protein is not expressed in pollen at detectable levels (Joseph and Hill, 2003). Therefore no non-target species of the Chrysomelidae or Curculionidae are likely to be harmed by mCry3A expressed in MIR604.

The Tenebrionidae are scavengers of plant material as both larvae and adults. Some species, known as mealworms, are pests of stored grain. Species known as darkling beetles or false wireworms can be pests of crops such as cotton and cucurbits. The most serious damage caused by darkling beetles is the cutting of stems of young seedlings just below the soil surface. Further feeding can also occur on flowers or the undersides of leaves, but this is rarely a cause of economic loss.

Darkling beetles are unlikely to be exposed to mCry3A through eating fresh tissue of MIR604 because they are not recorded as a pest species in corn (*e.g.*, Steffey *et al*., 1999). From what is known of the ecology of darkling beetles, we cannot eliminate the possibility that some species may be exposed to mCry3A by feeding on senescent tissue of MIR604 within cornfields.

Wu and Dean (1996) measured the LD_{50} of a native Cry3A to *Tenebrio molitor* (yellow mealworm), which we can regard as a representative species of the Tenebrionidae, and *Leptinotarsa decemlineata* (Colorado potato beetle), a species of Chrysomelidae commonly used in bioassays for native Cry3A because of its sensitivity to this protein (*e.g.*, Wu *et al*., 2000). The LD_{50} for *T. molitor* was 11.4 µg Cry3A/larva and that for *L. decemlineata* was 91 ng Cry3A/larva (*i.e.,* each *T. molitor* larva is about 125-fold less sensitive than each Colorado potato beetle larva). The concentration of mCry3A in senescing leaves of MIR604 hybrids is 4.2 µg/g fresh weight; corrected for 77.1% extraction efficiency for leaves, this amounts to 5.5 µg/g fresh weight (Joseph and Hill,

2003). Assuming the activity of mCry3A to these species is roughly the same as the native Cry3A used by Wu and Dean (1996), *T. molitor* larvae would need to ingest roughly 2.1 g of senescing leaf tissue of MIR604 to receive the median lethal dose of mCry3A. This represents about 3500X the body weight of a first instar and about 15X the body weight of a final instar of *T. molitor* (body weight data for *T. molitor* obtained from National University of Singapore [1998]). Given the omnivorous diet of the Tenebrionidae and the likely rapid degradation of mCry3A in soil (Raybould, 2004b), any darkling beetle species in cornfields are extremely unlikely to be exposed to sufficient mCry3A to result in a significant impact, even if they were sensitive to mCry3A.

F. ENDANGERED SPECIES CONSIDERATIONS

F.1. Endangered Coleoptera

Studies of non-target organisms and pest species have revealed no unexpected effects of mCry3A to Coleoptera. Therefore the expectation is that the activity of mCry3A will be limited to certain species within the Chrysomelidae, Curculionidae and Tenebrionidae families of beetles (Garcia-Alonso and Vlachos, 2003). There are currently no endangered or threatened species in any of these families (U.S. Fish and Wildlife Service Endangered Species Program, http://endangered.fws.gov/); this includes the salt creek tiger beetle (*Cicindela nevadica lincolniana*; family Cicindelidae), which is a new addition to the federal Endangered Species list with effect from 7th November 2005. In addition, there are no endangered or threatened beetle species in habitats in which corn is grown (US EPA, 2003). Pollen of MIR604 has no detectable mCry3A and corn pollen is not known to disperse into the habitats or breeding grounds of endangered beetles (US EPA, 2003). Therefore no endangered or threatened beetles are expected to be harmed by mCry3A expressed in MIR604 hybrids.

F.2. Other endangered species

Extensive testing of mCry3A, native Cry3A and other Cry3 proteins (Section D above; Garcia-Alonso and Vlachos, 2003; US EPA, 2001; US EPA, 2003) has shown no harmful effects to taxa outside the Coleoptera; groups tested include mammals, birds, fish, earthworms and several orders of insects. The lack of detectable expression of mCry3A in pollen of MIR604 inbred lines and hybrids, the lack of weediness of corn, the absence of sexually compatible wild relatives of corn in the USA and the very low probability that mCry3A will show significant dispersal in soil (Raybould, 2003) means that endangered and threatened species could only be exposed to mCry3A by eating MIR604 plants or organisms that had fed on MIR604 tissue.

EPA reviewed the likelihood of exposure of endangered bird and bat species to Cry3Bb1 (a coleopteran-active protein) expressed in corn Event MON863 (US EPA, 2003). EPA concluded that endangered birds and bats rarely forage in agricultural fields, and that insectivorous species tend to take flying insects rather than larvae (the life stage most likely to contain significant amounts of Cry protein). Indirect effects of MON863 could

occur through changes in prey abundance, however such changes would most likely be beneficial to rare species as their prey will increase due to reductions in pesticide use (US EPA, 2003). Similar changes are expected for MIR604 and therefore cultivation of MIR604 is extremely unlikely to have harmful effects on any endangered or threatened species.

F.3. Risks to the Monarch Butterfly

Although the monarch butterfly is not an endangered species, there are well-known reports that transgenic corn may harm populations of this insect (e.g. Losey *et al*., 1999). Laboratory studies have shown that first instar monarch larvae could be exposed to toxic concentrations of Cry1Ab through eating milkweed leaves (their primary plant food source) dusted with pollen from transgenic corn events expressing Cry1Ab (Hellmich *et al.*, 2001). However, a risk assessment using estimates of the proportion of the monarch population exposed to potentially harmful amounts of pollen showed that Cry1Ab posed no significant risk to monarchs (Sears *et al.*, 2001). Exposure of the first and second instars to corn pollen is low because the vast majority of monarch larvae do not develop on milkweed in or adjacent to cornfields during anthesis (pollen shed) of corn.

Event MIR604-derived hybrids pose no detectable risk to monarchs. First, mCry3A as expressed in MIR604 hybrids is extremely unlikely to be hazardous to monarchs. Extensive safety testing data indicate that activity of mCry3A is limited to species in 3 families of Coleoptera; monarchs are Lepidoptera. Secondly, monarchs are unlikely to be exposed to mCry3A; in addition to the low exposure of monarch larvae to corn pollen, mCry3A has not been detected in MIR604 pollen (see Chapter 6. **Quantification of mCry3A and PMI Proteins in Event MIR604**). Therefore, there is neither detectable hazard nor exposure of mCry3A to monarch larvae and Event MIR604-derived hybrids will pose negligible risk to this species.

Chapter 8. Food and Feed Safety

A. INTRODUCTION

This chapter represents a summary of data and information relevant to the food and feed safety of a modified Cry3A (mCry3A) insect control protein and the genetic material required for its production (*via* pZM26) in transgenic maize (corn) plants derived from Syngenta Seeds' transformation Event MIR604. Event MIR604 also expresses phosphomannose isomerase (PMI) as a selectable marker. A permanent exemption from the requirement of tolerances has been established for the PMI protein in all crops (US EPA, 2004a). This tolerance exemption was granted in response to a petition (EPA File Symbol PP 3E6748) submitted to the US Environmental Protection Agency by Syngenta Seeds. A large body of PMI protein characterization and mammalian safety data accompanied the PMI tolerance petition. Accordingly, the experimental details and results of this safety assessment, which included an evaluation of the potential for PMI to become a food allergen, are not presented in the present petition for deregulation. However, pertinent summary information can be found in Vlachos and Joseph, 2003, and US EPA, 2004a.

In connection with a December 15, 2003 application for an Experimental Use Permit for MIR604 corn (granted in May 2005), Syngenta Seeds petitioned the US EPA to grant a temporary exemption from tolerances (EPA File Symbol 4G6808; US EPA, 2004b) for the mCry3A protein. Syngenta Seeds has also petitioned the US EPA (EPA File Symbol PP 4F6838; US EPA, 2004b) to grant a permanent exemption from tolerances for mCry3A in all corn. That petition was submitted on April 30, 2004, concurrently with a FIFRA Section 3 registration application for MIR604 corn (US EPA, 2004c). These EPA applications are currently pending.

The scientific studies listed below (Table 20) contain data and detailed information that are relevant to the characterization and mammalian safety of Event MIR604 plants and/or the transgenic proteins produced therein. Those studies with EPA MRID #s were submitted to the US EPA concurrently with previous regulatory applications for MIR604 corn and the tolerance exemptions for mCry3A protein, or the tolerance exemption for PMI.

Table 20. List of Data Volumes Supporting the Food and Feed Safety of Event MIR604 Corn

Study Name	EPA MRID #	Reference
Characterization of Modified Cry3A Protein Produced in Event MIR604-Derived Maize (Corn) and Comparison with Modified Cry3A Protein Expressed in Recombinant *Escherichia coli*	46155603	Joseph and Graser, 2003a
Quantification of Modified Cry3A and PMI Proteins in Transgenic Maize (Corn) Tissues, Whole Plants, and Silage Derived from Transformation Even MIR604	46155604	Joseph and Hill, 2003
Characterization of Modified Cry3A Test Substance (MCRY3A-0102) and Certificate of Analysis	46155605	Joseph and Graser, 2003b
Further Characterization of Modified Cry3A Test Substance MCRY3A-0102	46155606	Moffatt, 2003
In vitro Digestibility of Modified Cry3A Protein (MCRY3A-0102 and IAPMIR604-0103) Under Simulated Mammalian Gastric Conditions	46155607	Joseph and Graser, 2003c
Effect of Temperature on the Stability of Modified Cry3A Protein (MCRY3A-0102)	46155608	Joseph, 2003
Analysis for the Presence of Modified Cry3A Protein in Wet and Dry Milled Fractions, Corn Oil and Corn Chips from Corn (Maize) Event MIR604	46155609	Joseph, R. and Kramer, C, 2003
Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Mouse	46155610	Johnson, 2003
Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Known Toxins[2]	NA[1]	Hart and Rabe, 2004a
Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Allergens[2]	NA	Hart and Rabe, 2004b
Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Known Toxins[2]	NA	Hart and Rabe, 2004c
Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Sequence Homology with Allergens[2]	NA	Hart and Rabe, 2004d
Characterization of Phosphomannose Isomerase (PMI) Produced in Maize (Corn) Plants Derived from Event MIR604 and Comparison to PMI as Contained in Test Substance PMI-0198	NA	Hill, 2004

[1]NA = not assigned.

[2]Previous versions of the four reports of amino acid sequence homology searches were submitted to the US EPA in 2003 in support of an Experimental Use Permit application for Event MIR604 corn (67979-EUP-U) or a petition for permanent exemption from tolerances for the PMI protein in all crops (Petition File Symbol PP 3E6748). The present reports represent the results of more recent bioinformatics searches of updated databases, however, the conclusions of the initial assessments (Zawodny, 2003a, 2003b, 2003c, 2003d, 2003e and 2003f) remain essentially unchanged.

B. HUMAN EXPOSURE CONSIDERATIONS: MODIFIED CRY3A PROTEIN

B.1. Dietary Exposure to Nucleic Acids

The nucleic acids (DNA and the RNA encoded by it) present in Event MIR604-derived corn plants as a result of transformation will not present a dietary safety concern. Based on the ubiquitous occurrence and known safety of nucleic acids in the food supply, the World Health Organization (WHO; FAO/WHO, 1991), and the US FDA (US FDA, 1992) have stated that the consumption of DNA from all sources, including genetically modified crops, poses no safety issue. In addition, a tolerance exemption under the Federal Food Drug and Cosmetic Act regulations has been established for residues of nucleic acids that are part of plant-incorporated protectants or associated inert ingredients (US EPA, 2001b).

B.2. Prior Human Exposure to Modified Cry3A Protein

Prior dietary exposure to the mCry3A protein has not occurred. However, it is conceivable that a low level of dietary exposure to the native Cry3A protein has occurred, as it is registered for use in the U.S. in NewLeaf® Bt potatoes and has been registered as a component of various *Bacillus thuringiensis*-based microbial insecticides (see Table 21), and is permanently exempt from food and feed tolerances (US EPA 2004b).

B.3. Expression Levels of mCry3A Protein in Event MIR604 Plants

Quantifiable levels of mCry3A protein were detected in all Event MIR604-derived plant tissues analyzed except pollen (Joseph and Hill, 2003). Across all growth stages, mean mCry3A levels measured in leaves, roots and whole plants ranged from *ca.* 3 - 23 µg/g fresh wt. (4 - 94 µg/g dry wt.), *ca.* 2 - 14 µg/g fresh wt. (7 - 62 µg/g dry wt.), and *ca.* 0.9 - 11 µg/g fresh wt. (3 - 28 µg/g dry wt.), respectively. Mean mCry3A levels measured in kernels at seed maturity and senescence ranged from *ca*. 0.6 – 1.4 µg/g fresh wt. (0.8 – 2.0 µg/g dry wt.). Mean mCry3A levels measured in silk tissue at anthesis were below the lower limit of quantification (LOQ), <0.1 µg/g fresh wt. (<1.0 µg/g dry wt.). Mean mCry3A levels measured in silk tissue at seed maturity ranged from *ca*. 0.6 – 1.9 µg/g fresh wt. (1 – 3 µg/g dry wt.). No mCry3A protein was detectable in pollen from either the inbred MIR604-A or the hybrids MIR604-B and MIR604-C [limit of detection (LOD) = 0.07 µg/g fresh wt., 0.15 µg/g dry wt.].

The stability of mCry3A protein expression over multiple generations was evaluated. Overall, levels were similar across the four generations analyzed and there was no evidence of any significant trend either up or down, indicating that the expression of mCry3A protein is stable.

A more detailed summary of the Event MIR604 expression data can be found in Chapter 6, **Quantification of mCry3A and PMI Proteins in Event MIR604.**

Table 21. List of Commercial Products Containing *Bacillus thuringiensis* subsp. *tenebrionis* or Cry3A delta endotoxin.

