BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis modified Cry1Ab (SYN-IR67B-1) and Vip3Aa19 (SYN-IR102-7) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton

U.S. Environmental Protection Agency
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Table of Contents

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Insect Resistance Management

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Benefit Assessment

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I. Overview

A. Executive Summary

EPA has conditionally registered a new pesticide product containing Syngenta Seeds Inc.'s new active ingredients, *Bacillus thuringiensis* Vip3Aa19 (OECD Unique Identifier SYN-IR102-7) and modified Cry1Ab (OECD Unique Identifier SYN-IR67B-1) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton. Syngenta has trademarked this product as VipCot -- the trademark name of VipCot will be used in this document to describe COT102 X COT67B cotton. The Agency has determined that the use of this pesticide is in the public interest and that it will not cause any unreasonable adverse effects on the environment during the time of conditional registration.

The new cotton plant-incorporated protectant, VipCot, produces its own insecticidal proteins within the cotton plant. These proteins were derived from *Bacillus thuringiensis* (Bt), a naturally occurring soil bacterium. The modified Cry1Ab and Vip3Aa19 proteins used in this product control lepidopteran pests of cotton.

On June 26, 2008, tolerance exemptions under 40 CFR Part 174 were approved for *Bacillus thuringiensis* modified Cry1Ab protein as identified under OECD Unique Identifier SYN-IR67B-1 in cotton (40 CFR 174.529) and Vip3Aa proteins in corn and cotton (40 CFR 174.501). The exemption for Vip3Aa is inclusive of the Vip3Aa19 protein and its use in cotton.

Benefits

Results of efficacy trials conducted in 2005 and 2006 show that VipCot cotton and its single event cotton isolines provide good protection against three major cotton pests: tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossypiella*). The Vip3Aa19 protein expressed in VipCot cotton has not been previously registered and provides a unique mode of action. When coupled with modified Cry1Ab in VipCot, the proteins have the potential to provide benefits for insect resistance management including: high-dose (for both proteins expressed together) against the major target pests, lack of cross-resistance (Vip3Aa19), and the potential to delay development of resistance in other cotton varieties expressing Cry toxins. As an additional registered Bt cotton product, VipCot will likely result in direct and indirect human and environmental health benefits by providing growers with an additional choice of Bt cotton option and the potential to increase grower choice and price competition, resulting in lower seed prices for consumers and higher adoption rates. Registration of VipCot may also result in further reduction of chemical insecticide use by growers.

Public Interest Finding

To grant a conditional registration under Section 3(c)(7)(C) of FIFRA, EPA must determine that such conditional registration will, *inter alia*, be in the public interest. EPA determines whether conditional registration of a pesticide is in the public interest in accordance with the criteria set forth at 51 Fed. Reg. 7628 (*Conditional Registration of New Pesticides*, March 5 1986). On the basis of analysis utilizing these criteria, EPA concludes that the use of VipCot protected cotton will be in the public interest, because it results in direct and indirect human and environmental health benefits by providing growers with an additional Bt cotton product which has the potential to extend the useful life of Bt cotton technology generally due to a novel mode of action (Vip3Aa19) and low likelihood of cross-resistance with other Bt Cry proteins.

Product Characterization

VipCot (COT102 x COT67B) was developed by conventional breeding of COT102 (Vip3Aa19) plants with COT67B (modified Cry1Ab) plants.

Event COT102 cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of a vector referred to as both pNOV3001 and pCOT1, expresses the insecticidal protein, Vip3Aa19 as well as a selectable marker, hygromycin B phosphotransferase (APH4). The Vip3Aa19 protein is intended to control several lepidopteran pests of cotton including *Heliothis virescens* (tobacco budworm, TBW), *Helicoverpa zea* (cotton bollworm, CBW), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper). Vip3A is a vegetative (i.e., produced during the vegetative stage of bacterial growth) insecticidal protein from *Bacillus thuringiensis* (*Bt*), a gram positive bacterium commonly found in soil.

Event COT67B cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of vectors pNOV4641 and pNOV1914, expresses the insecticidal protein, modified Cry1Ab. This protein contains an additional 26 amino acid sequence at the C-terminus (termed the 'Geiser motif'). The modified Cry1Ab protein is intended to control several lepidopteran pests of cotton including *Heliothis virescens* (tobacco budworm), *Helicoverpa zea* (cotton bollworm), *Pectinophora gossypiella* (pink bollworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper).

DNA characterization (i.e., Southern blot analysis) was used to confirm the integrity of the COT102 and COT67B inserts in the stacked product COT102 x COT67B. Samples from COT102 x COT67B cotton gave the same results as those observed for the individual events, indicating that the molecular characterization data provided for the individual events are also applicable to COT102 x COT67B.

Protein expression data, together with data indicating that there is no evidence of either a synergistic or antagonistic interaction between Vip3Aa19 and modified Cry1Ab in cotton bollworm or tobacco budworm, demonstrate that data on the individual events and individual proteins can be used to support the safety of the COT102 x COT67B (VipCot) combined product.

Human Health Assessment

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the modified Cry1Ab and Vip3Aa19 proteins. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because no toxicity to mammals has been observed, nor any indication of allergenicity potential for the plant-incorporated protectant.

Syngenta submitted four acute oral toxicity studies conducted on mice, which all indicated that Vip3Aa is non-toxic to humans. Three of the studies were conducted with microbially-produced Vip3Aa proteins with slight variations in amino acid sequence (1-2 amino acid differences), and one study was conduced with transgenic corn leaf tissue as the test material. No treatment-related adverse effects were observed in any of the studies. The oral LD_{50} for mice (males, females, and combined) was greater than 3675 mg Vip3Aa/kg body weight (the highest dose tested). For modified Cry1Ab, an acute oral toxicity study in mice indicated that the protein is non-toxic to humans. Groups of five male and five female mice were given 0 or 1830 mg/kg bodyweight microbially-produced modified Cry1Ab by oral gavage as a single dose. There were no effects on clinical condition, body weight, food consumption, clinical pathology, organ weight, or macroscopic or microscopic pathology that were attributed to the test substance.

Since Vip3Aa and modified Cry1Ab are proteins, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for Vip3Aa and modified Cry1Ab is as follows:

1. Source of the trait. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of Vip3Aa19 and modified Cry1Ab with known allergens showed no significant sequence identity over 80 amino acids or identity at the level of eight contiguous amino acid residues.
3. Digestibility. The Vip3Aa and modified Cry1Ab proteins were digested rapidly in simulated

gastric fluid containing pepsin.

4. Glycosylation. Vip3Aa and modified Cry1Ab (expressed in cotton) were shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for Vip3Aa and modified Cry1Ab to be food allergens is minimal.

Environmental Assessment

The Agency concludes that for the VipCot cotton breeding stack (COT102 x COT67B, containing modified Cry1Ab and Vip3Aa19) no unreasonable adverse effects will result to the environment or any federally-listed threatened or endangered species from commercial cultivation of COT102 x COT67B cotton. This conclusion is based on prior assessments conducted on Vip3Aa and Cry1Ab proteins individually. Furthermore, the Agency has determined that Events COT102, COT67B, and VipCot cotton will have No Effect (NE) on endangered and/or threatened species listed by the US Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds, terrestrial and aquatic plants, and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

The Agency believes that cultivation of VipCot cotton may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, Bt cotton requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of nonspecific conventional chemical pesticides is known to have an adverse effect on non-target beneficial organisms found living in the complex environment of an agricultural field. Many of these beneficial organisms are important integrated pest management controls (IPM) for secondary pests such as aphids and leafhoppers. Therefore, the overall result of cultivation of VipCot cotton, expressing Vip3Aa19 and modified Cry1Ab proteins, is that the number of chemical insecticide applications for non-target pest control will be reduced for management of multiple pest problems.

Insect Resistance Management

In order to reduce the possibility of the target pests developing resistance to Vip3Aa19 and modified Cry1Ab (as expressed in VipCot cotton), EPA is requiring Syngenta Seeds, Inc. to ensure that a portion of the planted acreage of this product be set aside where non-Bt cotton will be grown to serve as a "refuge." Under the established refuge strategy for Bt cotton, growers can choose from three structured refuge options:

Option 1: 95:5 external structured, unsprayed refuge; 150 ft wide, within ½ mile of edge of field.
Option 2: 80:20 external sprayed refuge; within 1 linear mile, preferably ½ mile, of edge of field.
Option 3: 95:5 embedded refuge; contiguous or within 1 mile^2 of field and 150 ft wide.

In addition to the refuge options above, growers of VipCot may participate in a community refuge plan in which multiple growers contribute to the overall required refuge acres by planting 20% external, sprayed or 5% external, unsprayed refuge.

BPPD has concluded that based on the modeling, dose, and efficacy studies, the requested refuge options 1-3 and community refuge plan are acceptable for VipCot cotton. Syngenta will also be required to develop and conduct a resistance monitoring program for Vip3Aa19 and modified Cry1Ab with the major target pests (cotton bollworm, tobacco budworm, and pink bollworm). Additional requirements for remedial action (in the event of resistance), grower education, compliance assurance, and annual reported will also be implemented for VipCot as terms of registration.

B. Use Profile

Pesticide Name: *Bacillus thuringiensis* Vip3Aa19 (OECD Unique Identifier SYN-IR102-7) and modified Cry1Ab (OECD Unique Identifier SYN-IR67B-1) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton

Trade and Other Names: VipCot Cotton; COT102 X COT67B Cotton

OPP Chemical Code: 006499 (Vip3Aa19) and 006529 (modified Cry1Ab)

Basic Manufacturers: Syngenta Seeds, Inc.

Type of Pesticide: Plant-Incorporated Protectant

Uses: Cotton

Target Pest(s): tobacco budworm, cotton bollworm, pink bollworm

C. Regulatory History

Syngenta Seeds, Inc. was issued an Experimental Use Permit (EUP) for VipCot Bt cotton containing Vip3Aa19 (Event COT102) and modified Cry1Ab (Event COT67B) on April 26, 2007 (EPA Reg. No. 67979-EUP-7). These proteins were selected to provide protection of cotton from feeding damage caused by major lepidopteran pests including tobacco budworm, cotton bollworm, and pink bollworm. On April 26, 2007, EPA established a temporary exemption from the requirement of a tolerance for Vip3Aa19 (72 FR 26300, amended 72 CFR 40752; 40 CFR 174.501) in the food and

feed commodities of cotton. For the purpose of the EUP, modified Cry1Ab was determined to be covered under the permanent tolerance exemption for Cry1Ab in all crops (40 CFR 174.511). Both the EUP and temporary tolerance exemption were originally set to expire on May 1, 2008. However, Syngenta was granted an extension of both the EUP and temporary tolerance exemption (72 FR 68744) on November 27, 2007 which expire on May 1, 2009.

A separate EUP (EPA Reg. No. 67979-EUP-5) was previously issued to Syngenta for two Bt cotton events (Event COT202 and COT203) containing Vip3A. These two events were not part of the more recent VipCot EUP and have not been proposed for commercial registration. This EUP expired on March 31, 2006.

On December 14, 2006, Syngenta submitted an application (EPA Reg. No. 67979-O) to register VipCot (Event COT 102 x Event COT67B) under Section 3 of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). On April 5, 2007, Syngenta submitted a second application for a seed increase registration (EPA Reg. No. 67979-RR). This application was subsequently withdrawn by the registrant on January 7, 2008.

On September 6, 2007, Syngenta submitted a petition to EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Food Quality Protection Act of 1996 (FQPA), requesting a permanent tolerance exemption for Vip3Aa in all plants (PP 7F7254). A separate petition was submitted to request a permanent exemption for modified Cry1Ab in all plants on November 9, 2007 (PP 7F7290). After review of the supporting data, EPA determined that the permanent tolerance exemptions would be limited to corn and cotton (Vip3Aa) and cotton (modified Cry1Ab).

On June 26, 2008 (73 FR 45620 and 73 FR 40760), the Agency established permanent exemptions from the requirement of a tolerance for residues of the *Bacillus thuringiensis* Vip3Aa proteins in corn and cotton (40 CFR 174.501) and modified Cry1Ab protein as identified under OECD Unique Identifier SYN-IR67B-1 in cotton (40 CFR 174.529) when used as plant–incorporated protectants.

On June 26, 2008, a conditional registration was issued for VipCot Bt Cotton (EPA Reg. No. 67979-9).

II. Science Assessment

The classifications that are found for each data submission are assigned by EPA science reviewers and are an indication of the usefulness of the information contained in the documents for risk assessment. A rating of "ACCEPTABLE" indicates the study is scientifically sound and is useful for risk assessment. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. The studies may have certain aspects determined not to be scientifically acceptable ("SUPPLEMENTAL: UPGRADABLE"). If a study is rated as "SUPPLEMENTAL: UPGRADABLE," EPA always provides an indication of what is lacking or what can be provided to change the rating to "ACCEPTABLE." If there is simply a "SUPPLEMENTAL" rating, the reviewer will often state that the study is not required by the current 40 CFR Part 158. Both "ACCEPTABLE" and "SUPPLEMENTAL" studies may be used in the risk assessment process as appropriate. An "UNACCEPTABLE" rating indicates that new data need to be submitted.

II.A. Product Characterization

II.A.1. Event COT102 Cotton (OECD Unique Identifier: SYN-IR102-7) Expressing Vip3Aa19

Event COT102 cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of a vector referred to as both pNOV3001 and pCOT1, expresses the insecticidal protein, Vip3Aa19 as well as a selectable marker, hygromycin B phosphotransferase (APH4). The Vip3Aa19 protein is intended to control several lepidopteran pests of cotton including, but not limited to, *Helicoverpa zea* (cotton bollworm/corn earworm), *Heliothis virescens* (tobacco budworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper). Vip3A is a vegetative (i.e., produced during the vegetative stage of bacterial growth) insecticidal protein from *Bacillus thuringiensis* (*Bt*), a gram positive bacterium commonly found in soil.

Transformation System:
COT102 cotton was produced by *Agrobacterium tumefaciens*-mediated transformation of hypocotyls of *Gossypium hirsutum* L. cultivar Coker 312 with plasmid pNOV3001 (also referred to as pCOT1). Plasmid pNOV3001 (pCOT1) contains T-DNA with the *vip3Aa19* and *aph4* expression cassettes. The *vip3Aa19* expression cassette contains the *vip3Aa19* coding sequence under the regulation of the Act2 promoter and intron (derived from *Arabidopsis thaliana*), and NOS terminator (derived from *Agrobacterium tumefaciens*). The *aph4* expression cassette contains the *aph4* coding sequence under the regulation of the Ubq3 promoter and intron (derived from *Arabidopsis thaliana*) and the NOS terminator (derived from *Agrobacterium tumefaciens*). The *vip3Aa19* gene encodes a protein that differs from the Vip3Aa1 protein from *Bacillus thuringiensis* strain AB88 by one amino acid at position 284 (The *vip3Aa1* gene encodes lysine at position 284, and the *vip3Aa19* gene encodes glutamine). Vip3Aa19 confers resistance to several lepidopteran pests. The *aph4* gene encodes

hygromycin B phosphotransferase (APH4), an enzyme that catalyzes the phosphorylation of hygromycin and some related aminoglycosides. Expression of APH4 allows growth in the presence of hygromycin and was used as a selectable marker, enabling selection of transformed cells.

Characterization of the DNA Inserted in the Plant and Inheritance and Stability:
Characterization of the DNA isolated from event COT102 cotton using restriction enzyme digests and Southern blot analysis as well as DNA sequencing indicates that the DNA was inserted in the cotton genome at a single locus, and the insert contains one copy each of the *vip3Aa19* and *aph4* expression cassettes. There were no other detectable elements other than those associated with the respective cassettes. No backbone sequences from plasmid pNOV3001 (pCOT1) were detected in the cotton genome. Southern blot analysis and protein expression data also demonstrated the stability of the insert over multiple generations.

Protein Characterization:

The insecticidal protein produced in event COT102 cotton, designated as Vip3Aa19[a], is a variant of the naturally occurring Vip3Aa1 protein isolated from *Bacillus thuringiensis* strain AB88, differing from the Vip3Aa1 protein by one amino acid (Vip3Aa19 contains a glutamine at position 284, while Vip3Aa1 contains a lysine). Both proteins are 789 amino acids in length and have a molecular weight of approximately 89 kDa. Syngenta has also developed a transgenic corn variety, MIR162, that produces another variant, designated as Vip3Aa20, differing from the naturally occurring Vip3Aa1 protein by two amino acids; at position 284, Vip3Aa20 has the same amino acid substitution as Vip3Aa19 (i.e., K284Q), and in addition, at position 129, Vip3Aa20 contains an isoleucine, while Vip3Aa1 contains a methionine (M129I).

The following techniques were used to characterize and compare the plant-produced and the *E. coli*-produced Vip3Aa proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, mass spectrometry, glycosylation analysis, N-terminal amino acid sequencing, and insecticidal activity assays. Glycoslyation analysis indicated that the proteins are not glycoslyated. These analyses demonstrated the structural and functional similarity between the plant-produced Vip3Aa19 and the *E. coli*-produced Vip3Aa19, Vip3Aa20, and Vip3Aa1 proteins and justified the use of *E. coli*-produced proteins in toxicity studies.

Analytical Detection Methods:

Syngenta has provided a validation study for SeedChek Vip3A/FLCry1Ab, a lateral flow test kit that detects both Vip3A and Cry1Ab. The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and

[a] Prior to receiving the Crickmore designation of Vip3Aa19, the protein produced in COT102 was referred to as Vip3A or Vip3Aa.

cotton leaf. No unexpected cross reactivity with other transgenic varieties or nontransgenic controls was observed. An independent lab validation of this method is still needed.

Protein Expresson:
Expression level data were provided for Vip3Aa19 and APH4 in different plant tissues and at different growth stages in COT102.

Table 1. Mean Expression Levels of Vip3Aa19 and APH4 from COT102 Plant Tissues

Tissue Type	Vip3Aa19 (µg/g dry weight ± standard deviation)	APH4 (µg/g dry weight ± standard deviation)
Leaves*	44 ± 10 - 277 ± 41	< 0.42 – 8.2 ± 1.4
Squares	116 ± 22	2.2 ± 0.4
Flowers	162	1.68
Pollen	3.47	64.3
Bolls	19 ± 4	< 0.39
Whole Plants	25 ± 4	< 0.37
Seed	7 + 2	1.4 ± 0.3
Roots	16 ± 2	0.53 ± 0.11

*Ranges reflect means at different growth stages for leaves

The data submitted for product characterization for event COT102 cotton are summarized in Table 2 below.

Table 2. Product Characterization Data Submitted for Event COT102 Cotton (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Expression Levels/ Quantitation of VIP3A and APH4 Protein in Cotton Tissues and Whole Plants Derived from Transformation Event COT102[b]	Transgenic cotton plants (COT102) and a non-transgenic isoline (Coker 312) were grown concurrently in 2001 in Camilla, GA; Maricopa, AZ; and Idalou, TX. Ten whole transgenic plants (including roots) and two control plants were harvested approximately 2, 4, 9, 13, 15, and 22 week post-emergence (stages: four-leaf, squaring, first white bloom, peak bloom, first open boll, pre-harvest, respectively). Tissue extracts were analyzed for VIP3A and APH4 by ELISA. VIP3A protein was detected in COT102 whole plants, leaves, roots, squares, and bolls at all six developmental stages examined. VIP3A levels varied in all plant tissues, generally declined with time, but stayed constant in the roots. The highest levels were found in	45835801

[b] Study submitted with EUP request and reviewed in memorandum from C. Wozniak to L. Cole dated March 24, 2004.

Study Type/Title	Summary	MRID #
	leaves at the squaring stage (mean of 8.56 to 10.78 µg VIP3A/g fresh tissue). Low VIP3A levels were found in seed (mean of 2.51 to 3.23 µg VIP3A/g) and in pollen (1.09 µg VIP3A/g). VIP3A was not detected in cotton fiber or nectar. The protein marker, APH4, was detected in COT102 plants at low, non-quantifiable levels at some developmental stages in leaves, roots, bolls, squares, and whole plants and at quantifiable levels in pollen (2.25 µg APH4/g air-dried pollen). APH4 was not detected in cotton fiber or nectar. Geographic location appeared not to have a significant effect on VIP3A levels, but no statistical analysis was done. APH4 levels appears to be similar across locations, but the lack of data points in many instances and the detectable levels falling below the level of quantitation (LOQ) do not allow for any definitive conclusions to be made. The estimated amount of VIP3A/acre cotton varied considerably among the developmental stages with the greatest mean level found at the peak bloom stage (105.80 g VIP3A/acre based on whole plant VIP3A levels). **Classification: ACCEPTABLE**	
Characterization of Inserted DNA/Molecular Characterization and Genetic Stability of Event COT102[b]	Southern blot analysis and DNA sequencing suggest that event COT102 has one transgene insertion site with a single copy of intact *vip3A(a)* and *aph4* expression cassettes (containing one copy of the *vip3A(a)* gene, *aph4* gene, actin-2 promoter, and ubq3 promoter). DNA sequence alignment revealed an exact sequence match between the pCOT-1 vector and event COT102, and showed the lack of *Agrobacterium* sequence beyond the T-DNA borders. VIP3 protein expression measurement (by ELISA) of five generations of COT102 seedlings (F1, BC1F2, BC2F1, BC2F2, and BC3F1) showed that the *vip3A(a)* gene was stable across generations and segregated in a Mendelian fashion, consistent with a single transgene insertion site. MRID 458358-02 provided very scant experimental details. Insufficient experimental methods details were provided for the Southern blots, DNA cloning and sequencing, PCR analysis, and protein detection and segregation analysis by ELISA, precluding confirmation of their appropriateness by an independent reviewer. Sample Southern blots demonstrating the integration copy number and lack of rearrangements through appropriate restriction analyses must be provided in order to assess the results of this study. Further information is required regarding the number of plants utilized in the segregation and heritability analysis. **Classification: SUPPLEMENTAL,** upgradeable to acceptable pending submission of additional methods details and correction/clarification of typographic errors in Figure 1, Figure 2, and/or the text of MRID 458358-02. **Superseded by MRID 47017603**	45835802
Characteristics of *Bacillus thuringiensis* VIP3A Protein and VIP3A Cotton Plants Derived from Event COT102[b]	The *Bacillus thuringiensis* (*Bt*) VIP3A insect control protein as expressed in transgenic cotton seed confers protection against the bollworm complex and other lepidopteran cotton pests. The seeds are derived from transgenic cotton event COT102, which contains the insecticidal gene via plasmid vector pCOT1. The product active ingredient is ≤0.0015 % dry weight *Bacillus thuringiensis* VIP3A Protein and the genetic material necessary for its production (pCOT1 in cotton). The product also contains ≤0.0001% dry	45766501

Study Type/Title	Summary	MRID #
	weight marker protein and the genetic material necessary for its production (pCOT1 in cotton). VIP3A protein in transgenic cotton plants derived from Event COT102, is produced by a synthetic *vip3A(a)* gene, which encodes a polypeptide of 789 amino acids. The VIP3A toxin is proteolytically activated to a toxin core in the lepidopteran larval midgut and forms pores in the gut membranes of sensitive species. Several formulated microbial *Bt* products containing VIP3A-like proteins and the genetic components in plasmid pCOT1, as well as its expression analysis, are described in MRID 457665-01. **Classification: ACCEPTABLE.** The wide certified limits of the active ingredient need to be explained, although they are within the bounds covered by the acute oral toxicity studies submitted for review.	
Characterization of the active ingredient/Characterization of VIP3A Protein Produced in COT102-Derived Cotton and Comparison with VIP3A Protein Expressed in Both Maize (Corn) Derived from Event PACHA and Recombinant *Escherichia coli*[b]	VIP3A protein produced in cotton plants derived from transgenic cotton event "COT102" was characterized for its biochemical and functional similarity with VIP3A expressed in recombinant *Escherichia coli* and "Pacha" derived transgenic maize plants. Samples of purified VIP3A protein from *E. coli* and maize were dissolved in buffer for analysis by SDS-PAGE and Western blotting. VIP3A from cotton leaves was extracted following published procedures and prepared for SDS-PAGE and Western blotting. VIP3A proteins from all three sources were determined to have the predicted molecular weight of ca. 89,000 and cross-reacted immunologically with the same anti-VIP3A antibody. No evidence of any post-translational modification of VIP3A was observed in any of the three Vip3A protein sources. Peptides representing *ca*. 85% (673/789) of the complete VIP3A amino acid sequence were identified by mass spectral analysis of cotton produced VIP3A protein. Amino acid sequences corresponded identically to the predicted amino acid sequence of the VIP3A protein. Comparisons of bioactivity of *E. coli*-expressed and cotton-expressed VIP3A protein in larvae of four lepidopteran species demonstrated comparable activities, with the exception of the tobacco budworm bioassays (TBW). A 35% difference in mortality was noted in TBW assays comparing these two sources of test substance. In the absence of an in-depth statistical analysis, it is not possible to assign a particular factor as the causal agent in delimiting this result. Given that both test substances contain other constituents, it is difficult to assess the reason for this observation. TBW is considered as one of the least sensitive species of lepidopteran insects evaluated. A similar rank order of species sensitivity was found for both test solutions; FAW was the most sensitive to VIP3A, while CBW and TBW were the least sensitive. These data indicate that VIP3A proteins from recombinant *E. coli*, Pacha-derived maize and event COT102-derived cotton are substantially equivalent. **Classification: ACCEPTABLE**	45835812
Expression Level/ Analysis of Processed COT102 Cottonseed Products for Yield and Presence of Gossypol	Processing transgenic COT102 and control Coker 312 cotton seeds resulted in similar yields for the hulls, lint, kernels, refined oil, and de-fatted meal. Analysis of the refined oil and de-fatted meal (non-toasted and toasted) by ELISA detected VIP3A protein in COT102 meal but not in oil, and not in meal or oil from control seeds. Analysis of both COT102 and Coker 312	45835803

Study Type/Title	Summary	MRID #
and Vip3A Protein[b]	de-fatted meal for the plant toxin gossypol detected free gossypol (HPLC method) and total gossypol (free + protein-bound; spectrophotometric method). Refined oil had >100-fold lower levels of total gossypol than meal. MRID 45835803 provided inadequate and/or conflicting details for some experimental methods and results. **Classification: ACCEPTABLE. Submission of additional methods details and correction and/or clarification of the MRID 458358-03 text as listed under "Deficiencies" is, however, recommended to ensure adequate recording in the official record.** **The additional information was subsequently determined to be unnecessary because no adverse effects were observed in the nontarget studies.**	
The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab delta-endotoxin[c]	This publication (Lee *et al.*, 2003), which examined the differences in the mechanism of insecticidal activity of Cry1Ab and Vip3A, was submitted by the registrant to provide additional product characterization data, specifically Vip3A's mode of action. The submitted publication examined differences in the mechanism of insecticidal activity of Cry1Ab and Vip3A proteins. Ligand blotting showed that activated Cry1Ab and Vip3A-G (Vip3A proteolytically cleaved with lepidopteran gut juice) bound different receptor molecules in midgut of Tobacco hornworm (*Manducta sexta*, Linnaeus) and that Vip3A-G did not bind Cry1A receptors. Voltage clamping assays showed that Vip3A-G formed distinct pores in dissected midgut from *M. sexta* but not in the monarch butterfly (*Danaus plexippus*, Linnaeus). Cry1Ab and Vip3A both formed voltage-independent and cation-selective stable ion channels in planar lipid bilayers, but their primary conductance state and cation specificity differed. **Classification: ACCEPTABLE**	46880801
Characterization of Test Substance/Re-Characterization of Vip3A Protein Test Substance (Vip3A-0204)	The purpose of this study was to re-characterize the microbially produced test substance, VIP3A-0204. The purity, integrity, and bioactivity of the test substance were determined and compared with previous analyses after being stored *ca.* 15 months under desiccation at -20 °C. Total protein in VIP3A-0204 was quantified spectrophotometrically, and the purity was determined using SDS-PAGE followed by densitometric analysis. The integrity of the Vip3Aa19 protein in test substance VIP3A-0204 was determined using Western blot analysis, and bioactivity was assessed in insect feeding assays using freshly hatched first-instar *S. frugiperda* (fall army worm) larvae. This re-characterization study demonstrated that VIP3A-0204 largely retained its insecticidal activity (LC_{50} of 34 ng Vip3A/cm^2 diet surface vs. 45.1 initially) after storage for 15 months. The purity of test substance VIP3A-0204 was determined to be *ca.* 92% Vip3Aa19 by weight. Western blot analysis revealed a dominant immunoreactive band corresponding to the predicted molecular weight of Vip3Aa19 of *ca.* 89 kDa. These results are similar to those obtained in previous analyses, demonstrating that the	47017602

[c] Study submitted with EUP request and reviewed in memorandum from A. Waggoner to M. Mendelsohn dated February 8, 2007.

Study Type/Title	Summary	MRID #
	test substance is stable when stored desiccated at -20 °C for approximately 15 months. **Classification: ACCEPTABLE**	
Characterization of the inserted DNA/ Additional Molecular Characterization of Event COT102 Cotton by Southern Analysis	Molecular analysis of event COT 102 was performed using restriction enzyme digestion and Southern blot analysis to determine the number of inserts, copy number of functional elements, and the presence or absence of plasmid backbone sequences. This study also assessed the inheritance and stability of the insert. Data from the Southern analyses demonstrated that the BC4F1 generation of COT102 cotton: (1) contains a single intact insert; (2) contains a single copy of the *vip3Aa19* gene and the *aph4* gene; (3) contains a single copy of the Act2 promoter; (4) contains a single copy of the Ubq3 promoter; (5) does not contain any detectable backbone sequences from the transformation plasmid pCOT1; and (6) the insert is stably integrated into the cotton genome. These results are consistent with results from previous molecular analysis studies on event COT 102. **Classification: ACCEPTABLE**	47017603
Inheritance and Stability/ Stability of Vip3Aa19 and APH4 Protein Expression Across Multiple Generations of Event COT102 Cotton	The purpose of this study was to use ELISA to analyze the levels of expression of the Vip3Aa19 and hygromycin B phosphotransferase (APH4) proteins in leaves (collected at the 1st white bloom stage) of three generations (F1, BC1F1, and BC4F1) of Event COT102 cotton. The levels of Vip3Aa19 protein measured were comparable (ca. 60 µg/g dry weight) in all three generations analyzed. APH4 protein was detectable in all three generations analyzed, but the concentrations were below the limit of quantification (LOQ). The consistency of Vip3Aa19 and APH4 protein concentrations demonstrate the stability of transgenic protein expression across multiple generations of COT102 cotton at the 1st white bloom stage. **Classification: ACCEPTABLE**	47017609

II.A.2. Event COT67B Cotton (OECD Unique Identifier: SYN-IR67B-1) Expressing Modified Cry1Ab

Event COT67B cotton, which was developed by *Agrobacterium*-mediated transformation of cotton using elements of vectors pNOV4641 and pNOV1914, expresses the insecticidal protein, modified Cry1Ab. This protein contains an additional 26 amino acid sequence at the C-terminus (termed the 'Geiser motif'). The modified Cry1Ab protein is intended to control several lepidopteran pests of cotton including, but not limited to, *Helicoverpa zea* (cotton bollworm/corn earworm), *Heliothis virescens* (tobacco budworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper).