Product Name	Company Name	Strain or Host	Percent AI	EPA ID#	Date EPA Approved	Pests	US States Approved
Trident	Certis USA, LLC	Bacillus thuringiensis subsp. tenebrionis	14.32	70051-64	12/12/1988	Colorado Potato Beetle (larvae)	No data available
Trident II Biological Insecticide	Sandoz Agro, Inc.	Bacillus thuringiensis subsp. tenebrionis	0.64	55947-138	3/1/1990	Colorado Potato Beetle (larvae); Elm Leaf Beetle	No data available
Foil Bfc Oil Flowable Bioinsecticide	Ecogen, Inc.	Bacillus thuringiensis subsp. kurstaki strain EG2424	10	55638-10	2/5/1991	Colorado Potato Beetle (larvae)	NC, RI (Discontinued, all states)
Ditera Technical Powder	Abbott Laboratories	Bacillus thuringiensis subsp. tenebrionis	99.9999	275-79	2/7/1991	No data available	No data available
Ditera Wettable Powder	Abbott Laboratories	Bacillus thuringiensis subsp. tenebrionis	3.65	275-80	2/7/1991	Colorado Potato Beetle (larvae); Elm Leaf Beetle; Mexican Bean Beetle	No data available
M-Trak Bioinsecticide	Ecogen, Inc.	Delta endotoxin of Bacillus thuringiensis subsp. san diego encapsulated in killed Pseudomonas fluorescens	10	55638-44	6/27/1991	Colorado Potato Beetle (larvae); Elm Leaf Beetle; Elm Caligrapha (larvae); Cottonwood Leaf Beetle; Imported Willow Leaf Beetle; Mealworms (larvae)	No data available
Raven	Certis USA, LLC	Bacillus thuringiensis sub. kurstaki strain EG7673 Lepidopteran active toxin	25	70051-82	1/18/1995	Colorado Potato Beetle (larvae); Elm Leaf Beetle; Cottonwood Leaf Beetle; Imported Willow Leaf Beetle; Mexican Bean Beetle	DE, NC, OK (Cancelled or Dropped, all states)

Plant Pesticide B.t. Subsp. Tenebrionis Colorado Potato Beetle Protein	Monsanto Company	Bacillus thuringiensis subspecies tenebrionis delta endotoxin as produced in potato by Cry IIIA gene and its controlling sequences and found in the following constructs:PV-STBT02, PV-STBT04, and PV-STMT01, IR-22,	0.2	524-474	5/2/1995	Colorado Potato Beetle	DE, IA, NC, OK, RI (Cancelled, Inactive or Dropped, all states)
New Leaf Plus Potatoes	Monsanto Company	Bacillus thuringiensis subspecies tenebrionis delta endotoxin as produced in potato by Cry IIIA gene and its controlling sequences and found in the following constructs:PV-STBT02, PV-STBT04, and PV-STMT01, IR-22; Potato leafroll virus (PLRV) replicase protein as produced in potato plants.	CryIIIA - 0.2; PLRV - 0.03	524-498	11/18/1998	Colorado Potato Beetle; Potato Leaf Roll Virus	No data available
Novodor Technical	Valent Biosciences Corporation	Bacillus thuringiensis subspecies tenebrionis, strain NB-176	92.6	73049-47	4/29/2000	No data available	No data available
Novodor Flowable Concentrate	Valent Biosciences Corporation	Bacillus thuringiensis subspecies tenebrionis, strain NB-176	10	73049-48	4/29/2000	Colorado Potato Beetle (larvae); Elm Leaf Beetle (larvae)	DE, ID, MD, MN, NC, NV, NY, RI, SD, VA, WA, WI
Bacillus Thuringiensis Subsp Tenebrionis Slurry	Valent Biosciences Corporation	Bacillus Thuringiensis Subsp Tenebrionis, strain NB-176	17	73049-64	3/3/2006	No data available	No data available

Source: Kelly Registration Systems, Inc., 10115 Highway 142 N., Covington, Georgia 30014; http://www.krsnetwork.com/home.asp

B.4. Fate of Modified Cry3A Protein in Processed Corn Products

Modified Cry3A protein (mCry3A) was quantitatively analyzed in wet and dry milled fractions generated from standard food processing procedures carried out on maize (corn) grain derived from Event MIR604, together with a corresponding non-transgenic control (Joseph and Kramer, 2003). In addition, flaking grits and flour produced during dry milling were further processed to oil and corn chips, respectively. All milling fractions, oil, and corn chips were analyzed for mCry3A by enzyme linked immunosorbent assay (ELISA).

The mCry3A level measured in a sample of the starting grain material derived from Event MIR604 was *ca*. 1.1 µg mCry3A/g. Among the wet milled fractions, the medium fiber (0.46 µg mCry3A/g), fine fiber (0.26 µg mCry3A/g), and gluten meal fractions (0.24 µg mCry3A/g) yielded quantifiable amounts of mCry3A. The coarse fiber, germ, and starch fractions had detectable but not quantifiable levels of mCry3A (<0.06 µg mCry3A/g). The steep water fraction showed no detectable mCry3A.

The analysis of different samples derived from the dry milling process resulted in quantifiable levels of mCry3A protein in all 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. Modified Cry3A levels measured 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 measured in the flour used in the preparation of corn chips was 0.32 µg mCry3A/g, no mCry3A was detected in the corn chips. Similarly, mCry3A was not detectable in oil, whereas the mCry3A concentration measured in the starting material, flaking grits, was 2.12 µg/g.

C. MAMMALIAN TOXICOLOGY ASSESSMENT OF MODIFIED CRY3A PROTEIN

C.1. Target Organism Specificity of Modified Cry3A Protein

The observed activity spectrum of the mCry3A protein (see Chapter 7. **Environmental Safety**), its mode-of-action and its structural similarity to Bt proteins for which human safety has previously been established (see Chapter 1. **Syngenta Seeds Petition for the Determination of Non-Regulated Status of Corn Event MIR604**) support the prediction that no adverse health effects will result from exposure to mCry3A protein from Event MIR604 corn. Nevertheless, as described below, Syngenta has conducted an extensive characterization and safety assessment of the mCry3A protein.

C.1.a. Test Substance Used for Safety Studies

The mCry3A protein cannot be reasonably extracted in sufficient quantities from Event MIR604 corn plants to deliver the high doses typically employed in standard acute oral toxicity studies. Therefore, mCry3A was produced and purified from a recombinant microbe (Joseph and Graser, 2003b).

The mCry3A test substance was prepared by expressing the same modified *cry3A* gene that was used for plant transformations in an *E. coli* over-expression system. The modified *cry3A* gene was linked to the *Bacillus thuringiensis cry1Ac* native promoter in a Bluescript™ vector and transformed into *E. coli* strain DH5α. The *cry1Ac* promoter permits expression of the modified *cry3A* gene in the stationary phase of bacterial cell growth. The resulting lyophilized protein preparation, designated test substance MCRY3A-0102, was insecticidally active and had a 144-hour LC_{50} of 1.4 μg/ml (95% confidence interval: 0.7 - 2.2 μg/ml) against larvae of western corn rootworm (WCRW). The purity of mCry3A in the test substance was determined by densitometric analysis of a Coomassie blue stained gel. The results of this analysis showed that test substance MCRY3A-0102 contained *ca.* 90.3% mCry3A by weight. Western blot analysis of the test substance revealed a single immunoreactive band corresponding to the predicted molecular weight of *ca*. 67,700 daltons. Re-analysis of test substance MCRY3A-0102 *ca*. nine months after its initial characterization, demonstrated that it was substantially stable under the conditions of storage (-20°C).

In a separate study (Moffatt, 2003), mass spectral analysis revealed that test substance MCRY3A-0102 contained two closely related proteins in a ratio of 2:3. The lesser of these two components (with the lower molecular weight) corresponded to the intended mCry3A protein (598 amino acids). The other component also contained these same 598 amino acids but was shown to contain an additional 16 amino acids at the N-terminus. As part of the test substance characterization study, small amounts of each of these two proteins, designated mCry3A-SF and mCry3A-LF, respectively, were prepared and their bioactivity determined against WCRW. The results of the bioassay demonstrated that, as expected, both proteins were of similar activity. On this basis, and also taking account of their high degree of structural homology (97.4 % amino acid identity), the two forms of mCry3A in test substance MCRY3A-0102 can be considered to be equivalent. It was demonstrated that these two forms of the protein in sample MCRY3A-0102 differed by 16 amino acid residues due to translation of the protein occurring from two different initiation codons in the vector sequence used for production of MCRY3A-0102 in *E. coli*. It is important to note that this only occurred in production of this test substance and could not have occurred in transformed plants. The extra initiation codon in the vector sequence used for the test substance production was present in the *cry1Ac* gene promoter used to express the *mcry3A* gene in *E. coli*. There is no additional initiation codon present in the gene promoter in the vector used for plant transformation.

C.1.b. Equivalence of mCry3A Protein in Test Substance MCRY3A-0102 and mCry3A Protein as Produced in Event MIR604 Plants

To justify the use of *E. coli*-expressed mCry3A protein as a surrogate for mCry3A as produced in Event MIR604 corn, an extensive "bridging" study was conducted (Joseph and Graser, 2003a). *E. coli*-produced test substance MCRY3A-0102 was compared by analysis of various functional and biochemical parameters to mCry3A protein produced in transgenic corn event MIR604. The mCry3A proteins from both sources were demonstrated to have the predicted molecular weight of *ca*. 67,700 Da and immunologically cross-reacted with the same anti-mCry3A antibody. No evidence of any post-translational glycosylation of mCry3A protein from either source was observed. Comparisons of the biological activity of *E. coli*-expressed and corn-expressed mCry3A

protein in a larval diet bioassay using western corn rootworm showed very similar activities (LC_{50} values). Based on these results, it can be concluded that mCry3A proteins from recombinant *E. coli* and MIR604-derived corn are substantially equivalent and that the microbial test substance MCRY3A-0102 is a suitable surrogate for mCry3A protein produced in transgenic corn Event MIR604.

Additional evidence supporting the equivalence of mCry3A in test substance MCRY3A-0102 and Event MIR604 plants is contained in Chapter 3 of this submission, **Molecular Analysis of Event MIR604**, and in the report, "Further Characterization of Modified Cry3A Test Substance MCRY3A-0102" (Moffatt, 2003).

C.2. Evaluation of mCry3A Amino Acid Sequence Homology with Known Toxins

Although the report of a previous toxin homology search (Zawodny, 2003a) was submitted to the U.S. EPA in support of mCry3A safety, due to the continuing deposition of new sequence entries into the database upon which this analysis relies, a new updated report of the toxin homology search is provided with this petition. However, this new report does not substantially alter the conclusions of the previous report submitted to EPA.)

To determine whether the mCry3A protein has any significant amino acid homology with protein sequences identified as toxins, the amino acid sequence was systematically compared to the latest posting of the National Center for Biotechnology Information (NCBI) Database (NCBI, 2004) containing all publicly available protein sequences (Hart and Rabe, 2004a). The procedure used allowed a determination of (1) whether any proteins in the database showed significant homology to the mCry3A protein, indicating they may be closely related to modified Cry3A, and (2) whether any sequences with significant homology to mCry3A were known to be toxins. The mCry3A query sequence showed no significant amino acid homology to any non-Bt proteins identified as, or known to be, toxins.

C.3. Evaluation of mCry3A Amino Acid Sequence Homology with Known or Putative Allergens

Although the report of a previous allergen homology search was submitted to the U.S. EPA in support of mCry3A safety (Zawodny, 2003b), due to the subsequent deposition of new protein sequence entries into the allergen database upon which this analysis relies, a new updated report of the *allergen homology search is provided with this petition. However, this new report has not substantially altered the conclusions of the previous report submitted to EPA.*

An extensive bioinformatics search was performed to determine whether the amino acid sequence of the mCry3A protein shows homology with proteins known or suspected to be allergens (Hart and Rabe, 2004b). Two different similarity searches were performed comparing the mCry3A protein to the entries in the Syngenta Biotechnology Incorporated (SBI) Allergen Database. This database was compiled from entries identified as allergens or putative allergens in public protein databases, and was

supplemented with additional amino acid sequences identified from the scientific literature.

First, overall homology was examined by comparing sequential 80-amino acid peptides of the mCry3A protein sequence to the allergen sequences using the FASTA search algorithm. Each successive ‘window’ of 80 amino acids was offset from the previous window by one residue, such that each peptide overlapped the previous peptide by 79 amino acids. Any 80-amino acid peptide of the query sequence having greater than 35% amino acid identity to an allergen sequence was defined as having significant homology to the allergen sequence. Second, the mCry3A protein sequence was screened for matches of eight or more contiguous amino acids using a program that compared every possible peptide of eight contiguous amino acids between the mCry3A sequence and the allergen sequences. The purpose of this analysis was to screen for short, local regions of amino acid identity that might indicate the presence of common IgE-binding epitopes.

The results of these analyses revealed that there was no significant similarity between any of the sequential mCry3A 80-amino acid peptides and any entries in the SBI Allergen Database. Additionally, there were no alignments of eight or more contiguous identical amino acids between the mCry3A protein and any of the proteins in the allergen database. Thus, the mCry3A protein shows no significant amino acid homology to known or putative allergenic proteins.

C.4. Acute Oral Mouse Toxicity Study of Modified Cry3A Protein

An acute mouse oral toxicity study was conducted at the Syngenta Central Toxicology Laboratory (Alderley Park, Macclesfield, Cheshire, UK) according to US EPA Test Guideline OPPTS 870.1100 (Johnson, 2003). Test substance MCRY3A-0102 (see description and analyses of test substance in Section C.1.a., above) was administered to 5 male and 5 female mice [strain Alderley Park albino mouse (AP_fCD-1); 8 - 9 weeks old] *via* a gavage dose of 2632 mg/kg body weight. The test substance contained *ca.* 90.3% mCry3A protein by weight. Therefore, the mice received *ca.* 2377 mg mCry3A/kg body weight. A negative control group (5 mice/sex) concurrently received the dosing vehicle alone, a suspension of 1% methylcellulose, at the same dosing volume as used for the test material mixture. Food was provided *ad libitum*, except during the *ca.* 3.5 hours prior to dosing, when the animals were fasted. Water was provided *ad libitum* throughout the study. Observations for mortality and clinical/behavioural signs of toxicity were made frequently on the day of dosing and daily[7] thereafter for 14 days. Detailed clinical observations were made for each animal at each observation time. Body weights were recorded daily[1] and food consumption was recorded, in most cases, at daily intervals throughout the study. Surviving animals were euthanized 14 days post dosing and subjected to gross necropsy. Organ weights (brain, liver with gall bladder, kidneys and spleen) were recorded and principal tissues (brain, cecum, colon, duodenum, ileum, jejunum, rectum, stomach, heart, kidney, liver with gall bladder, lung and spleen) were processed for microscopic examination.

[1] Except for day 13 of the study when, in error, observations were not made

No test substance-related mortalities occurred during the study, and no clinical signs attributable to the test substance were observed. There were no treatment-related effects on body weight, food consumption, or organ weights, nor were any treatment-related effects observed following macroscopic or microscopic examination of tissues. One female mouse in the test substance group was euthanized on day 2 of the study due to clinical signs consistent with a dosing injury, which was confirmed upon post-mortem examination.

MCRY3A-0102 was not acutely toxic to mice. There was no evidence of toxicity of the test substance at 2632 mg MCRY3A-0102/kg body weight, representing *ca*. 2377 mg mCry3A protein/kg body weight. The estimated LD_{50} value for pure mCry3A protein in male and female mice is >2377 mg/kg body weight, the single dose tested.

C.5. *In vitro* Digestibility Study of Modified Cry3A Protein

The susceptibility of mCry3A protein to proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin (Joseph and Graser, 2003c). mCry3A from two sources, transgenic maize (corn) and recombinant *E. coli* (test substance MCRY3A-0102), was readily degraded in SGF. No intact mCry3A (*ca.* 67,700 molecular weight) or immunoreactive fragments were detected following digestion in SGF for 2 minutes, as assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis. These data support a conclusion that mCry3A expressed in transgenic plants will be readily digested as conventional dietary protein under typical mammalian gastric conditions.