Transformation System:

COT67B cotton was produced by *Agrobacterium tumefaciens*-mediated cotransformation of *Gossypium hirsutum* L. cultivar Coker 312 using transformation vectors pNOV4641 and pNOV1914, each carrying one T-DNA. Plasmid pNOV4641 contains a full-length *cry1Ab* gene that encodes a full-length Cry1Ab protein that is identical to the Cry1Ab protein produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, except that it contains an additional 26 amino acids, which Syngenta describes as the 'Geiser motif,' in the C-terminal portion of the protein. The *cry1Ab* gene is under the regulation of the Act2 promoter and intron (derived from *Arabidopsis thaliana*) and NOS terminator (derived from *Agrobacterium tumefaciens*). Plasmid pNOV1914 contains a *hygromycin B phosphotransferase* gene (aph4) derived from *Escherichia coli* that confers resistance to the antibiotic hygromycin B and was used as a selectable marker. The two-T-DNA system enabled Syngenta to separate the two inserts by traditional breeding. COT67B cotton contains only the T-DNA from plasmid pNOV4641 encoding the modified Cry1Ab protein; the T-DNA from pNOV1914 containing the *aph4* gene is absent.

Characterization of the DNA Inserted in the Plant and Inheritance and Stability:
Characterization of the DNA isolated from event COT67B cotton using restriction enzyme digests and Southern blot analysis as well as DNA sequencing indicates that the DNA was inserted in the cotton genome at a single locus, and the insert contains one copy of the *cry1Ab* gene. No backbone sequences from the transformation plasmid pNOV4641 were found in COT67B. The left border and the adjacent 13 bp of the insert along with 24 bp of the right border (RB) were deleted during the insertion of the T-DNA. However, such deletions are common during transformation and do not affect the functioning of the T-DNA itself. Additionally, the analysis showed that COT67B cotton does not contain the selectable marker gene, *hygromycin B phosphotransferase* (*aph4*), the Ubq3 promoter from the transformation plasmid pNOV1914, or any backbone sequences from pNOV1914. Inheritance and stability studies of the *cry1Ab* gene in COT67B verified that it is stably integrated into the cotton genome, segregating in an expected Mendelian fashion of 1:1.

Protein Characterization:
Event COT67B expresses a full-length Cry1Ab protein that is identical to the Cry1Ab protein produced by *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, except that it contains an additional 26 amino acids (described by Syngenta as the 'Geiser motif') in the C-terminal portion of the protein. Syngenta states that the additional amino acids have been included because the insertion made fermentation in *Bacillus thuringiensis* more efficient, but they have no impact on insecticidal activity.

The following techniques were used to characterize and compare the plant-produced and the *E. coli*-produced modified Cry1Ab proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, mass spectrometry, glycosylation analysis, N-terminal amino acid sequencing, and insecticidal activity assays. Glycoslyation analysis indicated that the proteins are not glycoslyated. These analyses demonstrated the structural and functional similarity between the plant-produced and the *E. coli*-produced modified Cry1Ab proteins and justified the use of *E. coli*-produced protein in toxicity studies.

Analytical Detection Methods:
Syngenta has provided a validation study for SeedChek Vip3A/FLCry1Ab, a lateral flow test kit that detects both Vip3A and Cry1Ab. The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and cotton leaf, and no unexpected cross reactivity with other transgenic varieties or nontransgenic controls was observed. An independent lab validation of this method is still needed.

Protein Expresson:
Expression level data were provided for modified Cry1Ab in different plant tissues and at different growth stages in event COT67B cotton and summary results are provided in Table 3 below. The data were produced using an ELISA method.

Table 3. Mean Cry1Ab Expression levels in Event COT67B Cotton.

Tissue Type	Cry1Ab (µg/g dry weight ± standard deviation)*
Leaves	65 ± 9 – 158 ± 40
Squares	93 ± 13
Flowers	101
Pollen	12.1
Bolls	47 ± 7
Whole Plants	26 ± 2
Seed	29 ± 5
Roots	17 ± 1

*Range reflects means at different growth stages for leaves

Table 4 provides summaries of the product characterization studies and data provided.

Table 4. Product Characterization Data Submitted for Event COT67B (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Characterization of Inserted DNA/ Harper, B. (2006). Molecular characterization of	The purpose of this study was to determine the DNA sequence and contiguousness of the full length *cry1Ab* (*flcry1Ab*) gene present in Syngenta's COT67B cotton and its inheritance ratio across generations. COT67B cotton plants express a modified full length Cry1Ab *Bacillus*	46885901

[d] Reviewed in a memorandum from S. Matten to A. Reynolds dated April 4, 2007.

Study Type/Title	Summary	MRID #
Event COT67B cotton. Report No. SSB-125-06.[d]	*thuringiensis* protein (FLCry1Ab) that contain an additional 26 amino acids in the C-terminal portion of the protein described as the "Geiser motif." FLCry1Ab confers resistance to certain lepidopteran insects in cotton. The T-DNA insert (via the pNOV4641 plasmid) in COT67B cotton was analyzed by Southern blots and DNA sequencing. These analyses confirmed that there was a single, contiguous copy of the *flcry1Ab* gene present in COT67B. No backbone sequences from the transformation plasmid pNOV4641 were found in COT67B. The left border (LB) and the adjacent 13 bp of the insert along with 24 bp of the right border (RB) were deleted during the insertion of the T-DNA. However, such deletions are common during transformation and do not affect the functioning of the T-DNA itself. Additionally, COT67B cotton did not contain the selectable marker gene, hygromycin B phosphotransferase (*aph4*), or the Ubq3 promoter from the transformation plasmid pNOV1914 and was also free of any backbone sequences from pNOV1914. Inheritance studies of the *flcry1Ab* gene in COT67B verified that it is segregating in an expected Mendelian fashion of 1:1. **Classification: ACCEPTABLE**	
Expression Levels/ Hill, K. (2006). Quantification of Cry1Ab protein in Event COT67B cotton tissues and whole plants. Report No.SSB-022-06[d]	The purpose of this study was to quantify expression of Cry1Ab protein in Event COT67B-derived cotton plants. Quantifiable levels of Cry1Ab protein in Event COT67B-derived cotton plants were determined by enzyme-linked immunosorbent assay (ELISA) for various plant tissues and whole plants at five developmental stages in four locations. Corresponding, near-isogenic, non-transgenic control cotton plants were analyzed in parallel. As expected, Cry1Ab protein was detected in all COT67B plant tissues (i.e., young leaves, old leaves, roots, flowers, pollen, bolls) except fiber and nectar. The concentrations of Cry1Ab in COT67B were similar between the four locations for each tissue type at each time point, although no specific conclusions about differences between locations can be made from the data. Where the concentrations of Cry1Ab appeared variable, there were no consistent trends to indicate that the plants grown in a given location had higher or lower Cry1Ab concentrations. No statistical analysis was performed. Cry1Ab concentrations in most of the near-isogenic, nontransgenic control samples were either below the limit of detection (LOD) or below the limit of quantification (LOQ). The negative control seed from Quitman, GA was determined to have a low level of Cry1Ab (0.24 µg/g dw) that was likely due to contamination during processing or extraction. The average relative extraction efficiency for the various plant tissues analyzed varied between 70.7% for whole plants to 78.5% for pollen. The absolute amount of Cry1Ab in the cotton tissue samples is unknown and some Cry1Ab may be unextractable with the methods used. Extraction efficiency for the purposes of satisfying the analytical method would need to use a spike-recovery method. Several deviations from the protocol were noted by the study authors, but none of these affected the overall conclusions of the study. Across all growth stages, mean Cry1Ab concentrations (averaged across locations) measured in young leaves, old leaves and roots of COT67B	46885902

Study Type/Title	Summary	MRID #
	cotton ranged from 87.70 - 323.84, 194.02 - 255.74, and 12.61 - 56.56 µg/g dry weight (dw), respectively. Mean Cry1Ab concentrations measured in bolls (collected at 1st open boll), whole plants (collected at pre-harvest), and seed (collected at pre-harvest) averaged 45.24, 42.87, and 25.17 µg/g dw across locations, respectively. Cry1Ab concentrations in flowers and pollen collected at the Winnsboro, LA site at peak-bloom averaged 161.74 and 5.45 µg/g dw, respectively. Cry1Ab concentrations in nectar taken from the same cotton plants was not detectable (limit of detection = 0.0002 µg/mL). Cry1Ab concentrations in fiber samples collected at this site at pre-harvest was <0.02 µg/g dw The average Cry1Ab protein per acre and per hectare in pre-harvest COT67B plants collected from 4 sites was determined assuming a planting density of 50,000 plants/acre (123,500 plants/hectare). The average Cry1Ab protein concentration ranged from 46 to 183 g/acre (115 to 451 g/hectare). **Classification: ACCEPTABLE for the purposes of supporting the Experimental Use Permit**. Statistically-valid trends in the data (e.g., expression level differences between tissue types, across developmental stages, between locations) cannot be made. For a quantitative analysis, it is recommended that the expression data submitted to support the Section 3 registration include an appropriate statistical analysis. **Superseded by MRID 47017607.**	
Characterization of test substance/Characterization of Cry1Ab Test Substance FLCRY1AB-0103 and Certificate of Analysis	The purity, integrity, and bioactivity of *E. coli*-produced test substance FLCRY1AB-0103, containing modified full-length Cry1Ab, were determined initially and after *ca.* 5 months of being stored under desiccation at -20 °C. The purity of test substance FLCRY1AB-0103 was determined to be *ca.* 86%, both before and after storage, and Western blot analysis of the test substance showed a dominant immunoreactive band corresponding to the predicted molecular weight of *ca.* 133.5 kDa before and after storage. N-terminal sequencing confirmed that the first 12 amino acids of the test protein corresponded to the predicted N-terminal sequence of Cry1Ab. The test substance was insecticidally active and had a 72-hour LC_{50} of 3.7 ng Cry1Ab/cm^2 diet surface against first instar larvae of the European corn borer. Re-analysis of FLCRY1AB-0103 *ca.* 5 months after the initial analysis demonstrated that the test substance retained insecticidal activity when stored desiccated at -20 °C. **Classification: ACCEPTABLE**	47017604
Characterization of test substance/Re-Characterization of Cry1Ab Test Substance FLCRY1AB-0103	The purpose of this study was to re-characterize the purity, integrity, and bioactivity of microbially produced test substance FLCRY1AB-0103 (containing modified full-length Cry1Ab) after storage at -20 °C for *ca.* 14 months. Total protein in test substance FLCRY1AB-0103 was quantified spectrophotometrically by measuring its absorption at 280 nm (A_{280} method). The purity of test substance FLCRY1AB-0103 was calculated from the total sample weight and the total protein as determined by the A_{280}	47017605

Study Type/Title	Summary	MRID #
	method in conjunction with densitometry data after electrophoretic separation. The integrity of the Cry1Ab protein in test substance FLCRY1AB-0103 was determined using Western blot analysis. Bioactivity of the Cry1Ab protein in FLCRY1AB-0103 was assessed in insect feeding assays using freshly hatched first-instar *O. nubilalis* (European corn borer) larvae. The results demonstrated that the test substance remained intact and retained insecticidal activity during this storage period. **Classification: ACCEPTABLE**	
Characterization of Expressed Substance/ Characterization of the Cry1Ab Protein Produced in Event COT67B-Derived Cotton Plants and Comparison with Cry1Ab Protein Produced in Recombinant *Escherichia coli*	The purpose of this study was to use various biochemical and functional parameters to demonstrate the biochemical equivalence between the Cry1Ab protein expressed in transgenic Event COT67B cotton and the Cry1Ab protein contained in test substance FLCRY1AB-0103 prepared from an *E. coli* over-expression system. Cry1Ab protein was extracted from COT67B cotton plant tissue and its apparent molecular weight, immunoreactivity, glycosylation status, and bioactivity were compared to the Cry1Ab protein from test substance FLCRY1AB-0103. In addition, the microbial- and plant-derived Cry1Ab proteins were analyzed by peptide mass mapping and the N-terminal amino acid sequence of Cry1Ab from test substance FLCRY1AB-0103 was determined. The Cry1Ab proteins from COT67B and from microbially-derived test substance FLCRY1AB-0103 both had an apparent molecular weight of *ca.* 133.5 kDa, and both reacted with anti-Cry1Ab antibodies, as shown by Western blot analysis. Also, both the protein extract from COT67B and FLCRY1AB-0103 showed strong insecticidal activity against *O. nubilalis* (European corn borer). There was no evidence of post-translational glycosylation of Cry1Ab protein from COT67B or from microbially-derived test substance FLCRY1AB-0103. Peptide mass mapping analysis provided additional evidence of the identity of the insecticidal protein expressed in COT67B cotton and in test substance FLCRY1AB-0103. Based on the results of this study it can be concluded that Cry1Ab protein produced in recombinant *E. coli* (test substance FLCRY1AB-0103) is a suitable surrogate for Cry1Ab expressed in COT67B cotton. **Classification: ACCEPTABLE**	47017608
Expression levels/Stability of Cry1Ab Protein Expression Across Multiple Generations of Event COT67B Cotton	The purpose of this study was to use ELISA to analyze the levels of expression of the modified Cry1Ab protein in leaves (collected at open boll stage) of the F1, BC1F1, and BC4F1 generations of Event COT67B cotton. Identical plant tissues from two near-isogenic, nontransgenic cotton plants (cotton line 2429) from the BC1F1 and BC4F1 generations were concurrently sampled and analyzed to identify any potential background effects of the plant matrix on the ELISA. The levels of Cry1Ab protein measured in the three generations of COT67B cotton were comparable (~60 µg/g dry weight). The consistency of the Cry1Ab protein concentrations demonstrates the stability of transgenic protein expression across multiple generations of COT67B cotton at the open boll stage. **Classification: ACCEPTABLE**	47017610

Study Type/Title	Summary	MRID #
Expression Levels/ Analysis for the Presence of Cry1Ab Protein in Linters, Toasted Cottonseed Oil from Processed Seed of Event COT67B Cotton Expressing Full-Length Cry1Ab Protein	The purpose of this study was to quantify Cry1Ab protein in linters, defatted toasted cottonseed meal, and once-refined cottonseed oil derived from COT67B, and to determine Cry1Ab protein concentrations in the fuzzy seed used to generate these processed fractions. Quantification was carried out using an enzyme-linked immunosorbent assay (ELISA). The Cry1Ab extraction efficiencies were >69% for fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B. The mean concentrations of Cry1Ab protein (corrected for extraction efficiency) in fuzzy seed, linters, and defatted toasted cottonseed meal from COT67B were 25.1, 9.6, and 47.5 µg Cry1Ab/g, respectively. Cry1Ab was not detectable in the once-refined oil from COT67B (limit of detection = 0.003 µg Cry1Ab/ml). Cry1Ab concentrations in all cottonseed samples from Coker 312 (negative control) were below the limit of detection. **Classification: ACCEPTABLE**	47017611

II.A.3. COT102 x COT67B Cotton (OECD ID No SYN-IR102-7 x OECD ID No. SYN-IR67B-1) Expressing Vip3Aa19, APH4, and Modified Cry1Ab

COT102 x COT67B was developed by conventional breeding of COT102 plants with COT67B plants.

DNA characterization (*i.e.*, Southern blot analysis) was used to confirm the integrity of the COT102 and COT67B inserts in the stacked product COT102 x COT67B. Samples from COT102 x COT67B cotton gave the same results as those observed for the individual events, indicating that the molecular characterization data provided for the individual events are also applicable to COT102 x COT67B.

Analytical Detection Methods:
Syngenta has provided a validation study for SeedChek Vip3A/FLCry1Ab, a lateral flow test kit that detects both Vip3A and Cry1Ab. The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and cotton leaf, and no unexpected cross reactivity with other transgenic varieties or nontransgenic controls was observed. An independent lab validation of this method is still needed.

Protein Expresson:
Protein expression levels were provided for Vip3Aa19, APH4, and modified Cry1Ab in different plant tissues from COT102 x COT67B cotton, and means are shown below in Table 5. The protein levels are similar to those observed in plant tissue from cotton from the individual events.

Table 5. Mean Expression Levels of Vip3Aa19, APH4, and Modified Cry1Ab from COT102 x COT67B Plant Tissues

Tissue Type	Vip3Aa19 (µg/g dry weight ± standard deviation)*	APH4 (µg/g dry weight ± standard deviation)*	Cry1Ab (µg/g dry weight ± standard deviation)*
Leaves	55 ± 7 – 239 ± 46	<0.41 – 6.3 ± 1.3	70 ± 14 – 185 ± 63
Squares	132 ± 18	2.1 ± 0.5	94 ± 10
Flowers	148	1.80	121
Pollen	3.06	74.7	10.7
Bolls	21 ± 4	< 0.43	42 ± 7
Whole Plants	25 ± 7	< 0.40	29 ± 7
Seed	7 ± 1	1.6 ± 0.4	27 ± 4
Roots	11 ± 3	0.46 ± 0.05	20 ± 4

*Ranges reflect means at different growth stages for leaves

These data, together with data indicating that there is no evidence of either a synergistic or antagonistic interaction between Vip3Aa19 and modified Cry1Ab in cotton bollworm or tobacco budworm (reviewed in the ecological risk assessment memo for this product), demonstrate that data on the individual events and individual proteins can be used to support the safety of the COT102 x COT67B combined product.

Table 6. Product Characterization Data Submitted for COT102 x COT67B (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Characterization of Inserted DNA/ Comparative Southern Analysis of Stacked COT102 x COT67B	Molecular analyses (restriction enzyme digests and Southern blots) were performed to compare the integrity of the transgenic inserts in the cotton lines Event COT102 cotton and Event COT67B cotton with the transgenic inserts in stacked COT102 x COT67B cotton, which was produced by conventional plant breeding of COT102 and COT67B. The Southern blot data demonstrated the predicted molecular organization of the *vip3Aa19 and aph4* genes from COT102 cotton and the *cry1Ab* gene from COT67B cotton. The DNA hybridization patterns from each single event cotton line were identical to those in stacked COT102 x COT67B cotton, demonstrating that the integrity of the transgenic inserts was retained when the component lines were combined into the COT102 x COT67B cotton. **Classification: ACCEPTABLE**	47017606
Expression Levels/ Comparison of Transgenic Protein Expression in Event	The purpose of this study was to use an enzyme-linked immunosorbent assay (ELISA) to analyze tissues from cotton plants derived from transformation Event COT102, Event COT67B and from COT102 x COT67B in order to compare the concentrations of Vip3Aa19, hygromycin	47017607

Study Type/Title	Summary	MRID #
COT102, Event COT67B, and Stacked COT102 x COT67B Cotton Lines	B phosphotransferase (APH4), and Cry1Ab proteins produced in the transgenic plants. For the Vip3Aa19 and APH4 proteins, the concentrations and patterns of expression were generally similar between the COT102 line and the COT102 x COT67B line. Likewise, for the modified Cry1Ab protein, the concentrations and patterns of expression were generally similar between the COT67B line and the COT102 x COT67B line. Some statistically significant differences were seen in some tissues at certain sampling stages, but these differences were not consistent by genotype and/or were not consistent across the growing season. **Classification: ACCEPTABLE**	
Analytical Detection Method/Analytical Detection Method for the Detection of Vip3A and FLCry1Ab Protein in Cotton Tissues Derived from COT102 x COT67B Cotton (VipCot Cotton)	The SeedChek Vip3A/FLCry1Ab lateral flow test kit was tested for the qualitative detection of modified Cry1Ab and Vip3A proteins in cotton seed and cotton leaf. The study showed that the SeedChek kit is able to detect Vip3A and Cry1Ab in both cotton seed and cotton leaf, and no unexpected cross reactivity with other transgenic varieties was observed. **Classification: ACCEPTABLE**	47074101

II.B. Human Health Assessment

II.B.1. Human Health Assessment of Vip3Aa

Note: EPA's human health assessment was conducted for Vip3Aa proteins, which include the Vip3Aa19 protein as expressed in cotton.

A. Mammalian Toxicity and Allergenicity Assessment

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

Syngenta has submitted acute oral toxicity data demonstrating the lack of mammalian toxicity at high levels of exposure to Vip3Aa proteins. These data demonstrate the safety of Vip3Aa at a level well above maximum possible exposure levels that are reasonably anticipated in the crops. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity testing and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR Sec. 158.2140) For microbial products, further toxicity testing (Tiers II & III) and residue data are triggered by significant adverse acute effects in studies such as the mouse oral toxicity study, to verify the observed adverse effects and clarify the source of these effects.

Syngenta submitted four acute oral toxicity studies conducted on mice. Three of the studies were conducted with microbially-produced Vip3Aa proteins (Vip3Aa1, Vip3Aa19, and Vip3Aa20) with slight variations in amino acid sequence (1-2 amino acid differences), and one study was conducted with transgenic corn leaf tissue expressing Vip3Aa19 as the test material. No treatment-related adverse effects were observed in any of the studies. The results of these studies showed that the oral LD_{50} for mice (males, females, and combined) was greater than 3675 mg/kg body weight (the highest dose tested) for the tested Vip3Aa proteins.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad et al., 1992). Therefore, since no acute effects were shown to be caused by the Vip3Aa19 and Vip3Aa20 proteins, even at relatively high dose levels, they are not considered toxic. (This is also true of the Vip3Aa1 protein that was tested.) Further, amino acid sequence comparisons showed no similarities between Vip3Aa19 and Vip3Aa20, on the one hand, and known toxic proteins in protein databases, on the other hand, that would raise a safety concern.

Since Vip3Aa is a protein, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for Vip3Aa follows:

1. Source of the trait. *Bacillus thuringiensis,* the microorganism from which Vip3Aa proteins are derived, is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of Vip3Aa19 and Vip3Aa20 with known allergens showed no significant sequence identity over 80 amino acids or identity at the level of eight contiguous amino acid residues.
3. Digestibility. Both Vip3Aa19 and Vip3Aa20 proteins are digested rapidly in simulated gastric fluid containing pepsin.
4. Glycosylation. Both Vip3Aa19 and Vip3Aa20 were shown not to be glycosylated.

Considering all of the available information on Vip3Aa19 and Vip3Aa20, EPA concludes that the potential for these specific proteins to be food allergens is minimal. Moreover, as further explained below, EPA believes these data and the other submitted data demonstrating a lack of mammalian toxicity at high levels of exposure to Vip3Aa19 and Vip3Aa20 can be extrapolated to cover Vip3Aa more generally.

Vip3Aa is the designation assigned to a closely-related group of similar insecticidal proteins isolated from Bacillus thuringiensis. The specific variants referred to throughout this document (i.e., Vip3Aa19 and Vip3Aa20) are isolates of Vip3Aa protein. All Vip3Aa proteins (there are 25 known Vip3Aa proteins and there are sequences available for 19 of these) are highly related. Indeed, the amino acid sequence of all the Vip3Aa proteins can only vary up to 5% to be considered a part of the Vip3Aa group. With respect to the 19 Vip3Aa proteins for which sequences are available, they vary by less than 28 amino acids out of the 789 amino acids that make up the protein. This level of sequence similarity makes that group of 19 Vip3Aa protein variants 96% identical overall. The sequence identity between any two individual sequences is even higher. For example, the sequences of the protein variants tested by Syngenta (i.e., Vip3Aa19 and Vip3Aa20) are over 99.7% identical. Finally, as to the few amino acid differences that do exist between the Vip3Aa variants, these differences do not alter the surrounding sequence, rarely occur as contiguous amino acids, and are often substitutions with similar chemical side groups indicating similar chemical functionality. Therefore, EPA finds that none of the Vip3Aa variants would be expected to have significant amino acid sequence identity -- which is defined as either 35% identity over an 80 amino acid stretch and, for allergens, at the level of eight contiguous amino acids -- with a toxin, an anti-nutrient or an allergen.

This conclusion is further supported by EPA's overall safety assessment that includes other considerations such as the source of the trait, digestibility and glycosylation. As noted above, *Bacillus thuringiensis* (from which the Vip3Aa proteins are derived) is not considered to be a source of allergenic proteins. Furthermore, since all the Vip3Aa proteins have extremely homogenous structural similarities (as explained above), they are highly likely to show similar biochemical characteristics in terms of digestibility and glycosylation. So, as is the case for both Vip3Aa19 and Vip3Aa20, EPA expects that all Vip3Aa proteins will be rapidly digested under simulated gastric conditions and will not be glycosylated. The Vip3Aa proteins were only shown not to be glycosylated in cotton and corn, similarly it is unlikely to be glycosylated in any other crops because in order for a protein to be glycosylated, it needs to contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms of protein glycosylation are similar in different plants (Lerouge et al., 1998). Thus, EPA reasonably expects that because the data on Vip3Aa in cotton and corn demonstrate a lack of protein glycosylation, it will not be glycosylated in any other plants.

Finally, it is also highly relevant here that microbial pesticide products, which are distinct from plant-incorporated protectant pesticide products, containing *Bacillus thuringiensis* and its components (which could include microbially-expressed Vip3Aa proteins) are already exempt from the requirement for a tolerance under 40 CFR part 180.1011.

Accordingly, EPA believes that the foregoing supports EPA's reasonable certainty of no harm finding not only for the Vip3Aa19 and Vip3Aa20 protein variants, but also for all other closely-related members of the Vip3Aa designation as described using the Crickmore classification system (Crickmore et al., 2007).

B. Aggregate Exposures

Pursuant to FFDCA section 408(b)(2)(D)(vi), EPA considers available information concerning aggregate exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue (i.e., the Vip3Aa proteins) and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectant's chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. In addition, even if exposure can occur through inhalation, the potential for Vip3Aa to be an allergen is low, as discussed above. Although the allergenicity assessment focuses on potential to be a food allergen, the data also indicate a low potential for Vip3Aa to be an inhalation allergen. Exposure via residential or lawn use to infants and children is also not expected because the use sites for Vip3Aa proteins are agricultural. Oral exposure, at very low levels, may occur from ingestion of processed products and, theoretically, drinking water. However oral toxicity testing showed no adverse effects.

C. Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity from exposure to Vip3Aa proteins, we conclude that there are no cumulative effects for the Vip3Aa proteins.

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

1) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for Vip3Aa proteins includes the characterization of representative Vip3Aa proteins, as well as the acute oral toxicity studies, amino acid sequence comparisons to known allergens and toxins, and in vitro digestibility of the representative Vip3Aa proteins. The results of these studies were used to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were also considered.

Adequate information was submitted to show that the Vip3Aa test materials derived from microbial cultures were biochemically and functionally equivalent to the proteins produced by the plant-incorporated protectant ingredient in the plants. Microbially produced proteins were used in the studies so that sufficient material for testing was available.

The acute oral toxicity data submitted for the representative Vip3Aa proteins support the prediction that Vip3Aa proteins will be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad et al., 1992). Since no treatment-related adverse effects were shown to be caused by the representative Vip3Aa proteins, even at relatively high dose levels, Vip3Aa proteins are not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing or residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.2140). For microbial products, further toxicity testing (Tiers II and III) and residue data are triggered when significant adverse effects are seen in studies such as the acute oral toxicity study. Further studies verify the observed adverse effects and clarify the source of these effects.

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. However, data submitted demonstrated low levels of the representative Vip3Aa proteins in corn and cotton tissues.

Since Vip3Aa are proteins, potential allergenicity is also considered as part of the toxicity assessment. Considering all of the available information, including that (1) Vip3Aa originates from a non-allergenic source; (2) Vip3Aa19 and Vip3Aa20 have no sequence similarities with known allergens; (3) Vip3Aa19 and Vip3Aa20 are not glycosylated; (4) Vip3Aa19 and Vip3Aa20 are rapidly digested in simulated gastric fluid; and (5) the data developed for Vip3Aa19 and Vip3Aa20 can be extrapolated to all Vip3Aa proteins due to the extremely high level of structural similarity that exists between and among Vip3Aa proteins, EPA has concluded that the potential for Vip3Aa to be an allergen is minimal.

Neither available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) nor safety factors that are generally recognized as appropriate for the use of animal experimentation data were evaluated. The lack of mammalian toxicity at high levels of exposure to representative Vip3Aa proteins, as well as the minimal potential to be a food allergen, demonstrates the safety of Vip3Aa at levels well above possible maximum exposure levels anticipated.

The genetic material necessary for the production of the plant-incorporated protectant active ingredient include the nucleic acids (DNA, RNA) that encode these proteins and regulatory regions. The genetic material (DNA, RNA), necessary for the production of Vip3Aa proteins has been exempted from the requirement of a tolerance under 40 CFR 174.507 (“Nucleic acids that are part of a plant-incorporated protectant”).

2) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408(b)(2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the database unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for Vip3Aa proteins. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the considerations of consumption patterns, special susceptibility, and cumulative effects do not apply.

3) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to Vip3Aa proteins. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed, nor any indication of allergenicity potential for Vip3Aa proteins.

E. Other Considerations

1) Endocrine Disruptors

The pesticidal active ingredient is a protein, derived from a source that is not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of the plant-incorporated protectant at this time.

2) Analytical Method(s)

A validated lateral flow enzyme-linked immunosorbent assay (ELISA) protocol has been provided to the Agency for detecting Vip3Aa in cotton as well as a qualitative ELISA method for detecting Vip3Aa in corn.

3) Codex Maximum Residue Level

No Codex maximum residue level exists for the plant-incorporated protectant *Bacillus thuringiensis* Vip3Aa proteins and the genetic material necessary for their production in corn and cotton.

F. Tolerance Exemptions

The data submitted and reviewed for Vip3Aa support the petition for an exemption from the requirement of tolerance for *Bacillus thuringiensis* Vip3Aa proteins when used as plant–incorporated protectants in or on the food and feed commodities of corn and cotton.

G. Supporting Data

The human health studies submitted to support the safety of Vip3Aa are summarized in Table 7 below.