The observation that some food allergens exhibit proteolytic stability has led to the common belief that proteins that are resistant to gastric digestion are more likely to become food allergens. However, some researchers (Veiths *et al.,* 1999; Kenna and Evans, 2000; Fu, 2002; reviewed by Fu *et al.,* 2002) have questioned the validity of digestion stability as a criterion for protein allergenicity assessment. However, at the present time, information on gastric digestibility may be utilized in a comprehensive weight-of-evidence approach to assessing allergenic potential.

C.6. Effect of Heat on the Stability of Modified Cry3A Protein

The effect of temperature on mCry3A protein was determined by incubating test substance MCRY3A-0102 for 30 minutes at a range of temperatures (4ºC, 25ºC, 37ºC, 65ºC and 95ºC) followed by bioassay against WCRW larvae (Joseph, 2003). At 95ºC mCry3A was completely inactivated. At 4ºC, 25ºC, and 37ºC there was little or no effect on mCry3A bioactivity, whereas at 65ºC there was some reduction in the bioactivity.

D. EVIDENCE THAT MODIFIED CRY3A PROTEIN IS UNLIKELY TO BECOME A FOOD ALLERGEN

While virtually all allergens are proteins, only a few of the many proteins found in foods are allergenic. Although the probability that any specific novel protein will become a food allergen is, therefore, small, the potential allergenicity of the mCry3A

protein was evaluated using an extensive weight-of-evidence approach. The methods employed generally followed a decision tree for allergenicity assessments, as recommended and described by several international organizations[8] and other experts (Metcalfe *et al*., 1996; FAO/WHO, 2001; Taylor, 2002).

Four general concerns regarding potential allergenicity arise in the context of producing novel proteins in food plants:

- Is the novel protein derived from a source known to produce allergenic proteins and, therefore, might individuals previously sensitized to one or more of these allergens be inadvertently exposed *via* food from the modified crop?
- Is the novel protein sufficiently similar to known protein allergens such that it might elicit an allergic cross-reaction in sensitized individuals?
- Does the novel protein have particular physio-chemical characteristics that would make it more likely to sensitize some individuals, if sufficient dietary exposure occurred?
- Would the novel protein be present in sufficiently high concentrations in food to promote sensitization in the minority of individuals who might be predisposed to sensitization?

The following discussion presents specific test results and information regarding the mCry3A protein to address each of these concerns. Based on the weight of evidence from diverse sources, it can be concluded that mCry3A is very unlikely to represent a potential allergen in food. Additionally, no reports of food allergenicity have been reported to be associated with the use of Bt Cry3A-based insecticidal products in the several years that they have been used on food crops or as a plant-incorporated protectant.

D.1. Low Potential for Exposure to Modified Cry3A *via* Food

Many allergenic proteins, especially those in commonly allergenic foods, are abundant in the offending food, and are present at concentrations typically ranging between 1% and 80% of total protein (Metcalfe *et al.*, 1996). The average mCry3A concentration measured in unprocessed corn grain from Event MIR604 represents less than 0.0001% of the total protein (this calculation is based on corn grain containing 10% total protein by weight, and assumes less than 1 ppm (1 µg mCry3A/g) in the grain; see Chapter 6. **Quantification of mCry3A and PMI Proteins in Event MIR604**)

D.2. Cry3A is Not Derived from a Known Source of Oral Allergens

The source of native Cry3A protein is *Bacillus thuringiensis*. Bacteria have no history of allergenicity (Taylor and Hefle, 2001; FAO/WHO, 2001). Additionally, despite decades of widespread use of Bt insecticides on food crops, there have been no reports of oral allergies to these preparations, and the US EPA has stated that laboratory

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[8] The International Food Biotechnology Council (IFBC), the Allergy and Immunology Institute of the ILSI, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO)

animal studies submitted to the Agency have not indicated any potential for allergic reactions to Bt or its components (US EPA, 2001a).

D.3. Modified Cry3A Does Not Have Amino Acid Sequence Homology to Known Allergens

As described above (Chapter 8, Part C.3.), an extensive bioinformatics search determined that the mCry3A protein shows no significant amino acid homology to known or putative allergenic protein sequences.

D.4. Modified Cry3A is Unstable to Heat and Food Processing

Many food allergens are stable to heat and processing. However, the mCry3A protein has been demonstrated to lose all bioactivity upon heating at 95°C (see Chapter 8, Part C.6., above), and is substantially degraded or eliminated by standard corn processing methods that result in protein degradation, *e.g.* wet milling and the alkaline process used in making corn chips (see Chapter 8, Part B.4., above).

Disulfide bonds between cysteine residues can stabilize some proteins and appear to contribute to their allergenicity, as evidenced by the mitigating effect of the reducing agent thioredoxin on wheat and milk allergenicity (Buchanan *et al.,* 1997; del Val *et al*., 1999). The three-dimensional structure of native Cry3A protein does not include disulfide bonds (Li *et al.,* 1991), therefore it is highly unlikely that the mCry3A protein is stabilized by disulfide bonds. The amino acid sequence of the mCry3A protein contains only three cysteine residues, the same number as the native Cry3A protein. The very low likelihood of disulfide bonds within the mCry3A molecule, in addition to its lability to heat and processing, suggests that the mCry3A protein does not have features that contribute to high stability and, presumably, higher allergenic potential.

D.5. Modified Cry3A is Susceptible to Gastric Digestion

As previously described above (Chapter 8, Part C.5.) the mCry3A protein is readily degraded by pepsin under simulated gastric conditions. No intact mCry3A or immunoreactive fragments were detected following digestion in SGF for 2 minutes.

D.6. Modified Cry3A is Not Glycosylated

As previously described above (Chapter 8, Part C.1.b.), analysis of the mCry3A protein revealed no evidence of post-translational glycosylation.

E. MAMMALIAN SAFETY ASSESSMENT OF PMI MARKER PROTEIN

E.1. Permanent Exemption from Tolerances for PMI in All Crops

A permanent exemption from tolerances for the PMI protein as an inert ingredient in all plants has been granted by the U.S. EPA (U.S. EPA, 2004a) and details of the

mammalian safety assessment for PMI can be found elsewhere (Vlachos and Joseph, 2003).

E.2. Identification of Changes in PMI Protein as Expressed in Event MIR604

The maize transformant that gave rise to the MIR604 transgenic line was transformed using *Agrobacterium* and the plasmid pZM26, which contains the *E. coli*-derived *manA* gene that encodes PMI. As described in Chapter 3. **Molecular Analysis of Event MIR604**, post-transformation analysis of the DNA sequence encoding PMI in Event MIR604 plants determined that two unintended amino acid changes had occurred in the PMI protein as expressed in this event. Valine-61 has been substituted by alanine, and glutamine-210 has been substituted by histidine. To establish whether PMI from Event MIR604 plants was substantially equivalent to PMI as contained in test substance PMI-0198 (*E. coli*-produced PMI), several biochemical and functional parameters were evaluated; PMI proteins from both sources were compared by their apparent size, immunoreactivity with the same anti-PMI antibody, enzymatic activity and the presence or absence of glycosylation (Hill, 2004).

Western blot analysis determined that as expected, PMI extracted from Event MIR604 maize leaf tissue is of similar size and immunoreactivity to *E. coli*-produced PMI. The enzymatic activity of PMI from both sources was comparable, confirming that the two unintended amino acid changes in PMI protein as expressed in Event MIR604 had no functional effect on the PMI protein. Additionally, there was no evidence of post-translational glycosylation of PMI from either source. On the basis of these comparisons, it can be concluded that the PMI proteins from these two sources are substantially equivalent (Hill, 2004).

E.3. PMI as Expressed in Event MIR604 Plants: Evaluation of Amino Acid Homology with Known Toxins

Although the reports of previous toxin homology searches were submitted to the U.S. EPA in support of the safety of PMI (Zawodny, 2003e) and PMI as expressed in MIR604 plants (Zawodny, 2003c), due to the continuing deposition of new protein sequence entries into the databases upon which these analyses rely, a new updated report of the toxin homology search is provided with this petition.

To determine whether the PMI protein as expressed in Event MIR604 corn ("MIR604 PMI") has any significant amino acid homology with protein sequences identified as toxins, its amino acid sequence was systematically compared to the latest posting of the National Center for Biotechnology Information (NCBI) protein database (NCBI, 2004) containing all publicly available protein sequences (Hart and Rabe, 2004c). The procedure used allowed a determination of (1) whether any proteins in the database showed significant homology to the MIR604 PMI protein, indicating they may be closely related to MIR604 PMI, and (2) whether any sequences with significant homology to MIR604 PMI were known to be toxins. The MIR604 PMI query sequence showed no significant amino acid homology to any proteins identified as, or known to be, toxins.

E.4. PMI as Expressed in Event MIR604 Plants: Evaluation of Amino Acid Homology with Known or Putative Allergens

Although the reports of previous allergen homology searches were submitted to U.S. EPA in support of the safety of PMI (Zawodny, 2003f) and PMI as expressed in MIR604 plants (Zawodny, 2003d), due to the subsequent deposition of new protein sequence entries into the allergen database upon which these analyses rely, a new updated report of the allergen homology search is provided with this petition.

Two different similarity searches were performed comparing the MIR604 PMI protein to the entries in the Syngenta Biotechnology Incorporated (SBI) Allergen Database (Hart and Rabe, 2004d). This database was compiled from entries identified as allergens or putative allergens in public protein databases, and was supplemented with additional amino acid sequences identified from the scientific literature. The SBI Allergen Database is updated annually.

First, overall homology was examined by comparing sequential 80-amino acid peptides of the MIR604 PMI protein sequence to the allergen sequences using the FASTA search algorithm. Each successive 'window' of 80 amino acids was offset from the previous window by one residue, such that each peptide overlapped the previous peptide by 79 amino acids. Any 80-amino acid peptide of the query sequence having greater than 35% amino acid identity to an allergen sequence was defined as having significant homology to the allergen sequence. Second, the MIR604 PMI protein sequence was screened for matches of eight or more contiguous amino acids using a program that compared every possible peptide of eight contiguous amino acids between the MIR604 PMI sequence and the allergen sequences. The purpose of this analysis was to screen for short, local regions of amino acid identity that might indicate the presence of common IgE-binding epitopes.

The results of these analyses revealed that there was no significant similarity between any of the sequential MIR604 PMI 80-amino acid peptides and any entries in the SBI Allergen Database. As indicated above, the SBI Allergen Database is updated annually and subsequent to these searches, results from a recent allergen homology search (Hart and Rabe, 2004d) detected one region of sequence homology of eight contiguous identical amino acids between MIR604 PMI and a recently described allergen, α-parvalbumin (110 amino acids; Accession Number CAC83047) from *Rana species* (frog) CH2001. This allergen was identified and reported by Hilger *et al.,* 2002. The specific amino acid sequence in common was "DLSDKETT", which occurs at positions 327 – 334 of MIR604 PMI and positions 77 – 84 of the allergen sequence (Hart and Rabe, 2004d). It is of note that the two amino acid substitutions, described in Section E.2. above, that were identified in the Event MIR604-derived PMI protein do not occur within the above identified common sequence and thus are not fundamentally related to the results of the sequence homology search.

Hilger *et al.* (2002) described a severe case of food-induced anaphylaxis in a single individual who consumed frog legs of Indonesian origin. Using the patient's serum, Hilger *et al*. proceeded to identify the causative agent of this anaphylactic response as α-parvalbumin from an unidentified frog (Rana) described as *Rana species* CH2001. The

response of this patient appeared to be quite specific to the frog leg sample of Indonesian origin, in that the patient's serum showed no cross-reactivity to related parvalbumins from *Rana esculenta*, the common edible frog. To determine if IgE antibodies present in this patient's serum recognized PMI, Syngenta sent two protein samples to the Hilger group for analysis of cross-reactivity. The first protein, designated "POI1" (Protein Of Interest 1), was a PMI test substance, PMI-0198, produced from an *E. coli* over-expression system and used in previous safety testing as a surrogate for PMI as expressed in transgenic plants. As discussed above, the two amino acid changes that occurred in Event MIR604 PMI do not lay within the common sequence indicated above and thus the PMI test substance PMI-0198, which does not contain these substitutions, is appropriate for these cross-reactivity studies. As an internal check, an unrelated protein designated "POI2" (bovine serum albumin) and a positive control extract from *Rana species* CH2001 were also included in the experiments performed in Dr. Hilger's laboratory for cross-reactivity analysis.

The results of the serum screening analysis demonstrated no cross-reactivity between the human serum IgE and PMI (POI1). Negative control serum screening analysis demonstrated no cross-reactivity with BSA (POI2). The positive control extract from *Rana species* CH2001 demonstrated cross-reactivity as expected. This indicates that the allergic patient's serum IgE does not recognize any portion of the PMI protein as an allergenic epitope. Therefore, the observed low degree of sequence identity between MIR604 PMI and α-parvalbumin from *Rana species* CH2001 is not biologically relevant.

F. NON-HUMAN EXPOSURE AND SAFETY CONSIDERATIONS

Field corn (maize) is readily consumed by livestock in the United States, with roughly 80% of the crop fed to livestock. Livestock that feed on maize include cattle, pigs, poultry, sheep and goats. The safety of Event MIR604 maize as livestock feed is supported by its compositional and nutritional equivalence to non-transformed control and commercially available maize lines, the absence of any unintended or toxic effects associated with Event MIR604 maize grain when incorporated into the diets and fed to broiler chickens, the rapid digestibility in mammalian gastric fluid and lack of toxicity of the introduced mCry3A insecticidal protein.

F.1. Prior Livestock Exposure to Modified Cry3A Protein

Prior dietary exposure of livestock to mCry3A via feed has not occurred. However, it is conceivable that a low level of exposure to the native Cry3A protein has occurred, as it is registered for use in the U.S. in NewLeaf Bt potatoes and as a component of encapsulated *Bacillus thuringiensis*-based microbial insecticides (see Table 17), and is permanently exempt from food and feed tolerances (US EPA 2004a). Livestock consuming feed containing Event MIR604 maize may receive low exposure to the mCry3A protein, as quantifiable levels of mCry3A were detected in all Event MIR604-derived plant tissues except pollen (see part B.3. above). However, the rapid degradation of mCry3A observed under *in vitro* conditions simulating the gastric environment of mammals (see section C.5. above) indicates that any protein consumed by livestock will be rapidly digested and metabolised as conventional dietary protein.

F.2. Livestock Exposure to Nucleic Acids

All whole foods and feedstuffs contain DNA and humans and animals, including livestock, routinely consume significant quantities of DNA from a variety of sources. The nucleic acids (DNA and the RNA encoded by it) present in Event MIR604-derived corn plants as a result of transformation will not present a dietary safety concern. Based on the ubiquitous occurrence and known safety of nucleic acids in the food supply, the World Health Organization (WHO; FAO/WHO, 1991), and the US FDA (US FDA, 1992) have stated that the consumption of DNA from all sources, including genetically modified crops, poses no safety issue. In addition, a tolerance exemption under the Federal Food Drug and Cosmetic Act regulations has been established for residues of nucleic acids that are part of plant-incorporated protectants or associated inert ingredients (US EPA, 2001b).

F.3. Nutritional Data

The nutritional composition of MIR604-derived maize plants and isogenic non-transgenic control plants was assessed (See Chapter 5, **Compositional Analysis of Event MIR604 Corn** for details and data). Key nutritional components in maize grain and whole plants (forage) derived from Event MIR604 and near isogenic non-transgenic control plants were compared (Kramer, 2004). The whole plants and grain analyzed were from hybrid pairs grown at 12 locations in the USA over two growing seasons (2002 and 2003). As would be expected from an analysis of this size, sporadic statistically significant differences were observed for some parameters between the MIR604 transgenic and near isogenic controls. All components evaluated in this study were within the range of reported literature values for maize with the exception of potassium in forage and phytosterols in grain.

At the time the forage potassium data were generated, Syngenta was unable to identify and provide a control range for concentrations of potassium in conventional corn forage. Syngenta subsequently conducted a study to measure potassium concentrations in non-transgenic forage using the same methodology that was employed in the original analysis of the MIR604 forage (de Fontes and Kramer, 2006). In addition, further literature investigations revealed historical data that was previously overlooked. Forage potassium concentrations for MIR604 and its controls were compared and found to be within both the newly measured and literature ranges.

Average phytosterol levels in both control and transgenic grain samples were below the average concentration reported in the literature. Syngenta subsequently conducted a study to measure campesterol and stigmasterol concentrations in non-transgenic grain using the same methodology that was employed in the original analysis of the MIR604 grain (de Fontes and Kramer, 2005). Grain campesterol and stigmasterol concentrations for MIR604 and its controls were compared and found to be within the newly measured ranges.

For all other analytes, no consistent patterns emerged to suggest that biologically significant changes in composition or nutritive value of the grain or forage had occurred as an unintended result of the transformation process or expression of the transgene. The

conclusion based on these data is that there is strong evidence that the genetically modified MIR604 hybrids are substantially equivalent in composition to the isogenic controls, and other commercial hybrids.

The nutritional quality of Event MIR604 maize grain was assessed in a 49-day feeding trial in broiler chickens (Brake, 2004). Broiler chickens are highly sensitive to small nutrient changes within their diets because of their extremely rapid growth. Male and female broiler chickens were fed diets containing Event MIR604 maize grain or non-transformed control or commercially available ("NC 2003") maize lines. The diets were formulated based on the individual nutrient analyses for each of the grains to meet standard nutritional recommendations for poultry.

The transgenic MIR604 hybrid diets supported rapid broiler chicken growth at low mortality rates and excellent feed conversion ratios without significant impact on overall carcass yield or quality. The absence of biologically relevant differences in growth and feed conversion parameters confirms the nutritional equivalence of Event MIR604 corn to its isogenic control, the absence of any unintended effects and the absence of toxicity and supports the conclusion that consumption of MIR604 maize in animal feed does not pose a safety concern.

F.4. Potential Toxicity of Introduced Protein

As described above, the lack of observed toxicity in rodents acutely exposed to high oral doses of the mCry3A protein indicates that any residues of mCry3A in MIR604 used in animal feeds will not pose a safety concern (section C.4.). In addition, as described in Section C.2. above, the potential toxicity of the mCry3A and PMI proteins was also assessed by comparing the amino acid sequences against the latest posting of the National Center for Biotechnology Information (NCBI) Entrez Protein Database (NCBI, 2004) containing all publicly available protein sequences identified as toxins. The mCry3A query sequence showed no significant amino acid homology to any non-delta endotoxin proteins identified as, or known to be, toxins. The absence of toxicity of the protein introduced into Event MIR604 maize supports the safety of Event MIR604 maize as livestock feed.

G. CONCLUSIONS

The large body of data and information described herein support the conclusion that the modified Cry3A protein as expressed in Event MIR604 corn will pose no hazard to humans or domestic animals upon commercial approval of the use of Event MIR604 corn. Additionally, the PMI protein has been granted a permanent exemption from tolerance in all crops from the U.S. EPA based on an extensive body of data that demonstrate this selectable marker presents no risk to humans, animals or the environment.

Chapter 9. Environmental Consequences of Introduction

A. INTRODUCTION

As described in Chapters 1 and 6, mCry3A has demonstrated very specific, targeted and enhanced pesticidal activity toward certain maize coleopteran pest species: northern, western and Mexican corn rootworm. Corn plants transformed with the modified *cry3A* gene display resistance to these pests (Chen and Stacy, 2003). Expression of mCry3A in Event MIR604-derived hybrids has been shown to occur in root and other tissues, and during the course of normal agricultural practice, the active principle mCry3A could theoretically enter neighboring environments through establishment of weedy populations of MIR604 plants outside fields, through transfer and expression of the mCry3A gene in other organisms via sexual hybridization or horizontal gene transfer, through off-crop movement of MIR604 pollen or via degradation of plant tissue and subsequent movement of mCry3A through soil. Below are arguments and reviews of studies that indicate that introduction of Event MIR604-derived hybrids will lead to minimal exposure of the environment to mCry3A. In addition, when coupled with the data described in Chapter 7 (**Environmental Safety**) demonstrating no detectable hazard of mCry3A to non-target organisms that might feed on MIR604 growing in cornfields, it is clear that Event MIR604-derived hybrids present very little environmental risk and may provide substantial benefits over current traditional corn rootworm control practices.

B. EXPRESSION LEVELS OF MCRY3A IN HYBRIDS DERIVED FROM MIR604

Details of the study that determined expression levels of mCry3A in MIR604 tissues are provided in the Chapter 6. **Quantification of mCry3A and PMI Proteins in Event MIR604.** A summary of the study report (Joseph and Hill, 2003) is provided here.

Corn tissues derived from three genotypes were sampled at four growth stages: whorl stage, 6 weeks after planting; anthesis, 10-11 weeks after planting; seed maturity, 18-20 weeks after planting; and senescence, about 24 weeks after planting. The concentrations of mCry3A in leaves, roots, kernels, silks and pollen were determined by ELISA. Measurements of mCry3A concentrations in whole plants and in silage were also made.

With one exception, all tissues at all sampling times contained detectable amounts of mCry3A. The only tissue in which mCry3A was not detected was pollen (limit of detection = 0.07 µg/g fresh weight). The finding of effective absence of mCry3A in pollen from MIR604 hybrids was supported by the lack of detectable mCry3A protein in pollen from the inbred line; all pollen grains are expected to contain the *mcry3A* gene in the MIR604 inbred, whereas only 50% of pollen grains of MIR604 hybrids are expected to contain the gene.

The stability of expression profiles over multiple generations was investigated in four successive backcross generations under greenhouse conditions. Leaf tissue was sampled at anthesis and the amount of mCry3A was measured using ELISA. The concentrations of mCry3A in all generations were similar and no trend towards increased or decreased

expression was observed. An independently validated detection method for mCry3A as expressed in Event MIR604 hybrids has been developed and submitted to the US EPA (Steiner and Larkin, 2004).

C. DEGRADATION OF mCRY3A IN SOIL

C.1. Background

The EPA has recently reviewed data concerning the degradation in the soil of various Cry proteins registered as plant-incorporated protectants (US EPA, 2001a). Laboratory soil degradation studies on the lepidopteran-active proteins Cry1Ab, Cry1Ac or Cry1F in field-collected soils, as either pure protein or in plant material, indicate that these proteins are degraded rapidly. The period for the Cry protein concentration or bioactivity to fall to half its initial value (the DT_{50}) is typically between 2 and 22 days for Cry1A proteins. The DT_{50} for Cry1F is just over 3 days (US EPA, 2001a).

Subsequent to the EPA review, a further plant-incorporated protectant, Cry3Bb1, which is active against certain Coleoptera, has been registered (US EPA, 2003). The DT_{50} of this protein was determined in the laboratory in a range of soil types and found to be between less than 1 day and up to 9 days.

The weight of evidence is that Cry proteins, like virtually all proteins, are inherently degradable in live soils *via* enzymatic degradation by ubiquitous soil proteases. Therefore the expectation is that mCry3A is also inherently degradable in live soil; a laboratory study was carried out to test this hypothesis.

C.2. Laboratory study of the degradation of mCry3A in soil

To test the hypothesis that mCry3A will degrade rapidly in live soil, mCry3A was incorporated into a sample of field-collected soil and its degradation was measured by bioassay against larvae of the Colorado potato beetle (CPB), which are known to be sensitive to mCry3A (Kramer and Joseph, 2004).

Soil was obtained from a farm in Grundy County, Iowa, USA in October 2003. Agvise Laboratories (Northwood, North Dakota, USA) identified the soil as silty clay loam with a moisture holding capacity at 1/3 bar of 27.7%. The soil was sieved and acclimated at 25ºC ± 1ºC under constant moisture levels for 10 days prior to the start of the test. Biomass determinations indicated that microbial activity was maintained during the period of the study and that the soil was suitable for use in this study.

The source of mCry3A for the study was the test substance MCRY3A-0102, a 90.3% pure sample of microbially expressed mCry3A. The preparation and characterization of MCRY3A-0102 have been described (Joseph and Graser, 2003b). In a separate study, test substance MCRY3A-0102 has been shown to be a suitable surrogate for mCry3A protein produced in transgenic corn Event MIR604, as assessed by various biochemical and functional parameters (Joseph and Graser, 2003a).

Soil was weighed and transferred to flasks such that 50 g dry weight equivalent of soil at 18.25% moisture was added to each flask. Soil samples were treated with 5 ml of a solution of MCRY3A-0102 to give a concentration of 230 µg mCry3A/g dry weight soil; this concentration is about 18X the highest concentration of mCry3A in whole plants of MIR604. This is a considerably higher concentration of mCry3A than would result from incorporation of residues of MIR604 hybrids in the field as the residues will be diluted in a large volume of soil (US EPA, 2001a).

Treated soil samples were capped, shaken and then weighed to determine actual moisture levels in the sample flasks. Moisture levels were adjusted and maintained throughout incubation at 75% ± 12% of field moisture capacity (FMC) at 1/3 bar, to simulate average field conditions. The samples were kept in a constant temperature room at 25°C ± 1 °C. Duplicate samples were removed from the constant temperature room at 1, 3, 7, 12 and 30 days after treatment and, along with two samples taken immediately after treatment, were frozen and stored at –20°C before analysis.

The DT_{50} for the bioactivity of mCry3A in the treated soil was measured using a bioassay. For each incubation period, a sample of treated soil was incorporated into a standard CPB diet at 10% w/w. Triplicate aliquots of each duplicate soil sample were taken to prepare 6 treatment diets; 2 bioassay plates, comprising 10 first instar CPB each, were prepared from each diet, giving 12 bioassay plates for each incubation period. Bioassay plates were kept at room temperature and mortality of the CPB larvae was measured after 72 hours.

For soil samples collected up to and including day 7 post soil treatment, mortality in the bioassays of mCry3A-treated soil was high (48 - 54%; 13-27% std. error). A positive control, consisting of CPB diet treated with MCRY3A-0102 to a concentration of 23 µg mCry3A/g diet (*i.e.* the nominal concentration of mCry3A in the treated soil diet), gave 53% (12% std. error) mortality. Mortality in the 12- and 30-day incubation treatments was 11% (13% std. error) and 9% (9% std. error), respectively. Mortality was 18% (15% std. error) in a water-treated soil control.

The reductions in mortality of CPB in the bioassay were modeled using simple first-order kinetics, and indicated that the DT_{50} of mCry3A in soil was 7.6 days. The results of this study confirm that mCry3A will be rapidly degraded in soil.

D. PERSISTENCE AND SPREAD OF THE *mcry3a* GENE

A full assessment of the environmental fate of mCry3A requires not only consideration of the expression and degradation of the protein in fields during and immediately after cultivation of MIR604 corn, but also the theoretical possibility that mCry3A could persist or spread more widely because of gene flow or the establishment of weedy populations of MIR604 (Raybould, 2004a). Additional details can be found in Chapter 2. **The Corn (Maize) Family**.

D.1. Gene Flow

Corn (*Zea mays* ssp. *mays*) will hybridize with a group of taxa collectively called teosinte. Several types of teosinte are classified as subspecies of *Zea mays*, whereas others are regarded as separate species of *Zea*. Teosinte species are natives of Central America and have co-existed with cultivated corn for several thousand years. They have remained genetically distinct from cultivated varieties despite occasional introgression (US EPA, 2001a). Teosinte species are not natives of the USA, but isolated populations have been recorded in Florida and Texas, the former a possible remnant of the use of annual teosinte as a forage grass. These populations are apparently now extinct in both states (US EPA, 2001a). Teosinte species are grown in botanical gardens, but fertilization of these plants with pollen from MIR604 is extremely unlikely.

Species of the genus *Tripsacum* are considered close relatives of corn. There are sixteen species of *Tripsacum* worldwide, of which three occur in the USA: *T. dactyloides*, a widespread forage grass; *T. floridanum*, known from southern Florida; and *T. lanceolatum*, which is present in Arizona and possibly New Mexico (US EPA, 2001a).

Corn breeders view *Tripsacum* as a potential source of useful genes for traits including apomixis, pest and disease resistance and drought tolerance (OECD, 2003) and therefore substantial effort has been made to obtain and characterize corn X *Tripsacum* hybrids. Hybrids between corn and *Tripsacum* species are difficult to obtain outside the laboratory or greenhouse and are often sterile. Only one record exists of an open-pollinated hybrid between *Zea* and *Tripsacum*, which involved species native to Guatemala. After consultation with experts on improvement of forage grasses, US EPA (2001a) concluded that the chance of natural introgression of genes from corn to *Tripsacum* was 'extremely remote' and that no other species in the continental USA would interbreed with commercial corn.

The data reviewed above indicate the very low probability of transfer of the *mcry3A* gene from MIR604 to wild relatives of corn. Species of *Zea* other than corn are not recorded outside botanical gardens in the USA. *Tripsacum dactyloides* is widespread, but does not hybridize readily with corn, and the probability of backcross or F_2 progeny of *Tripsacum* X *Zea* hybrids being produced in the field is negligible. Therefore, mCry3A protein is unlikely to spread from cultivated MIR604 corn and persist in the environment as the result of gene flow.

D.2. Potential for Horizontal Gene Transfer

In its recent reassessment of the environmental safety of Bt plant-incorporated protectants, EPA conducted an extensive review of information relevant to the theoretical risks of horizontal gene transfer (HGT). Studies reviewed by EPA showed no evidence for HGT under field conditions, and only equivocal evidence for HGT under laboratory conditions designed to maximize the recovery of transformants (US EPA, 2001a). Conner *et al*. (2003) also reviewed the literature and found very few examples where HGT had been demonstrated convincingly, and these cases relied on artificially high sequence homology between the transgene and the potential recipient (*e.g*., de Vries *et al*., 2001). Conner *et al*. (2003) also reported that new data comparing full genomic

sequences of various prokaryotes and eukaryotes have identified putative HGT events, however alternative interpretations of these data are possible (see references in Conner *et al*., 2003). There is no reason to suppose that corn derived from Event MIR604 is likely to transfer genes by HGT at a higher rate than any other plant, and therefore the likelihood of exposure to mCry3A through microorganisms expressing the *mcry3A* gene from MIR604 is minimal.