Table 7. Summary of Vip3Aa Human Health Data (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Summary of Mammalian Toxicology Data for the VIP3A and APH4 Proteins Produced by Transgenic VIP3A Cotton Event COT102[2]	No significant adverse effects were observed in male and female mice dose by gavage at approximately 3675 mg VIP3A/kg body weight (the highest dose tested) and the LD_{50} for pure VIP3A protein was >3675 mg/kg body weight. The LD_{50} for pure APH4 protein in male and female mice was >774 mg/kg body weight. The allergen database compiled by Syngenta needs to be better defined or described in order to ascertain the number and types of allergens searched for homology. **Classification: SUPPLEMENTAL.** **Note: this is a summary of multiple studies and is therefore superseded by the individual studies summarized below, which provide additional information, including the requested information on the SBI allergen database.**	45766502
Acute Oral Toxicity/ Acute Oral Toxicity of Vip3A Protein in Mice[2]	Eleven male and 11 female HSD:ICR albino mice were dosed with VIP3A protein (Lot no. VIP3A-0196 containing ~ 32% by weight VIP3A protein). The mice were quarantined for 5 days and fasted approximately 16 hours prior to dosing. The test material (5050 mg/kg body weight) was dosed as a 12.5 % w/v suspension in 2 % w/v carboxymethyl cellulose (CMC) in distilled water by gavage (Table 1). The dose volume was 40.4 mL/kg and was divided into 2 parts administered approximately one hour apart. The control group was treated with 2 % w/v CMC in the same manner as the test animals. Body weights were recorded prior to dosing, on days 7 and 14 or at death. The test animals were observed for clinical signs of toxicity at least three times post-dosing and at least daily thereafter for 14 days. All decedent or euthanized animals were necropsied. One control male (No. 17-M) was found dead on day 2. All other mice survived the study. With the exception of one female (No. 10-F) that failed to gain weight during the first week, all surviving animals gained weight during the study. In the	45766503

Study Type/Title	Summary	MRID #
	vehicle control group (*i.e.*, CMC treated), there was no affect on weight gain. The oral LD_{50} for males, females, and combined was greater than 5050 mg/kg (or > 1616 mg VIP3A protein/kg body weight). **Classification: SUPPLEMENTAL.** The VIP3A protein used in this study differs from the VIP3A protein present in COT102 cotton by a two amino acids, one at position 2 (aspartate replaces asparagine), another at position 284 (lysine replaces glutamine). **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	
Acute Oral Toxicity/ Single Dose Oral Toxicity Study with VIP3A-0199 in Mice[2]	Twenty-seven male and 27 female CD-1® (ICR)BR mice were dosed with VIP3A protein (Batch VIP3A-0199 containing ~ 54% by weight VIP3A protein), produced in an *E. coli* over-expression system. The VIP3A protein used as the test material differs from that present in cotton Event COT102 by a single amino acid; glutamine substituted for lysine (Q284K). The mice were quarantined for 16 days and fasted approximately 4 hours prior to dosing. The test material (5000 mg/kg body weight) was dosed as a suspension of 200 mg/mL in 0.5% w/v carboxymethyl cellulose (CMC) in deionized water by gavage (Table 1). The dose volume was 25 mL/kg. The control group was treated with 0.5 % w/v CMC in the same manner and volume as the test animals. Body weights were recorded prior to dosing, and on day 8 for animals designated to be sacrificed on day 15, and on each animals's respective day of necropsy (days 1, 2, or 15). The animals were observed for clinical signs of toxicity approximately 1, 2.5, 4, and 6 hours post dosing and at least daily until sacrifice. Animals were observed for any abnormal behavior, changes in posture or clonic / tonic movements. Mortality was observed twice daily. All animals were necropsied after sacrifice. The organ weight of the brain, kidneys, liver with drained gallbladder, and stomach were recorded and organ to body weight and organ to brain weight were calculated. Histopathology was performed on brain, gallbladder, heart, intestines (cecum, colon, duodenum, ileum, jejunum, and rectum), kidneys, lesions, liver, lung, and stomach. All animals survived prior to the scheduled sacrifice. All animals sacrificed on day 15 had normal body weight gains. All control and a few test animals sacrificed on day 1 and one male test and some control animals sacrificed on day 2 lost weight prior to sacrifice. No significant differences considered to be test material related in organ/body weight or organ/brain weight between control and test animals were found. The oral LD_{50} for males, females, and combined was greater than 5000 mg/kg (or > 2700 mg VIP3A protein/kg body weight). **Classification: SUPPLEMENTAL -** The test material for this study, VIP3A-0199, differs in sequence by one amino acid (Q284K) from that form of the protein which is present in COT102. **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	45766504
Acute Oral Toxicity/ Acute Oral Toxicity	The test animals (Sixteen male and 16 female Crl-1® (ICR)BR mice) were quarantined for 9 days and fasted approximately 4 hours prior to dosing.	45766505

Study Type/Title	Summary	MRID #
Study with Test Substance VIP3A-0100 Protein in Mice[2]	The test material (5000 mg/kg body weight) was dosed as a suspension of 196 mg/mL in 0.5% w/v carboxymethyl cellulose (CMC) in deionized water by gavage. The dose volume was 25.5 mL/kg. The control group was treated with 0.5% w/v CMC in the same manner as the test animals. Body weights were recorded prior to dosing and on days 8 and 15 for animals designated to be sacrificed on day 15. The animals were observed for clinical signs of toxicity approximately 1, 2.5, 4, and 6 hours post dosing and at least daily until sacrifice. Mortality was observed twice daily. All animals were necropsied after sacrifice. The organ weight of the brain, kidneys, liver with drained gallbladder, and stomach were recorded and organ to body weight and organ to brain weight were calculated. Histopathology was performed on brain, gallbladder, heart, intestines (cecum, colon, duodenum, ileum, jejunum, and rectum), kidneys, lesions, liver, lung, and stomach. All animals sacrificed on day 15 had normal body weight gains. No test material related macroscopic alterations were noted. In addition, no significant differences related to the test material in organ/body weight or organ/brain weight between control and test animals were found. The oral LD_{50} for males, females, and combined was greater than 5000 mg/kg (or > 3675 mg VIP3A protein/kg body weight). **Classification: Acceptable**	
Acute Oral Toxicity/ Single Dose Oral Toxicity Study with VIP3A-Enriched Maize (Corn) Leaf Protein (LPPACHA-0199) in Mice[2]	VIP3A-Enriched Maize (Corn) Leaf Protein (Sample Lot. No. LPPACHA-0199 containing ~ 0.36% by weight VIP3A protein) was prepared from transgenic VIP3A maize (corn) leaves. The mice were quarantined for at least 7 days and fasted approximately 4 hours prior to dosing. The test material (5000 mg/kg body weight) was dosed as a suspension of 250 mg/mL in 0.5% w/v carboxymethyl cellulose (CMC) in deionized water by gavage (Table 1). The dose volume was 20 mL/kg. The control group was treated with Control Maize (Corn) Leaf Protein, Batch LPPACHA-0199C in 0.5% w/v CMC in deionized water at a concentration of 250 mg/mL in the same manner as the test animals. Body weights were recorded prior to dosing, and on days 7, 14, or at death. The test animals were observed for clinical signs of toxicity at least three times post-dosing and at least daily thereafter for 14 days. All decedent or euthanized animals were necropsied. All mice survived the study, gained weight and appeared normal during the study. The oral LD_{50} for males, females, and combined was greater than 18 mg/kg VIP3A protein/kg body weight. The net concentration of VIP3A (18 mg / kg body weight) is significantly lower than the prescribed 2000 to 5000 mg / kg body weight suggested in the guideline requirements. At this concentration and with the mix of other proteins present in the leaf preparation, no toxicity was evident in the test animals. **Classification: SUPPLEMENTAL.** Information is supportive, but not part of guideline requirements; no further information required. **Note: this study provides additional support for the conclusion that Vip3Aa proteins are non-toxic to mammals.**	45766506
In Vitro Digestibility of VIP3A Protein Under	VIP3A from recombinant maize (field corn) plants was prepared as sample LPPACHA-0199 by extracting the leaves of recombinant corn plants and	45835805

Study Type/Title	Summary	MRID #
Simulated Mammalian Gastric Conditions [2]	concentrating the VIP3A by ammonium sulfate precipitation, dialysis of the resulting salt, and lyophilization of the collected protein. ELISA showed VIP3A constituted ~0.36 % by weight of the sample and retained insecticidal activity against sensitive lepidopteran species. VIP3A from *E. coli* was prepared as sample VIP3A-0100 in an *E. coli* strain BL21DE3pLysS over-expression system. The synthetic *vip3A(a)* gene was cloned into the inducible over-expression pET-3a® vector. Following collection, purification, dialysis, and lyophilization, the sample was estimated by ELISA to contain ~73.5% VIP3A by weight and it retained its insecticidal activity against sensitive lepidopteran species. The reactions were initiated by the addition of 80 µL of LPPACHA-0199 or VIP3A-0100 to 320 µL of simulated gastric fluid containing pepsin incubated at 37°C. Immediately after sample addition, an aliquot was removed and quenched with an equal volume of Laemmli buffer (pH not reported) and inactivated at >75°C for 10 minutes. Additional aliquots were removed and treated as above following 2, 5, 10, 20, 30, and 60 minutes of incubation. Digestion of the protein samples was evaluated using SDS-PAGE and Western blotting. The digestion of VIP3A protein in a simulated gastric environment proceeds at a rapid rate and demonstrates the lability of this protein to conditions typical of a monogastric mammalian stomach. The presence of a small amount of immunoreactive protein (approximately 6 to 9 kD) indicates that a portion or domain of the protein is less readily digested in this environment, although these bands do degrade beyond the point of immunorecognition with time. Results of this study indicate VIP3A protein, whether isolated from recombinant corn plants or from genetically modified *E. coli*, will be rapidly digested in a simulated gastric environment. **Classification: ACCEPTABLE**	
Amino acid sequence comparison/ Vip3Aa19: Assessment of amino acid sequence homology with known toxins. Report No. SSB-122-06[4]	The purpose of this study was to determine if Vip3Aa19 had any significant amino acid sequence homology to known protein toxins. No relevant similarities between the Vip3Aa19 query sequence and known protein toxins were found other than with other insect-specific vegetative insecticidal proteins of *B. thuringiensis*. **Classification: Acceptable; Supersedes MRID 457665-02**	46885903
Amino acid sequence comparison/ Vip3Aa19: Assessment of amino acid sequence homology with known allergens. Report No. SSB-130-06[4]	The purpose of this study was to determine if Vip3Aa19 had any significant amino acid sequence homology to known protein allergens. Vip3Aa19 had no significant amino acid sequence homology to known or putative allergenic proteins. **Classification: Acceptable; Supersedes MRID 457665-02**	46885906
Amino acid sequence comparison/ Vip3A as	The purpose of the study was to determine if Event MIR162 Vip3A protein had any significant amino acid sequence homology to known or putative	46864808

Study Type/Title	Summary	MRID #
expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known toxins[3]	protein toxins. The database identified 32 entries with E values below 6 x 10^{-6}, of which 30 were vegetative insecticidal proteins of *B. thuringiensis* and had E values of 0.0 to 1 x 10^{-10}. Two proteins were identified as rhoptry proteins from *Plasmodium yoelii*, a pathogen that causes malaria in rodents *via* erythrocyte binding and invasion (Ogun and Holder, 1996). Despite the pathogenic nature of *P. yoelii*, the low overall sequence similarity between MIR162 Vip3A and the rhoptry proteins (3.9 or 11.4% overall amino acid sequence identity) suggests that the E values are of no biological significance (Doolittle, 1990). Furthermore, a global protein alignment (Myers and Miller, 1988) demonstrates that there are no more than three contiguous identical amino acids between Vip3A and the rhoptry proteins. Therefore, no relevant similarities between the Event MIR162 Vip3A query sequence and known protein toxins were found. **Classification: ACCEPTABLE**	
Amino acid sequence comparison/ Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known allergens[3]	The purpose of this study was to determine if Event MIR162 Vip3Aa20 had any significant amino acid sequence homology to known or putative protein allergens. No significant sequence homology was found between any sequential MIR162 Vip3A 80-amino acid peptides and any entry in the SBI Allergen Database. No alignments of eight or more contiguous identical amino acids were identified between MIR162 Vip3A and proteins in the SBI Allergen Database. Therefore, no significant amino acid sequence homology was found between the MIR162 Vip3A and any known or putative protein allergens. **Classification: ACCEPTABLE**	46864809
Analysis of Vip3A or Vip3A-Like Proteins in Six Different Commercial Microbial *Bacillus thuringiensis* Products	The purpose of this study was to determine whether Vip3A or Vip3A-like proteins are detectable and quantifiable in commercial formulations of *Bacillus thuringiensis* (Bt)-based microbial insecticide products. ELISA (enzyme-linked immunosorbent assay) and Western blot analyses were used to detect and analyze Vip3A or Vip3A-like proteins in the formulations. Vip3A or Vip3A-like proteins were detected in all six commercial products, with concentrations ranging from a low of *ca*. 2.0 µg/g product to a high of *ca*. 209 µg/g. Those products showing the highest protein concentrations were all derived from the *kurstaki* subspecies of *B. thuringiensis*. **Classification: ACCEPTABLE**	47017613
Amino acid sequence comparison/ Vip3Aa19: Assessment of amino acid sequence homology with known allergens	Two amino acid sequences comparisons of Vip3Aa19 with known allergens were conducted using the Syngenta Biotechnology, Inc (SBI) Allergen Database. The results indicate that Vip3Aa19 has no significant amino acid sequence homology to known or putative allergenic proteins based on a search for greater than 35% sequence identity over successive 80-amino acid peptides and a search for eight or more contiguous identical amino acids. **Classification: ACCEPTABLE**	47017617

II.B.2. Human Health Assessment of Modified Cry1Ab Containing 26 Additional Amino Acids

A. Mammalian Toxicity and Allergenicity Assessment

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

Syngenta has submitted acute oral toxicity data demonstrating the lack of mammalian toxicity at high levels of exposure to the pure modified Cry1Ab protein containing the additional 26 amino acid 'Geiser motif'. The 26 amino acid sequence is found at the C-terminus of the pro-toxin portion of the modified Cry1Ab protein. The pro-toxin is enzymatically cleaved in the insect gut to produce active Cry1Ab. These toxicity data demonstrate the safety of the product at a level well above maximum possible exposure levels that are reasonably anticipated in the crop. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity testing and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.2140). For microbial products, further toxicity testing (Tiers II & III) and residue data are triggered by significant adverse acute effects in studies such as the acute oral toxicity study, to verify the observed adverse effects and clarify the source of these effects.

An acute oral toxicity study in mice indicated that modified Cry1Ab is non-toxic to humans. Groups of five male and five female mice were given 0 or 1830 mg/kg bodyweight microbially-produced modified Cry1Ab by oral gavage as a single dose. There were no effects on clinical condition, body weight, food consumption, clinical pathology, organ weight, or macroscopic or microscopic pathology that were attributed to the test substance.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad et al., 1992). Therefore, since no acute effects were shown to be caused by modified Cry1Ab, even at relatively high dose levels, the modified Cry1Ab protein is not considered toxic.

Since modified Cry1Ab is a protein, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for modified Cry1Ab follows:

1. Source of the trait. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of modified Cry1Ab with known allergens showed no significant sequence identity over 80 amino acids or identity at the level of eight contiguous amino acid residues.
3. Digestibility. Modified Cry1Ab was rapidly digested in simulated gastric fluid containing pepsin.
4. Glycosylation. Modified Cry1Ab expressed in cotton was shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for modified Cry1Ab to be a food allergen is minimal.

Although modified Cry1Ab was only shown not to be glycosylated in cotton, it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it needs to contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms of protein glycosylation are similar in different plants (Lerouge et al., 1998).

B. Aggregate Exposures

Pursuant to FFDCA section 408(b)(2)(D)(vi), EPA considers available information concerning aggregate exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectants chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. In addition, even if exposure can occur through inhalation, the potential for modified Cry1Ab to be an allergen is low, as discussed above. Although the allergenicity assessment focuses on potential to be a food allergen, the data also indicate a low potential for modified Cry1Ab to be an inhalation allergen. Exposure via residential or lawn use to infants and children is also not expected because the use sites for the modified Cry1Ab protein is agricultural. Dietary exposure may occur from ingestion of processed cotton products but is expected to be very low because the already low expression levels in the seed and would be reduced further by the heat and pressure used for processing. Also, dietary exposure may theoretically occur through exposure in drinking water because plant stubble may release modified Cry1Ab protein into ground water upon decay. This protein would not be expected to survive in the soil due to microbial degradation, adherence to soil components and removal upon drinking water treatment procedures. In addition, oral toxicity testing showed no adverse effects.

C. Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity from the plant-incorporated protectant, EPA concludes that there are no cumulative effects for the modified Cry1Ab protein.

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

1) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the modified Cry1Ab protein includes the characterization of the expressed modified Cry1Ab protein in cotton, as well as the acute oral toxicity study, amino acid sequence comparisons to known allergens, and in vitro digestibility of the protein. The results of these studies were used to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were also considered.

Adequate information was submitted to show that the modified Cry1Ab test material derived from microbial culture was biochemically and functionally equivalent to the protein in the plant. Microbially produced protein was used in the safety studies so that sufficient material for testing was available.

The acute oral toxicity data submitted support the prediction that the modified Cry1Ab protein would be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad et al., 1992). Since no treatment-related adverse effects were shown to be caused by the Cry1Ab protein, even at relatively high dose levels, the modified Cry1Ab protein is not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.2140). For microbial products, further toxicity testing and residue data are triggered when significant adverse effects are seen in studies such as the acute oral toxicity study. Further studies verify the observed adverse effects and clarify the source of these effects.

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. However, data submitted demonstrated low levels of the modified Cry1Ab protein in cotton tissues.

Since Cry1Ab is a protein, potential allergenicity is also considered as part of the toxicity assessment. Considering all of the available information (1) modified Cry1Ab originates from a non-allergenic source; (2) modified Cry1Ab has no sequence similarities with known allergens; (3) modified Cry1Ab is not glycosylated; and (4) modified Cry1Ab is rapidly digested in simulated gastric fluid; EPA has concluded that the potential for modified Cry1Ab to be an allergen is minimal.

Neither available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) nor safety factors that are generally recognized as appropriate for the use of animal experimentation data were evaluated. The lack of mammalian toxicity at high levels of exposure to the modified Cry1Ab protein, as well as the minimal potential to be an allergen, demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated.

The genetic material necessary for the production of the plant-incorporated protectant active ingredient include the nucleic acids (DNA, RNA) that encode these proteins and regulatory regions. The genetic material (DNA, RNA) necessary for the production of the modified Cry1Ab protein has been exempted from the requirement of a tolerance under 40 CFR 174.507 ("Nucleic acids that are part of a plant-incorporated protectant").

2) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408(b)(2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the database unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for the modified Cry1Ab protein. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the considerations of consumption patterns, special susceptibility, and cumulative effects do not apply.

3) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the modified Cry1Ab protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as

discussed above, no toxicity to mammals has been observed, nor any indication of allergenicity potential for the plant-incorporated protectant.

E. Other Considerations

1) Endocrine Disruptors

The pesticidal active ingredient is a protein, derived from a source that is not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of this plant-incorporated protectant at this time.

2) Analytical Method(s)

A lateral flow enzyme-linked immunosorbent assay (ELISA) protocol has been provided to the Agency for detecting modified Cry1Ab in cotton. This analytical method will be independently validated as a condition of registration for cotton product(s) containing modified Cry1Ab.

3) Codex Maximum Residue Level

No Codex maximum residue level exists for the plant-incorporated protectant *Bacillus thuringiensis* modified Cry1Ab protein.

F. Tolerance Exemptions

The data submitted and reviewed for modified Cry1Ab support the petition for an exemption from the requirement of tolerance for *Bacillus thuringiensis* modified Cry1Ab protein containing the additional 26 amino acid sequence when used as a plant-incorporated protectant in or on the food and feed commodities of cotton.

G. Supporting Data

The human health studies submitted to support the safety of modified Cry1Ab are summarized in Table 8 below.

Table 8. Summary of Modified Cry1Ab Human Health Data (reviewed in Edelstein 2008 unless otherwise noted)

Study Type/Title	Summary	MRID #
Acute oral toxicity (OPPTS 870.1100)/ FLCRY1AB-0103: Single Dose Oral	Groups of five male and five female mice were given 0 or 1830 mg/kg bodyweight microbially-produced modified Cry1Ab (FLCRY1AB-0103) by oral gavage as a single dose. There were no effects on clinical condition, body weight, food consumption, clinical pathology, organ weight, or	47017614

Toxicity Study in the Mouse (AM7516/Regulatory/Report)	macroscopic or microscopic pathology that were attributed to the test substance. **Classification: ACCEPTABLE**	
In vitro digestibility/ *In vitro* digestibility of full-length Cry1Ab protein (test substances FLCRY1AB-0103 and IAPCOT67B-0106) under simulated mammalian gastric conditions	The *in vitro* digestibility in simulated gastric fluid of the modified Cry1Ab protein as expressed in COT67B and from a bacterial source was investigated. No intact full-length modified Cry1Ab protein from bacterial- or plant-derived sources was found one minute after incubation in simulated gastric fluid. An immunoreactive polypeptide fragment (~ 60,000 Da) in the digestion mixture was visible in the 5 minute sample in the plant-derived source and in the 10 minute sample in the bacterial-derived source. The study results indicate that the full-length Cry1Ab protein is rapidly digested in simulated gastric fluid; a 60 kDa fragment is formed, which also appears to be digestible, but at a slower rate. **Classification: ACCEPTABLE**	47017615
Heat stability/ Effect of temperature on the stability of full-length Cry1Ab protein	The effect of temperature on the bioactivity of modified Cry1Ab was investigated. Heating of *E. coli*-derived modified Cry1Ab (FLCRY1AB-0103) at 65˚C or 95˚C for 30 minutes substantially decreased or eliminated the insecticidal activity of the protein. No significant effect on the protein's insecticidal properties was found following incubation for 30 minutes at temperatures ≤37˚C. **Classification: ACCEPTABLE**	47017616
Amino acid sequence comparison/ Full-length Cry1Ab as expressed in Event COT67B: Assessment of amino acid sequence homology with known allergens	Two amino acid sequences comparisons of modified Cry1Ab with known allergens were conducted using the Syngenta Biotechnology, Inc (SBI) Allergen Database. The results indicate that modified Cry1Ab has no significant amino acid sequence homology to known or putative allergenic proteins based on a search for greater than 35% sequence identity over successive 80-amino acid peptides and a search for eight or more identical contiguous amino acids. **Classification: ACCEPTABLE**	47017619

II.B.3. Human Health Assessment of Hygromycin B Phosphtransferase (APH4)

The hygromycin B phosphotransferase (APH4) protein expressed in COT102 x COT67B is covered by the exemption from the requirement of a tolerance at 40 CFR 174.526 Hygromycin B phosphtransferase (APH4) marker protein in all plants; exemption from the requirement of a tolerance.

Summary of new data submitted for APH4

MRID# 47017618—APH4 (Entrez Database accession No. CAA85741): Assessment of Amino Acid Sequence Homology with Known Allergens:
Two amino acid sequences comparisons of APH4 with known allergens were conducted using the Syngenta Biotechnology, Inc (SBI) Allergen Database. The results indicate that APH4 has no

significant amino acid sequence homology to known or putative allergenic proteins based on a search for greater than 35% sequence identity over successive 80-amino acid peptides and a search for eight or more contiguous amino acids.
Classification: ACCEPTABLE

II.B.4. References

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Waggoner, A., 2007. Memorandum from A. Waggoner to M. Mendelsohn dated February 8, 2007.

Wozniak, C. 2004. EPA Review of the product characterization, environmental fate and human health toxicity studies for the insecticidal protein VIP3A, as expressed in upland cotton, for control of lepidopteran insect pests, and the selective marker protein APH4. Memorandum from C.

Wozniak to L. Cole dated March 24, 2004.

II. C. Environmental Hazard Assessment

Note: EPA's environmental assessment was conducted for Vip3Aa proteins, which include the Vip3Aa19 protein as expressed in cotton. "Full-length Cry1Ab" (FLCry1Ab) refers to the modified Cry1Ab (OECD Unique Identifier SYN-IR67B-1) protein in VipCot.

Background

Vip3A is a novel class of recently discovered insecticidal proteins that occur naturally in *Bacillus thuringiensis* (*Bt*), a gram-positive soil bacterium (Estruch, *et al.* 1996). The vegetative insecticidal proteins are produced during vegetative bacterial growth and are secreted as soluble proteins into the extracellular environment. Syngenta Seeds, Inc. has developed Event COT102, a cotton line that expresses an insect control protein, known as Vip3Aa. In addition, Syngenta Seeds, Inc. has also developed Event COT67B, a cotton line that expresses a *Bt* insect control Cry protein, known as full-length Cry1Ab. These proteins are intended to control several lepidopteran pests of cotton including: *Helicoverpa zea* (cotton bollworm), *Heliothis virescens* (tobacco budworm), *Spodoptera frugiperda* (fall armyworm), *Spodoptera exigua* (beet armyworm), and *Trichoplusia ni* (cabbage looper).

Syngenta Seeds, Inc. is requesting a Sec. 3 registration for *Bt* insect control protein Vip3Aa as expressed in Event COT102 cotton, full-length Cry1Ab (hereafter, referred to as FLCry1Ab) as expressed in Event COT67B cotton, and its associated breeding stack, COT102 x COT67B [also known as VipCot, EPA Reg. No. 67979-O] cotton (which combines Vip3Aa and FLCry1Ab proteins), crossed via traditional breeding. An experimental use permit (EUP) was granted by the Agency to conduct field tests on Event COT102, Event COT67B, and its associated breeding stack COT102 x COT67B (Matten, 2007).

Event COT102 cotton specifically expresses Vip3Aa19[e], a variant of the naturally occurring Vip3Aa1 protein isolated from *Bacillus thuringiensis* strain AB88, differing from the Vip3Aa1 protein by one amino acid. The same protein variant present in Event COT102 cotton is also expressed as Vip3Aa19 in Syngenta's experimental Event Pacha corn. The Agency previously determined that "all proteins designated as Vip3Aa are more than 95% identical," and "there is sufficient information to support the safety of all Vip3Aa proteins, provided that they do not have any significant sequence similarity with known allergens" (Edelstein, 2008). Therefore, in addition to the data reviewed in this report, all the previously submitted data developed for Vip3Aa protein can be cited in support of the registration of Event COT102.

Although Vip3Aa protein shares no homology with FLCry1Ab or other known Cry proteins, extensive testing by Syngenta has established that Vip3Aa has demonstrated a similar toxicity

[e] Prior to receiving the Crickmore designation of Vip3Aa19, the protein produced in Events COT102 and Pacha were referred to as VIP3A, Vip3A or Vip3Aa.

against larvae of certain lepidopteran species, including key pests of cotton. While the modes of action differ between the two proteins, the general symptoms displayed by sensitive lepidopteran larvae following ingestion of Vip proteins resembles that caused by Cry proteins (i.e., cessation of feeding, loss of gut peristalsis, overall paralysis of the insect, and death) (Yu, *et al*, 1997). Since the effects of Vip and Cry proteins are considered similar, the studies submitted on non-target organisms for Event COT102 were conducted and evaluated according to the same environmental risk assessment criteria of previously reviewed PIP products containing Cry protein.

FLCry1Ab protein expressed in COT67B cotton and native Cry1Ab protein are both derived from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (*B.t.k.*). FLCry1Ab differs from the naturally occurring Cry1Ab protein in that FLCry1Ab contains 26 additional consecutive amino acids (described as the 'Geiser motif") in the C-terminal portion (Geiser *et al*., 1986). The 'Geiser motif' is also expressed in another registered PIP cotton product containing Cry1Ac. FLCry1Ab protein in Event COT67B is also similar to the truncated protein variants of Cry1Ab as expressed in transgenic maize. The Agency previously determined that Syngenta's Event Bt11 corn produces a truncated Cry1Ab protein that has the same insecticidal active region of amino acids as FLCry1Ab produced in COT67B cotton (Matten, 2007). In addition, there are numerous laboratory studies, field studies, and scientific literature on the mode of action of Cry1Ab protein, Cry1Ab-expressing maize and Cry1Ac-expressing cotton (US EPA, 2001b; Naranjo *et al*., 2005; Romeis *et al*., 2006; Cattaneo *et al*., 2006; and Torres and Ruberson, 2007). These data provide a large weight-of-evidence that these proteins demonstrate very similar insecticidal activity against several lepidopteran cotton pests at concentrations found in transgenic plants. Furthermore, the Agency also determined that field efficacy data submitted with the registration application (MRID No. 470176-33) and reports provided with the Public Interest Document (MRID No. 470176-35) demonstrate a similar insecticidal spectrum of the truncated and full-length Cry1Ab proteins (Martinez, 2008). Therefore, the effects of truncated Cry1Ab proteins are considered predictive of the effects of FLCry1Ab protein as expressed in COT67B cotton to non-target organisms for the purposes of the environmental risk assessment.

The Agency has conducted an environmental risk assessment of COT102 and COT67B cotton lines expressing Vip3Aa and FLCry1Ab proteins. The general topics covered include gene flow to related wild plants, potential of weediness, effects on wildlife, and fate of Vip3Aa and Cry1Ab proteins in the environment. The assessment is based on data submitted to the Agency during the development of the cotton lines, additional data submitted for registration, Federal Insecticide Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) recommendations, consultations with scientific experts, and public comments on Plant-Incorporated Protectant (PIP) regulation.

II. C. 1. Environmental Risk Assessment for COT102 and COT67B (lepidopteran active)

A. Tiered Testing and Risk Assessment Process

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

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

The EPA uses a tiered (Tiers I-IV) testing system to assess the toxicity of a PIP to representative non-target organisms that could be exposed to the toxin in the field environment. Tier I high dose studies reflect a screening approach to testing designed to maximize any toxic effects of the test substance on the test (non-target) organism. The screening tests evaluate single species in a laboratory setting with mortality as the end point. Tiers II – IV generally encompass definitive hazard level determinations, longer term greenhouse or field testing, and are implemented when unacceptable effects are seen at the Tier I screening level.

Testing methods which utilize the tiered approach were last published by the EPA as Harmonized OPPTS Testing Guidelines, Series 850 and 885 (EPA 712-C-96-280, February 1996)[g]. These

[f] Non-target invertebrate hazard tests often are conducted at exposure concentrations several times higher than the maximum concentrations expected to occur under realistic exposure scenarios. This has customarily allowed an endpoint of 50% mortality to be used as a trigger for additional higher-tier testing. Lower levels of mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic exposure scenarios. Thus, it follows that the observed proportion of responding individuals can be compared to a 50% effect to determine if the observed proportion is significantly lower than 50%. For example, using a binomial approach, a sample size of 30 individuals is sufficient to allow a treatment effect of 30% to be differentiated from a 50% effect with 95% confidence using a one-sided Z test. A one-sided test is appropriate because only effects of less than 50% indicate that further experiments are not needed to evaluate risk.

[g] OPPTS Testing Guidelines, Series 850 and 885 website:
http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/885Microbial_Pesticide_Test_Guidelines/Series

guidelines, as defined in 40 CFR 152.20, apply to microbes and microbial toxins when used as pesticides, including those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation. Therefore, PIPs containing microbial toxins are also covered by these testing guidelines.

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

Failing the Tier I (10 X EEC) screening at the MHD dose does not necessarily indicate the presence of an unacceptable risk in the field but it triggers the need for additional testing.[i] A less than 50% mortality effect at the MHD is taken to indicate minimal risk. However, greater than 50% mortality does not necessarily indicate the existence of unacceptable risk in the field, but it does trigger the need to collect additional dose-response information and a refinement of the exposure estimation before deciding if the risk is acceptable or unacceptable. Where potential hazards are detected in Tier I testing (i.e. mortality is greater than 50%), additional information at lower test doses is required which can serve to confirm whether any effect might still be detected at more realistic field [1X EEC] concentrations and routes of exposure[j].

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

[h] The dose margin can be less than 10x where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits or other factors. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the test concentration used must exceed 1X EEC.

[i] It is notable that that the 10 X EEC MHD testing approach is not equivalent to what is commonly known as "testing at a 10X SAFETY FACTOR" where any adverse effect is considered significant. Tier I screen testing is not 'safety factor testing'. In a "10X safety factor" test any adverse effect noted is a "level of concern", whereas in the EPA environmental risk assessment scenario any adverse effect is viewed as a concern only at 1X the field exposure.

[j] The 1X EEC test dose is based on plant tissue content and is considered a high worst case dose (sometimes referred to as HEEC). This 1X EEC is still much greater than any amount which any given non-target organism may be ingesting in the field because most non-target organisms do not ingest plant tissue.

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

Data that shows less than 50 % mortality at the maximum hazard dosage level – (i.e. LC_{50}, ED_{50}, or LD_{50} >10 X EEC) is sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. It is also notable that the recommended >10X EEC maximum hazard dose level is a highly conservative factor. The published EPA Level of Concern [LOC] is 50% mortality at 5X EEC [k] (US EPA, 1998).
Validation: The tiered hazard assessment approach was developed for the EPA by the American Institute of Biological Sciences (AIBS) and confirmed in 1996 as an acceptable method of environmental hazard assessment by a FIFRA Scientific Advisory Panel (SAP) on microbial pesticides and microbial toxins. The December 9, 1999 SAP agreed that the Tiered approach was suitable for use with Plant-Incorporated Protectants (PIPs); however, this panel recommended that, for PIPs with insecticidal properties, additional testing of beneficial invertebrates closely related to target species and/or likely to be present in GM crop fields should be conducted. Testing of *Bt* Cry proteins on species not closely related to the target insect pest was not recommended, although it is still performed to fulfill the published EPA non-target species data requirements. In October 2000, another SAP also recommended that field testing should be used to evaluate population-level effects on non-target organisms. The August 2002 SAP, and some public comments, generally agreed with this approach, with the additional recommendation that indicator organisms should be selected on the basis of potential for field exposure to the subject protein (US EPA, 2000, 2001a, 2002, and 2004).

Chronic studies: Since delayed adverse effects and/or accumulation of toxins through the food chain are not expected to result from exposure to proteins, protein toxins are not routinely tested for chronic effects on non-target organisms. However, the 30 day test duration requirement does amount to subchronic testing when performed at field exposure test doses. Proteins do not bioaccumulate. The biological nature of proteins makes them readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports that some proteins (Cry proteins) bind to soil particles, it has also been shown that these proteins are degraded rapidly by soil microbial flora upon elution from soil particles.

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

Conclusion: The tiered approach to test guidelines ensures, to the greatest extent possible, that the Agency requires the minimum amount of data needed to make scientifically sound regulatory decisions. The EPA believes that maximum hazard dose Tier I screening testing presents a reasonable approach for evaluating hazards related to the use of biological pesticides and for identifying negative results with a high degree of confidence. The Agency expects that Tier 1 testing for short-term hazard assessment will be sufficient for most studies submitted in support of PIP registrations. However, if long range adverse effects must be ascertained, then higher-tier longer-term field testing will be required As noted above, the October 2000 SAP and the National Academy of Sciences (NAS, 2000) recommended testing non-target organisms directly in the field. This approach, with an emphasis on testing invertebrates found in corn fields, was also recommended by the August 2002 SAP and was supported by several public comments. Based on these recommendations, the Agency has required field studies on long term invertebrate population/community and Cry protein accumulation in soils as a condition of registration due to the lack of baseline data on the potential for long-term environmental effects from the cultivation of PIP-producing plants.

Since the commercialization of *Bt* crops, the number of field studies published in scientific literature in combination with the post-registration field studies submitted to the Agency has accumulated to a level where empirical conclusions can be made. As a result, the issue of long range effects of cultivation of these Cry proteins on the invertebrate community structure in *Bt* crop fields has since been adequately addressed. Specifically, a meta-analysis[1] of the data collected from 42 field studies indicated that non-target invertebrates are generally more abundant in *Bt* cotton and *Bt* maize fields than in non-transgenic fields managed with insecticides (Marvier, *et al*., 2007). In addition, a comprehensive review of short and long term field studies on the effects of invertebrate populations in *Bt* corn and cotton fields indicated that no unreasonable adverse effects are taking place as a result of wide scale *Bt* crop cultivation (Sanvido, *et al.* 2007). Another review of field tests published to date concluded that the large-scale studies in commercial *Bt* cotton have not revealed any unexpected non-target effects other than subtle shifts in the arthropod community caused by the effective control of the target pests (Romeis *et al*., 2006). Slight reductions in some invertebrate predator populations are an inevitable result of all pest management practices, which result in reductions in the abundance of the pests as prey.

Overall, the Agency is in agreement with the conclusions of these studies and collectively, these results provide extensive data to support that *Bt* crops have not caused long term environmental effects on a population level to organisms not targeted by *Bt* proteins. Based on these considerations,

[1] This research was funded by Environmental Protection Agency grant CR-832147-01. The *Bt* crop non-target effects database can be found on the National Center for Ecological Analysis and Synthesis (NCEAS). Website. (http://delphi.nceas.ucsb.edu/btcrops/).

regulatory testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

B. Environmental Exposure Assessment

The EPA risk assessment is centered only on adverse effects at the field exposure rates (1X EEC), and not on adverse effects at greater concentrations. Although it is recommended that non-target testing be conducted at a test dose 10 X the EEC whenever possible, the test dose margin can be less than 10X where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the concentration used must exceed 1X EEC. It is important to note that Tier I screen testing is not "safety factor testing". In a traditional "10X safety factor" test any adverse effect noted is a "level of concern", whereas in the EPA environmental risk assessment scenario any adverse effect is viewed as a concern only at 1X the field exposure.

For the purposes of the non-target organism (NTO) studies submitted in support of Event COT102 and Event COT67B, the test material dose levels were based on the estimated concentration of Vip3Aa and full-length Cry1Ab protein expressed in the tissue(s) that NTO would most likely be exposed to in the environment (see Matten, 2007; Edelstein, 2008 for protein expression levels). The Agency has determined that the NTOs most likely to be exposed to the Vip3Aa and FLCry1Ab protein in transgenic cotton fields were beneficial insects feeding on cotton pollen. Consequently, test material dose levels were based on the maximum level of measured protein expression in pollen (3.47 ug/g dwt for Vip3Aa and 12.1 ug/g dwt for Cry1Ab). The principal route of Vip3Aa and full-length Cry1Ab protein exposure for soil-dwelling organisms (such as collembola, earthworms, and/or rove beetles) is assumed to be from decomposing plant tissue and plant exudates in soil. Consequently, the dose levels of the test material were based on the maximum level of estimated protein expression in the soil environment.

C. Non-Target Wildlife Hazard Assessments for Event COT102 and Event COT67B

Two separate SAP reports (October 2000 and August 2002) recommended that non-target testing of *Bt* Cry proteins should focus on invertebrate species exposed to the crop being registered. Following SAP recommendations, the EPA determined that non-target organisms with the greatest exposure potential to Cry protein in transgenic corn fields are beneficial insects, which feed on corn pollen and nectar, and soil invertebrates, particularly Lepidoptera species. The Agency recommended using this same approach for testing the effects of Vip protein in Event COT102 and Cry protein in Event COT67B on beneficial insects in transgenic cotton fields. Therefore, toxicity testing using the maximum hazard dose on representative beneficial organisms from several taxa was performed in support of both Section 3 FIFRA cotton registrations. The toxicity of the Vip3Aa and Cry1Ab have been evaluated on several species of invertebrates including the lady beetle, minute pirate bug,

collembola, daphnia, honey bee, rove beetle, and/or earthworm. Reproductive and developmental observations were also examined in the lady beetle, rove beetle, minute pirate bug, and honeybee studies.

As previously noted, Vip3Aa protein in Event COT102 and Cry1Ab protein in Event COT67B are very host specific, conferring toxic effects on cotton bollworm, tobacco budworm, fall armyworm, beet armyworm, and cabbage looper. Despite the October 2000 and August 2002 SAP's recommendations against testing of non-target species not related to susceptible target pests, EPA has completed a risk assessment on a range of non-target wildlife to comply with the Agency's published non-target data requirements. In the absence of PIP-specific risk assessment guidance, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins. These requirements include birds, mammals, plants, and aquatic species. In addition, earthworm, springtail, and/or rove beetle studies were voluntarily submitted to the Agency to ascertain the potential effects of Vip3Aa and FLCry1Ab proteins on beneficial decomposer species.

The October 2000 SAP recommended that while actual plant material is the preferred test material, bacterial-derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume cotton plant tissue and where large amounts of Cry protein (Cry protein concentrations that exceed levels present in plant tissue) are needed for maximum hazard dose testing. For Event COT102, an insect feeding study, which compared the relative potency of plant-derived Vip3Aa protein in both Event COT102 cotton and Event Pacha corn to the microbial-derived proteins, indicated that plant-derived protein was similar in toxicity to the microbial-derived protein (MRID No. 458358-12 and Edelstein, 2008). Similarly, for Event COT67B, an insect feeding study, which compared the relative potency of plant-derived FLCry1Ab protein in COT67B cotton to the microbial-derived protein, indicated that plant-derived protein was similar in toxicity to the microbial-derived protein (MRID No. 470176-08 and Edelstein, 2008). Therefore, these data indicate that the microbial-derived proteins for each event are substantially equivalent to the plant-derived proteins expressed in cotton plants based on the similar insecticidal activity for studying any potential toxicity on NTOs for the purposes of the environmental risk assessment.

The Agency has also determined that toxicity studies using corn-derived plant material rather than cotton-derived plant material is acceptable because cotton contains gossypol and other possible plant toxicants that may adversely affect non-target organisms. Furthermore, the non-target species in the cotton agroecosystem are comparable to those in corn; Specifically for Vip3Aa protein toxicity tests, Event COT102 cotton expresses the same *vip3A(a)* gene as is expressed in Event Pacha corn, and the expression level of pollen of Event Pacha corn is much higher than that of Event COT102 cotton.

In support of the COT102 registration, test substances used in the submitted studies included bacterial-produced purified Vip3Aa19 and Vip3Aa1 protein, in addition to Vip3Aa19 as expressed in COT102 cotton pollen and Event Pacha maize grain, pollen, and leaves. Likewise, in support of

the COT67B registration, test substances used in the submitted studies included bacterial-produced purified full-length Cry1Ab and truncated Cry1Ab protein, in addition to Cry1Ab protein as expressed in Event Bt11 maize grain, pollen, and leaves. The individual results for each study on ecological effects for Vip3Aa and Cry1Ab are summarized in Tables 9 and 10, respectively. The results are also presented in a more descriptive format in subsequent sections of the risk assessment document. Full reviews of each study for each event can be found in the individual Data Evaluation Reports (DERs/MRID#s).

Table 9. Summary of environmental effects studies and waiver justifications for COT102 submitted to comply with data requirements published in 40 CFR § 158.2150(d).

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
Avian dietary testing, broiler chicken, *Gallus domesticus*	885.4050	Vip3Aa19 maize grain (Event Pacha)	A 49-day dietary study showed no adverse affects to broiler chickens when fed a 50% diet composed of Event Pacha maize grain (containing VIP3A). Therefore, the NOEC was 0.588 µg VIP3A/g corn feed and the LC_{50} was > 0.588 µg VIP3A/g feed corn grain. **Classification: Acceptable**	470176-23
Avian injection testing	885.4100	N/A	**Acceptable waiver rationale**	N/A
Avian oral testing, bobwhite quail, *Colinus virginianus*	850.2100	Microbial Vip3Aa1 (VIP3A-0198)	A 14-day study showed no adverse effects to bobwhite quail from VIP3A-0198, after a single oral dose via gavage. The NOEL was 400 mg VIP3A/kg and the LD_{50} was > 400 mg VIP3A/kg bird body weight. **Classification: Acceptable**	457665-08
Wild mammal testing	885.4150	N/A	**Acceptable bridging rationale to acute oral toxicity test on mice (MRID No. 457665-05).**	N/A
Freshwater fish testing, channel catfish, *Ictalurus punctatus*	885.4200	Vip3Aa19 maize grain (FFPACHA-0100)	A 30-day study showed no adverse effects on juvenile catfish after exposure to Vip3Aa protein from Event Pacha corn grain. Therefore, the NOEC was 7.10 µg Vip3Aa19/g fish feed and the LC_{50} was > 7.10 µg Vip3Aa19/g **Classification: Acceptable**	470176-24
Freshwater aquatic invertebrate testing, water flea, *Daphnia magna*	885.4240	Vip3Aa19 maize pollen (PHOPACHA-0199)	In a 48-hour static renewal limit bioassay, VIP3A maize pollen (containing 83.8 µg VIP3A protein/g) had no adverse effects on the survival of *Daphnia magna,* when suspended in 120 mg pollen/L. The LC_{50} was > 83.8 µg VIP3A protein/g. **Classification: Unacceptable.** The 885 Series Guidelines call for a 21 day study. The submitted 48 hour acute study is inadequate.	457921-01
Estuarine and marine animal	885.4280	N/A	**Acceptable waiver rationale**	N/A

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
testing				
Non-target plant testing	885.4300	N/A	**Acceptable waiver rationale**	N/A
Non-target insect testing, minute pirate/insidious flower bug , *Orius insidiosus*	885.4340	Microbial Vip3Aa19 (VIP3A-0104)	*Orius insidiosus* nymphs fed a meat-based diet containing microbial-derived 7.25 mg Vip3Aa19 protein/ g diet showed no adverse effects after 21 days. The NOEC was 7.25 mg Vip3Aa19 protein/ g and the LC_{50} was > 7.25 mg Vip3Aa19 protein/ g. **Classification: Acceptable**	468648-14
Non-target insect testing, pink-spotted lady beetle, *Coleomegilla maculata*	885.4340	Vip3Aa19 maize pollen (PHOPACHA-0100)	*Coleomegilla maculata* adults were fed a diet containing 5.0% VIP3A maize pollen (containing 144.8 µg VIP3A protein/g pollen) for 21 days with no adverse effects observed. The NOEC was 7.24 µg VIP3A protein/g pollen and the LC_{50} was > 7.24 µg/g VIP3A/g pollen. **Classification: Acceptable**	457665-09
Non-target insect testing, seven-spotted ladybird beetle, *Coccinella septempunctata*	885.4340	Microbial Vip3Aa19 (VIP3A-0204)	*C. septempunctata* adults fed a 50% sucrose diet containing 7250 µg Vip3Aa19 protein/g diet for showed no adverse effects after 15 days. The NOEC was 7250 µg Vip3Aa19 protein/g diet and the LC_{50} was > 7250 µg/g Vip3Aa19 protein/g diet. **Classification: Acceptable**	468848-02
Non-target insect testing, green lacewing, *Chyrsoperla carnea*	885.4340	Microbial Vip3Aa19 (VIP3A-0104)	*Chyrsoperla carnea* larvae fed a meat-based diet containing 7250 µg Vip3Aa19 protein/g diet showed no adverse effects. The NOEC of 7250 µg Vip3Aa19 protein/g diet and the LC_{50} was > 7250 µg Vip3Aa19 protein/g diet at day 14, when the control mortality reached 20%. There were no statistically significant differences between the VIP3A-0104 group and the negative control group. **Classification: Acceptable**	468848-15
Non-target insect testing, collembolan, *Folsomia candida*	885.4340	Vip3Aa19 maize leaves (LLPACHA-0100)	Collembola were fed a diet containing 50% yeast and 50% leaf tissue for 28 days. No statistically significant effects on survival or reproduction were found among the test and negative control groups. The NOEC was 43.2 µg Vip3Aa19 protein/g diet and the LC_{50} was > 43.2 µg Vip3Aa19 protein/g diet. **Classification: Acceptable**	458358-10
Honeybee testing, Honeybee larvae, *Apis mellifera*	885.4380	Vip3Aa19 maize pollen (PHOPACHA-0199)	Three-to-five day old honeybee larvae were administered a single dose of *ca.*2 mg of pollen moistened with a drop of 30% sucrose solution (containing the equivalent of 168 µg of Vip3Aa) in their individual brood cells. After 19 days, there were no significant differences between the treatment and control groups in survival to capping, survival to emergence of adults, and the behavior and morphology of the emerged adults. The NOEL was	458358-09

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
			83.8 µg Vip3Aa19 protein/g diet and the LD_{50} was > 83.8 µg Vip3Aa19 protein/g diet. **Classification: Acceptable**	
Earthworm toxicity, *Eisenia foetida*	OECD Guideline 207, 850.6200	Vip3Aa19 maize leaves (LPPACHA-0199)	Adult earthworms were exposed to artificial soil containing 3.60 µg VIP3A protein/g soil for 14 days. No mortality or differences in body weights were observed in the test group. The NOEC was 3.60 µg VIP3A protein/g soil and the LC_{50} > 3.60 µg VIP3A protein/g soil. **Classification: Acceptable**	457921-02
Soil fate and degradation	885.5200	Vip3Aa19 maize leaves (LPPACHA-0199)	Results of this degradation study indicate that the DT_{50} of 16 mg/g concentration of the Vip3Aa19 test material protein do not persist in various types of soil from 6 days to 12.6 days via measuring the loss of bioactivity in black cutworm. **Classification: Acceptable**	470176-30

Table 10. Summary of environmental effects studies and waiver justifications for COT67B submitted to comply with data requirements published in 40 CFR § 158.2150 (d).

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
Avian dietary testing, broiler chicken, *Gallus domesticus*	885.4050	Bt11 maize grain	A 42-day dietary study showed no deleterious effects on broiler chicken survival or carcass yield when fed a 50% diet composed of Bt11 maize grain (containing Cry1Ab). **Classification: Acceptable**	4565251-01
Avian injection testing	885.4100	N/A	**Acceptable waiver rationale**	N/A
Avian oral testing, bobwhite quail, *Colinus virginianus*	850.2100	Bt176 Maize leaf protein (LP176-0194)	A 14-day study on bobwhite quail showed no adverse effects after a single oral dose of Bt176 grain, containing Cry1Ab. The NOEL was 140 mg Cry1Ab/kg bodyweight and the LD_{50} was > 140 mg Cry1Ab/kg bodyweight. **Classification: Acceptable**	433236-09
Wild mammal testing	885.4150	N/A	**Acceptable bridging rationale to acute oral toxicity test on mice (MRID No. 47017614)**	N/A
Freshwater fish testing, channel catfish, *Ictalurus punctatus*	885.4200	Microbial FLCry1Ab (FLCRY1AB-0103)	A 30-day study showed no adverse effects to juvenile channel catfish. The NOAEC was 7.10 µg FLCry1Ab/g fish feed and the LC_{50} was > 7.10 µg FLCry1Ab/g fish feed. **Classification: Acceptable**	470176-25
Freshwater aquatic invertebrate,	885.4240	Bt176 maize pollen (PHO176-0194)	In a 48-hour static renewal limit bioassay, Event 176 maize pollen containing 12.36 µg Cry1Ab protein/g had no adverse effects on the survival of *Daphnia magna*,	433236-10

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
water flea neonate, *Daphnia magna*			when suspended in 150 mg pollen/L. The LC_{50} was > 12.36 µg Cry1Ab protein/L. **Classification: Unacceptable.** The 885 Series Guidelines call for a 21 day study. The submitted 48 hour acute study is inadequate	
Estuarine and marine animal testing	885.4280	N/A	**Acceptable waiver rationale**	N/A
Non-target plant testing	885.4300	N/A	**Acceptable waiver rationale**	N/A
Non-target insect testing, predatory bug, *Orius laevigatus*	885.4340	Microbial FLCry1Ab (FLCRY1AB-0103) and Microbial Vip3Aa19 (VIP3A-0204)	*Orius laevigatus* had no adverse effects after fed meat-based artificial diets, containing either 1.0039 mg FLCry1Ab/g diet or 1.0039 mg FLCry1Ab + 0.1950 mg Vip3Aa19/g diet for 14 days. Only the results from the first study were valid. The NOEC for *O. laevigatus* was 1003.9 µg FLCry1Ab/g diet and 1003.9 µg FLCry1Ab + 195.0 µg Vip3Aa19/g diet for Event COT67 and Event COT102 x COT67B cotton leaves, respectively. Furthermore, the LC_{50} was greater than 1003.9 µg FLCry1Ab/g diet and 1003.9 µg FLCry1Ab + 195.0 µg Vip3Aa19/g diet for COT67B and COT102 x COT67B cotton, respectively. **Classification: Supplemental- see discussion below (Section III.2.e.ii)**	470176-28
Non-target insect testing, pink-spotted lady beetle, *Coleomegilla maculata*	885.4340	Microbial FLCry1Ab (FLCRY1AB-0103) and Microbial Vip3Aa19 (VIP3A-0204)	*Coleomegilla maculata* larvae were fed prepared diets containing bee pollen, *Ephestia* eggs, and either FLCRY1AB-0103 (containing 1000 µg FLCry1Ab protein/g diet) or FLCRY1AB-0103 and VIP3A-0204 (containing 1000 µg FLCry1Ab and 250 µg Vip3Aa protein/g diet) for 21 days. No adverse effects were observed on larval, pupal, or adult survival from either test material diet. The NOAEC for FLCry1Ab was 1000 µg FLCry1Ab/g diet and the LC_{50} was greater than 1000 µg FLCry1Ab/g diet. The NOAC for FLCry1Ab + Vip3Aa19 proteins tested in combination was 1000 µg FLCry1Ab/g diet + 250 µg Vip3Aa19/g diet and the LC_{50} was greater than 1000 µg FLCry1Ab/g diet + 250 µg Vip3Aa19/g diet. **Classification: Acceptable**	470176-26
Non-target insect testing, rove beetle, *Aleochara bilineata*	885.4340	Microbial FLCry1Ab (FLCRY1AB-0103)	*A. bilineata* adults were fed a meat diet containing 1298.7 g FLCry1Ab protein/g diet for 35 days with a LC_{50} > 1298.7 g FLCry1Ab /g. Reproductive effects were also assessed by counting the number of second-generation adult beetles emerging from parasitized pupae of the onion fly (*Delia antique).* There were no differences noted between the treatment and negative control groups. **Classification: Acceptable**	470176-27

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
Non-target insect testing, collembolan, *Folsomia candida*	885.4340	Lyophilized Bt11 maize leaf (LLBt11-0100)	Collembola were fed a diet containing 50% yeast and 50% Bt11 leaf tissue for 28 days. No statistically significant effects on survival or reproduction were found among the test and negative control groups. The NOEC for the survival and reproduction of *F. candida* was 17.1 µg Cry1Ab protein/g diet and the LC_{50} was > 17.1 µg Cry1Ab protein/g diet. **Classification: Acceptable**	458358-10
Honeybee testing, *Apis mellifera* larvae, adults, and whole hive conditions	885.4380	Microbial FLCry1Ab (FLCRY1AB-0103)	Honeybees were exposed via oral ingestion to microbial-derived FLCry1Ab test material in a sucrose solution, using in-hive commercial bee feeders. The treatments consisted of: a sucrose solution containing 107.82 mg/L FLCRY1AB-0103 test material/g sucrose solution (representing 92.4 µg FLCry1Ab/mL and 10X EEC in FlCry1Ab in Event COT67B pollen), a negative control of 50% w/v sucrose solution, or a positive control of 6.35 g/L diflubenzuron insect growth regulator in sucrose solution. The test consisted of a single application of one liter of the appropriate solution per hive and the hives were observed for 24 days for percent successful brood development to adults and colony conditions. There was no significant difference in mortality between the test and negative control groups for cells with eggs and young or old larvae. There was also no significant difference in pre- and post-test hive conditions between the test and negative control treatments. Results for the positive control treatment were significantly different from the other treatments. Adult bees were not affected by any of the treatments. Despite some experimental shortcomings, there is enough certainty to indicate exposure of the FLCry1Ab to adult worker honeybees and larvae, via direct and incidental oral ingestion. Furthermore, the results of the study may be considered as weight-of-evidence for determining effects on honeybees for the purposes of the environmental risk assessment. Therefore, the NOEL was 92.4 µg FLCry1Ab/mL and the LD_{50} was greater than 92.4 µg FLCry1Ab/mL. **Classification: Acceptable-for the purposes of the environmental risk assessment**	470176-29
Soil fate and degradation	885.5200	Microbial FLCry1Ab (FLCRY1AB-0103)	The degradation of FLCry1Ab protein (incorporated at a rate equivalent to 80 µg FLCry1Ab/g dry wt soil) in a sandy loam soil was assessed by measuring the loss of bioactivity to European corn borer. The estimated DT_{50} and DT_{90} values were 17 and 52 days, respectively, indicating that FLCry1Ab protein in plant residues incorporated into sandy loam soil is not likely to persist or accumulate in soil. **Classification: Acceptable**	470176-31

Data Requirement	OPPTS Guideline	Test Substance	Results Summary and Classification	MRID No.
3-year Soil Degradation	885.5200	Soil from Bt11 corn cultivated fields	Soil samples were collected from five fields, representing four different soil types, in five different states, in which *Bt* corn expressing Cry1Ab had been grown for three consecutive years. Results showed that European corn borer (ECB) larvae exhibited no toxic response to a diet mixture, containing 15% *Bt* corn soil. Overall, results support use of corn expressing the Cry1Ab protein does not result in the accumulation and persistence of this protein in soil. **Classification: Acceptable**	460224-01

1. Non-target Wildlife Study Summaries for COT102 expressing Vip3Aa

a. Avian species

Published data and studies on file at EPA show that consumption of *Bt* plants have no measurable deleterious effects on avian species. However, to comply with published data requirements, the following studies were submitted to EPA in support of Vip3Aa protein as expressed in Event COT102 product registration. The broiler chicken study was published in a peer-reviewed journal and not subject to GLP standards, while the Northern Bobwhite quail study was GLP compliant. When considered together, these studies meet EPA data requirements for avian species risk assessment.

i. Broiler Chicken (MRID No. 470176-23)

For the first 49 days of life, commercial broiler chickens (*Gallus domesticus*) were fed a prepared diet based on 50% corn grain from transgenic Event Pacha containing VIP3A protein, grain from an isoline non-transgenic corn, or grain from one of two locally grown reference corns. There were no treatment-related differences for mortality, body weight, feed conversion ratio, carcass yield, or clinical chemistry parameters. The diet containing VIP3A had no deleterious effects on broiler performance or carcass yield. A separate study determined the concentration of the transgenic Event Pacha grain as 0.588 µg Vip3Aa19/g feed for this study (MRID No. 470176-20). Therefore, the NOEC was 0.588 µg VIP3A/g feed and the 49-day LC_{50} for broilers is greater than 0.588 µg VIP3A/g feed.

Conclusions/Recommendations: No adverse effects were observed on *Gallus domesticus* after a 49-day chronic dietary study after exposure to a 50% diet containing Event Pacha corn grain, expressing VIP3A. The NOEC was 0.588 µg VIP3A /g feed and the LC_{50} for broilers is greater than 0.588 µg VIP3A /g feed. Based on the information presented, this study is acceptable.

ii. Northern Bobwhite Quail (MRID No. 457665-08)

Five male and five female (*Colinus virginianus*) quails were administered a single oral dose of 2000 mg VIP3A-0198 /kg, via gelatin capsules. The VIP3A-0198 test substance (microbial-derived protein) represented 400 mg VIP3A /kg body weight. No mortalities occurred during the study period. There were no clinical signs of toxicity in any birds during the study. There were no statistically significant changes in body weights after dosing. Additionally, gross pathological examinations of all birds at study termination revealed no abnormalities. The results indicate that the NOEL was 400 VIP3A mg/kg and the 14-day LD_{50} was > 400 VIP3A mg/kg body weight for northern bobwhite for 14 days.

Conclusions/Recommendations: No adverse effects or mortalities were found after a 14-day acute oral study after exposure to the test substance (VIP3A-0198, microbial-derived containing Vip3Aa1). The NOEL was 400 VIP3A mg/kg and the 14-day LD_{50} was > 400 VIP3A mg/kg body weight for northern bobwhite for 14 days. Based on the information presented, this study is acceptable.

b. Wild mammalian species

Mammalian wildlife exposure to Vip3Aa protein is considered likely; however, mammalian toxicology information gathered to date on *Bt* Cry and Vip proteins does not show a hazard to wild mammals. In addition, acute oral toxicity studies submitted to EPA in support of the COT102 registration indicated that no significant toxicity was seen when rodents were exposed to microbial-derived Vip3Aa19 (VIP3A-0100) protein at the maximum hazard dose level (MRID No. 457665-05). Therefore, no hazard from COT102 cotton expressing Vip3Aa protein to mammalian wildlife is anticipated and data on wild mammal testing is not required for this registration.

c. Aquatic species

There is no reported toxicity to aquatic organisms from exposure to anti-coleopeteran Cry proteins in *Bt* plants. However, a published laboratory study with lepidopteran-active Cry proteins has revealed that the leaf shredding (caddis fly) trichopteran, *Lepidostoma liba*, had 50% lower growth rate when fed *Bt* corn litter (Rosi-Marshall, et al. 2007). Two previous field study reports by the same authors did not find adverse effects on head stream invertebrates. The Agency's position on this matter is that until Tier III and Tier IV field studies are performed, there is not enough information to assert that sufficient corn plant litter enters streams to cause unreasonable adverse effects on stream invertebrate populations or communities (See Section B.I. above - Tiered Testing Hazard and Risk Assessment Process). Two years ago the Iowa State University and the University of Maryland received Research grants to study the effects of *Bt* corn cultivation on streams and to develop methods for aquatic hazard assessment. The results of these studies are pending. When the study reports are reviewed the Agency will respond with action commensurate with the outcome of the

studies. Therefore, the Agency's current position is that there is no evidence to conclude that there is sufficient aquatic exposure to Cry proteins in corn plant litter to result in adverse effects on stream invertebrate populations or communities. In regards to *Bt* cotton plant litter expressing lepidopteran-active Vip proteins, the Agency maintains the same position at this time.

Farmed fish may be exposed to *Bt* protein in fish feed. However, *Bt* protein activity is generally destroyed during typical fish food manufacturing processes due to protein degradation from with the high temperatures. Consequently, exposure of farmed fish to active *Bt* proteins is not expected. Overall, aquatic animal exposure to *Bt* crops is extremely small.

i. Freshwater fish - Channel Catfish (MRID No. 470176-24)

The objective of this study was to determine the potential for adverse effects of Vip3Aa protein to freshwater fish, using the channel catfish, *Ictalurus punctatus*, as a representative test species, in a 30-day feeding study. The study compared survival and growth of juvenile channel catfish fed commercial fish feed formulated with transgenic maize grain with test substance FFPACHA-0100 (containing 7.1 µg Vip3Aa19 protein/g diet) or with non-transgenic maize grain for 30 days. Both feeds contained approximately 50% maize grain by weight. The diet was formulated using a "cold pelleting" process to minimize exposure to temperatures that might degrade VIP3A protein. The formulation, nutrient composition, characterization, homogeneity, and stability of the fish feed test substance was also analyzed. After 30 days, there was no test material-related mortality. Fish fed either the VIP3A maize grain or the control maize grain gained equal amounts of weight, and no abnormal behavior was observed in either group. The activity and stability of VIP3A in grain and fish feed was confirmed via fall armyworm insect bioassay and analyzed by ELISA to confirm the presence and amount of the test material. There were no adverse effects on growth or behavior of juvenile catfish exposed for 30 days. Therefore, the NOEC was 7.1 µg Vip3Aa19/g diet and the 30-day LC_{50} was greater than 7.1 µg Vip3Aa19/g diet fish feed made from Event Pacha maize grain.