EPA also concluded that there were no significant hazards should a *cry* gene be transferred from a transgenic plant to a microorganism. *Bacillus thuringiensis* is common in soil and *cry* genes have been available for HGT to other species for long periods and no harmful events appear to have resulted from this prolonged exposure.

Soil microorganisms have not previously been exposed to the *mcry3A* gene contained in MIR604. While native and modified Cry3A proteins have a high degree of sequence homology, the DNA sequence of *mcry3A* was altered substantially from native *cry3A* to optimize codon use for expression in plants (Rabe, 2004). The change to the plant-preferred pattern of codon usage means that the *mcry3A* gene likely has lower homology to potential recombination sites in soil microorganisms than has the native *cry3A* gene.

Should *mcry3A* be integrated into a plasmid or chromosome of a bacterium, mCry3A protein is extremely unlikely to be produced because the maize metallothionein promoter linked to *mcry3A* in MIR604 (Rabe, 2004) is unlikely to function in bacteria and codon use in *mcry3A* is optimized for expression in corn, not bacteria (*e.g.,* Baneyx, 1999; Chen and Stacy, 2003). Laboratory testing of the effects of mCry3A protein on a variety of non-target organisms revealed no adverse effects (Chapter 7. **Environmental Safety**). Therefore in the extremely unlikely event that *mcry3A* is stably integrated and expressed in a soil microorganism, no harmful effects are expected.

D.3. Weediness

Corn has lost the ability to survive outside cultivation (OECD, 2003). It can over-winter and germinate in a subsequent crop as a volunteer weed. For example, corn is a common volunteer in soybeans. However, several features of corn make it unlikely to form self-sustaining weedy populations in agriculture: it is easily controlled in subsequent crops with selective herbicides; seed dispersal is limited because seeds are held inside the husks of the cob; and the seeds lack dormancy so that young plants are exposed to harsh winter conditions. Corn does not persist in habitats outside agriculture because, in addition to the features listed above, it requires disturbed ground to germinate and it is very uncompetitive against perennial vegetation.

Volunteers of MIR604 in following crops can be controlled in the same way as volunteers of any other corn variety; enhanced resistance to corn rootworm is highly unlikely to increase the frequency of volunteers. Furthermore, enhanced resistance to corn rootworm is unlikely to increase the invasiveness of corn in uncultivated land because ease of weed control, lack of dormancy and low competitiveness are the main reasons why corn is not a persistent weed. The lack of invasiveness of corn, particularly in non-agricultural habitats, means that mCry3A will not spread from sites of cultivation and persist in the environment as weedy populations of MIR604.

E. ENVIRONMENTAL FATE AND EXPOSURE OF mCRY3A

The data reviewed above indicate that exposure to mCry3A protein will be limited to the fields in which MIR604 corn will be grown; mCry3A is unlikely to persist in fields for a long period after grain from MIR604 has been harvested.

E.1. Exposure During Cultivation

No mCry3A protein was detected in pollen of MIR604 hybrids or inbreds and therefore ecologically significant exposure to mCry3A outside cultivation is unlikely to occur through contact with pollen. Furthermore, the rapid degradation of mCry3A in soil means that the protein is very unlikely to spread from cultivation into surface or ground water. These conclusions are particularly pertinent to the potential exposure of aquatic organisms to mCry3A (Section E.3 below). In short, exposure to mCry3A during cultivation will be limited to direct contact with living, senescent or dead MIR604 tissues in cornfields and possible short-term exposure to exuded proteins in soil.

E.2. Exposure Following Cultivation

Persistence of mCry3A protein following planting of MIR604 hybrids is likely to be for a limited period and be confined to sites of cultivation. After harvest, some protein will remain in MIR604 plant material (for example, roots and vegetative material plowed into the soil) until the plant tissues degrade. mCry3A protein is expected to degrade rapidly and not move off site. Control of volunteers will minimize the potential for prolonged exposure to mCry3A following cultivation of MIR604. Expression of mCry3A in soil microorganisms that have been transformed with the *mcry3A* gene by HGT is highly improbable and is also very unlikely to be hazardous.

Corn is not a weedy species and in the unlikely event that any feral plants of MIR604 corn result from accidental seed spillage, they are not expected to survive to flowering outside cultivation. There are no sexually compatible wild relatives of corn in the USA and therefore mCry3A protein will not spread and persist through introgression of the *mcry3A* gene into wild plants.

E.3. Organisms Likely to be Exposed to mCry3A

Aquatic organisms are unlikely to be exposed because of the low probability that mCry3A will enter watercourses through movement of soil particles, pollen dispersal or seed spillage (Vlachos, 2004); corn fields may become flooded during heavy rain, though degradation and dilution of mCry3A, and uncertainty about the immigration of aquatic organisms into flooded fields makes quantification difficult. Therefore other than humans and animals that eat corn grain, or animals that eat corn forage or silage, the only organisms likely to be exposed to mCry3A as a result of cultivation of MIR604 hybrids are pests and non-target organisms that feed on corn tissue, predators and parasitoids of these animals, and soil organisms. Environmental exposure will be limited spatially to areas where MIR604 is grown. Temporal exposure will be limited to the period of cultivation for non-target organisms feeding on the above ground parts of corn. Soil organisms may be exposed to mCry3A during the cultivation and *via* plant material

incorporated into the soil after harvest. The rapid degradation of mCry3A in the soil indicates that the duration of potential post-harvest exposure of soil organisms to significant amounts of mCry3A protein will be limited to a few weeks. A second period of exposure to all non-target organisms will occur if MIR604 volunteers arise, although volunteers should result in much lower exposure than planting of the crop. The precise routes of exposure and the safety of mCry3A to these non-target organisms are assessed in Chapter 7 (**Environmental Safety**).

F. CURRENT CORN ROOTWORM CONTROL PRACTICES

Insect pest management practices employed to reduce rootworm damage generally fall into two categories: (1) crop rotation and (2) application of chemical insecticides. As the preferred diet of rootworm larvae is corn roots and corn rootworm beetles lay their eggs in a diapause condition, farmers have adopted a crop rotation strategy that can significantly interrupt the corn rootworm life cycle. By following a first year corn crop with soybeans or other non-corn crop in the second season's planting, the population of emerging beetles is reduced as the primary food source (corn roots) for the hatching larvae is not available. With the pest population reduced, corn can then be planted again in the third season with little risk of economic impact. This crop rotation (primarily corn and soybeans) has been used since the mid-70s as an effective non-chemical control strategy for corn rootworm.

In areas where crop rotation has been used extensively, rootworm variants have evolved which have minimized the effectiveness of the crop rotation strategy. The first adaptation is referred to as the 'extended diapause' variant and was first documented in northwestern Iowa. In this instance, eggs laid by Northern corn rootworm (CRW) beetles remain in diapause two or more winters before hatching. In 2002, USDA-NASS (USDA, 2002) estimated that this extended diapause variant is present in 9.8 million acres of corn in the U.S.

The second adaptation, referred to as the 'soybean variant', was first documented in east central Illinois. In this case the Western CRW adult beetle deposit its eggs in neighboring soybean fields, which hatch in the following year's corn crop. According to USDA-NASS estimates, in 2002, 7.1 million acres of corn were planted in the heart of the soybean variant infestation zone that covers large portions of Illinois and Indiana. According to Payne *et al*. (2003) this new variant of CRW has also spread through most of northern Indiana, eastern Illinois, southern Michigan and western Ohio. Given historic movement patterns, the new variants may soon spread as far west as eastern Iowa (Onstad *et al*., 1999). This expanded area is equivalent to 16 million acres with the westward expansion being the greatest threat. Western CRW have also been detected in soybean fields located in counties extending to the Wisconsin border. As these new CRW variants spread throughout the U.S. corn belt, the effectiveness of this non-chemical control strategy will diminish.

In 2003, over 16 million acre treatments occurred using 6 organophosphates (OPs), 1 carbamate, 2 pyrethroids, and fipronil comprising the top ten chemical pesticides used to prevent corn rootworm damage (Steiner *et al*., 2004). Including all chemical alternatives

used in 2003, over 21 million acre treatments occurred (Table 22). On a percentage basis, OPs accounted for 72% of the pounds of active ingredient applied in 2003 (Table 23); OPs and OP-pyrethroid combinations accounted for 63% of the acre treatments. Pyrethroids accounted for only 12% of the pounds but 32% of the acre treatments for corn rootworm.

Table 22. Corn Rootworm Pesticides by Class

Chemical Class	Pounds of Active Ingredient	Acre Treatments	Grower Costs (US dollars)
OP	4,306,445	4,601,773	50,611,707
Pyrethroid	728,276	6,948,614	93,171,730
OP-Pyrethroid	554,693	8,864,582	55,919,425
Carbamate	216,175	266,934	3,745,043
Other	150,575	870,264	12,659,437
TOTALS	5,956,164	21,552,167	216,107,342

Table 23. Corn Rootworm Pesticides Percent of Use by Class

Chemical Class	Percent of Use Based on Pounds of AI	Percent of Use Based on Acre Treatments
OP	72	22
Pyrethroid	12	32
OP-Pyrethroid	9	41
Carbamate	4	1
Other	3	4
TOTALS	100	100

A number of products that comprise these classes of chemical pesticides have been classified for restricted use by the U.S. EPA (US EPA 2004d). EPA pointed out that phorate and terbufos (OPs) had been reviewed under the Agency's special review process because use of those pesticides may result in unreasonable adverse effects. In addition, EPA referenced its work on the avian risks presented by granular pesticides used on corn. Specifically, the Agency noted that phorate, terbufos, and chlorpyrifos are still used to combat corn rootworm. Also, EPA reported that, while carbofuran (a carbamate) was no longer used as a granular product, the non-granular formulation was still of concern. Finally, EPA noted that many of the corn rootworm pesticides were restricted use compounds because of human health and environmental concerns. These restrictions include extensive personal protective equipment that must be used by workers in order to use the pesticides, as well as significant personnel training to ensure compliance. Regardless of whether the restricted use pesticides are used in or out of compliance, many raise ecological concerns. Additionally, non-selective insecticides are expected to be toxic to many non-target arthropods, including beneficial arthropods, occurring in or near the treated fields.

Due to the wide spread use of chemical insecticides to control corn root worm infestations in the U.S. corn belt, both larval and adult CRW resistance has been reported (Scharf, *et al*., 2001; Zhou, *et al*., 2003). Control failures linked to corn rootworm resistance to carbaryl (Zhu, et al., 2001), methyl parathion (an organophosphate; Meinke, et al., 1998) and fipronil (Scharf, *et al*., 2000) have been reported. Continued extensive use of these and other chemical insecticides and spread of resistant phenotypes will most likely result in an increase of control failures and increase the demand for alternative methods of corn rootworm control.

G. BENEFITS OF INTRODUCTION OF EVENT MIR604-DERIVED HYBRIDS

Introduction of Event MIR604-derived hybrids could reduce many of the environmental risks associated with extensive use of conventional pesticides. mCry3A protein in Event MIR604 corn presents little if any risk to man and other non-target organisms. In contrast, the major chemical alternatives present numerous risk concerns. In addition, a potential 4.5 million acre treatments and about 1.25 million pounds of active ingredients could be eliminated during the first five years of sales of MIR604 corn (Steiner *et al*., 2004). From a comparative risk perspective, MIR604 corn presents a significant reduced risk profile compared to the chemical alternatives. Unlike chemical treatments, the plant-based delivery of active ingredient afforded by MIR604 hybrids offers consistent performance under varying environmental conditions. Unpredictable environmental factors can influence the efficacy of chemical treatments. In addition, with MIR604 corn, there will be reduced potential for crop injury resulting from herbicide/insecticide interactions and provide a viable alternative to crop rotation which has become less effective because of variant CRW strains. Finally, transgenic plant technology is easier to use than chemicals that require equipment calibration and application decisions concerning timing and whether to use the chemicals.

In addition to being safer to use, MIR604 hybrids deliver at a minimum comparable efficacy and yield performance compared to the chemical alternatives (see Chapter 4, **Agronomic Performance of Event MIR604**). Syngenta's efficacy data show that MIR604 corn performs significantly better than non-treated and chemically treated controls. Under environmental conditions of drought or heavy CRW pressure that can result in significantly reduced yield, the use of MIR604 can result in a positive yield impact. Under such conditions, grower monetary benefits from increased yield will be maximized. In less severe conditions, the benefits will be reduced.

While some benefits may be difficult to quantify and while environmental factors will influence efficacy and yield of any corn hybrid, the use of MIR604 corn will provide positive benefits for growers. Finally, as discussed above, the use of MIR604 corn provides substantial human health and environmental benefits that growers can achieve at the same time they accrue the economic advantages of MIR604 corn. Considering risks and benefits, including economic benefits, the deregulation of MIR604 corn will have significant public benefits, including direct benefits to US agriculture.

H. IRM AND STEWARDSHIP

Because of the clear agronomic, environmental and economic benefits of Event MIR604-derived hybrids outlined in this petition, it is essential that, once this technology is deployed, its continued utility is protected through the implementation of an appropriate and effective insect resistance management (IRM) program. Such a plan is founded on the best available understanding of the action of MIR604, the biology of the target CRW pests, the nature of the potential selection for resistance to mCry3A and a clear appreciation of resistance management options available to mitigate against any resistance development (FIFRA SAP, 1998). Moreover, an IRM plan for MIR604 must be compatible with existing IRM plans that include other CRW control such as YieldGard Rootworm® (Cry3Bb, Monsanto) and other corn pest (Lepidoptera) control (e.g., Bt11; Cry1A(b), Syngenta) technologies. Syngenta has developed an IRM plan based on the above principles and this plan will employ strategies that will include: a minimum structured refuge of non-CRW-control corn, complementary and harmonized IRM practices and an aggressive stewardship program that will maintain the long-term efficacy of MIR604 maize by reducing the potential for pests to develop resistance to the mCry3A protein (McCaffery, 2004).

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APPENDIX 1

EVENT MIR604 HYBRID 2002-2003 AGRONOMIC DATA

APPENDIX 1A

MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRID EFFICACY DATA

APPENDIX 1A (CONT'D)

TABLE 1A

CORN ROOTWORM ROOT DAMAGE RATING 0-3.9 SCALE[1]

0 - 3 Rating	Description of Rootworm Damage
0.01	No damage to 1-2 light surface scars on roots
0.02	3+ light surface scars ≤ 4 heavy scars
0.05	5+ heavy scars (long, deep scars), but **NO** root pruning
0.10	One root pruned to ≤ 2 inches accompanied with heavy scars, typically.
0.25	2+ roots pruned to ≤ 2 inches (up to 1/4 nodes, equivalent, pruned back)
0.50	Equivalent of 0.50 node of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
0.75	Equivalent of 0.75 node of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.00	Equivalent of 1.00 node of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.25	Equivalent of 1.25 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.50	Equivalent of 1.50 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
1.75	Equivalent of 1.75 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.00	Equivalent of 2.00 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.25	Equivalent of 2.25 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.50	Equivalent of 2.50 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
2.75	Equivalent of 2.75 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
3.00	Equivalent of 3.00 nodes of roots pruned. (No. of roots pruned varies for inbred vs. hybrid).
3.90	Total Root Crown Destruction - No roots left

[1]At the VT to R1 stage (approximately 7 weeks following infestation) plants were manually dug from the ground, washed free of soil and rated for rootworm damage using a damage rating derived from the Iowa State 0-3 node-injury rating scale (http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html). According to the upper range of this scale, a rating of 3.00 denotes '*three or more nodes eaten'*, a high degree of root damage, whereas a rating of 0.0 is no damage. In some instances of extreme corn rootworm pressure, total root crown destruction can occur such that no roots are left on the corn plant. Depending on the degree of root crown destruction, this level of root damage is assigned by Syngenta a rating as high as 3.9.

APPENDIX 1A (CONT'D)

TABLE 2A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2,], 2002

LOCATION, YEAR	ENTRY[4]	MEAN CRW ROOT DAMAGE RATING[5]	# OF REPS
Stanton, MN, 2002 Trial SYN137[3]	MIR604 early hybrid A1	1.092[7]	3
	MIR604 early hybrid A2	1.087	3
	Control early hybrid 1A	2.988	3
	LSD (0.05) [6]	**1.080**	
Bloomington, IL, 2002 Trial SYN177[3]	MIR604 early hybrid A1	0.949	3
	Control early hybrid 1A	3.130	3
	LSD (0.05)	**0.367**	
Bloomington, IL, 2002 Trial SYN178[3]	MIR604 early hybrid A2	0.798[8]	2
	MIR604 early hybrid A1	1.142	3
	Control early hybrid 1A	3.555	3
	LSD (0.05)	**0.318**	

[1]Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm plus artificial infestation with western corn rootworm.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3]Individual corn plants were artificially infested with freshly deposited Western corn rootworm eggs at an estimated calculated density of 1182 eggs per plant for trial SYN137 and 1462 eggs per plant for trials SYN177 and SYN178. Additional details can be found in Chapter 4, Section B.

[4]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid A1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data

[5]CRW root damage rating scale 0-3 (see Table 1A).

[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. [7]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level.

[8]One replication was mistakenly treated with insecticide for this MIR604 hybrid, and was not included in the LSD calculation.

APPENDIX 1A (CONT'D)

TABLE 3A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2,], 2003

LOCATION, YEAR	ENTRY[4]	MEAN CRW ROOT DAMAGE RATING[5,11]	# OF REPS
Stanton, MN, 2003 Trial SYN142[8]	MIR604 early hybrid G3	0.11	2
	MIR604 early hybrid G8	0.09	2
	Control early hybrid 1D[6]	2.11	2
	LSD (0.05) [7]	**1.07**	
Willmar, MN, 2003 Trial SYN143[9]	MIR604 early hybrid G3	0.05	4
	MIR604 early hybrid G8	0.05	4
	Control early hybrid 1D	1.41	4
	LSD (0.05)	**0.20**	
Bloomington, IL, 2003 Trial SYN180[3]	MIR604 early hybrid A1	0.10	3
	Control early hybrid 1A	2.54	3
	MIR604 early hybrid G3	0.26	3
	Control early hybrid 1D	2.62	3
	LSD (0.05)	**0.38**	
Bloomington, IL, 2003 Trial SYN181[3]	MIR604 early hybrid G3	0.05	4
	MIR604 early hybrid G5	0.10	4
	Control early hybrid 1D	1.73	4
	LSD (0.05)	**0.22**	
Bloomington, IL, 2003 Trial SYN182[8]	MIR604 late hybrid E1	0.27	4
	MIR604 late hybrid E4	0.29	4
	Control late hybrid 2E	2.46	4
	LSD (0.05)	**0.49**	
Bloomington, IL, 2003 Trial SYN183[8]	MIR604 late hybrid E1	0.20	4
	MIR604 late hybrid E4	0.17	4
	Control late hybrid 2E	1.86	4
	LSD (0.05)	**0.38**	
Champagne, IL, 2003 Trial SYN001[10]	MIR604 late hybrid E5	1.04	4
	MIR604 late hybrid E6	0.87	4
	Control late hybrid 2E	2.37	4
	LSD (0.05)	**0.49**	
York, NE, 2003 Trial SYN002[10]	MIR604 late hybrid E5	0.02	4
	MIR604 late hybrid E6	0.04	4
	Control late hybrid 2E	1.79	4
	LSD (0.05)	**0.38**	

APPENDIX 1A (CONT'D)

TABLE 3A (CONT'D)

Fortescue, MO, 2003 Trial SYN003[10]	MIR604 late hybrid E5	0.32	4
	MIR604 late hybrid E6	0.17	4
	Control late hybrid 2E	2.65	4
	LSD (0.05)	**0.40**	
Monteno, IL, 2003 Trial SYN004[10]	MIR604 late hybrid E5	0.28	4
	MIR604 late hybrid E6	0.24	4
	Control late hybrid 2E	1.51	4
	LSD (0.05)	**0.28**	
Janesville, WI, 2003 Trial SYN005[10]	MIR604 late hybrid E5	1.63	4
	MIR604 late hybrid E6	1.36	4
	Control late hybrid 2E	2.71	4
	LSD (0.05)	**0.63**	
Richland, IA, 2003 Trial SYN006[10]	MIR604 late hybrid E5	0.54	4
	MIR604 late hybrid E6	0.75	4
	Control late hybrid 2E	2.27	4
	LSD (0.05)	**0.33**	
Champagne, IL, 2003 Trial SYN007[10]	MIR604 late hybrid E5	0.88	4
	MIR604 late hybrid E6	1.01	4
	Control late hybrid 2E	2.64	4
	LSD (0.05)	**0.45**	
Norway, KS, 2003 Trial SYN008[10]	MIR604 late hybrid E5	0.36	4
	MIR604 late hybrid E6	0.38	4
	Control late hybrid 2E	0.29	4
	LSD (0.05)	**0.48**	
York, NE, 2003 Trial SYN009[10]	MIR604 late hybrid E5	0.02	4
	MIR604 late hybrid E6	0.04	4
	Control late hybrid 2E	1.88	4
	LSD (0.05)	**0.28**	
Minnehaha, SD, 2003 Trial SYN010[10]	MIR604 late hybrid E5	0.09	4
	MIR604 late hybrid E6	0.05	4
	Control late hybrid 2E	0.29	4
	LSD (0.05)	**0.22**	

APPENDIX 1A (CONT'D)

TABLE 3A (CONT'D)

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm plus artificial infestation with western corn rootworm unless noted otherwise in the table.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3]Individual corn plants were artificially infested with freshly deposited Western corn rootworm eggs at a density of 594 eggs per plant for trial SYN180 and 405 eggs per plant for trial SYN181. Additional details can be found in Chapter 4, Section B.

[4]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid D3) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data.

[5]CRW root damage rating scale 0-3 (see Table 1A).

[6]Only control hybrids 2E were negative segregants of their respective MIR604 hybrids. All other control hybrids were non-transgenic parental inbred lines.

[7]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[8]Trial was not artificially infested.

[9]Trial was infested primarily with naturally occurring northern corn rootworm.

[10]These trials were conducted in a randomized complete block design, 2 x 20 ft. rows, 4 replicates, 25 seed per row. 10 root masses per plot (5 randomly selected plants per row) were evaluated at the VT to R1 stage (approximately 7 weeks following infestation). Plants were manually dug from the ground, washed free of soil and rated for rootworm damage according to the Iowa State 0-3 node-injury rating scale (see Table 1A above). (http://www.ent.iastate.edu/pest/rootworm/nodeinjury/nodeinjury.html).

[11]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level except for trial SYN008, Norway, KS.

APPENDIX 1A (CONT'D)

TABLE 4A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS IN GLASSHOUSE TRIALS[1]

LOCATION, YEAR	ENTRY[2]	MEAN CRW ROOT DAMAGE RATING[3]	# OF REPS
Stanton, MN, 2003 Trial SYN160	MIR604 early hybrid D1	0.019[6]	20
	MIR604 early hybrid D2	0.022	20
	Control early hybrid 1D[4]	0.730	20
	LSD (0.05) [5]	**0.092**	
Stanton, MN, 2003 Trial SYN165	MIR604 early hybrid D1	0.024	12
	MIR604 early hybrid D2	0.015	12
	Control early hybrid 1D	0.638	12
	LSD (0.05)	**0.138**	

[1]Individual potted corn plants (V2 to V3 leaf stage) were artificially infested with freshly deposited Northern corn rootworm eggs at a density of 43 eggs per plant for trial SYN160 and 58 eggs per plant for trial SYN165. Additional details can be found in Chapter 4, Section B.
[2]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid D1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data
[3]CRW root damage rating scale 0-3 (see Table 1A).
[4]All control hybrids were negative segregants of their respective MIR604 hybrids.
[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.
[6]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level.

APPENDIX 1A (CONT'D)

TABLE 5A

COMPARISON OF ROOT DAMAGE RATINGS (0-3) OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

LOCATION, YEAR	ENTRY[2]	MEAN CRW ROOT DAMAGE RATING[3]	# OF REPS
Monoville, TX, 2003 Trial SYN011[1]	MIR604 late hybrid E5	1.37[7]	4
	MIR604 late hybrid E6	0.94	4
	Control late hybrid 1E[4]	2.71	4
	LSD (0.05) [5, 6]	**0.56 – 0.60**	
Abbott, TX, 2003 Trial SYN012[1]	MIR604 late hybrid E5	0.91	4
	MIR604 late hybrid E6	0.42	4
	Control late hybrid 1E	2.81	4
	LSD (0.05)[5]	**0.63**	

[1]Trials comprised single row plots using a randomised complete block design with natural populations of Mexican corn rootworm.

[2]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid E5) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2003 data

[3]CRW root damage rating scale 0-3.9 (see Table 1A).

[4]All control hybrids were negative segregants of their respective MIR604 hybrids.

[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[6]Missing plot value in replicate 3 of the Negative Control 1E, causes an unbalanced design; LSD is dependent upon n = no. of reps and is variable for unbalanced design.

[7]All the mean values for MIR604 hybrids are significantly different from the negative control at the 0.05% level.

APPENDIX 1B

YIELD DATA FOR MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS

APPENDIX 1B (CONT'D)

TABLE 1B

YIELD COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2], 2002

LOCATION(S), YEAR	ENTRY[3]	YIELD (BUSHELS/ACRE)	# OF REPS
Stanton, MN, 2002 Trial SYN653[7]	MIR604 early hybrid B1 (Bt)[4]	145.8	5
	MIR604 early hybrid B2 (Bt)	133.2	5
	Control early hybrid 1B (Bt)[5]	140.7	5
	LSD (0.05)[6]	**13.90**	
Bloomington-1, Bloomington-2, Bondville, St. Joseph, IL 2002 Trial SYN673[7]	MIR604 late hybrid C1 (Bt)	**110.1**[8]	5
	MIR604 late hybrid C2 (Bt)	**102.2**	5
	Control late hybrid 1C (Bt)	45.6	5
	LSD (0.05)	**19.42**	

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid B1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data.

[4]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, both MIR604-derived and control hybrids expressing the Cry1Ab protein (Bt) were included in the trials.

[5]All control hybrids were negative segregants of their respective MIR604 hybrids.

[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.

[7] Randomized complete block design for trial SYN653 and Split plot design at all locations for trial SYN673.

[8]MIR604 hybrid mean values in **bold** are significantly different from the negative control at the 0.05% level.

APPENDIX 1B (CONT'D)

TABLE 2B

YIELD COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

LOCATION (S), YEAR	ENTRY[2]	YIELD (BUSHELS/ ACRE)	# OF REPS
Faribault, LeRoy, Mankato, Owatonna, Stanton, MN, 2003 Trial SYN652	MIR604 early hybrid G3	114.6	5
	MIR604 early hybrid G4	115.8	5
	Control early hybrid 2G[3]	116.2	5
	MIR604 early hybrid H6[4] (Bt)	122.2	5
	MIR604 early hybrid H7 (Bt)	118.9	5
	Control early hybrid 3H (Bt)	116.6	5
	LSD (0.05) [5]	**8.4**	
Hampton, IA; Brookings, SD; Janesville, WI, Rochelle, IL 2003 Trial SYN656	MIR604 early hybrid G3	144.4	3
	MIR604 early hybrid G4	143.6	3
	Control early hybrid 2G	140.4	3
	LSD (0.05)	**16.5**	
Bondville, Chillicothe, Hudson, Leroy, Shirley, IL, 2003 Trial SYN672	MIR604 late hybrid E2	103.1	5
	MIR604 late hybrid E3	101.7	5
	Control late hybrid 1E	104.5	5
	MIR604 late hybrid F4 (Bt)	107.9	5
	MIR604 late hybrid F3 (Bt)	108.5	5
	Control late hybrid 2F (Bt)	98.3	5
	LSD (0.05)	**11.0**	
Glidden, Washington, IA; Leesburg, IN; Henderson, KY; Seward, NE, 2003 Trial SYN676	MIR604 late hybrid E2	102.6	3
	MIR604 late hybrid E3	97.2	3
	Control late hybrid 1E	109.1	3
	LSD (0.05)	**18.1**	
Hampton, IA; Stanton, MN, 2003 Trial SYN653[7]	MIR604 early hybrid H6 (Bt)	151.3	4
	Control early hybrid 3H (Bt)	150.2	4
	LSD (0.05)	17.4	4
Bondville, Chillicothe, Hudson, Leroy, IL; Seward, NE, 2003 Trial SYN673[7]	MIR604 late hybrid F4[4] (Bt)	**120.5**[6]	4
	MIR604 late hybrid F3 (Bt)	112.0	4
	Control late hybrid 2F (Bt)	106.4	4
	LSD (0.05)	**9.6**	

[1] Trials comprised single row plots using a randomised complete block design with natural populations of western and northern corn rootworm.

[2]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid D3) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data.

[3]All control hybrids were negative segregants of their respective MIR604 hybrids.

[4]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 hybrids H and F and the controls 3H and 2F expressing the Cry1A(b) protein (Bt) were included in the trials.