Conclusions/Recommendations: No observed adverse effects were noted in *Ictalurus punctatu* after exposure to Vip3Aa via commercial feed formulated from Event Pacha grain. The NOEC was 7.1 µg Vip3Aa19/g diet and the LC_{50} was greater than 7.1 µg Vip3Aa19/g diet. Based on the information presented, this study is acceptable.

ii. Freshwater aquatic invertebrates (MRID No. 457921-01)

The objective of this study was to determine the potential for acute effects to the aquatic organism, *Daphnia magna*, during a static renewal exposure to VIP3A via the Pacha maize pollen. The test was conducted as a limit test using test substance PHOPACHA-0199, containing 83.8 µg VIP3A protein/g pollen. Daphnids were exposed to a single nominal test concentration of 120 mg pollen/L for 48 hours with renewal of the test solution at approximately 24 hours. Two control groups were included: a group in water exposed to pollen (120 mg/L) from non-transgenic, near-isogenic maize, and an assay control group exposed to water only. Each treatment was replicated three times and

each replicate contained 10 neonate daphnids. Observations of mortality, immobility and other sub-lethal effects were made during the test. At test termination, there was 100% survival in each group with no sign of immobilization or any other adverse effects. Therefore, the NOEC was 120 mg VIP3A pollen/L and the LC_{50} was greater than 120 mg VIP3A pollen/L.

Conclusions/Recommendations: Results of the 48-hour limit test showed the LC_{50} was greater than 120 mg PHOPACHA-0199/L, representing 10.1 µg VIP3A /L. Based on the information presented, this study is unacceptable. The 48 hour test duration is not sufficient to show mortality for *Bt* toxins. The mode of action of the toxin would take more than 48 hours for target insect pests to succumb to Cry proteins, therefore, mortality or reproductive effects to aquatic invertebrates (e.g., daphnids) are not expected to show within 48 hours. Because Vip proteins are also derived from *Bt* and susceptible species display similar symptoms upon ingestion, a 7-14 day *Daphnia* study (OPPTS Guideline 885.4240 Series) must be performed. This study can be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the 7-14 day *Daphnia* study.

iii. Estuarine and marine animals - *Waiver granted*

Estuarine and marine animal studies were not required for this product, because of the low probability that estuarine or marine systems will be exposed to Vip3Aa protein produced in event COT102 cotton plant tissues and pollen.

d. Terrestrial and aquatic plant species - *Waiver granted*

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

e. Invertebrate species

The Vip3Aa protein is meant to target species within the order Lepidoptera (moths and butterflies). *Bt* toxins are known to have a limited host range, however, to address any unforeseen change in activity spectrum as a result of laboratory protein synthesis and to fulfill the published registration data requirements EPA requires that test species used for non-target insect evaluations should include several species that are not related to the target pests. Earthworm studies are also recommended.

i. Ladybird beetle

MRID No. 457665-09

The purpose of this study was to determine the potential dietary effects of the Vip3Aa protein on the mortality and development of the ladybird beetle, *Coleomegilla maculata*. The protocol for the non-target lady beetle study was based on OPPTS Guideline 885.4340. Eight- to nine-day old ladybird beetles were exposed to Vip3Aa via Pacha maize pollen test substance (PHOPACHA-0100), incorporated into an artificial diet at 5% weight by weight (w/w). The negative control diet comprised 5% w/w pollen from non-transgenic, near-isogenic maize, and a positive control diet contained 50 µg thiobendacarb/g diet. The treatment and control groups each comprised three replicates of 25 beetles, which received fresh diet daily. After 21 days, there were no statistically significant differences in survival, development, and growth between the treatment and negative control groups ($P \leq 0.05$), while there was 100% mortality in the positive control group. Therefore, the NOEC was 7.24 µg Vip3Aa19/g diet and the LC_{50} was greater than 7.24 µg Vip3Aa19/g diet.

Conclusions/Recommendations: The results indicate that Vip3Aa protein had no adverse effect on the survival, development, and growth of the ladybird beetles. The NOEC was 7.24 µg Vip3Aa19/g of diet and the LC_{50} was greater than 7.24 µg Vip3Aa19/g of diet. This study was previously reviewed and found acceptable (Rose and Vaituzis, 2003).

MRID No. 468848-02

The objective of this study was to determine the potential dietary effects of Vip3Aa protein on the mortality and development of the five-spotted ladybird beetle, *Coccinella septempunctata.* The test substance, VIP3A-0204, was produced by recombinant *E. coli* fermentation system and contained 7.25 mg Vip3Aa19/g before addition to a 50% sucrose diet. The negative control diet comprised of sucrose only, and a positive control diet contained 0.3333 mg dimethoate/g diet. Treatment and control groups, each comprising of 40 beetles, were fed fresh diet daily and the endpoints evaluated were survival and development through 15 days. At study end, mortality in the Vip3Aa-treated group was not statistically significantly different from that of the untreated controls (0% vs. 5%, respectively). Positive control mortality was 100%. The NOEC was 7.25 mg Vip3Aa19 protein/g diet and the LC_{50} was > 7.25 mg Vip3Aa19 protein/g diet.

Conclusions/Recommendations: No adverse effects were seen in *C. septempunctata* after exposure to Vip3Aa protein in a sucrose diet. The NOEC was 7.25 mg Vip3Aa19 protein/g diet and LC_{50} was > 7.25 mg Vip3Aa19 protein/g diet. This study was previously reviewed and found acceptable (Milofsky and Vaituzis, 2007).

ii. Minute pirate bug (MRID No. 468848-14)

The purpose of this study was to determine the potential dietary effects of Vip3Aa protein on mortality and development of *Orius insidiosus*, the minute pirate bug or insidious flower bug.

The test substance was VIP3A-0104, a 63.1 % pure preparation of microbial-derived Vip3Aa19. The test substance was dissolved in buffer and incorporated at a rate of 11.49 mg/g diet (7.25 mg Vip3Aa19/g of artificial diet -- approximately 310X the highest mean concentration of Vip3Aa in COT102) and was continuously supplied to predatory bug (*Orius insidiosus*) nymphs for 21 days. Control nymphs were fed untreated diet, and positive control nymphs were fed diet treated with 10 µg teflubenzuron/g of diet. At study end, mortality in the Vip3Aa treated nymphs was not significantly different from that of the untreated controls (15% vs. 13%, respectively). Positive control mortality was 100%. The NOEC was 7.25 mg Vip3Aa19 protein/g diet and the LC_{50} value was determined to be greater than 7.25 mg Vip3Aa19 protein/g diet.

Conclusions/Recommendations: No adverse effects were seen in *Orius insidiosus* after exposure to Vip3Aa protein in an artificial diet. The NOEC was 7.25 mg Vip3Aa19 protein/g diet and the LC_{50} value was determined to be greater than 7.25 mg Vip3Aa19 protein/g diet. This study was previously reviewed and found acceptable (Milofsky and Vaituzis, 2007).

iii. Green Lacewing (MRID No. 468848-15)

The purpose of this study was to determine the potential dietary effects of Vip3Aa protein on mortality and development of *Chrysoperla carnea* larvae, the green lacewing. The test substance, VIP3A-0104, consisted of 7.25 mg aVip3Aa19/g of artificial diet was continuously supplied to green lacewing (*Chrysoperla carnea*) larvae for 21 days. Control larvae were fed untreated diet, and positive control larvae were fed diet treated with 10 µg teflubenzuron/g diet. At study end, mortality in the Vip3Aa-treated larvae was not statistically significantly different from that of the untreated controls (37.5% vs. 35.0%, respectively). Positive control mortality was 100%. Although the control mortality exceeded the 25% criterion for the test to be considered valid, mortality did not differ significantly between the test and control groups. Furthermore, the control mortality was <25% through day 21, which was judged to be a sufficient exposure period to observe acute and developmental effects on lacewing larvae. Therefore, the NOEC was 7.25 mg Vip3Aa19 protein/g diet and the LC_{50} value was greater than 7.25 mg Vip3Aa19 protein/g diet.

Conclusions/Recommendations: No adverse effects were seen in *Chrysoperla carnea* after exposure to Vip3Aa protein mixed in an artificial diet. The NOEC was 7.25 mg Vip3Aa19 protein/g diet and the LC_{50} value was determined to be greater than 7.25 mg Vip3Aa19 protein/g diet. This study was previously reviewed and found acceptable (Milofsky and Vaituzis, 2007).

iv. Collembola (MRID No. 458358-10)

The purpose of this study was to determine the potential dietary effects of Vip3Aa protein on mortality and reproduction on *Folsomia candida* (springtail; Collembola). The test substances included: LLPACHA-0100, containing 43.4 µg Vip3Aa19 protein/g leaf tissue diet from Event Pacha, distilled water as a negative control and thiodicarb as a positive control. There were 4

replicates of 10 juvenile collembola per replicate per treatment and fresh diet was provided daily. Vip3Aa protein had no detectable impact on the survival or reproduction of the collembola after 28 days of continuous exposure. The NOEC of lyophilized Vip3Aa protein from Event Pacha corn leaves was 50% of the diet which was the highest concentration tested. Therefore, the NOEC was 43.4 µg Vip3Aa19 protein/g diet and the LC_{50} was greater than 43.4 µg Vip3Aa19 protein/g diet.

Conclusions/Recommendations: No adverse effects were seen on *Folsomia candida* after exposure to Vip3Aa protein in Event Pacha maize leaf tissue. The NOEC was 43.4 µg Vip3Aa19 protein/g diet and the LC_{50} was greater than 43.4 µg Vip3Aa19 protein/g diet. This study was previously reviewed and found acceptable (Rose and Vaituzis, 2003).

v. Honeybee (MRID No. 458358-09)

The objective of this study was to evaluate potential dietary effects of transgenic Vip3Aa pollen from Event Pacha corn on honeybee larvae (*Apis mellifera*) survival and adult emergence in a single dose study. The test substance (PHOPACHA-0199) contained 2 mg of pollen moistened with 30% sucrose solution and was estimated to contain 83.8 µg Vip3Aa19/g pollen. The study included three controls: one group of larvae were fed 2 mg inbred maize pollen (PIPACHA-0299C) moistened with 30% sucrose solution, one group received 2 mg inbred maize pollen (PIPACHA-0299C) moistened with 30% sucrose solution and mixed with potassium arsenate at 1000 ppm (positive control), and the third group received no treatment at all. Eighty, three- to five-day old larvae (four replicates of 20) were allowed to consume the pollen and then returned to their source hives for capping of the brood cells by nurse bees. The hives were maintained under natural environmental conditions. After 19 days, mean survival to capping and mean survival to adult emergence were 76.3% in the Vip3Aa corn pollen group and 77.5% in the control corn pollen group. Mean survival to capping and mean survival to adult emergence were 87.5% for the negative control group. There were no statistically significant differences among these three study groups. Mean survival to capping and mean survival to adult emergence were 20% in the positive control group, which was statistically significantly lower than in the other two study groups. No behavioral or morphological abnormalities were noted among the emerged adult bees, and no differences in mean emergence times were observed. Therefore, no adverse effects from Vip3Aa pollen in Event Pacha corn were seen on honeybee larvae and adult emergency. The NOEC was 83.8 µg Vip3Aa19/g pollen and the LC_{50} was greater than 83.8 µg Vip3Aa19/g pollen.

Conclusions/Recommendations: No adverse effects from Vip3Aa pollen in Event Pacha corn were seen on the survival of *Apis mellifera* honeybee larvae and adult emergence. The NOEC was 83.8 µg Vip3Aa19/g pollen and the LC_{50} was greater than 83.8 µg Vip3Aa19/g pollen. This study was previously reviewed and found acceptable (Rose and Vaituzis, 2003).

vi. Earthworm (MRID No. 457921-02)

The objective of this study was to evaluate the potential effects of Vip3Aa from Event Pacha administered to earthworms (*Eisenia fetida*) via an artificial soil substrate during a 14-day exposure period. The testing was conducted based on OPPTS Series 850.6200 Earthworm Sub-chronic Toxicity Test and OECD Guideline 207. In the test, earthworms were exposed to a single concentration of VIP3A protein derived from Event Pacha maize leaf (test substance LPPACHA-0199) and incorporated into an artificial soil substrate at 3.60 µg VIP3A/g soil. There were no mortalities in the assay control group, buffer control group, or VIP3A protein group. Analysis of the test soil showed that VIP3A was present in the soil and was biologically active against *Agrotis ipsilon* (black cutworm). Therefore, no adverse effects on earthworms were observed after exposure to VIP3A protein via Event Pacha maize leaf tissue. The NOEC was 3.60 µg VIP3A protein/kg dry soil and the14-day LC_{50} for earthworms was determined to be greater than 3.60 µg VIP3A protein/kg dry soil.

Conclusions/Recommendations: No adverse effects from Vip3Aa maize leaf tissue in Event Pacha were seen on the survival of *Eisenia fetida,* via an artificial soil substrate after 14 days. The NOEC was 3.60 µg VIP3A protein/kg dry soil and the14-day LC_{50} for earthworms was determined to be greater than 3.60 µg VIP3A protein/kg dry soil. Based on the information presented, this study is acceptable.

2. Non-target Wildlife Study Summaries for COT67B expressing FLCry1Ab

a. Avian species

Published data and studies on file at EPA show that consumption of *Bt* plants have no measurable deleterious effects on avian species. However, to comply with published data requirements, the following studies were submitted to EPA in support of Event COT67B registration. The broiler chicken study was published in a peer-reviewed journal and not subject to GLP standards, while the Northern Bobwhite quail study was GLP compliant. When considered together, these studies meet EPA data requirements for avian species.

i. Broiler Chicken (MRID No. 456521-01)

For the first 42 days of life, commercial broiler chickens (*Gallus domesticus*) were fed a prepared diet based on 50% grain from either transgenic Bt11 corn containing Cry1Ab protein, transgenic Bt11 corn sprayed with Liberty herbicide, grain from an isoline, non-transgenic corn, or grain from a locally grown reference corn. There were no treatment-related differences for mortality, body weight, feed conversion ratio, carcass yield, or clinical chemistry parameters. The corn diet containing the test substance had no deleterious effects on broiler performance or carcass yield in this study. It should also be noted that the concentration of Cry1Ab in Bt11 grain used to formulate

the diet was 0.8 µg/g seed, however, the concentration of Cry1Ab in the feed was not determined. In a similar broiler chicken study, the concentration of Cry1Ab in Bt176 corn was less than 0.005 µg/g grain. Therefore, while an official NOEC was not determined, exposure to Cry1Ab was very likely during the experiment since it is expected that Cry1Ab in Bt11 grain would behave similarly to Cry1Ab in Bt176 grain during preparation of broiler diets.

Conclusions/Recommendations: No adverse effects were found in the 42-day dietary study with *Gallus domesticus* when fed transgenic Bt11 grain, containing Cry1Ab. This study was previously reviewed and found acceptable (Hunter and Vaituzis, 2007).

ii. Northern Bobwhite Quail (MRID No. 433236-09)

Five male and five female juvenile bobwhite quails (*Colinus virginianus*) were administered a single oral dose of 140 mg of Cry1Ab protein/kg body weight, via oral gavage. The test substance was LP176-0194 (Bt176 maize leaf protein). No mortalities occurred during the study period. There were no clinical signs of toxicity in any birds during the study. There were no statistically significant changes in body weights at any weighing interval (3, 7 or 14 days) after dosing. Additionally, gross pathological examinations of all birds at study termination revealed no abnormalities. The results indicate that the NOEL was 140 mg of Cry1Ab protein/kg body weight and the 14-day LD_{50} was greater than 140 Cry1Ab mg/kg body weight for bobwhite quail.

Conclusions/Recommendations: No adverse effects were found in the 14-day dietary study with *Colinus virginianus* when administered a single oral dose of Vip3A. The NOEL was 140 mg of Cry1Ab protein/kg body weight and the 14-day LD_{50} was greater than 140 Cry1Ab mg/kg body weight for bobwhite quail. This study was reassessed in the 2001 *Bt* PIPs Reassessment BRAD (US EPA, 2001b).

b. Wild mammalian species

Mammalian wildlife exposure to Cry1Ab protein is considered likely; however, mammalian toxicology information gathered to date on *Bt* Cry proteins does not show a hazard to wild mammals. In addition, acute oral toxicity studies submitted to EPA in support of the COT67B registration indicated that no significant toxicity was seen when rodents were exposed to microbial-derived full-length Cry1Ab (FLCRY1AB-0103) protein at the maximum hazard dose level (MRID No. 470176-14). Therefore, no hazard to mammalian wildlife is anticipated and data on wild mammal testing is not required for this registration.

c. Aquatic species

There is no reported toxicity to aquatic organisms from exposure to anti-coleopteran Cry proteins in *Bt* plants. However, a published laboratory study with lepidopteran-active Cry proteins has revealed that the leaf shredding (caddis fly) trichopteran, *Lepidostoma liba*, had 50% lower growth rate when

fed *Bt* corn litter (Rosi-Marshall, et al. 2007). Two previous field study reports by the same authors did not find adverse effects on headwater stream invertebrates. The Agency's position on this matter is that until Tier III and Tier IV field studies are performed, there is not enough information to assert that sufficient corn plant litter enters streams to cause unreasonable adverse effects on stream invertebrate populations or communities (See Section B.I. above - Tiered Testing Hazard and Risk Assessment Process). Two years ago the Iowa State University and the University of Maryland received Research grants to study the effects of *Bt* corn cultivation on streams and to develop methods for aquatic hazard assessment. The results of these studies are pending. When the study reports are reviewed, the Agency will respond with action commensurate with the outcome of the studies. Therefore, the Agency's current position is that there is no evidence to conclude that there is sufficient aquatic exposure to Cry proteins in corn plant litter to result in adverse effects on stream invertebrate populations or communities. In regards to lepidopteran-active *Bt* cotton plant litter, the Agency maintains the same position at this time.

Farmed fish may be exposed to *Bt* protein in fish feed. However, *Bt* protein activity is generally destroyed during typical fish food manufacturing processes due to protein degradation in high temperatures associated and consequently, exposure of farmed fish to active *Bt* proteins is not expected. Overall, aquatic animal exposure to *Bt* crops is negligible.

i. Freshwater fish- Channel Catfish (MRID No. 470176-25)

The objective of this study was to determine the potential for adverse effects of full-length Cry1Ab to freshwater fish, using the channel catfish, *Ictalurus punctatus*, as a representative test species in a 28 day feeding study. The study compared survival and growth of juvenile channel catfish fed commercial catfish diet containing a purified preparation of FLCRY1AB-0103 (a microbial-derived test substance, representing 15.4 µg FLCry1Ab protein/g diet) or standard untreated diet for 28 days. The diet was formulated using a "cold pelleting" process to minimize exposure to temperatures that might degrade FLCry1Ab protein. After 28 days, no mortalities or abnormalities were seen in fish either fed the test material or control diet. The activity and stability of FLCry1Ab fish feed was confirmed via European corn borer insect bioassay and analyzed by ELISA to confirm the presence and amount of the test material in a separate study. Overall, there were no adverse effects and no mortality observed for juvenile catfish fed with the commercial catfish diet containing FLCry1Ab after 28 days. The NOAEC was 15.4 µg FLCry1Ab/g fish food diet and the LD_{50} was greater than 15.4 µg FLCry1Ab/g diet for juvenile channel catfish.

Conclusions/Recommendations: No observed adverse effects were noted in *Ictalurus punctatus.* Therefore, the NOAEC was 15.4 µg FLCry1Ab/g fish food diet and the LD_{50} was greater than 15.4 µg FLCry1Ab/g diet for juvenile channel catfish. Based on the information presented, this study is acceptable.

ii. Freshwater aquatic invertebrates (MRID No. 433236-10)

The objective of this study was to determine the potential for acute effects to the aquatic organism, *Daphnia magna*, during a static renewal exposure to Cry1Ab via the Bt176 maize pollen test substance (PHO176-0194- containing 12.36 µg Cry1Ab/g). The test was conducted as a limit test using one test concentration, representing 1.85 µg Cry1Ab/L. Daphnids were exposed to a single nominal test concentration of 150 mg pollen/L for 48 hours with renewal of the test solution at approximately 24 hours. Two control groups were included: a group in water exposed to pollen (150 mg/L) from non-transgenic, near-isogenic maize, and an assay control group exposed to water only. Each treatment was replicated three times and each replicate contained 10 neonate daphnids. Observations of any mortality, immobility and other sub-lethal effects were recorded. At test termination there was 100% survival in each group with no sign of immobilization or other effects. The NOEC was 150 mg PHO176-0194/L and the LC_{50} was greater than 150 mg PHO176-0194/L, representing 1.85 µg Cry1Ab/L.

Conclusions/Recommendations: After 48 hours, the results of the limit test showed no adverse effects to Daphinia. The NOEC was 150 mg PHO176-0194/L and the LC_{50} was greater than 150 mg PHO176-0194/L, representing 1.85 µg Cry1Ab/L. However, based on the information presented, this study is unacceptable. The 48 hour test duration is not sufficient to show mortality for *Bt* toxins. The mode of action of the toxin would take more than 48 hrs. for target insect pests to succumb to Cry proteins, therefore, mortality or reproductive effects to aquatic invertebrates e.g., daphnids, are not expected to show within 48 hours. Therefore, a 7-14 day Daphnia study (OPPTS Guideline 885.4240 Series) needs to be performed. This study can be submitted as a condition of registration. Alternatively, a dietary study of the effects Cry1Ab on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, may be performed and submitted in lieu of the 7-14 day Daphnia study.

iii. Estuarine and marine animals-*Waiver granted*

Estuarine and marine animal studies were not required for this product, because of the low probability that estuarine or marine systems will be exposed to Cry1Ab protein produced in event COT67B cotton plant tissues and pollen.

d. Terrestrial and aquatic plant species-*Waiver granted*

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

e. Invertebrate species

The Cry1Ab protein is meant to target species within the order Lepidoptera (moths and butterflies). *Bt* toxins are known to have a limited host range; however, to address any unforeseen change in activity spectrum as a result of laboratory protein synthesis and to fulfill the published registration

data requirements, EPA requires that test species used for non-target insect evaluations should include several species that are not related to the target pests. Earthworm studies are also recommended.

i. Ladybird beetle (MRID No. 470176-26)

The purpose of this study was to determine the potential dietary effects of FLCry1Ab protein test alone and FLCry1Ab and Vip3Aa19 tested in combination on the survival and development of the pink-spotted ladybird beetle (*Coleomegilla maculata*). The protocol for the non-target lady beetle study was based on OPPTS Guideline 885.4340. Five-day old, second instar ladybird beetles were exposed to a diet of bee pollen, *Esphestia* (moth) eggs, and either FLCRY1AB-0103 test material (at 1000 µg FLCry1Ab/g diet) or FLCRY1AB-0103 + VIP3A-0204 test materials (at 1000 µg FLCry1Ab/g diet + 250 µg Vip3Aa19/g diet). The negative control diet was the pollen and moth egg diet only, and a positive control diet contained 250 µg potassium arsentate/g diet. The treatment and control groups each comprised of 40 beetles, which received fresh diet every other day. After 21 days, there were no statistically significant differences in larval, pupal, and adult survival between the treatment and negative control groups ($P \leq 0.05$), while there was 100% mortality in the positive control group. Therefore, the NOAEC for FLCry1Ab was 1000 µg FLCry1Ab/g diet and the LC_{50} was greater than 1000 µg FLCry1Ab/g diet. The NOAC for FLCry1Ab + Vip3Aa19 proteins tested in combination was 1000 µg FLCry1Ab/g diet + 250 µg Vip3Aa19/g diet and the LC_{50} was greater than 1000 µg FLCry1Ab/g diet + 250 µg Vip3Aa19/g diet.

Conclusions/Recommendations: The results indicate that the FLCry1Ab protein tested alone or in combination with Vip3Aa19 had no adverse effect on the survival, development, and growth of the ladybird beetles. In conclusion, the NOAEC for FLCry1Ab was 1000 µg FLCry1Ab/g diet and the LC_{50} was greater than 1000 µg FLCry1Ab/g diet. The NOAC for FLCry1Ab + Vip3Aa19 proteins tested in combination was 1000 µg FLCry1Ab/g diet + 250 µg Vip3Aa19/g diet and the LC_{50} was greater than 1000 µg FLCry1Ab/g diet + 250 µg Vip3Aa19/g diet. Based on the information presented, this study is acceptable.

ii. Minute pirate bug (MRID No. 470176-28)

The purpose of this study was to determine the potential dietary effects of FLCry1Ab protein as expressed in Event COT67B and FLCry1Ab and Vip3Aa19 proteins tested in combination, as expressed in Event COT102 X COT67B, on mortality and development of *Orius laevigatus*, a predatory bug which is closely-related and ecologically very similar to *O. insidiosus*.

The report contained two dietary studies studying the effects on *O. laevigatus*, after exposure via meat-based artificial diets containing either FLCry1Ab insecticidal protein alone or in combination with Vip3Aa19 insecticidal protein. Only the results of the second run of the first sudy were considered valid and are presented in this summary. After 14 days, *O. laevigatus* fed 1.0039 mg

FLCry1Ab/g diet (7X the maximum concentration in COT67B cotton leaves) had pre-imaginal mortality of 17.95%. *O. laevigatus* fed the combined proteins of 1.0039 mg FLCry1Ab + 0.1950 mg Vip3Aa19/g diet (corresponding to 10X the highest mean concentrations of FLCry1Ab and Vip3Aa19 found in COT67B and COT02 cotton leaves) had pre-imaginal mortality of 39.47%, which was a statistically significant increase in mortality. The control pre-imaginal mortality was 12.82%, while the toxic reference standard had 100% mortality, as expected. The NOEC for *O. laevigatus* was 1003.9 µg FLCry1Ab/g diet and 1003.9 µg FLCry1Ab + 195.0 µg Vip3Aa19/g diet for Event COT67 at 7X EEC and Event COT102 x COT67B at 10X EEC cotton leaves, respectively. Furthermore, the LC_{50} was greater than 1003.9 µg FLCry1Ab/g diet and 1003.9 µg FLCry1Ab + 195.0 µg Vip3Aa19/g diet for COT67B and COT102 x COT67B cotton, respectively.

Conclusions/Recommendations: The overall results of the two studies were inconsistent due to the high control mortality, implicating the use of *Orius laevigatus* is equivocal, as a representative indicator species for studying the effects of *Bt* PIP proteins. In the only valid study, there was a statistically significant increase in mortality of *O. laevigatus* exposed to FLCYR1AB-0103 + VIP3A-0204 at 10X EEC for COT102 x COT67B cotton leaves, which may represent an interaction between FLCry1Ab and Vip3Aa19. However, the EPA established Level of Concern (LOC) is 50% mortality when tested at 5X EEC and a less than 50% mortality effect at the MHD is indicative of a minimal risk for the purposes of the environmental risk assessment (US EPA, 1998). Therefore, no adverse effects on *O. laevigatus* are expected at concentrations encountered in field crops. The NOEC for *O. laevigatus* was 1003.9 µg FLCry1Ab/g diet and 1003.9 µg FLCry1Ab + 195.0 µg Vip3Aa19/g diet for Event COT67 at 7X EEC and Event COT102 x COT67B at 10X EEC cotton leaves, respectively. Furthermore, the LC_{50} was greater than 1003.9 µg FLCry1Ab/g diet and 1003.9 µg FLCry1Ab + 195.0 µg Vip3Aa19/g diet for COT67B and COT102 x COT67B cotton, respectively. Based on the information submitted, this study is supplemental for the purposes of the environmental risk assessment.

In addition, a three-year field study conducted on Event Bt11 x Event Pacha maize (expressing Cry1Ab and VIP3A proteins) showed no differences on densities of non-target arthropod communities, including *Orius insidiosus,* when compared with an isogenic conventional corn control (Dively *et al.* 2005). The results also showed that biodiversity and community-level responses were not significantly affected by expression of the stacked VIP3A and Cry1Ab proteins.

When the results of the second run of the first study on *O. laevigatus* are considered in combination with the three-year field Dively, *et al.* (2005) study, the weight-of-evidence indicates there are no adverse effects on *Orius* species from FLCry1Ab protein as expressed in COT67B or its associated stacked product, Event COT102 x COT67B cotton. The Agency also notes that there are several published field studies on the effects of *B*t crops on insect predators showing no significant differences in the density of beneficial insects, including *Orius insidiosus* (Pilcher *et a*l., 1997a, 1997b, and 2005; and Al-Deeb *et al*., 2001).

iii. Rove Beetle (MRID No. 470176-27)

The purpose of this study was to determine any reproductive effects of FLCry1Ab protein on *Aleochara bilineata* (rove beetle). In a laboratory bioassay, adult rove beetles (*Aleochara bilineata*) were exposed to a prepared meat diet containing 1298.7 g FLCRY1AB-0103/g of diet for 35 days. The FLCry1Ab concentration fed to the beetles was approximately 10 times that which occurs in fresh leaf tissue of Event COT67B cotton plants. A negative control diet and a reference control diet were also included in the test. To assess reproduction of the beetles, onion fly (*Delia antique*) pupae were provided to be parasitized by the beetles during the test. Second-generation beetles emerging from the parasitized pupae were counted until emergence stopped on test day 86. The results of the reproductive success of the beetles showed no statistically significant differences between the number of beetles that emerged from the FLCry1Ab test treatment, when compared to the control. The IOBC validity criteria were met (Grimm, *et al.,* 2000) and the stability and bioactivity of the test material in the prepared diet were also confirmed. Therefore, no adverse effects were noted on the reproductive effects of FLCry1Ab protein on *A. bilineata*. Furthermore, the NOEC was 1000 µg FLCry1Ab/g diet for the reproduction of *Aleochara bilineata* and the LC50 was greater than 1000 µg FLCry1Ab/g diet, when exposed orally via a treated meat-based diet

Conclusions/Recommendations: No adverse effects were noted on the reproductive effects of FLCry1Ab protein on rove beetles. Therefore, the NOEC was 1000 µg FLCry1Ab/g diet for the reproduction of *Aleochara bilineata* and the LC_{50} was greater than 1000 µg FLCry1Ab/g diet, when exposed orally via a treated meat-based diet. Based on the information presented, this study is acceptable.

iv. Collembola (MRID No. 458358-10)

The purpose of this study was to determine the potential dietary effects of Cry1Ab protein on mortality and reproduction on *Folsomia candida* (springtail; Collembola). The treatments included: 17.1 µg Cry1Ab/g diet of equal parts LLBt11-0100 test substance (lyophilized leaf from Bt11 maize) and yeast, a control diet containing equal parts yeast and lyophilized leaves of non-transgenic, near-isogenic mazie, a diet to control the effects of maize leaves consisting of yeast only, and a positive control of yeast with 500 µg thiodicarb/g diet. There were 4 replicates of 10 juvenile collembola per replicate per treatment and fresh diet was provided daily.

After 28 days, mean survival was 83%, 78%, and 80% in the LLBt11-0100-treated group, the non-transgenic maize leaf-treated group, and the group fed yeast only, respectively. The mean survival for the positive control group was 3%, which was statistically significant from the other treatment groups. The mean number of juveniles was 446.5, 343.5, and 218.5 in the LLBt11-0100-treated group, the non-transgenic maize leaf-treated group, and the group fed yeast only, respectively. The positive control was significantly different from the other groups. Therefore, Cry1Ab protein had no detectable impact on the survival or reproduction of the collembola after 28 days of continuous

exposure. The NOEC for the survival and reproduction of *F. candida* of lyophilized Bt11 corn leaves was 17.1 µg Cry1Ab protein/g diet and the LC_{50} was greater than 17.1 µg Cry1Ab protein/g diet.