APPENDIX 1B (CONT'D)

TABLE 2B (CONT'D)

[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means.
[6]Mean values in **bold** are significantly different from the negative control at the 0.05% level.
[7]These trials were conducted with trap crop populations of CRW. Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

APPENDIX 1C

AGRONOMIC DATA FOR MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS

APPENDIX 1C (CONT'D)

TABLE 1C
AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1, 2], 2002

Location	Bloomington-1, Bloomington-2, Bondville, St. Joseph, IL 2002 Trial SYN673					Stanton, MN 2002 Trial SYN653				
Reps	5					5				
Agronomic Trait[3]	MIR604 late hybrid C1[4] (Bt)	MIR604 late hybrid C2 (Bt)	Control late hybrid 1C[5] (Bt)	**LSD (0.05)[6]**	Locations w/ data	MIR604 early hybrid B1 (Bt)	MIR604 early hybrid B2 (Bt)	Control early hybrid 1B (Bt)	**LSD (0.05)**	Locations w/ data
DROPP	0	0	0.4	NA[7]	4	-	-	-	-	-
EMRGP	**76.5**[8]	81.8	81.3	1.3	4	-	-	-	-	-
ERHTN	-[9]	-	-	-	-	109	97	95	15	1
GMSTP	19.9	**19.2**	19.9	0.5	4	**28.1**	**28.0**	31.2	1.4	1
HAVPN	**24523**	26205	26048	417	4	30492	30492	30492	450	1
HU5SN	-	-	-	-	-	**1298**	**1302**	1360	24	1
HU5PN	-	-	-	-	-	**1298**	**1292**	1334	24	1
INTLR	**8.1**	**8.3**	5.9	0.6	4	-	-	-	-	-
LRTLP	59	59	37	23	4	**26**	**24**	6	13	1
PLHTN	-	-	-	-	-	275	268	277	17	1
POL5N	-	-	-	-	-	**61**	**61**	63	1	1
PSTSP	74	92	64	30	4	-	-	-	-	-
SLK5N	-	-	-	-	-	**61**	62	63	1	1
STKLP	**5.9**	**6.3**	3.3	2.0	4	1	3	3	4	1
TWSMN	**61.7**	**62**	52.8	2.4	4	46.9	47.6	46.9	2.1	1

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm.

[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.

[3] See Chapter 4, Table 1 for list and description of agronomic parameters.

[4]All control hybrids were negative segregants of their respective MIR604 hybrids.

[5]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.

[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.

[7]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.

[8]Mean values in **bold** are significantly different from the negative control at the 0.05% level.

[9]No data collected for this trait at these trial sites.

APPENDIX 1C (CONT'D)

TABLE 2C

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

Location	Glidden, Washington, IA; Leesburg, IN; Henderson, KY; Seward, NE, 2003 Trial SYN676					Hampton, IA; Brookings, SD; Janesville, WI, Rochelle, IL 2003 Trial SYN656				
Reps	3					3				
Agronomic Trait[2]	MIR604 late hybrid E2	MIR604 late hybrid E3	Control late hybrid 1E[3]	**LSD (0.05)**[4]	Locations w/ data	MIR604 early hybrid G3	MIR604 early hybrid G4	Control early hybrid 2G	**LSD (0.05)**	Locations w/ data
DROPP	0	0	0	NA[5]	3	-[6]	-	-	-	-
EMRGP	86.6	87.1	87.0	NA	2	100.3	96.9	99.4	NA	2
ERHTN	80	72	85	17	1	113	113	113	10	1
GMSTP	**18.6**[7]	19.4	20.2	0.9	4	19.9	20.2	20.0	1.5	3
HAVPN	30126	30322	30479	NA	4	29756	29450	29705	NA	3
HU5SN	1291	1327	1321	33	1	958	1118	1129	NA	2
HU5PN	1200	1218	1226	48	1	1110	1104	1116	NA	2
INTLR	5.2	5.0	4.8	1.0	2	5.3	5.0	5.7	NA	1
LRTLP	0	4	0	NA	1	-	-	-	-	-
PLHTN	243	257	237	25	1	248	252	237	19	1
POL5N	57	58	58	2	1	-	-	-	-	-
PSTSP	62	45	48	36.2	2	-	-	-	-	-
SLK5N	61	63	62	NA	1	-	-	-	-	-
STKLP	8.1	2.4	1.9	NA	2	2.5	1.7	1.6	NA	3
TWSMN	58.5	58.8	59.3	NA	4	**57.2**	55.6	54.5	1.8	3

[1] Trials comprised single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm.
[2] See Chapter 4, Table 1 for a list and description of agronomic parameters.
[3] All control hybrids were negative segregants of their respective MIR604 hybrids.
[4] Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[5] NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[6] No data collected for this trait at these trial sites.
[7] Mean values in **bold** are significantly different from the negative control at the 0.05% level.

APPENDIX 1C (CONT'D)

TABLE 3C

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

Location	Faribault, LeRoy, Mankato, Owatonna, Stanton, MN, 2003 Trial SYN652							
Reps	5							
Agronomic Trait[2]	MIR604 early hybrid H7[3] (Bt)	MIR604 early hybrid H6 (Bt)	Control early hybrid 3H[4] (Bt)	MIR604 early hybrid G3	MIR604 early hybrid G4	Control early hybrid 2G	**LSD (0.05)[5]**	Locations w/ data
EMRGP	86.7	87.1	87.1	86.3	87.2	86.7	NA[6]	3
ERHTN	102	103	102	98	100	103	12.1	2
GMSTP	22.0	20.7	20.6	19.3	20.3	19.2	1.4	4
HAVPN	29379	29524	29524	29234	29532	29379	NA	4
HU5SN	1249	**1239**[7]	1264	1249	1234	1244	23	1
HU5PN	1244	1239	1249	1239	1234	1244	25	1
PLHTN	298	303	297	307	304	304	11.0	2
STKLP	11.0	12.7	11.8	14.0	20.1	10.8	10.4	4
TWSMN	38.4	**40.2**	38.2	**39.2**	37.3	37.6	1.7	4

[1] Trials comprised single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm.

[2] See Chapter 4, Table 1 for a list and description of agronomic parameters.

[3] To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 hybrids D7 and D6 and control hybrid 3D expressing the Cry1A(b) protein (Bt) were included in the trials.

[4] All control hybrids were negative segregants of their respective MIR604 hybrids.

[5] Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.

[6] NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.

[7] Mean values in **bold** are significantly different from the negative control at the 0.05% level.

APPENDIX 1C (CONT'D)

TABLE 4C

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[1], 2003

Location	Bondville, Chillicothe, Hudson, Leroy, Shirley, IL, 2003 Trial SYN672							
Reps	5							
Agronomic Trait[2]	MIR604 late hybrid F4[3] (Bt)	MIR604 late hybrid F3 (Bt)	Control late hybrid 2F[4] (Bt)	MIR604 late hybrid E2	MIR604 late hybrid E3	Control late hybrid 1E	**LSD (0.05)[5]**	Loc. w/ data
EMRGP	1.2	1.2	1.2	1.0	1.2	1.0	1.9	5
ERHTN	106	110	107	106	106	110	7	5
GMSTP	21.7	**23.7**[7]	22.5	**21.0**	22.7	23.1	0.8	5
HAVPN	25783	25435	25618	25783	25252	25526	585	5
HU5SN	1514	1509	1509	1499	1534	1529	53	1
HU5PN	1509	1499	1504	1494	1534	1509	56	1
INTLR	7.2	6.3	7.0	7.6	7.2	6.8	0.9	5
LRTLP	7	6	2	2	6	5	NA[6]	1
PLHTN	220	232	224	217	226	219	8	5
STKLP	22.9	18.4	24.1	24.2	15.2	17.1	11.6	5
TWSMN	**58.7**	57.9	57.5	57.6	57.6	57.6	1.0	5

[1] Trials comprised single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm.
[2] See Chapter 4, Table 1 for a list and description of agronomic parameters.
[3]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 hybrids F4 and F3 and control hybrid 2F expressing the Cry1A(b) protein (Bt) were included in the trials.
[4]All control hybrids were negative segregants of their respective MIR604 hybrids.
[5]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[6]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[7]Mean values in **bold** are significantly different from the negative control at the 0.05% level.

APPENDIX 1C (CONT'D)

TABLE 5C

FOLIAR DISEASE RATING 1-9 SCALE USED BY SYNGENTA AGRONOMISTS

Rating	Description
1	No lesions or one or two restricted lesions on inoculated leaf. About One (1) percent of leaf surface area is affected.
2	A few scattered lesions on inoculated leaf. About two (2) - four (4) percent of leaf surface area.
3	Few lesions on inoculated leaf. 5-15% of leaf surface area affected. A few scattered lesions on leaves above and/or below inoculated leaf (less than 5% of leaf area affected).
4	Few lesions on inoculated leaf. Approximately 15-25 percent of leaf surface area affected. A few scattered lesions on leaves above and/or below inoculated leaf (less than 10% of leaf area affected).
5	Light-moderate number of lesions on inoculated leaf (25-35 percent of leaf surface area affected). Some lesions may be coalesced. Leaves above and/or below inoculated leaf with light lesion development. Less than 15 percent leaf surface area affected.
6	Moderate numbers of lesions on inoculated leaf (35-50 percent of leaf surface area affected). Some lesions may be coalesced. Leaves above and/or below inoculated leaf with light- moderate lesion number (15-30 percent of leaf surface area).
7	Lesions abundant on inoculated leaf (50-75 percent of leaf surface area affected). Some lesions coalesced. Lower and upper leaves with moderate lesion numbers (30-50 percent of leaf surface area). Some lesions may be coalesced
8	Lesions abundant on inoculated leaf (75-100 percent of leaf surface affected). Numerous lesions coalesced. Lower and upper leaves with moderate-heavy lesion number (50-75 percent of leaf surface affected). Numerous lesions may be coalesced.
9	Lesions highly abundant on all leaves. Lesions highly coalesced. Plants may be prematurely killed.

APPENDIX 1C (CONT'D)

TABLE 6C

COMPARISON OF DISEASE RATINGS[1] OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS[2], 2002-2003

	Disease	Gray Leaf Spot	Northern Corn Leaf Blight	Southern Corn Leaf Blight	Eyespot
	Reps	4	4	4	4
Location	**Hybrid**				
Stanton, MN, 2002 SYN135 & SYN136	MIR604 Early Hybrid B1[3] (Bt)	4.25	7.5	-	-
	MIR604 Early Hybrid B2 (Bt)	3.75	6.5	-	-
	Early Control Hybrid 1B (Bt)	3.75	6.0	-	-
	LSD (0.05)	**1.15**	**1.5**	-	-
Bloomington, IL, 2002 SYN175 & SYN176	MIR604 Late Hybrid C2 (Bt)	7.0	-	7.3	-
	MIR604 Late Hybrid C1 (Bt)	7.0	-	7.5	-
	Late Control Hybrid 1C (Bt)	7.0	-	7.3	-
	LSD (0.05)	**0.3**	-	**0.6**	-
Stanton, MN, 2003 SYN136 & SYN137	MIR604 Early Hybrid H6 (Bt)	-	2.5	-	5.25
	MIR604 Early Hybrid H7 (Bt)	-	2.5	-	6.00
	Early Control Hybrid 3H (Bt)	-	2.5	-	5.50
	LSD (0.05)	-	**1.4**	-	**NA**
Bloomington, IL, 2003 SYN177	MIR604 Late Hybrid F1 (Bt)	6.2		-	-
	MIR604 Late Hybrid F2 (Bt)	7.2		-	-
	Late Control Hybrid 1F (Bt)	8.0		-	-
	LSD (0.05)	0.9		-	-

[1] Disease ratings (see table 5C above) were assigned according to a standard 1-9 foliar disease rating scale (Ullstrup et al., 1945).

[2]Trials were comprised of single row plots replicated three times using a randomised complete block design with natural populations of western and northern corn rootworm. Plots relied upon natural disease pressure and were rated multiple times during the growing season on an individual plant basis.

[3]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.

APPENDIX 1D

EFFICACY, YIELD AND AGRONOMIC DATA FOR MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS WITH INSECTICIDE TREATMENT

APPENDIX 1D (CONT'D)

TABLE 1D

EFFICACY COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1, 2], 2002

LOCATION, YEAR	ENTRY[4]	INSECTICIDE TREATMENT	MEAN CRW ROOT DAMAGE RATING[5]	# OF REPS
Stanton, MN, 2002 Trial SYN137	MIR604 early hybrid A1	Force 3G[6]	0.083	3
	MIR604 early hybrid A2	Force 3G	0.133	3
	Control early hybrid 1A[7]	Force 3G	0.193	3
	LSD (0.05) [8]		**0.185**	
Bloomington, IL, 2002 Trial SYN179	MIR604 early hybrid A1	Force 3G	0.045	3
	MIR604 early hybrid A2	Force 3G	0.077	3
	Control early hybrid 1A	Force 3G	0.335	3
	LSD (0.05)		**0.105**	

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm plus artificial infestation with western corn rootworm.
[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.
[3]Individual corn plants were artificially infested with freshly deposited Western corn rootworm eggs at a density of 1182 eggs per plant for trial SYN137 and 1462 eggs per plant for trials SYN179. Additional details can be found in Chapter 4, Section B.
[4]Genotype designations assigned to hybrids (e.g., MIR604 early hybrid A1) distinguish different genetic backgrounds and maturity groups within a trial, and across trials only for 2002 data
[5]CRW root damage rating scale 0-3 (see Appendix 1A, Table 1A above).
[6]Force 3G applied at 1.12 g ai/100 row meters
[7]All control hybrids were non-transgenic parental inbreds.
[8]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.

APPENDIX 1D (CONT'D)

TABLE 2D

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1], 2002

Location	Hudson, Newman, St. Joseph, Shirley IL, 2002 Trial SYN672					Delavan, Faribault, LeRoy, Owatonna, MN 2002 Trial SYN652				
Treatment[2]	Force 3G					Force 3G				
Reps	4					4				
Agronomic Trait[3]	MIR604 late hybrid C2[4] (Bt)	MIR604 late hybrid C1 (Bt)	Control late hybrid 1C[5] (Bt)	**LSD (0.05)**[6]	Loc. w/ data	MIR604 early hybrid B1 (Bt)	MIR604 early hybrid B2 (Bt)	Control early hybrid 1B (Bt)	**LSD (0.05)**	Loc. w/ data
YGSMP	**106.8**[7]	**110.8**	119.6	8.6	4	118.1	125.4	125.7	16.6	4
DROPP	0	0	0	NA[8]	3	0	0	0	NA	1
EMRGP	81.6	**78.0**	81.5	2.7	4	94.7	92.4	93.4	3	4
ERHTN	82	72	78	6	4	100	103	103	NA	1
GMSTP	**18.2**	**19.1**	19.6	0.4	4	**24.0**	**25.3**	26.9	1.4	4
HAVPN	26140	**24995**	26117	859	4	30952	30395	30686	611	4
HU9SN	**1530**	**1541**	1558	14	1	-[9]	-	-	-	-
HU9PN	**1452**	1490	1496	25	1	-	-	-	-	-
INTLR	7.4	7.4	7.0	1.1	4	-	-	-	-	-
LRTLP	74	80	60	20	1	5	7	10	7	4
PLHTN	185	181	182	7	4	258	259	260	NA	1
PSTSP	70	56	56	20	1	-	-	-	-	-
STKLP	7	4	3	5	4	4.4	**9.9**	3.7	5.9	4
TWSMN	**61**	**61.4**	62.5	0.8	4	**45.2**	46.7	47.4	1.4	4

[1] Trials comprised single row plots using a randomised complete block design with natural populations of western and northern corn rootworm.
[2]Force 3G applied at 1.12 g ai/100 row meters.
[3] See Chapter 4, Table 1 for a list and description of agronomic parameters.
[4]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.
[5]All control hybrids were negative segregants of their respective MIR604 hybrids.
[6]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[7]Mean values in **bold** are significantly different from the negative control at the 0.05% level.
[8]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[9]No data collected for this trait at these trial sites.