Conclusions/Recommendations: No adverse effects of Cry1Ab were observed on *Folsomia candida* from Bt11 corn leaf tissue. The NOEC for the survival and reproduction of *F. candida* of lyophilized Bt11 corn leaves was 17.1 µg Cry1Ab protein/g diet and the LC_{50} was greater than 17.1 µg Cry1Ab protein/g diet. This study was previously reviewed and found acceptable (Vaituzis.and Rose, 2000).

v. Honeybee (MRID No. 470176-29)

A semi-field whole-hive feeding study was conducted based on the recommendations in EPPO Bulletin 22 (Oomen, *et al.,* 1992), in accordance with UK Good Laboratory Practice regulations of 1999 and OECD principles [Revised 1997].

The objective of this study was to evaluate potential dietary effects of transgenic microbial-derived full-length Cry1Ab on honeybee (*Apis mellifera*) larvae survival, adult emergence, exposed adult worker bee survival, and whole-hive conditions in a semi-field study. Honeybees were exposed, via oral ingestion using in-hive commercial bee feeders. The treatments consisted of: a sucrose solution containing 107.82 mg/L FLCRY1AB-0103 test material/g sucrose solution (representing 92.4 µg FLCry1Ab/mL and 10X EEC in FlCry1Ab in Event COT67B pollen), a negative control of 50% w/v sucrose solution, or a positive control of 6.35 g/L diflubenzuron insect growth regulator in sucrose solution. The test consisted of a single application of one liter of the appropriate solution per hive and the hives were observed for 24 days for percent successful brood development to adults and colony conditions. There was no significant difference in mortality between the test and negative control groups for brood development. There was also no significant difference in pre- and post-test hive conditions between the test and negative control treatments. Results for the positive control treatment were significantly different from the other treatments for brood development and hive condition (as indicated by the significantly reduced mean percentage of comb covered by life stages). Adult bees were not affected by any of the treatments. These results indicate direct and incidental ingestion of FLCry1Ab proteins did not adversely affect brood development, exposed worker bees, and the hive condition. Therefore, the NOEL was 92.4 µg FLCry1Ab/mL and the LD_{50} was greater than 92.4 µg FLCry1Ab/mL.

Conclusions/Recommendations: No adverse effects were observed after a single-dose application of FLCRY1AB-0103 test material mixed with a sucrose solution were observed on *Apis mellifera* honeybee larvae, adult emergence, exposed adult worker bee survival, and whole-hive conditions after 24 days. Despite some experimental shortcomings, there is enough certainty to indicate exposure of the FLCry1Ab to adult worker honeybees and larvae, via direct and incidental oral ingestion. Therefore, the NOEL was 92.4 µg FLCry1Ab/mL and the LD_{50} was greater than 92.4 µg FLCry1Ab/mL.Therefore, the NOEL was 92.4 µg FLCry1Ab/mL and the LD_{50} was greater than

92.4 µg FLCry1Ab/mL. Therefore, this study is rated acceptable for the purposes of the environmental risk assessment.

In addition to this study, a recent meta-analysis of 25 studies that independently assessed potential effects of *Bt* Cry proteins on honeybee survival showed that *Bt* Cry proteins used in genetically modified crops commercialized for control of lepidopteran and coleopteran pests do not negatively affect the survival of either honeybee larvae or adults in laboratory settings (Duan, *et al.,* 2008). A semi-field study also showed no adverse effects of *Bt* corn pollen containing high levels of Cry1Ab protein on adult honeybee survival, foraging frequency, behavior or brood development during the 7-day period of pollen shed and no adverse effects on brood development after an additional 30 days following pollen shed (Schur *et al.,* 2000).

Therefore, the weight-of-evidence demonstrates that there are no adverse effects of FLCry1Ab protein on honeybee brood development and adults in either the laboratory or field setting. This conclusion was determined by the two semi-field studies (showing no adverse effects of FLCry1Ab and *Bt* Cry1Ab on brood development, adult survival, and whole hive conditions) in combination with the meta-analysis of various laboratory studies (demonstrating no adverse effects of *Bt* Cry proteins on honeybee larvae and adults).

3. Soil Fate

Soil organisms may be exposed to Vip3Aa and FLCry1Ab protein through contact with cotton plant roots (by direct feeding), cotton plant root exudates, incorporation of above-ground plant tissues into soil following harvest, or by soil-deposited pollen. Some evidence suggests that soils which are high in clays and humic acids are more likely to bind Cry protein. However, neutral pH soils tend to have high microbial activity and microbes contribute to Cry protein degradation. In addition, a study on the release of Cry proteins in the root exudates of *Bt* cotton has shown that no Cry proteins were detected immunologically or by larvicidal assay in any soil or hydroponic solution in which *Bt* cotton had been grown (Saxena and Stotzky, 2001). The weight of evidence indicates that Cry proteins do not accumulate in soil to arthropod-toxic levels. Because Vip and Cry proteins are both toxins derived from soil-inhabiting bacteria, *Bacillus thuringiensis* and found in commercial microbial insecticides (De Maagd *et al.*, 2003 and Graser and Song, 2006), Vip protein degradation would also be similar to Cry protein degradation. Nonetheless, the Agency required the following soil fate evaluations to support the Event COT102 and COT67B *Bt* cotton registrations.

MRID No. 470176-30
The purpose of this study was to investigate the degradation of Vip3Aa protein in various types of soils (clay, sandy clay loam, sandy loam, silt loam, and artificial soils) by assessing the loss of bioactivity, via insect bioassay. The test substance LPPACHA-0199 (maize leaf protein, containing ca. 0.36% Vip3Aa19) was incorporated at concentrations of 16 or 4 Vip3Aa19 mg/g of soil and incubated under controlled conditions for 29 days. During the incubation, soil samples were collected

weekly and used in black cutworm (BCW, *Agrotis ipsilon*) bioassays to determine biological activity of the test substance against the insect over time. The loss of bioactivity was measured by BCW mortality, which was used to estimate the DT_{50} (time to dissipation of 50% of the initial bioactivity) of the 16 mg/g concentration of the test material in each soil. The estimated DT_{50} values ranged from 6.0 days in the silt loam to 12.6 days in one of the clays, indicating that Vip3Aa protein in plant residues incorporated into soil is not likely to persist or accumulate in soil.

MRID No. 470176-31
The purpose of this study was to investigate the degradation of FLCry1Ab protein in a viable microbial agricultural soil typical of a cotton-growing region by assessing the loss of bioactivity, via insect bioassay. The test substance FLCRY1AB-0103 (microbial-derived protein, containing 103 µg FLCRYCRY1AB-0103/g soil) was applied to sandy loam soil at a rate equivalent to 80 µg FLCry1Ab/g dry wt of soil, which would be 160 times the estimated soil concentration that would result from incorporation of pre-harvest stage COT67B cotton crop residue in the field. The soil was incubated under controlled conditions for 0, 1, 3, 7, 14, 30, 62, 94, or 120 days after dosing, with samples collected at each time point for use in the bioassays. The dosed soil samples were incorporated into insect diet at a concentration of 10% (w/v) and provided to first instar European corn borer (ECB, *Ostrinia nubilalis*) larvae for approximately five days. Degradation of FLCry1Ab was assessed by the loss of bioactivity, measured by ECB mortality. Mortality was plotted against incubation time to estimate the DT_{50} and DT_{90} (time to dissipation of 50% and 90% of the initial bioactivity, respectively) of the test material in the soil. The estimated DT_{50} and DT_{90} values were 17 and 52 days, respectively, indicating that FLCry1Ab protein in plant residues incorporated into sandy loam soil is not likely to persist or accumulate in soil.

Conclusions/Recommendations: These studies utilized field soil spiked with purified insecticidal protein derived from either plant- or microbial-derived protein. This approach is useful because dose responses can be easily quantified. However, the degradation and accumulation of *Bt* Cry proteins found within decaying plant tissue may behave differently than proteins in artificially spiked soil. Because Vip protein is derived from *Bt* and display similar insecticidal activity, the behavior of Vip protein is expected to be similar to Cry proteins as well. Thus, the presence of low levels of *Bt* Cry and Vip proteins in the soil (at or below the level of detection) is anticipated until all plant tissue is 'mineralized'. However, the reviewed data show that Cry and Vip proteins will be quickly degraded upon release from decaying plant tissue. In addition, a study that evaluated Cry1Ab protein accumulation in a field with three years of continuous Cry1Ab field corn production showed that the protein had not accumulated in soil to a level that would elicit a toxic response from ECB larvae, a species that is highly susceptible to Cry1Ab protein (MRID No. 460224-01; Milofsky and Vaituzis, 2006).

Based on FIFRA Scientific Advisory Panel recommendations and public comments, the Agency has required three year soil fate studies for the currently registered Cry protein producing crops grown in a variety of soils and environmental conditions, as a condition of registration. The results of these

studies show that there is no detectable Cry protein accumulation in agricultural soils during commercial planting of currently registered Cry protein producing crops (Milofsky and Vaituzis, 2006).

More recently, a comprehensive review of all available scientific data on ecological effects of commercially grown GM crops over the last ten years was completed (Sanvido, *et al*. 2007). The review concluded "none of the laboratory or field studies suggest accumulation of *Bt*-toxins in soil over several years of cultivation" and "experience from commercial cultivation indicates that *Bt*-toxin will not persist for long periods under natural conditions." The Agency agrees with these conclusions.

Collectively, the long-term field studies for *Bt* crops also confirm the previous SAP conclusion that "bioaccumulation is not expected to occur with transgenic proteins because *biodegredation mechanisms for proteins are ubiquitous*" (US EPA, 2000). More importantly, the numerous laboratory studies that demonstrated rapid protein degradation in soil of *Bt* proteins produced in *Bt* crops (when performed under realistic environmental conditions) are can be considered predictive that *Bt* protein in soil is not likely to persist or accumulate in soil after continuous cultivation.

In light of these published findings and the rapid degradation of Vip3Aa and FLCry1Ab proteins in soil as demonstrated in the insect bioassays, there is no indication that the proteins expressed in Event COT102 and Event COT67B are likely to persist or accumulate in soil after continuous cultivation. Therefore, no additional long-term field studies are required for these PIP products.

4. Effects on Soil Microorganisms

Numerous published studies indicate that exposure to Cry protein produced in *Bt* PIP crop plants does not adversely affect soil microorganisms (Sanvido *et al*., 2007). Although a minimal transient increase and shift in microbial populations may result from the presence of transgenic plant tissue in soil, no adverse effects have been attributed to the Cry protein. In addition, comparisons of microbial biomass in FLCry1Ab dosed and undosed soil prior to and during the study showed that microbial activity was maintained throughout the test period. Vip protein had similar DT_{50} or degradation time to Cry proteins and these proteins are both *Bt* toxins.

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

With regard to the impact of genetically engineered crops on soil, it is important to note that agricultural practices themselves cause large changes in soil and soil microbial composition. Furthermore, factors such variations in seasons and weather, plant growth stage, and plant varieties,

independent of being genetically engineered, are also responsible for significant shifts in soil microbial communities. Most studies with genetically engineered crops to date have shown minor or no effects on soil microbes beyond the variation caused by the factors listed above.

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

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

As a result of these findings, which suggest that HGT is at most an artificial event, and the fact that the Bt toxins engineered into COT102 and COT67B were derived from soil-inhabiting bacteria, EPA has concluded that there is no risk of HGT from Vip3Aa or Cry1Ab producing cotton.

6. Gene Flow and Weediness Potential

Movement of transgenes from crop plants into weeds is a significant concern, due to uncertainty regarding the effect that a new pest resistance gene may have on plant populations in the wild. Under FIFRA, the Agency has reviewed the potential for gene capture and expression of Cry proteins in commercial *Bt* cotton by wild or weedy relatives of cotton in the United States, its possessions or territories. Because Vip proteins are *Bt* toxins and have similarities to Cry proteins in its insecticidal activity on similar target species, the Agency maintains the same approach in evaluation of gene flow and weediness potential.

There is a possibility for gene transfer in locations where wild or feral cotton relatives exist. Therefore, EPA requires stringent sales and distribution restrictions on *Bt* cotton within these areas to preclude outcrossing or hybridization from the crop to sexually compatible relatives. There are only four areas in the United States and its territories wherein cultivated cotton has the opportunity to outcross to wild or feral species, which are genetically compatible: (1) southern Arizona, (2) Hawaiian islands, (3) southern Florida and 4) Puerto Rico. *G. thurberi* (Arizona Wild Cotton) is present in the elevated regions of Arizona and does not grow in areas of commercial cotton production. *G. thurberi* is a diploid and produces sterile, triploid progeny when crossed with the tetraploids *G. hirsutum* or *G. barbadense*. In the very south of Florida, feral *G. hirsutum* exists in apparently self-sustaining populations. Since these would readily cross with cultivated cotton, sale of *Bt*-Cotton is restricted south of Interstate 60. There is currently no commercial cotton production in the southern part of Florida. Evidence from germplasm collections indicates that feral *G. barbadense*

and possibly *G. hirsutum* exist in the U.S. Virgin Islands. There is presently no production of commercial cotton in either of these places; hence, outcrossing is not an issue. For a detailed review of the Agency's assessment of the potential for gene capture and expression of *Bt* endotoxins by wild or weedy relatives of cotton in the U.S., its possessions or territories, see the EPA Biopesticides Registration Action Document (BRAD) for the *Bacillus thuringiensis* (*Bt*) Plant-Incorporated Protectants, dated October 15, 2001.

7. Impacts on Endangered Species

The primary route of exposure to Vip3Aa and FLCry1Ab proteins in cotton is through ingestion of cotton tissue or pollen. There are no reports of threatened or endangered species feeding on cotton plants; therefore, such species would not be exposed to cotton tissue containing these proteins. Since Vip3Aa and FLCry1Ab proteins have not been shown to have toxic effects on mammals, birds, plants, aquatic species, insects and other invertebrate species at the Estimated Environmental Concentration (EEC), a "may affect" situation for endangered land and aquatic species is not anticipated. As previously noted, there is a possibility for gene transfer in locations where wild or feral cotton relatives exist. As a result, EPA requires stringent sales and distribution restrictions on *Bt* cotton within these areas to preclude outcrossing or hybridization from the crop to sexually compatible relatives. Therefore, EPA does not expect that any threatened or endangered species will be affected by outcrossing to wild relatives or by competition with such entities.

There are extensive data that demonstrate the lack of hazard of Cry1Ab to non-Lepidoptera and the environmental safety of Bt11 corn (US EPA, 2001b). Because of the selectivity of Vip3Aa and FLCry1Ab proteins for lepidopteran species, endangered species concerns are mainly restricted to the order Lepidoptera. Examination of an overlay map showing the county level distribution of endangered/threatened lepidopteran species (currently listed by the U.S. Fish and Wildlife Service) relative to cotton production counties in the United States clearly indicated that any potential concern regarding range overlap with cotton production was mainly restricted to the Kern primrose sphinx moth (*Euproserpinus euterpe*). However, cotton is not a host plant for this species nor do host-range considerations place habitat in or near cotton fields.

Likewise, other insect species in the orders Diptera, Hemiptera, Coleoptera, Donata, and Orthoptera that are listed as endangered/threatened species are found in dune, meadow/prairie or open forest habitats and are not closely associated with row crop production, often times due to the specificity of the habitat of their host plants. Furthermore, the reviewed toxicological data shows the relative insensitivity of a range of insects in non-lepidopteran orders to the Vip3Aa and FLCry1Ab proteins, indicating that COT102 and COT67B cotton plants are not likely to have detrimental effects on non-lepidopteran insects included on the endangered/threatened species list.

In light of the above considerations (based on no spatial and temporal overlap), the Agency has determined that registered uses of Event COT102 and Event COT67B cotton plants will have No

Effect (NE), direct or indirect, on endangered and threatened species or their habitat as listed by the United States Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds or terrestrial and aquatic plants and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

II. C. 2. Environmental Risk Assessment for Event COT102 and Event COT67B

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

A. Direct effects

At present, the Agency is aware of no identified significant adverse effects of Vip3Aa and/or FLCry1Ab proteins on the abundance of non-target beneficial organisms in any population in the field environment, whether they are pest parasites, pest predators, or pollinators. Further, the EPA believes that cultivation of Event COT102 and/or Event COT67B cotton may have fewer adverse impacts on non-target organisms than use of chemical pesticides for cotton production, because under normal circumstances, COT102 and COT67B cotton requires substantially fewer applications of chemical pesticides, compared to production of non-*Bt* cotton. Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers. In addition, no adverse effect on Federally-listed endangered and threatened species is expected from the proposed lepidopteran-resistant cotton registration (see part 7 in the preceding section above). Furthermore, the EPA has determined that there is no significant risk of gene capture and expression of Vip3Aa and FLCry1Ab protein by wild or weedy relatives of cotton in the U.S., its possessions, or territories (see part 6 in the preceding section above). Available data do not indicate that Cry or Vip proteins have any measurable adverse effect on microbial populations in the soil (see part 4 in the preceding section above), nor has horizontal transfer of genes from transgenic plants to soil bacteria been demonstrated (see part 5 in the preceding section above). In conclusion, this risk assessment finds no hazard to the environment at the present time from cultivation of Event COT102 and Event COT67B cotton in support for the Sec. 3 registration.

B. Indirect effects:

The purpose of using PIP plants is the same as for any other pest management tactic, i.e., to reduce pest populations below economic injury levels. As a result, the abundance of pest insects should be significantly reduced and this will have corresponding implications for those organisms that exploit these pests as prey and hosts. Thus, the potential for these indirect ecological effects on biological control organisms should not be regarded as a unique ecological risk associated with the PIP crop. Some reductions, however, should be expected if the pest management strategy is effective. Since PIP crops are often grown in vicinity with conventional crops to prevent resistance build-up by the

target pest(s), specialist antagonists can persist in these 'refuges', in other crops and in non-crop habitats and retain the potential for recolonization of the PIP crop area. Based on these considerations, regulatory testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

II. C. 3. Supplemental Data Needed to Confirm COT102 and COT67B Non-Target Hazard Assessment

The Agency has sufficient information to believe that there is no risk from the proposed uses of Event COT102 and Event COT67B cotton to non-target wildlife, aquatic, and soil organisms. In previous Section 3 registrations of PIPs, the Agency required registrants to conduct post-registration long term invertebrate population/community studies and Cry protein accumulation in soils studies. However, the issue of long range effects of cultivation of these Cry proteins on the invertebrate community structure in corn and cotton fields has since been adequately addressed by the meta-analysis of field studies performed during the last 10 years (Marvier, *et al*. 2007; Sanvido, *et a*l. 2007). No unexpected adverse effects on invertebrate community structure were reported. The Agency is in agreement with these conclusions Likewise, no unexpected accumulation of Cry or Vip proteins in agricultural soils was seen in published studies (Icoz and Stotzky, 2007; Sanvido, *et al.* 2007) and in numerous studies submitted directly to the EPA for the currently registered Cry proteins (Milofsky, 2006; Part 3 in the preceding section above).

However, in light of recently published laboratory studies showing reduced growth in shredding caddis flies exposed to anti-lepidopteran Cry1A protein corn litter (Rosi-Marshall, et al. 2007), additional aquatic invertebrate data are required. The submitted *Daphnia magna* study is unacceptable because it is an 850 Series OPPTS Guideline study. The 48 hour duration of this study is not sufficient to detect mortality. It takes more than 48 hours for the target pests to succumb to *Bt* δ-endotoxins, such as Cry or Vip proteins, therefore 48 hours is also not expected to show mortality or reproductive effects on *Daphnia.* A 7-14 day *Daphnia* study as per the OPPTS Series 885.4240 guideline must be performed (see Tables 11 and 12) for Event COT102 and Event COT67B. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, may be performed and submitted in lieu of the 7-14 day Daphnia study. These studies can be submitted as a condition of registration.

Table 11. Supplemental non-target data requirements for COT102 expressing Vip3Aa

Testing Category	Type of Data
Aquatic invertebrate	A 7-14 day *Daphnia* study as per the OPPTS 885.4240 guideline has to be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the 7-14 day *Daphnia* study.

Table 12. Supplemental non-target data requirements for COT67B expressing FLCry1Ab

Testing Category	Type of Data
Aquatic invertebrate	A 7-14 day *Daphnia* study as per the OPPTS 885.4240 guideline has to be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the 7-14 day *Daphnia* study.

II. C. 4. Event COT102 X COT67B (VipCot) Environmental Risk Assessment

SUMMARY

Syngenta Seeds, Inc. developed COT102 x COT67B cotton (VipCot) by conventional breeding of transgenic event COT102 cotton and transgenic event COT67B cotton, which express the Vip3Aa and FLCry1Ab insecticidal proteins, respectively, for control of certain lepidopteran pests. Vip3Aa is a protein variant of Vip3Aa1 (originally identified in *Bacillus thuringiensis* strain AB88), from which it differs by one amino acid substitution. FLCry1Ab is a δ-endotoxin identical to a protein produced by *B. thuringiensis* subsp. *kurstaki* HD-1 except for an additional 26 amino acids at the C-terminal region. The VipCot stack expresses both the Vip3Aa and FLCry1Ab proteins.

It was previously established by the Agency that the relative potency of plant-produced Vip3Aa and full-length Cry1Ab proteins is similar to their corresponding microbial-produced proteins, indicated that plant-produced protein was similar in toxicity to the microbial-produced protein (Matten, 2007 and Edelstein, 2008). Each event also had comparable protein expression levels to the COT102 x COT67B breeding stack (MRID No. 470176-07 and Edelstein, 2008).

Although the general symptomatology of Vip3Aa displayed by sensitive lepidopteran larvae following ingestion of *Bt* δ-endotoxins resembles that of Cry proteins (Yu *et al.*, 1997), Vip3Aa contains significantly different receptor binding properties than the Cry proteins (Lee *et al.*, 2003). Therefore, since the proteins have different modes of action, the predicted effect of the mixture was calculated using a model called independent joint action[m] (Raybould, 2007; Colby, 1967). The observed and expected mortalities were compared over a range of concentrations. Since there is no test to identify statistical significance, the predicted dose response curves were compared with the expected dose response curves. If there is greater mortality than expected over the range of concentrations in a sensitive pest species, the hypothesis of synergism is falsified and subsequently it is likely that there will be no synergism of the mixture against non-target organisms.

[m] Model hypothesis: if a certain amount of protein A alone kills x% of a sample, and a certain amount of protein B kills y%, the predicted percentage kill of a mixture of these amounts of protein is given by x + y – (xy/100).

Syngenta submitted additional data on the potential synergistic interaction between Vip3Aa and FLCry1Ab proteins and are summarized in this report to support the hypothesis of no synergism between the two proteins. If no synergism is indicated, then development of new non-target species data are not required because the reviewed non-target data and the environmental risk assessments for the single indicated PIP lines are applicable to the COT102 x COT67B cotton line. The results of ecological effects studies[n] submitted in support of the Section 3 full-commercial registration of Event COT012 and Event COT67B were previously summarized in Tables 1 and 2, respectively, and presented in a more descriptive format in previous sections of this risk assessment document.

Synergism Studies

The purpose of these studies was to characterize the potential for interaction between the lepidopteran-active proteins Vip3Aa and FLCry1Ab. The Vip3Aa and FLCry1Ab proteins were tested alone and in combination against tobacco budworm (TBW, *Heliothis virescens*) and cotton bollworm (CBW, *Helicoverpa zea*), respectively, in diet incorporation studies.

MRID No. 470176-21
Four laboratory feeding bioassays were conducted to assess any synergistic or antagonistic interactions between Vip3Aa and full-length Cry1Ab proteins in a key lepidopteran pest, tobacco budworm (*Heliothis virescens*). Five dilution series of the test materials were prepared in buffer for each test: one series each of Vip3Aa and FLCry1Ab alone, and three series of the two proteins mixed together in different ratios (up to 1600 µg/mL Vip3Aa and 100 µg/mL FLCry1Ab together). The treatments were applied to non-transgenic cotton leaves which were fed to *H. virescens* larvae. Interaction between the two test materials was assessed by comparing the larval mortality observed for the mixed proteins with the predicted responses based on the bioassay of each protein individually. The predicted responses were calculated based on the assumption of "independent action" (Raybould, 2007) and there was no evidence of either a synergistic or an antagonistic interaction between Vip3Aa and FLCry1Ab in *H. virescens*, indicating that the effect of a mixture of Vip3Aa and FLCry1Ab on non-target Lepidoptera can be predicted from the effects of the individual proteins alone.

MRID No. 470176-22
Three laboratory feeding bioassays were conducted to assess any synergistic or antagonistic interactions between Vip3Aa and full-length Cry1Ab proteins in a key lepidopteran pest, cotton bollworm (*Helicoverpa zea*). Five dilution series of the test materials were prepared in buffer for each test: one series each of Vip3Aa and FLCry1Ab alone, and three series of the two proteins mixed in different ratios (up to 25,600 ng/cm^2 Vip3Aa and 12,800 ng/cm^2 FLCry1Ab together). The

[n] Bridging of data between the variants of Vip3Aa as well as the Cry1Ab proteins was addressed in the Agency' reviews of the VipCot™ Experimental Use Permit (see memoranda: Matten, 2006; Milofsky and Vaituzis, 2007b).

test materials were added to standard lepidopteran diet and fed to *H. zea* larvae. Interaction between the two test materials was assessed by comparing the larval mortality observed for the mixed proteins with the predicted responses based on the bioassay of each protein individually. Since previous evidence indicates that Vip3Aa and FLCry1Ab act at different binding sites, the predicted responses were calculated based on the assumption of "independent action" (Raybould, 2007). The results were compared and there was no evidence of either a synergistic or an antagonistic interaction between Vip3Aa and FLCry1Ab in *H. zea*, indicating that the effect of a mixture of Vip3Aa and FLCry1Ab on non-target Lepidoptera can be predicted from the effects of the individual proteins alone.

Conclusions/Recommendations: The results of the interaction studies of the combined proteins (Vip3Aa and FLCry1Ab) indicate that there is no change in the level of activity among susceptible insects. Collectively these data provide evidence that Vip3Aa and FLCry1Ab proteins do not interact in an antagonistic or synergistic manner. These studies, along with the single-species, NTO toxicity testing and the Vip3Aa and Cry1Ab protein long-term field studies, reviewed for the parental Event COT102 and Event COT67B, indicate its associated breeding stack, COT102 x COT67B cotton, will not result in any unexpected interaction related to an antagonistic or synergistic action to target and non-target insects. Therefore, it is extremely unlikely that the Vip3Aa and FLCry1Ab proteins contained in a single plant will impart any hazard to non-target organisms exposed to these hybrids in the environment. In conclusion, the Agency has determined that the environmental risk assessment of Event COT102 expressing Vip3Aa protein and Event COT67B expressing FLCry1Ab protein indicate there will be no unreasonable adverse effects to the environment, including federally-listed threatened and endangered species, by VipCot (COT102 x COT67B) cotton hybird, crossed via traditional breeding.

CONCLUSION

The environmental risk assessment indicates for the VipCot cotton breeding stack (COT102 x COT67B), based on prior assessments conducted on Vip3Aa and Cry1Ab proteins individually, that no unreasonable harm will result to the environment or any federally-listed threatened or endangered species from commercial cultivation of COT102 x COT67B cotton. Furthermore, the Agency has determined that Events COT102, COT67B, and VipCot cotton will have No Effect (NE) on endangered and/or threatened species listed by the US Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds, terrestrial and aquatic plants, and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

The Agency believes that cultivation of VipCot cotton may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, *Bt* cotton requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of nonspecific conventional chemical pesticides is known to have an adverse effect on non-target beneficial organisms found living in the

complex environment of an agricultural field. Many of these beneficial organisms are important integrated pest management controls (IPM) for secondary pests such as aphids and leafhoppers. Therefore, the overall result of cultivation of VipCot cotton, expressing Vip3Aa and FLCry1Ab proteins, is that the number of chemical insecticide applications for non-target pest control will be reduced for management of multiple pest problems.

II. C. 5. References

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II. D. Insect Resistance Management

1. Background

Syngenta's VipCot cotton is a pyramided transgenic cotton trait that expresses full-length Cry1Ab insecticidal protein (COT67B event) and Vip3Aa19 (COT102 event). The Vip3A is different from Cry proteins as it is produced during vegetative growth of the bacteria, does not form parasporal crystal proteins, and is secreted (but not processed upon secretion) from the cell as a soluble protein. Its physical manifestations of intoxication, however, resemble those of Cry proteins (gut paralysis and lysis of midgut epithelial cells) (Schnepf *et al.* 1998). Activated Vip3A does not bind to the same receptors as Cry1Ab (APN and cadherin-like receptor); the two Bt proteins do not seem to share binding sites. Lee et al. (2003) have investigated the mode of action of the Vip3A protein and determined that it involves a number of steps much like the mode of actions for the δ-endotoxins. Following ingestion by the lepidopteran target pest, the Vip3A protein becomes soluble in the gut and is then processed into four dominant bands (retaining activity). The authors propose that this processing is required for the bioactivity of the toxin (activation step). Interaction with the midgut epithelium is the next likely step in the mode of action of Vip3A. However, Vip3A does not bind to APN and cadherin-like glycoprotein receptors as does Cry1Ab (as already stated by Schnepf et al. (1998)) and supported by the researchers' competition study. Upon binding to midgut epithelial receptors, data supports the existence of a pore-forming step that creates ion channels which are structurally and functionally distinct from those of Cry1Ab. Direct structural information is missing for Vip3A; however, preliminary data do not support the notion that the two proteins share similar domain organization or an α-helical bundle region.

COT102 cotton expresses the vegetative insecticidal protein (VIP3A), which was isolated from *Bacillus thuringiensis* strain AB88. The cotton line Coker 312 (*Gossypium hirsutum* L. cv Coker 312) was transformed via *Agrobacterium* transformation procedures with synthetic *vip3A(a)* gene encoding VIP3A protein and the selectable marker gene *aph4* encoding the enzyme APH4. The transformation event that produced the transgenic cotton line, designated COT102, was transformed with plasmid pCOT1. COT102 is intended to protect cotton from feeding by the primary lepidopteran pests: tobacco budworm (*Heliothis virescens*, TBW), cotton bollworm (*Helicoverpa zea*, CBW), and pink bollworm (*Pectinophora gossypiella*, PBW). Based on cotton insect loss data from 1991-2000, the primary target pests, TBW, CBW, and PBW, account for more than 77% of the yield loss and 84% of the insecticide use due to lepidopteran infestation in cotton.

In 2004, BPPD reviewed an earlier IRM plan for COT102 and determined that the registrant did not provide sufficient data to formulate an IRM strategy (BPPD, 2004). Specifically, The BPPD IRM team concluded that data or published literature was needed to address the pest biology of each target pest; additional data was required to make high-dose claim for COT102 using two of the five techniques described by 1998 FIFRA Science Advisory Panel; baseline susceptibility and diagnostic concentrations needed to be established for all target pests; estimates of initial resistant allele frequency as well as models of evolution of resistance for Vip3A19 should be provided; additional

cross-resistance data was required for target pests using Vip3A19, Cry1Ac, and Cry2Ab2; and specific monitoring plans, remedial action strategies, grower education program, compliance assurance program and research activities for COT102 needed to be provided.

A revised IRM plan (MRID 470176-34) was submitted by Syngenta to support registration of VipCot in 2006 and has been reviewed by BPPD (see Martinez 2008 a, b). An additional e-mail correspondence clarifying aspects of the dose evaluation was submitted by Syngenta (Reed 2008). The details of resistance management plan as reviewed by BPPD are described below.