APPENDIX 1D (CONT'D)

TABLE 3D

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1, 2], 2002

Location	Bloomington 1, Bloomington 2, Bondville, St. Joseph, IL 2002 Trial SYN673					Stanton, MN 2002 Trial SYN653				
Reps	5									
Treatment[3]	Force 3G					Force 3G				
Agronomic Trait[4]	MIR604 late hybrid C1[5] (Bt)	MIR604 late hybrid C2 (Bt)	MIR604 late hybrid 1C[6] (Bt)	**LSD (0.05)**[7]	Locations w/ data	MIR604 early hybrid B1 (Bt)	MIR604 early hybrid B2 (Bt)	Control early hybrid 1B (Bt)	**LSD (0.05)**	Locations w/ data
YGSMP	**127.1**[8]	**125.5**	93.6	19.42	4	152.9	150.2	162.7	13.9	1
DROPP	0	0.3	0.4	NA[9]	4	-	-	-	-	-
EMRGP	77.1	81.7	83.5	1.3	4	-	-	-	-	-
ERHTN	-[10]	-	-	-	-	96	107	110	15	1
GMSTP	**20.0**	**19.4**	20.9	0.6	4	**27.4**	29.8	30.1	1.4	1
HAVPN	24698	26186	26758	417	4	30492	30492	30492	450	1
HU5SN	-	-	-	-	-	**1304**	1320	1334	24	1
HU5PN	-	-	-	-	-	**1293**	1316	1330	24	1
INTLR	7.9	**8.4**	7.3	0.6	4	-	-	-	-	-
LRTLP	24	22	3	23	4	15	**27**	11	13	1
PLHTN	-	-	-	-	-	**277**	**271**	295	17	1
POL5N	-	-	-	-	-	61	62	62	1	1
PSTSP	50	**86**	46	30	4	-	-	-	-	-
SLK5N	-	-	-	-	-	**61**	62	63	1	1
STKLP	**2.9**	5.8	5.3	2.0	4	1	3	3	4	1
TWSMN	61.9	61.7	62.2	2.4	4	46.9	47.6	46.9	2.1	1

[1]Trials comprised single row trap crop plots using a split plot design with natural populations of western and northern corn rootworm.
[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.
[3]Force 3G applied at 1.12 g ai/100 row meters.
[4] See Table 2D for a list and description of agronomic parameters.
[5]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.
[6]All control hybrids were negative segregants of their respective MIR604 hybrids.
[7]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[8]Mean values in **bold** are significantly different from the negative control at the 0.05% level.
[9]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[10]No data collected for this trait at these trial sites.

APPENDIX 1D (CONT'D)

TABLE 4D

AGRONOMIC COMPARISON OF MIR604-DERIVED AND NON-TRANSGENIC CONTROL HYBRIDS TREATED WITH FORCE 3G®[1, 2], 2003

Location	Bondville, Chillicothe, Hudson, Leroy, IL; Seward, NE, 2003 Trial SYN674					Hampton, IA; Stanton, MN, 2003 Trial SYN654				
Treatment[3]	Force 3G					Force 3G				
Reps	4					4				
Agronomic Trait[4]	MIR604 late hybrid F4[5] (Bt)	MIR604 late hybrid F3 (Bt)	Control late hybrid 2F[6] (Bt)	**LSD (0.05)[7]**	Loc. w/ data	MIR604 early hybrid H7 (Bt)	MIR604 early hybrid H6 (Bt)	Control early hybrid 3H (Bt)	**LSD (0.05)**	Loc. w/ data
YGSMN	119.6	113.3	111.3	11.0	4	154.3	152.0	146.9	18.4	4
DROPP	0	0	0	NA[8]	1	-	-	-	-	-
EMRGP	79.3	78.8	77.5	NA	5	98.5	98.9	100	NA	2
ERHTN	100	103	103	8	5	109	110	115	15	1
GMSTP	**21.5**[9]	22.9	22.6	0.9	5	24.2	23.0	22.6	1.8	2
HAVPN	25557	25387	24980	NA	5	30558	30558	30558	NA	2
HU5SN	**1324**	1313	1291	29	1	-[10]	-	-	-	-
HU5PN	1219	1218	1225	30	1	-	-	-	-	-
INTLR	7.9	6.9	7.2	1.5	4	-	-	-	-	-
LRTLP	0	2	0	NA	4	-	-	-	-	-
PLHTN	230	240	231	9.1	5	299	290	306	19	1
POL5N	58	58	58	1	1	-	-	-	-	-
PSTSP	28	**8**	38	16	1	-	-	-	-	-
SLK5N	63	62	61	NA	1	-	-	-	-	-
STKLP	11.5	10.9	14.4	NA	4	3.0	0.8	1.5	NA	2
TWSMN	59.2	58.2	58.5	NA	5	54.8	55.5	53.7	NA	2

[1] Trials comprised single row trap crop plots using a randomised complete block design with natural populations of western and northern corn rootworm.
[2]Trap crops are planted the previous year with continuous corn or cucurbits and are designed to enhance the natural population of CRW.
[3]Force 3G applied at 1.12 g ai/100 row meters.
[4] See Table 2D for a list and description of agronomic parameters.
[5]To eliminate the confounding effect on yield of insect damage caused by lepidopteran pests, MIR604 and control hybrids expressing the Cry1A(b) protein (Bt) were included in the trials.
[6]All control hybrids were negative segregants of their respective MIR604 hybrids.
[7]Least significant difference at the 5% significance level. Data were analysed using analysis of variance procedures where means were compared using the F-test. If the F-test was significant (alpha=0.05), the least significant difference (LSD, alpha=0.05) procedure was used to detect differences between the means. If the F-test was not significant at alpha=0.05, mean separation procedures were not used and are designated as NA.
[8]NA = Not Applicable, the f-test showed no significant difference between entries thus calculating LSD was not necessary.
[9]Mean values in **bold** are significantly different from the negative control at the 0.05% level.
[10]No data collected for this trait at these trial sites.

APPENDIX 2

UNPUBLISHED DATA REPORTS IN SUPPORT OF REGULATORY APPROVALS FOR EVENT MIR604 HYBRIDS

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ALL DATA AND INFORMATION IN THIS APPENDIX IS CONSIDERED CONFIDENTIAL BUSINESS INFORMATION

Appendix 2 – Section 1

[CBI-Deleted]

Molecular Characterization of Event MIR604 Maize (Corn) Expressing a Modified Cry3A *Bacillus thuringiensis* Protein for USDA Petition for Non-Regulated Status

Appendix 2 – Section 2

[CBI-Deleted]

Characterization and Safety of Modified Cry3A Protein and Maize (Corn) Plants Derived from Event MIR604.

Appendix 2 – Section 3

[CBI-Deleted]

Characterization of Phosphomannose Isomerase (PMI) Produced in Maize (Corn) Plants Derived from Event MIR604 and Comparison to PMI as Contained in Test Substance PMI-0198

Appendix 2 – Section 4

[CBI-Deleted]

Quantification of Modified Cry3A and PMI Proteins in Transgenic Maize (Corn) Tissues, Whole Plants, and Silage Derived from Transformation Event MIR604

Appendix 2 – Section 5

[CBI-Deleted]

Compositional Analysis of Grain and Whole Plants from Transgenic Maize (Corn) Event MIR604

Appendix 2 – Section 6

[CBI-Deleted]

Environmental Safety Assessment of Modified Cry3A Protein and Event MIR604 Corn to Non-Target Organisms

Appendix 2 – Section 7

[CBI-Deleted]

Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Mouse

Appendix 2 – Section 8

[CBI-Deleted]

Analysis for the Presence of Modified Cry3A Protein in Wet and Dry Milled Fractions, Corn Oil and Corn Chips from Processing of Event MIR604 Maize (Corn)

Appendix 2 – Section 9

[CBI-Deleted]

Phosphomannose Isomerase (Sample PMI-0198): Acute Oral Toxicity Study in Mice

Appendix 2 – Section 10

[CBI-Deleted]

Effect of Temperature on the Stability of Modified Cry3A Protein (MCRY3A-0102)

Appendix 2 – Section 11

[CBI-Deleted]

In vitro Digestibility of Modified Cry3A Protein (MCRY3A-0102 and IAMPMIR604-0103) Under Simulated Mammalian Gastric Conditions

Appendix 2 – Section 12

[CBI-Deleted]

In vitro Digestibility of PMI Protein Under Simulated Mammalian Gastric and Intestinal Conditions

Appendix 2 – Section 13

[CBI-Deleted]

Characterization and Safety of Phosphomannose Isomerase (PMI), a Selectable Marker Expressed in Event 3243M-Derived Maize (Corn) Plants

Appendix 2 – Section 14

[CBI-Deleted]

Summary of Data Demonstrating the Environmental Safety of Modified Cry3A *Bacillus thuringiensis* Insect Control Protein and Event MIR604-Derived Corn (Maize) to Non-Target organisms

Appendix 2 – Section 15

[CBI-Deleted]

An Acute Oral Toxicity Study of Modified Cry3A Protein (MCRY3A-0102) in the Northern Bobwhite

Appendix 2 – Section 16

[CBI-Deleted]

A 28-Day Laboratory Study to Evaluate the Effects of Modified Cry3A Maize Fish Feed (FFMIR604-0103) on the Growth of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

Appendix 2 – Section 17

[CBI-Deleted]

A Semi-Field Test to Evaluate the Effects of the Modified Cry3A Protein (MCRY3A-0102) on Brood Development of the Honybee, *Apis mellifera* (Hymenoptera: Apidae)

Appendix 2 – Section 18

[CBI-Deleted]

Characterization of Fish Feed Test Substance (FFMIR604-0103) Prepared from Event MIR604-Derived Maize Grain

Appendix 2 – Section 19

[CBI-Deleted]

A Laboratory Test of the Toxicity of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae)

Appendix 2 – Section 20

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Coccinella septempunctata* to Modified Cry3A Protein: Supplement to Report Titled 'A Laboratory Test of the Toxicity of Modified Cry3A Protein (MCRY3A-0102) to Larvae and Adults of the Ladybird Beetle *Coccinella septempunctata* (Coleoptera: Coccinellidae)'

Appendix 2 – Section 21

[CBI-Deleted]

A Laboratory Toxicity Test of the Modified Cry3A Protein (MCRY3A-0102) to Larvae of the Ground-Dwelling Beetle, *Poecilus cupreus* (Coleoptera: Carabidae)

Appendix 2 – Section 22

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Poecilus cupreus* to Modified Cry3A Protein: Supplement to Report Titled ‘A Laboratory Toxicity Test of the Modified Cry3A Protein (MCRY3A-0102) to Larvae of the Ground-Dwelling Beetle *Poecilus cupreus* (Coleoptera: Carabidae)’

Appendix 2 – Section 23

[CBI-Deleted]

A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylinidae)

Appendix 2 – Section 24

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Aleochara bilineata* to Modified Cry3A Protein: Supplement to Report Titled ‘A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Rove Beetle, *Aleochara bilineata* (Coleoptera: Staphylinidae)’

Appendix 2 – Section 25

[CBI-Deleted]

A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)

Appendix 2 – Section 26

[CBI-Deleted]

Analysis of Test Diet Used to Expose *Orius insidiosus* to Modified Cry3A Protein: Supplement to Report Titled 'A Laboratory Toxicity Test of Modified Cry3A Protein (MCRY3A-0102) to the Predatory Bug, *Orius insidiosus* (Heteroptera: Anthocoridae)'

Appendix 2 – Section 27

[CBI-Deleted]

Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the Earthworm *Eisenia foetida* in an Artificial Soil Substrate

Appendix 2 – Section 28

[CBI-Deleted]

Analysis of Artificial Soil Used to Expose *Eisenia foetida* to Modified Cry3A Potein: Supplement to Report Titled ‘Determination of Acute Toxicity of Modified Cry3A Protein (MCRY3A-0102) to the Earthworm *Eisenia foetida* in an Artificial Soil Substrate’

Appendix 2 – Section 29

[CBI-Deleted]

Environmental Fate Assessment of Modified Cry3A Protein in Event MIR604 Corn

Appendix 2 – Section 30

[CBI-Deleted]

Laboratory Soil Degradation of Modified Cry3A Protein (MCRY3A-0102)

Appendix 2 – Section 31

[CBI-Deleted]

Waiver Request: Freshwater Aquatic Invertebrate Testing of Modified Cry3A Protein as Expressed in Event MIR604 Corn (Maize)

Appendix 2 – Section 32

[CBI-Deleted]

Characterization of Modified Cry3A Protein Produced in Event MIR604-Derived Maize (Corn) and Comparison with Modified Cry3A Protein Expressed in Recombinant *Escherichia coli*

Appendix 2 – Section 33

[CBI-Deleted]

Characterization of Modified Cry3A Test Substance (MCRY3A-0102) and Certificate of Analysis

Appendix 2 – Section 34

[CBI-Deleted]

Further Characterization of Modified Cry3A Test Substance MCRY3A-0102

Appendix 2 – Section 35

[CBI-Deleted]

Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Toxins

Appendix 2 – Section 36

[CBI-Deleted]

Modified Cry3A Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Allergens

Appendix 2 – Section 37

[CBI-Deleted]

Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Toxins

Appendix 2 – Section 38

[CBI-Deleted]

Phosphomannose Isomerase Protein as Expressed in Transgenic Maize Event MIR604: Assessment of Amino Acid Homology with Known Allergens

Appendix 2 – Section 39

[CBI-Deleted]

Evaluation of Transgenic Corn (Maize) MIR604 in Broiler Chickens

Appendix 2 – Section 40

[CBI-Deleted]

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

Appendix 2 – Section 41

[CBI-Deleted]

Amended Public Interest Document in Support of Registration of the Plant-Incorporated Protectant Modified Cry3A Protein as Expressed in Event MIR604 Corn

Appendix 2 – Section 42

[CBI-Deleted]

Insect Resistance Management for Syngenta Event MIR604 Maize (Corn)

Appendix 2 – Section 43

[CBI-Deleted]

Effects of Temperature on the Stability of Phosphomannose Isomerase (PMI)

Appendix 2 – Section 44

[CBI-Deleted]

Natural Variation of Phytosterol Levels in Maize (Field Corn) Grain

Appendix 2 – Section 45

[CBI-Deleted]

Demonstration that the Potassium Levels in Forage Derived from MIR604 Maize (Field Corn) are within the Range of Potassium Levels in Conventional Maize Forage