2. Pest Biology

A clear understanding of pest biology and ecology is essential to the development of a sound IRM plan. The target pests of VipCot (TBW, CBW, and PBW) have been well studied since Bt cotton was first introduced and is available in the scientific literature. A summary of the biology and ecology for these insects can be found in the Agency's 2001 Bt crop reassessment document (EPA 2001).

3. Dose

The determination of dose, or the amount of toxin expressed by the transgenic crop relative to the susceptibility of the target pests, is a critical component of IRM. Models have shown that a high-dose of toxin, coupled with a non-transgenic refuge to provide a supply of susceptible insects, is the most effective strategy for delaying resistance in Bt crops. The high-dose/refuge strategy assumes that resistance to Bt is recessive and is conferred by a single locus with two alleles resulting in three genotypes: susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR). The high-dose/refuge strategy also assumes that there will be a low initial resistance allele frequency and extensive random mating between resistant and susceptible adults. In practice, a high-dose PIP should express sufficient quantities of toxin to kill all susceptible insects (SS) as well as heterozygous insects with one resistance allele (RS). Lower dose PIPs might allow for survival of insects with at least one susceptibility allele (SS or RS), although effective IRM may still be possible with a suitable refuge strategy. To be able to demonstrate high-dose, it is recommended that registrants generate data by at least two of the five laboratory and field approaches as outlined by the SAP (1998) and described by the Agency in the 1998 Bt Plant-Pesticides and Resistance Management document (EPA, 1998) and 2001 Biopesticide Registration Action document (EPA, 2001).

The 1998 SAP defined high-dose as a level of toxin 25 times greater than is needed to kill all susceptible insects. The SAP also outlined five techniques to determine high dose: 1) Serial dilution bioassay with artificial diet containing lyophilized tissues of Bt plants using tissues from non-*Bt* plants as controls; 2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar determined by quantitative ELISA or some more reliable technique; 3)

Survey large numbers of commercial plants in the field to make sure that the cultivar is at the $LD_{99.9}$ or higher to assure that 95% of heterozygotes would be killed (see Andow & Hutchison 1998); 4) Similar to #3 above, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} value similar to field strains; and 5) Determine if a later larval instar of the targeted pest could be found with an LD_{50} that was about 25-fold higher than that of the neonate larvae. If so, the later stage could be tested on the Bt crop plants to determine if 95% or more of the later stage larvae were killed.

It must be noted that both the high-dose definition and verification techniques were developed in 1998 when all of the registered Bt crops were single toxin products targeted against lepidopteran pests. In recent years, PIPs in Bt cotton have been approved that contain two genes targeted at the same insect pest. These "pyramided" products can be beneficial for IRM since target pests must overcome two toxins to develop field resistance to the PIP. The benefits are greatest for two toxins with unrelated modes of action (i.e. binding to different Bt receptor sites in the midgut) that are expressed at high-doses in the plant (Roush 1994).

For pyramided products, the dose of each toxin should be evaluated separately. This can be easily accomplished if the pyramided product is created through conventional breeding -- in this case, the dose of the single toxin products has already been established and the combined dose in the pyramided PIP can be determined with comparative efficacy studies. However, for pyramids created by non-conventional breeding (e.g. recombinant DNA techniques), defining the dose can be more complicated since single toxin lines may not be available (or commercialized) for comparisons. The dual toxins can also be evaluated collectively to determine an "effective" high-dose. In some examples, each toxin by itself may not supply a high-dose, but in combination a sufficient control (>95% of heterozygotes) is provided and can be considered high-dose.

To evaluate dose, Syngenta conducted a number of laboratory and field studies with diet bioassays and COT67B, COT102, and VipCot cotton plant material. Three sets of experiments were conducted: 1) bioassays with the single proteins expressed in lyophilized plant material and both proteins expressed in lyophilized plant material and combined as VipCot to determine target pest susceptibility (TBW, CBW, PBW), 2) field tests on VipCot and COT67B plants and control plants (artificial infestation) during the 2005 and 2006 growing seasons, and 3) tolerance assays with single proteins expressed in fresh plant material and both proteins expressed in fresh plant material and combined as VipCot to determine susceptibility of neonates as compared to fourth instar larvae (N.C. State University lab colony).

Verification Methods:

1) Bioassays (Syngenta's submission, MRID 470176-34, Appendix 1) using US EPA method #1 (serial dilution assays) were conducted by two laboratories (Jealott's Hill International Research Center and Syngenta Biotechnology, Inc) using lyophilized tissue from terminal leaves and bolls of

COT102, COT67B, VipCot, and Coker 312 cotton. Mortality data for *H. zea*, *H. virescens*, and *P. gossypiella* were collected.

At 25X dilution, percent mortality on COT102 for TBW ranges from 66.7- 95.0%, and for CBW mean corrected mortality reported (one lab only) is 72.4%. For PBW, mean corrected mortality reported (by one lab only) is 16.7%. These data seem to indicate that COT102 alone has minimal efficacy against PBW and an efficacy against TBW ranging from intermediate to high. COT102 efficacy against CBW does not appear to quite as high judging from these test results. Based on the range of susceptibility observed in CBW for other Bt PIPs, it can be expected that the range of susceptibility for COT102 in this target pest is rather wide as well and that 72% mortality likely represents an upper value in this potential range.

At 25X dilution, percent mortality on COT67B for TBW and PBW is reported to be 100% and for CBW ranges from 98.3- 100%. The dilution bioassay for VipCot was conducted at SBI only; their results suggest that at 25X dilution, mortality on VipCot for TBW, CBW, and PBW is 100%. Based on this verification method alone, VipCot provides an 'effective' high-dose against all three target pests, while COT102 provides no high-dose to all three target pests and COT67B expresses a high-dose against TBW and PBW and is nearly high dose for CBW.

2) Unlike the artificial diet bioassay, the second method to verify high-dose was conducted on VipCot and COT67B plants in the field (US EPA method #4) using artificial infestations of laboratory strains of TBW and CBW (MRID 470176-34, Appendix 2; additional clarification provided in Reed 2008). The purpose of this approach was to determine whether the dose of toxins expressed in VipCot and COT67B was at or greater than the LD_{99} for the key target pests. Specifically, TBW survival was measured on both COT67B and VipCot, while CBW survival was measured on VipCot cotton only.

In 2005, two field trials were conducted for COT67B of which one was held in Mississippi and the other in Florida. Unreplicated blocks of >1,000 plants were planted with one control block of Coker 312 plants at each site. Infestation with TBW was simulated by spraying eggs onto cotton plants (greater infestation was conducted on Bt variety due to low survival expectancy). Survival on control plants was estimated by collecting leaves containing TBW eggs from Coker 312 plants (50% and 75% of field inspected in MS and FL, respectively) and counting successful larval hatching. Survival of TBW larvae on COT67B plants was estimated by visually assessing Bt plants for larval presence in each field in MS and FL, respectively. When survivors were found, the plants were marked and reexamined for further larval survival after four to seven days. Syngenta states in their report that in MS and FL one and two surviving TBW larvae, respectively, were found after seven days of artificial infestation (7DAI) and zero survivors after 14 days (14DAI). The second set of observations (14 DAI) may not be as reliable as the initial assessment of survivors because it appears that bolls were not 'caged' to prevent larval loss or escape. Thus, BPPD will base its review and conclusion on the first set of observations (7DAI).

In 2006, six VipCot cotton field trials were held at three locations in the US (1,600 -2,000 plants each trial) and, like for COT67B, did not get replicated. Experimental design, artificial infestation for TBW and CBW, egg hatching, and larval survival estimation methods resembled those of 2005. The number of survivors of TBW and CBW (7DAI) were 32 and 21, respectively, at the Texas site and 14 and 5, respectively, at the Louisiana site. For Mississippi, no CBW and TBW survivors on VipCot plants were reported. In Tables 2 and 3 of Appendix 2 (MRID 470176-34), BPPD notes that there is large difference in number of insects observed in the control plots 7DAI (i.e. 363 TBW observed, TX) and 14DAI (i.e. 17 TBW observed, TX). Based on such a decrease of survivors in the control plots, BPPD concludes that other outside effects add to the natural mortality when laboratory reared insects are exposed to cotton plants (i.e. fed on artificial diets for many generations and no longer fit to survive on natural host). Thus, the low number of TBW survivors reported from Bt-plants 7DAI is very likely confounded by this additional 'non-Bt exposure' mortality. Similar results were reported for CBW, and therefore, the same conclusion is extended to this pest. Based on the discrepancy observed in survivorship on control plants, BPPD concludes that the 2006 data alone are inconclusive. However, given the results observed in 2005 for TBW, it can be concluded that COT67B likely provides a high-dose for TBW under this method.

3) The third verification method for high-dose was conducted as a tolerance bioassay with purified toxins as well as leaf disks to determine if a later larval instar of the targeted pest could be found with an LD_{50} that was approximately 25-fold higher than that of the neonate larvae (US EPA method #5) on VipCot, COT 102, and COT67B plants for CBW and on COT102 for TBW (MRID 470176-34, Appendix 3). The SBI lab conducted the purified toxin (with Vip3A and FLCry1Ab) and leaf disk study (on COT102, COT67B, and VipCot) for CBW; JH lab conducted the purified toxin (with Vip3A) and leaf disk study (on COT102) for TBW.

Leaf Disk Bioassays for CBW
While Syngenta reports 100% mortality for both neonates and older CBW larvae for leave disk bioassays (method #5), Syngenta does not mention that control mortality for neonates ranged from 0% to 81% for neonates. While neonates may have only served as a reference, it is inappropriate to list these results to support the conclusion that VipCot, as well as its individual events, is highly effective against CBW. However, control mortality for later instar larvae was much lower and ranged from 0% to 28% (only one control had very high mortality results). The results from leaf disk bioassays show that later instar larvae of CBW are experiencing 100% mortality when exposed to VipCot, COT102, and COT67B.

Leaf Disk Bioassays for TBW
Mortality for TBW later instar larvae was 100% when tested on COT102. Control mortality results for later instar larvae were 14%, 15%, and 27%; treatment mortality for later instars was 100%. The results from leave disk bioassays show that older larvae of TBW are experiencing 100% mortality when exposed to COT102.

Tolerance Assays for CBW
Syngenta reports that the tolerance assay conducted with the purified toxin Vip3A and tested on CBW did not allow estimating LC_{50} for later instars because the estimates were well in excess of the highest concentration tested. Syngenta concludes that later instars are at least 25X more tolerant to Vip3A than neonate larvae. BPPD recognizes that there are several issues with the tolerance assay data reported: 1) Mortality data for neonates are highly variable from bioassay to bioassay, and LC_{50} estimates for CBW neonates range from 504.6ng/cm^2 to 2669ng/cm^2. 2) Mortality data for neonates *within* any test are not steadily increasing with increasing toxin concentrations but show a fluctuating trend and data gaps at some concentrations tested. Mortality data for later instar larvae follow the same trend as described for neonates under 1 and 2. 3) Where an LC_{50} could be estimated for both CBW neonates and later instars, the difference between estimates is only 8 fold as opposed to the desirable 25-fold factor. The variability reported in estimated LC_{50}s for neonates and mortality data for neonates and older instars are most likely due to CBW's wide range in response to Bt toxins. Overall, the tolerance assay data support that it is difficult to achieve mortality in neonates and older instars of CBW when exposed to Vip3A.

Two tolerance bioassays were conducted with FLCry1Ab on CBW (Tables 6 and 7, Appendix 3). In these tests, mortality data for later instars and neonates were more consistently increasing with increasing concentrations. Later instar LC_{50} estimates were greater than neonate LC_{50} estimates by more than 25-fold; BPPD concludes that older CBW larvae are >25-fold more tolerant than neonates when exposed to FLCry1Ab.

Leaf Disk Assays for TBW
For the leaf disk assays, Syngenta reported 100% mortality for older TBW larvae. Although control mortality for older larvae was somewhat higher than desirable (ranging from 15%-27%), BPPD concludes that COT102 appears to have very good activity against TBW.

Tolerance Assays for TBW
Syngenta reports that the tolerance bioassays conducted with Vip3A on TBW neonate and 2nd instar larvae show a 36-fold difference in susceptibility/ LC_{50} estimates. BPPD would like to add that there were two estimated LC_{50}s for older larvae of which one showed a 21-fold and the other 36-fold difference from neonate susceptibility. Overall, BPPD concludes that older TBW larvae are approximately ≥25-fold more tolerant than neonates when exposed to Vip3A alone.

4) Method #4 was conducted to verify high-dose in the field on COT67B plants (and COT69D plants) under artificial infestation of laboratory strains of PBW (MRID 470176-34, Appendix 4). The purpose of this approach was to determine whether the dose of toxins expressed in COT67B was at or greater than the LD_{99} for this key target pest.

The experiment was conducted at the University of California, Desert Research and Extension Center. The experiment was set up as a randomized complete block design with four replicates,

COT67B plants as treatment, and Coker 312 plants as controls. One-hundred bolls per plot were artificially infested (twice) with PBW eggs supplied by USDA/ARS in Phoenix, AZ. Eight days after infestation, 75 bolls from each plot got harvested and evaluated for damage such as warts, mines, dead larvae, and exit holes (indicating survival of larva). Surviving larvae and exit hole data collected on Coker 312 plants served as a baseline to assess infestation levels and PBW populations.

No live larva larger than 1st instar was found on COT67B cotton bolls. One exit hole in one boll was found out of 1,120 bolls of COT67B towards the later time of the season and is possibly attributed to lower expression levels of the toxin in aged plants. Mortality on COT67B plants is estimated at 99.9%. Mortality in Coker 312 is significantly lower and estimated at 40.3% with first instar larvae included. These data support the conclusion that COT67B expresses a high-dose against PBW.

Overall, BPPD's conclusions for the VipCot dose trials are as follows:

- COT67B expresses a probable high-dose against TBW (methods 1 and 4) and CBW (methods 1 and 5).
- VipCot expresses a probable high-dose against TBW (using method 1; method 4 was considered inconclusive) and CBW (methods 1 and 5).
- COT102 does not appear to express a high-dose against any of three target pests when tested with method 1, but has a probable high dose against TBW and CBW with method 5.
- COT67B expresses a high-dose against PBW based on data from verification methods 1 and 4.
- VipCot expresses a high-dose against PBW based on data from verification method 1.

Table 13. BPPD's High-Dose Determinations for TBW, CBW, and PBW

Species	Method 1			Method 4		Method 5		
	COT102	COT67B	VipCot	COT67B	VipCot	COT102	COT67B	VipCot
TBW	No high-dose	High-dose	High-dose	Probable high-dose	***	Probable high-dose	---	---
CBW	No high-dose	Near high dose	High-dose	---	***	High-dose	High-dose	High-dose
PBW	No high-dose	High dose	High-dose	High dose	---	---	---	---

Shaded fields indicate high-dose determinations by BPPD for single toxins or stacked *Bt* product
*** indicates that results were inconclusive for a definitive dose determination
--- indicates *Bt* variety not tested under a particular method

4. Cross-Resistance Potential

Analyses of resistance to Bt Cry proteins indicate that cross-resistance occurs most often with proteins that are similar in structure (Tabashnik, 1994; Gould et al., 1995). While direct structural information of the Vip3A protein expressed in VipCot is missing (Lee et al. 2003), this novel Bt protein does not share any sequence homology with the known Bt Cry protein genes, and the predicted secondary structure give no indication of a similar domain organization or α-helical bundle

region within the polypeptide sequence of Vip3A as exists for the Cry proteins. Protein folding blasts reveal that Vip3A may be a pore forming protein that has a structure of β-barrels (Syngenta unpublished data). In order to further investigate the potential for cross-resistance of Vip3A to Cry proteins, Syngenta examined the mode of action of Vip3A at selected steps critical to the mode of action of Bt Cry proteins: proteolytic activation, receptor binding, and pore forming.

The first piece of analysis relates to the proteolytic activation in both Bt toxins and shows that Vip3A and Cry proteins are proteolytically activated upon solubilization in the midgut. Syngenta's experiments further demonstrate that both Vip3A and two Cry proteins (Cry1Ac and Cry2Ab2) can be processed by either trypsin or gut juice extracts. However, in Vip3A proteolysis occurs in susceptible as well as non-susceptible insects and alone does not appear to be a key factor in insect toxicity and specificity. Based on this information and published literature stating that high levels of resistance have not been found to correlate with the toxin activation step, Syngenta speculates that the theoretical risk of cross-resistance is very small at this particular step of the mode of action.

Second, Syngenta investigated whether Vip3A and Cry proteins (Cry1Ab, Cry1Ac, and Cry2Ab) shared the same receptor sites in Lepidoptera species (*M. sexta*, *H. virescens*, and *H. zea*) by conducting receptor binding studies with Amino Peptidase N and cadherin-like glycoproteins (identified as putative Cry1A protein receptors) as well as others identified to be Cry1Ac and Cry2Ab2 binding sites. Those studies show that the protease activated form Vip3A does not bind to APN, the ectodomaine of the cadherin-like protein, or other putative Cry1A toxin binding proteins. In yet another study with *H. zea* and *H. virescens*, the non-specific binding of Cry2Ab was not inhibited by the addition of unlabeled Vip3A indicating that Vip3A does not bind to the Cry2Ab binding sites. Syngenta further demonstrated that activated Vip3A bound to two proteins of ca. 80 and 110 kDa and not to APN and cadherin-like proteins. These binding studies demonstrate that there is little risk of cross-resistance between Vip3A and Cry1Ab, Cry1Ac, and Cry2Ab2.

The last piece of analysis relates to pore forming properties in both Bt toxin types. Syngenta states that Vip3A and Cry proteins share symptomological manifestations of intoxication, and yet, the pore forming properties of Vip3A are unique. The kinetics of Vip3A pore formation are more than 8 times slower than for equimolar Cry1Ab; pore channels were characterized by long open times and a predominant open state; channels formed by Vip3A differ considerably in their conductance state specificity from Cry1A protein. In addition, Domain I, modulated by Domain III interactions, has been considered responsible for the pore formation steps in the Bt Cry protein mode of action. Again, direct structural information is not available for the Vip3A protein, yet, as mentioned above, available information gives no indication of a similar domain organization or α-helical bundle region within the polypeptide sequence as exists for the Cry proteins.

BPPD agrees with Syngenta that the potential risk for cross-resistance between Cry1Ab (and other Cry1A proteins as well as Cry2Ab2) and Vip3A appears low considering that: 1) Vip3A does not bind to APN and cadherin-like proteins and thus, the two types of Bt toxins do not share binding

sites; and 2) Vip3A pore channels formed in the midgut of insects are structurally and functionally distinct from Cry1Ab (and maybe other Cry proteins).

5. Modeling

EPA has used predictive models to compare IRM strategies for Bt crops. Because models cannot be validated without actual field resistance, models have limitations and the information gained from the use of models is only a part of the weight of evidence used by EPA in assessing the risks of resistance development. It was the consensus of the 2000 SAP Subpanel that models were an important tool in determining appropriate Bt crop IRM strategies. They agreed that models were "the only scientifically rigorous way to integrate all of the biological information available, and that without these models, the Agency would have little scientific basis for choosing among alternative resistance management options." They also recommended that models must have an agreed upon time frame for resistance protection. For example, conventional growers may desire a maximum planning horizon of five years, while organic growers may desire an indefinite planning horizon. The Subpanel recommended that model design should be peer reviewed and parameters validated. Models should also include such factors as level of Bt crop adoption, level of compliance, economics, fitness costs of resistance, alternate hosts, spatial components, stochasticity, and pest population dynamics.

Syngenta commissioned Dr. Michael Caprio to evaluate the risk of resistance evolving to VipCot cotton. In the next few paragraphs, BPPD summarizes the most important features and assumptions of the model and simulation results for CBW and TBW. Later, BPPD comments on the input parameter assumptions and applicability of the simulation results.

Dr. Caprio used a spatially explicit, stochastic, two-locus population genetic and landscape model simulating a heterogeneous environment (wild hosts and other Bt crop hosts), incorporating pest biology/ecology, parameter uncertainty (max/min value, most likely value, assuming normal distribution), and allowing for parameter interactions. The first modeling approach and simulations estimated the time to resistance in CBW and TBW populations in the field when ≤100% of the Bt cotton planted was assumed to be VipCot. The rate at which resistance evolved was estimated by determining the amount of time required until the average resistance allele frequency across all fields exceeded 0.5. The second modeling approach explored the impact of VipCot on other single gene Bt cotton events such as for Cry1Ac. These second simulations assumed complete cross-resistance between Cry1A and VipCot (making Cry1Ac and Cry1Ab functionally the same because of reported cross-resistance) and no cross-resistance between Cry1A and Vip3A.

Simulation results for CBW indicate that there were few cases of resistance (0.3%) for Cry1A toxin over 1000 simulations when 80% of all cotton acres were assumed to be VipCot (with 10% of total corn acreage planted to Cry1A hybrid); no resistance to Vip3A evolved in any of the simulations. When 50% of the total corn acreage was planted to a pyramided Vip3A x Cry1A hybrid, resistance to either protein did not evolve after 400 model runs. In the first five years of the simulations, the

rate of increase for the Cry1A allele was lower than the rate of increase for the Vip3A resistance allele; however, the rate measure was strongly affected by the assumption of the initial resistance allele frequency. The overall simulation results suggest that the introduction of VipCot is not likely to select for resistance to Cry1A(b/c) and Vip3A within 20-25 years.

The second set of simulations compared the impact of VipCot on a Cry1A gene expressed in other Bt cotton products (mosaic ratio 1:1 vs. Cry1A only). The simulation outcomes and sensitivity analysis support the hypothesis that the introduction of VipCot delays to a small degree the evolution of resistance to the Cry1A toxin. Specifically, the sensitivity analysis determined that the dominance of the Cry1A resistance gene and mortality due to Vip3A were the main parameters that accounted for more than 83% of the unexplained variance, essentially confirming that Vip3A could delay the resistance to the Cry1A trait. In the simulations, 40% of the time, resistance evolved within 15 years in the Cry1A simulations, and 13% of the time it evolved in the mosaic (VipCot and Cry1A) within the same time frame. Under no circumstances did evolution to Vip3A occur, which means, product failure (resistance to both toxins) did not occur.

Simulation results for TBW indicate that there are few cases of resistance evolving to the Vip3A toxin and that most values (number of occurrences) clump around the frequency of 10^{-3}. There is a 0.2% chance of resistance evolving to Vip3A and Cry1Ab (product failure) within a 25 year time. The most likely outcome for the resistance allele was either 'no change' or 'slight decline' in frequency. Equilibrium for Vip3A resistance allele at around 0.0032 except for when resistance evolved for Cry1A allele leading to considerable variation in the final resistance allele frequency for Vip3A. This suggests that the equilibrium value for the Vip3A allele is dependent on interactions between two loci which generate an effect similar to overdominance. Whether this is an error in the model or an effect to multi-locus overdominance still needs to be further investigated. Dr. Caprio concludes that for the moment it appears that high-dose in combination with fitness cost may lead to unusual results.

The second simulations again compared the impact of VipCot on a Cry1A gene expressed in other Bt cotton products (mosaic ratio 1:1 vs. Cry1A only) and indicate that there may be rapid evolution of resistance to a Cry1A cotton product in absence of VipCot (despite high-dose against TBW). Like in the case of CBW, the introduction of VipCot is expected to decrease the risk of resistance in TBW to the Cry1A toxin in a single trait cotton product. In the simulations, there was a 1% chance of resistance evolving within 20 years and 4% chance that resistance would evolve in 25 years to VipCot.

For CBW and TBW, comparison of VipCot simulations alone versus the mosaic simulation results indicate that the presence of a Cry1A single gene cotton product may seriously reduce the effectiveness of resistance management strategies for dual gene products.

Syngenta provided Dr. Caprio with the following critical information based on interpretations of laboratory and field results: Vip3A mortality in CBW and TBW was assumed to have a maximum of 0.975 (near high-dose assumption) and minimum of 0.875 with the most likely mortality being at 0.92. Similarly, COT67B mortality in CBW and TBW was assumed to have a maximum of 0.999 (high-dose assumption for both species) and a minimum of 0.95 (for CBW). For CBW, the most likely mortality was chosen to be 0.99, a near high-dose. For TBW the assumption was that COT67B provides only a high-dose. These mortality assumptions may be reasonable even though high-dose for Vip3A against CBW was only demonstrated by verification method #5, but not by verification method #1. The assumed actual and minimum mortality for CBW may also be too high (see discussion below). In addition, the mortality assumption (0.99) for COT67B in CBW may be somewhat high in light of the data submitted. For high-dose verification method #1, the reported CBW mortality ranged from 98 -100%, although for method #5, high-dose expression of COT67B was demonstrated. Given what is known about variation in susceptibility of CBW towards other Bt toxins, it may not be realistic to assume that the actual CBW mortality due to COT 102 and COT67B will be 97.5% and 99%, respectively, though the data show at least a "near high dose" can be expected. Also, the COT67B high-dose assumption for TBW has been completely verified by method #1 only. The second verification method (#4) produced a *probable* high-dose for COT67B. Therefore, a more conservative assumption for 'most likely' mortality of 0.95 (instead of 0.999), together with a maximum and minimum mortality value of 0.999 and 0.90 respectively, may have been appropriate mortality input values for the model for TBW and COT67B.

Evolution of resistance to Cry1A toxin in CBW is predicted to occur well beyond the life-time expectancy for any Bt product. Likewise, evolution of resistance to Cry1A and Vip3A toxin in TBW (assuming high-dose and near high-dose, respectively) is also predicted to occur well beyond the life-time expectancy for any Bt product. As described above, BPPD has some questions about the COT67B dose data submitted by Syngenta. If COT67B is actually expressed at a level below those assumed in the model, it is unclear how much or how little such a change in mortality would affect the evolution of resistance in TBW and CBW.

BPPD reiterates from Dr. Caprio's report that the conclusion of delayed resistance to Cry1A toxins following the introduction of VipCot hinges greatly on the assumption that mortality caused by Vip3A (COT102) is high (ranging between 0.875 – 0.975 with most likely mortality being 0.92). BPPD notes that this particular assumption of high mortality was supported by some (but not all) of the submitted dose and efficacy studies. For CBW, the reported actual mortality on COT102 with verification method #1 was 72.4% (rather than 0.92), although data from method #5 indicated high dose and Syngenta supplied the modeler only with the highest mortality data supported by method #5. For TBW, the reported mortality on COT102 with verification method #1 ranged from 66.7 – 95.0% and with method #5 was near high-dose. There are indications that COT102 may not be very efficacious against TBW under some conditions.

Since the uncertainties regarding dose/mortality are relatively minor, BPPD does not request further modeling at this time and concludes that the provided simulation results are sufficient to support the

refuge strategies requested for VipCot cotton. The refuge options proposed by Syngenta match the presently in use cotton refuge strategies (see the next section). While BPPD has some minor reservations about the high-dose assumptions for some toxins against TBW and CBW, it appears that VipCot falls into the existing paradigm for what constitutes an 'effective' high-dose pyramided product. However, since the conclusion of delayed resistance in TBW to Cry1A toxins hinges greatly on the assumption that Vip3A mortality is high, the evolution of resistance in TBW following introduction of VipCot might be expected to occur in less time than predicted by the model. Similarly for CBW, the evolution of resistance to Cry1A toxins may not be delayed by the introduction of VipCot when Vip3A mortality is low.

6. Refuge Strategy

The size, placement, and management of the refuge are critical to the success of the high-dose/structured refuge strategy to mitigate insect resistance to Bt proteins produced in cotton (as well as corn and potatoes). The 1998 SAP Subpanel defined structured refuges to "include all suitable non-Bt host plants for a targeted pest that are planted and managed by people. These refuges could be planted to offer refuges at the same time when the Bt crops are available to the pests or at times when the Bt crops are not available." The 1998 Subpanel suggested that a production of 500 susceptible adults in the refuge for every adult in the transgenic crop area (assuming a resistance allele frequency of 5×10^{-2}) would be a suitable goal. The placement and size of the structured refuge employed should be based on the current understanding of the pest biology data and the technology. The 2000 SAP Subpanel echoed the 1998 SAP's recommendations that the refuge should produce 500:1 susceptible to resistant insects and that regional IRM working groups would be helpful in developing policies (US EPA, 2001).

Under the established refuge strategy for cotton, growers can choose from three structured refuge options, which are thoroughly described in the Agency's 2001 Bt crop reassessment document and briefly listed here:

Option 1: 95:5 external structured, unsprayed refuge; 150 ft wide, within ½ mile of edge of field.
Option 2: 80:20 external sprayed refuge; within 1 linear mile, preferably ½ mile, of edge of field.
Option 3: 95:5 embedded refuge; contiguous or within 1 mile^2 of field and 150 ft wide.

According to their IRM plan (MRID 470176-34), Syngenta has requested identical refuge requirements as for currently registered cotton PIPs. In addition, Syngenta requests that VipCot be considered for the community refuge plan that allows multiple growers to contribute to the overall required refuge acres by planting 20% external, sprayed or 5% external, unsprayed refuge.

BPPD notes that the simulations run by Dr. Caprio addressed refuge option 2 only, the 20% external sprayed refuge (Appendix 5). BPPD would like to expand on this apparent deficiency and clarify that the 20% refuge option may actually be considered the least conservative approach of all three

options, and thus, the modeling assumptions could potentially represent a worst case scenario for IRM because non-Bt cotton refugia are often sprayed with multiple applications of insecticides during a growing season. Shelton et al. (2000) indicate that great care should be used to ensure that refuges sprayed with highly efficacious insecticides produce adequate numbers of susceptible alleles; thus, the 20% external sprayed refuge option for non-Bt cotton, if over sprayed, may not produce a great amount of susceptible adults that could potentially mate with resistant survivors from the Bt-field. Gould & Tabashnik (1998) in their evaluation of Bt cotton IRM options commented that a 20% external refuge that can be extensively treated with insecticidal sprays may result in almost no refuge because all of the susceptible target larvae would be killed. Therefore, the 5% external unsprayed refuge as well as the embedded refuge can be expected to generate the greatest number of susceptible insects that are able to potentially mate with resistant survivors from adjacent Bt-fields in comparison with the worst case scenario for the 20% external sprayed cotton refuge. BPPD requests that in future reports Syngenta be clear as to why certain assumptions were not included in the modeling efforts.

BPPD concludes that based on the modeling, dose, and efficacy studies, the requested refuge options 1-3 and community refuge plan are acceptable for VipCot cotton.

7. Resistance Monitoring

The need for proactive resistance detection and monitoring is critical to the survival of Bt technology. The Agency mandates that registrants monitor for insect resistance (measurement of resistance-conferring alleles) to the Bt toxins as an important early warning sign to developing resistance in the field and whether IRM strategies are working. Grower participation (e.g., reports of unexpected damage) is also important for monitoring. Resistance monitoring is also important because it provides validation of biological parameters used in models. However, resistance detection/monitoring is a difficult and imprecise task. It requires both high sensitivity and accuracy. Good resistance monitoring should have well-established baseline susceptibility data prior to introduction of Bt crops. The chances of finding a resistant larva in a Bt crop depend on the level of pest pressure, the frequency of resistant individuals, the location and number of samples that are collected, and the sensitivity of the detection technique. Therefore, as the frequency of resistant individuals or the number of collected samples increases, the likelihood of locating a resistant individual increases (Roush & Miller 1986). If the phenotypic frequency of resistance is one in 1,000, then more than 3,000 individuals must be sampled to have a 95% probability of one resistant individual (Roush & Miller 1986). Current sampling strategies have a target of 100 to 200 individuals per location. Previous experience with conventional insecticides has shown than once resistant phenotypes are detected at a frequency >10%, control or crop failures are common (Roush & Miller 1986). Because of sampling limitations and monitoring technique sensitivity, resistance could develop to Bt toxins prior to it being easily detected in the field. (http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm)

Syngenta is working with to develop appropriate assay methods for PBW and as well as appropriate assay methods and baseline susceptibility data for TBW and CBW. Collection of insects to be used in bioassays to fulfill the Agency's annual monitoring requirement will focus on cotton growing regions where VipCot cotton sales are expected to be the highest.

Key features of Syngenta's monitoring plan include:

- EPA receives monitoring plan for approval, revised monitoring plan within three months of the date of product registration, and detailed resistance monitoring
- Development of diagnostic concentration assays by Jan 31 of the year after VipCot cotton is registered
- Follow-up on grower, extension specialist, or consultant reports of unexpected damage or control failure of the three main target pests

BPPD considers the monitoring plan adequate for this step of the VipCot registration process. In order to facilitate future communication between BPPD and the registrant, the IRM team makes the following recommendations for monitoring procedures: Syngenta should use the diagnostic concentration (LC_{99}) for both toxins and target pests where the approach has proven successful, and pests are susceptible and population variance is small. In addition, follow-up testing of larval survivors needs to be conducted for all toxins where field population survivorship (≥2 instar) is significantly different from lab/reference colony's survivorship.

Specifically for CBW (but not only), BPPD has the following recommendations for Syngenta: if a good amount of effort has been put into developing a discriminating or diagnostic concentration for CRW and FLCry1Ab and there is evidence that the diagnostic concentration cannot be achieved due to i.e. high-variability in response to the toxin, then a comparison in baseline susceptibility (i.e. LC_{50}s) may be a feasible approach to monitoring. Estimated LC_{50}s may serve well as a baseline monitoring tool for shifts in susceptibility to Bt toxins; however, the LC_{50} approach is not useful in discriminating resistant from susceptible individuals. Therefore, this approach must then be linked with follow-up testing of populations with elevated LC_{50}s relative to previously established baseline susceptibility. Furthermore, BPPD recommends that Syngenta consider head capsule width assay and DNA markers in lieu of mortality based diagnostic concentrations.

8. Grower Education

Syngenta proposes to use the following methods to educate growers which have already been established for other PIPs:

- Signing of grower agreement with purchase of VipCot

- Grower agreement and/or stewardship documents referenced in the grower agreement will set forth terms of current IRM program and contractually bind grower to comply with IRM requirements
- Annual affirmation system for VipCot cotton growers to ensure they understand that they are contractually bound to comply to requirements

Syngenta proposes to 1) submit within 90 days from product registration a copy of the grower agreement/stewardship documents and written description of a system assuring that growers will sign grower agreement; 2) revise and expand as necessary its education program to take into account the information collected through the compliance survey (discussed in Section 9); and 3) maintain records of all signed VipCot cotton grower agreements for three years.

BPPD concludes that the proposed grower education plan meets the Agency's present requirement and is acceptable.

9. Compliance

Grower compliance with refuge and IRM requirements is a critical element for resistance management. Significant non-compliance with IRM among growers may increase the risk of resistance for Bt crops. To minimize the effects of non-compliance, it is necessary to develop a broad compliance program as part of the IRM strategy. Such a program has to include 1) an understanding of the effect of non-compliance on IRM; 2) identification of compliance mechanisms to maximize adoption of IRM requirements; 3) measurement of the level of compliance; and 4) establishment of an enforcement structure to ensure compliance and penalize non-compliance.

Syngenta commits to implementing a compliance assurance program designed to 1) evaluate the extent to which growers of VipCot cotton are complying with the IRM requirements and 2) take reasonable actions necessary to assure that non-compliant growers become compliant with those requirements and submit within 90 days of the date of registration a written description of their compliance assurance program. Consistent with the registration of other cotton Bt PIPs, there are several key elements to the CAP that Syngenta will employ:

- Establish and publish a phased compliance approach that outlines instances of non-compliance to IRM terms and options of responding to non-compliant growers
- Annual survey conducted by third party will measure degree of compliance by growers in different cotton growing regions and consider potential impact of non-response
- Survey will obtain grower feedback on usefulness of educational tools and initiatives and provide understanding of any difficulties growers encounter with IRM requirements
- Annual on-farm assessment followed by appropriate action consistent with the 'phased compliance approach' for non-compliant growers

- 'Tips and complaints' line with follow-up investigations and appropriate actions taken consistent with the 'phased compliance approach' for non-compliant growers

Syngenta proposes to revise and expand, as necessary, its compliance assurance program to take into account information collected through the compliance survey and allow a review of the compliance records by EPA or by a State pesticide regulatory agency.

BPPD concludes that Syngenta has included the major requirements needed by a compliance program and outlined by Agency in the first paragraph of this section and the 2001 Bt crop reassessment document. Syngenta's proposed CAP resembles CAPs for other introduced Bt PIPs and meets the Agency's requirements at this time.

10. Remedial Action Plan

Remedial action plans are a potential response measure should resistance develop to Bt crops. Since resistance may develop in "localized" pest populations, it may be possible to contain the resistance outbreak before it becomes widespread. A specific remedial action plan should clearly indicate what actions the registrant will take in cases of "suspected" resistance (i.e., unexpected damage) and "confirmed" resistance. The remedial action plan can also include appropriate adaptations for regional variation and the inclusion of appropriate stakeholders. To fully mitigate resistance, a critical element of any remedial action plan should be that once pest resistance is confirmed, sales of all Bt cotton hybrids that express a similar protein or a protein in which cross-resistance potential has been demonstrated would be ceased in the affected region (http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm).

Syngenta states that it if resistance to any of the three major target pests is suspected, growers will be informed to use alternate pest control measures such as pesticide treatment, crop rotation the following year, or use of soil or seed insecticides the following year.

Syngenta states that the following steps in order of events will be taken if resistance to any of the three major target pests is confirmed:

- Notify the Agency within 30 days of resistance confirmation
- Notify affected customers and extension agents about confirmed resistance
- Encourage affected customers and extension agents to employ alternative lepidopteran control measures
- Cease sale and distribution of VipCot cotton in affected area
- Devise long-term resistance management action plan according to characteristics of resistance event and local agronomic needs

BPPD concludes that the steps outlined in the remedial action plan and the depth of detail are similar to remedial action plans for other Bt PIP products. In addition, BPPD would prefer if Syngenta 'directed' and 'instructed' affected growers (or have a clause in grower agreements) and extension agents to employ alternative control measures instead of 'encouraged' them when resistance to any of the target pests has been confirmed. Since it is in the economic interest of all parties involved, including the public, that the durability of VipCot cotton is preserved for a long time, Syngenta will need to play a more aggressive and supportive role in case of confirmed resistance to VipCot cotton. A final remedial action plan (based on the steps described above) for tobacco budworm and cotton bollworm will be needed as a term of the registration. This remedial action plan should include definitions of "suspected" and "confirmed" resistance and steps to take in the event of confirmed resistance. The existing remedial action plan developed by the Arizona Bt Cotton Working Group for pink bollworm should be used with VipCot cotton.

11. Reporting Requirements and IRM Conditions of Registration

Syngenta commits to providing an initial report to EPA summarizing activities carried out under the grower education program for the prior year with annual updates thereafter of any substantive changes.

Syngenta will provide 1) a final written summary of the survey results of the prior year to the Agency by January 31 of each year, beginning the year after VipCot cotton is registered; 2) an annual report to the Agency after January 31 of each year summarizing results of their CAP, activities carried out under the CAP for the prior year, and plans for the current year; 3) annual sales summed by state; 4) number of VipCot cotton seeds shipped or sold and not returned, and number of such units sold to persons who have signed grower agreements.

Syngenta will provide to the Agency an annual resistance monitoring report (by August 31 of each year beginning with the year after VipCot cotton is registered) conducted on populations collected the following year.

At this point in the VipCot registration process, the Agency is satisfied with Syngenta's commitment to fulfill the reporting requirements.

In addition to the annual reporting requirements, the following items will be required as terms and conditions of the VipCot registration:

1) A detailed resistance monitoring plan to be submitted within 90 days of the date of registration. The monitoring program description must include sampling (number of locations and samples per location), sampling methodology, bioassay methodology, standardization procedures, detection technique and sensitivity, and the statistical analysis of the probability of detecting resistance

2) A report on baseline susceptibility and diagnostic concentrations for TBW, CBW, and PBW to Vip3Aa19 and modified Cry1Ab by January 31, 2009. Syngenta must include the following testing scheme for survivors of the diagnostic or discriminating concentrations (or identified survivors of any resistance detection method): 1) Determine if the observed effect is heritable; 2) Determine if the increased tolerance can be observed in the field (i.e., survive on VipCot cotton plants); 3) Determine if the effect is due to resistance, 4) Determine the nature of resistance (dominant, recessive), 5) Determine the resistance allele frequency, 6) Determine, in subsequent years, whether the resistance allele frequency is increasing, and 7) Determine the geographic extent of the resistance allele (or alleles) distribution. Should the resistance allele frequency be increasing and spreading, the specific remedial action plan should be implemented to mitigate the extent of Bt resistance.

3) A copy of the grower agreement/stewardship documents and written description of a system assuring that growers will sign grower agreement to be submitted within 90 days of the date of registration.

4) A compliance assurance program (CAP) for VipCot to be submitted within 90 days of the date of registration. The CAP must include a "phased compliance approach" that outlines instances of non-compliance to the IRM requirements and options of responding to non-compliant growers. Options should include withdrawal of the right to purchase VipCot cotton for an individual grower or for all growers in a specific region. An individual grower found to be significantly out of compliance two years in a row should be denied sales of the product the next year. The CAP must also include a description of an annual survey conducted by third party to measure (statistically representative) the degree of compliance by growers in different cotton growing regions. In addition, an annual on-farm survey must be included in the CAP. Non-compliant growers identified in the on-farm survey should face actions consistent with the "phased compliance approach" for non-compliant growers. A program to investigate "tips and complaints" about non-compliant growers must also be included with the CAP.

5) A final remedial action plan (based on the steps described in Syngenta's IRM submission) for tobacco budworm and cotton bollworm to be submitted within 90 days of the date of registration. This remedial action plan should include definitions of "suspected" and "confirmed" resistance and steps to take in the event of confirmed resistance. The existing remedial action plan developed by the Arizona Bt Cotton Working Group for pink bollworm should be used with VipCot cotton (Dennehy, 2002).

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II. E. Benefits and EPA Public Interest Finding

Syngenta submitted two documents to support the benefits of VipCot which will be summarized, and analyzed here: an efficacy study for the 2005 and 2006 season (O'Reilly et al. 2006, MRID 470176-33) and Public Interest Document (Stone 2006, MRID 470347-01) with five sections, 1) public interest finding, 2) grower benefits, 3) human health and environmental benefits, 4) resistance management benefits, and 5) VipCot cotton marketing plan. The IRM chapter submitted (Kurtz et al. 2006, MRID 470176-34) is addressed in this review of the Public Interest Document (PID) as applicable. A complete discussion of IRM for VipCot is contained in section II.D. of this document.

1. Public Interest Finding

a) Summary of Syngenta's Submission (MRID 470347-01):

Syngenta believes that registration of the active ingredients in VipCot meets the criteria for a conditional registration. The registration is clearly in the public interest. Registration and market introduction of Syngenta's VipCot product will result in agronomic, economic, human health, environmental, and resistance management benefits that are highlighted here and discussed in greater detail in later sections of the PID (MRID 470347-01).

Syngenta's VipCot expresses high levels of the proteins, Vip3A and FLCry1Ab, through the combination of transgenic cotton events, COT102 and COT67B, respectively. The Vip3A protein is characterized by a range of properties that clearly distinguish it from the FLCry1Ab protein and the Cry proteins expressed by the Bt cotton varieties currently available to growers. The combination of the Vip3A and FLCry1Ab proteins offers effective protection from the principal Lepidopteran pests of cotton (TBW, CBW, and PBW). As discussed in the confidential marketing plan, VipCot will eventually include an herbicide resistant trait.

Efficacy trials (see section 2 in this chapter) show effective control of CBW, TBW, and PBW. Preliminary yield data demonstrates no negative agronomic factors that will impact a variety development program. Strong efficacy and yield potential combined with Syngenta marketing and field expertise will result in varieties that are very competitive with those varieties now on the market. Current *Bt*-based PIPs offer agronomic and economic benefits compared to the use of chemical pesticides. The introduction of VipCot will continue to enhance the agronomic and economic benefit stream by offering growers a new choice in germplasm, technology, and terms of use.

In addition, a comparison of human health and environmental factors clearly demonstrates both the same low risk potential for VipCot as the current *Bt* PIPs, and the strong reduced risk potential of VipCot cotton compared to the use of alternative conventional chemical pesticides. While VipCot will primarily replace other *Bt* products as it gains market share, its presence in the marketplace will

extend the useful life of Bt-based cotton technology generally, and thus, contribute to the continued human health and environmental benefits resulting from the use of Bt cotton compared to chemical alternatives.

Finally, VipCot will introduce Vip3A, a vegetative insecticidal protein that offers little chance for cross-resistance to its companion protein, FLCry1Ab, or the other *Bt* Cry insecticidal proteins currently marketed. Since VipCot expresses a protein with a unique mode of action, its combination with a viable competitive market presence will offer strong resistance management potential. As discussed in a later section of the report, risk assessment modeling of VipCot cotton confirms the low likelihood of cross-resistance and the potential to extend the useful life of *Bt* cotton technology generally.

b) BPPD's response

BPPD concludes that VipCot cotton is expected to provide similar human health, environmental, agronomic, economic, and IRM benefits also provided by other cotton PIP products already registered by the Agency. For a summary of these general *Bt* cotton benefits, refer to the 2001 Bt crop reassessment (U.S. EPA 2001, http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm).

To grant a conditional registration under Section 3(c)(7)(C) of FIFRA, EPA must determine that such conditional registration will, *inter alia*, be in the public interest. EPA determines whether conditional registration of a pesticide is in the public interest in accordance with the criteria set forth at 51 Fed. Reg. 7628 (*Conditional Registration of New Pesticides*, March 5 1986). On the basis of analysis utilizing these criteria, EPA concludes that the use of VipCot protected cotton will be in the public interest because it results in direct and indirect human and environmental health benefits by providing growers with an additional choice of *Bt* cotton product and the potential to extend the useful life of *Bt* cotton technology generally due to the Vip3A proteins novel mode of action and low likelihood of cross-resistance with other Bt Cry proteins.

BPPD disagrees with Syngenta's claim that VipCot is expected to provide equal or superior efficacy as Bollgard II because no comparative study and data have been submitted in support of this claim. At this point, this particular benefit is unverified.

Furthermore, Syngenta claims that efficacy trials show effective control against PBW; however, no efficacy data for PBW were submitted as stated in the efficacy section of BPPD's review. BPPD revises Syngenta's claim by stating that high-dose results/data for VipCot may support the notion that efficacy of VipCot against PBW may be similar to efficacy of VipCot against TBW and CBW although no field data is available at this time.

2. Efficacy Data

a) Summary of Syngenta's Efficacy Data and Review (MRIDs 470176-33):

Vip3A is effective against the two most economically important lepidopteran pests of cotton in the US (tobacco budworm and cotton bollworm). Likewise, the FLCry1Ab protein provides control of TBW and CBW as well as PBW (pink bollworm). The plant protection and insect control provided by the combined effect of these proteins is expected to be equivalent to or better than the cotton plant-incorporated protectants (PIPs) currently registered by the EPA.

Syngenta's first field trial in 2005 was conducted with cotton plants homozygous for both genes *vip3Aa19* and *flcrylab*. Syngenta planted seeds for Coker 312 (control), COT102, COT67B, and heterozygous VipCot (for both genes) cotton in three US locations (Leland - MS, Winnsboro - LA, Louisburg - NC) with four replicates for each treatment in a randomized complete block design. Field trials were conducted under artificial and/or natural infestation for TBW and CBW, respectively. Insect damage data was collected from each location and used in the statistical analysis (Analysis of Variance, ANOVA).

The second field trial in 2006 was conducted with cotton plants homozygous for both genes *vip3Aa19* and *flcrylab*. Data from trials were collected from eight US locations (Leland – MS, Bossier City – LA, Winnsboro – LA, Scott – AR, Jackson – TN, Seven Springs – NC, Beasley – TX) with four replicates for each treatment plot (Coker 312, COT102, COT67B, and VipCot) in a randomized complete block design. A plot consisted of four 40-foot rows with approximately three plants per foot. Duplicate plantings were made at six of the eight locations to collect data from separate infestations of TBW and CBW. Field trials were conducted under artificial and/or natural infestation for TBW and CBW, respectively. Insect damage data were collected from each location and used in the statistical analysis (ANOVA). While damage assessments were made on a plot-by-plot basis, damage in transgenic plots was often very low such that the data became unsuitable for statistical analysis at the plot level. Consequently, data were often averaged across replicates.

i) Infestation results for 2005

TBW damage (Average % Fruiting Structure) was assessed from artificially infested cotton plants at the three experimental locations in 2005, and the data are presented in Table 14[o]. At all locations, there was negligible damage to heterozygous VipCot, homozygous COT102, and homozygous COT67B cotton (no statistically significant difference detectable). All three treatments had significantly less damage than Coker 312 control plants at a significance level of 5%.

[o] All data tables contain results submitted by Syngenta (Public Interest Document - MRID 470347-01; Efficacy study - MRID 470176-33).

Table 14. Average % Fruiting Damage Following Artificial infestation on Bt Cotton and Control Plants with *H. virescens* in 2005

	Locations, average % damage			
Genotype	**L-MS**	**L –NC**	**W – LA**	**Overall Mean %**
Coker 312	57.8	41.0	4.8	34.5
COT102	3.5	1.0	0.7	1.7
COT67B	0.6	1.0	0.7	0.8
VipCot™	0.5	1.0	0	0.5

CBW damage (Average % Fruiting Structure) was assessed from naturally infested cotton plants at the experimental location in North Carolina. The data are presented in Table 15. No damage was reported on the heterozygous VipCot plants; low to moderate damage was reported on homozygous COT102 and COT67B cotton plants, and great damage on the control plants (Coker 312).

Table 15. Average % Fruiting Damage Following Natural Infestation on Bt Cotton and Control Plants with *H. zea* in 2005

	% damage[1]
Genotype	**L –NC**
Coker 312	88.3
COT102	8.3
COT67B	1.3
VipCot™	0

[1]It was not possible to carry out any formal statistical analysis on this dataset

Combined CBW and TBW damage (% fruiting structure damage) was assessed from naturally infested plots at the experimental location in Louisiana. The data are presented in Table 16. Low level damage was reported on heterozygous VipCot and both homozygous COT102 and COT67B cotton plants; substantial damage was reported on the control plants (Coker 312).

Table 16. Average % Fruiting Damage Following Natural Infestation of Bt Cotton and Control Plants with Mixed *H. zea* and *H. virescens* in 2005

	% damage[1]
Genotype	**W –LA**
Coker 312	26.0
COT102	5.1
COT67B	1.7
VipCot™	1.7

[1]it was not possible to carry out any formal statistical analysis on this dataset

ii) Infestation results for 2006

CBW damage (Average % Fruiting Structure) was assessed from infested cotton plants at seven experimental US locations in 2006, and the data are presented in Table 17 – 19. At all locations, there was low or zero damage to homozygous VipCot, low to moderate damage to homozygous COT102 and homozygous COT67B, and great damage to control plants. Specifically, Table 17 shows that overall mean 'square damage' results for homozygous VipCot cotton were significantly different from individual event results on homozygous COT102 and homozygous COT67B cotton at the 5% significance level. All three Bt treatments had significantly less damage than Coker 312 control plants. Table 18 shows that overall mean 'flower' damage for VipCot cotton was significantly lower than for COT67B (but not for COT102 alone) and that all Bt treatments had significantly less damage reported than for control cotton plants. Table 19 shows no significant difference in overall mean 'boll' damage reported between all three Bt treatments but a significant difference between Bt treatments and control cotton.

Table 17. Average % Square Damage Following Infestation of Bt Cotton and Control Plants with *H. zea* in 2006

	Locations[1], average % damage							
Genotype	**BC – LA**	**W – LA**	**S – AR**	**J – TN**	**SS – NC**	**J – NC**	**B - TX**	**Overall Mean %**
Coker 312	12.4	13.6	12.8	11.5	60.3	67.5	18.5	28.1
COT102	1.7	4.6	3.1	0	2.0	5.5	3.5	2.9
COT67B	1.3	1.6	2.0	0	0.5	5.0	2.0	1.8
VipCot™	0.4	0.6	0.4	0	0.8	0.5	0	0.4

[1] No infestation occurred at L – MS
Shaded number fields indicate locations where data are available for all damage assessments in all locations

Table 18. Average % Flower Damage Following Infestation of Bt Cotton and Control Plants with *H. zea* in 2006

	Locations[1], average % damage					
Genotype	**BC – LA**	**S – AR**	**SS – NC**	**J – NC**	**B – TX**	**Overall Mean %**
Coker 312	10.2	20.5	35.5	43.0	16.3	25.1
COT102	1.2	4.8	0	4.5	4.7	3.0
COT67B	1.8	5.0	0.8	3.5	6.7	3.6
VipCot™	0.4	1.5	0.8	1.0	0	0.7

Shaded number fields indicate locations where data are available for all types of damage assessments in all locations
[1]No infestation occurred at L – MS; no data was collected from W-LA and J-TN

Table 19. Average % Boll Damage Following Infestation of Bt Cotton and Control Plants with *H. zea* in 2006

	Locations[1], average % damage				
Genotype	**BC – LA**	**S – AR**	**SS – NC**	**J – NC**	**Overall Mean %**
Coker 312	15.3	21.0	34.5	67.2	34.5
COT102	1.3	7.3	0	21.7	7.6
COT67B	1.3	5.3	0	12.2	4.7
VipCot™	0.3	1.3	0	7.8	2.4

Shaded number fields indicate locations where data are available for all types of damage assessments in all locations
[1]No infestation occurred at L – MS; no data was collected from W-LA, B-TX, and J-TN

TBW damage was assessed by measuring square, flower, and boll damage following artificial infestation. The trends observed for TBW were similar to those reported above for CBW. At all locations, there was low or zero damage to homozygous VipCot, low to moderate damage to homozygous COT102 and homozygous COT67B, and great damage to control plants. VipCot consistently showed the highest level of protection against TBW damage, while COT102 and COT67B showed good protection against damage. However, the difference between mean damage from VipCot cotton and other Bt cotton plants was not statistically significant (see Tables 20 - 22 for results).

Table 20. Average % Square Damage Following Artificial Infestation of Bt Cotton and Control Plants *H. virescens* in 2006

	Locations[1], % damage					
Genotype	**L-MS**	**BC – LA**	**W – LA**	**S – AR**	**SS – NC**	**Overall Mean %**
Coker 312	29.0	21.5	32.5	20.0	44.5	29.5
COT102	0.5	0.4	9.0	4.9	2.5	3.5
COT67B	0	0.1	4.5	3.8	0.5	1.8
VipCot™	0	0.3	1.0	2.0	0.5	0.8

Shaded number field indicates location where data are available for all types of damage assessments in 2006
[1]No infestations were carried out at J-NC. Not enough damage was reported from J-TN and B-TX for results to be included in analysis

Table 21. Average % Flower Damage Following Artificial Infestation of Bt Cotton and Control Plants with *H. virescens* in 2006

	Average[1] % damage
Genotype	**S – AR**
Coker 312	16.5
COT102	5.8
COT67B	5.9
VipCot™	0.7

Shaded number field indicates location where data are available for all types of damage assessments in 2006
[1]Locations not included here did not get assessed for flower damage, did not receive artificial infestation, or did not have enough damage for inclusion in analysis.

Table 22. Average % Boll Damage Following Artificial Infestation of Bt Cotton and Control Plants with *H. virescens* in 2006

	Locations[1], average % damage			
Genotype	**L-MS**	**S – AR**	**SS – NC**	**Overall Mean %**
Coker 312	33.0	12.5	74.0	39.8
COT102	0.5	9.0	12.0	7.2
COT67B	0	5.0	7.0	4.0
VipCot™	0	0.3	1.0	0.4

Shaded number field indicates location where data are available for all types of damage assessments in 2006
[1]No infestation was carried out at J – NC. Infestations at BC – LA, J – TN, and B – TX did not result in enough damage for results to be included in analysis.

iii) Syngenta's Efficacy Conclusions

The efficacy trials performed in 2006 were designed to provide data on the efficacy of Syngenta's VipCot cotton against the heliothine pests, *H. virescens* and *H. zea*. Preliminary data obtained in 2005 demonstrated the efficacy of VipCot cotton against these pests (plants were heterozygous for vip3A and flcry1Ab genes). In contrast, the VipCot cotton tested in 2006 was homozygous for both traits. VipCot cotton efficacy data against *H. virescens* were mainly derived from artificial infested field trials, whereas, *H. zea* data were derived mostly from natural infestations supplemented by artificial infestations at only one location (Scott, Arizona).

Similar trends were observed following infestation by both *H. virescens* and *H. zea* in 2006. COT102 and COT67B cotton showed good levels of control under most circumstances, and results were consistent with the data obtained in 2005. In every case, VipCot cotton provided as good or better control of *H. virescens* and *H. zea* than either of the component events. These data confirm the preliminary indications obtained in 2005 that VipCot cotton has excellent activity to these key heliothine cotton pests in the US.

b) BPPD's response

BPPD concludes that efficacy benefits of VipCot cotton are like the efficacy benefits of other cotton PIPs already registered (i.e. a high level of protection against the three major Bt cotton target pests).

Overall, 2005 and 2006 efficacy data show that VipCot cotton and its two single event cotton plants provide good protection against TBW and CBW. In 2005, efficacy results for CBW on VipCot were statistically different from efficacy results of individual event cotton plants. It appears that environmental variables may have the potential to affect efficacy of VipCot as compared to the efficacy of the two single event plants.

Syngenta states that Vip3A is effective against tobacco budworm and cotton bollworm, while FLCry1Ab protein provides control of TBW and CBW as well as PBW (pink bollworm). BPPD clarifies here that efficacy data were submitted for CBW and TBW and not for PBW. However, evidence of high-dose was provided for VipCot and COT67B for PBW in the IRM submission (MRID 470176-34).

3. Grower Benefits

a) Summary of Syngenta's Submission and Expert Reports in the PID (MRID 470347-01):

i) Agronomic Benefits

The introduction of Bt cotton has transformed cotton production in the United States. The dramatic shift from conventional chemical insecticides to Bt cotton during the last 10 years has occurred because of the strongly positive agronomic and economic factors associated with Bt cotton technology. VipCot will continue in this tradition.

Syngenta asked experts from three cotton growing regions, Dr. Bradley of North Carolina University, Dr. Leonard of Louisiana State University and Jack Hamilton, Regents chair in cotton production, as well as Dr. Rummel of Texas A & M University, to comment on agronomics associated with Bt cotton generally and on VipCot specifically. The following is a short summary of the conclusions extracted by BPPD from the expert reports, which were submitted by Syngenta as separated appendices in the PID (MRID 470347-01):

The cotton growing region of southeastern US recovered from its demise with the boll weevil eradication in the 1980s. The introduction of Bt cotton in the 1990s was the second event that further boosted the cotton industry and enabled farmers to further increase the cotton acreage, for example, from 500 to 800 thousand acres annually (in NC) during the next decade. Bt crops in general have produced great economic benefits to US growers while leading to a reduced reliance on conventional insecticides. For cotton alone, EPA estimated that there was a 7.5-million acre

reduction in insecticide use due to planting of Bt varieties in 1999. During the first several years of the Bt cotton introduction, average (over years and locations) Bt cotton yields in the US exceeded those of conventional cotton with a mean profit advantage ranging from $16 to less than $173/acre. Aside from environmental benefits mentioned above, other extended benefits of Bt cotton are fuel savings and reduced labor expenditures. Specifically, in North Carolina, Bt cotton is now planted in areas where cotton could not be grown before because of insecticide drift concerns in school and housing areas, etc.

Preliminary field testing results suggest that Vip3A proteins may have superior efficacy compared to Widestrike and equal efficacy to Bollgard II. Dr. Bradley further writes that Cry1Ac resistant TBW have shown to be susceptible to Vip3A toxins and Vip3A producing plants in the lab and greenhouse. These data indicate that VipCot provides growers with viable alternatives to other Bt cotton varieties presently registered. In addition, VipCot offers a mechanism of resistance management.

In the mid-south states of the US (AR, LA, MS, TN), cotton is historically one of four crops that makes up the largest amount of acres planted (average range 530,000 – 1.22 million acres). Since the introduction of Bollgard (Cry1Ac toxin), losses to cotton growers have been reduced from damage caused by TBW and CBW. Losses were further reduced with the introduction of Bollgard II and Widestrike. Average yield loss to cotton growers was 4.1% from 1990-1995. Since 1999, losses from Bt and non-Bt varieties, dropped to <1% and 2 - 5%, respectively. Annual lint yields were the highest ever reported in these states from 2002 – 2006. More than 80% of cotton acreage in 2005 was planted with Bt varieties. Field trials across the mid-south States showed enhanced efficacy to single Cry proteins against bollworms and satisfactory control against other Lepidopteran pests. Cotton entomologists expect VipCot to provide equal or better protection against these pests than other currently registered Bt cotton varieties. Because Vip3A is the first transgenic non-Cry protein introduction into cotton varieties, VipCot is expected to have considerable value as a Bt resistance management tool.

The boll weevil eradication and introduction of Bt cotton allowed cotton production in Texas to rebound and also expand into previous non-cotton areas of the Texas Pan handle. Texas cotton growing regions are as diverse geographically (rainfall, soil type, length of growing season, available irrigation, and production techniques) as they are numerous, and production requirements from region to region vary greatly. Bt cotton varieties that provide optimum performance in one region, may exhibit marginal performance in another. Texas growers require access to numerous insect resistant cotton varieties to meet the requirements of the various production regions. Tests conducted across the cotton belt show good efficacy of VipCot, and efficacy at least as good to Bollgard II is expected. No cross-resistance between Cry1Ac and Vip3A was detected in lab resistant CBW. The novel mode of action of Vip3A differs in several respects compared to Cry1Ab, and its availability to growers is expected to serve as a Bt resistance management tool.

Insect efficacy is only one factor that must be considered when developing new transgenic technologies for use in commercial cotton. Yield potential and fiber quality traits must also be considered. In both cases, available data support the potential for viable VipCot varieties.

Concerning yield data (Appendix 4 of PID, MRID 470347-01), Syngenta's marketing partner evaluated VipCot in a multiple location trial series representative of the US cotton growing region. The results showed no significant differences in seed cotton yield between Cocker 312 and VipCot (see Table 23). No significant location by variety interaction occurred in the 2006 testing ($p = 0.1031$). The conclusion from this preliminary work is that VipCot demonstrates no negative agronomic characteristics that would impact a variety development program. Agronomic, efficacy, yield, and fiber quality data and information all support the strong potential for VipCot to become a quality competitive product in the Bt cotton market. These factors coupled with an aggressive marketing plan and strong market presence will provide growers with additional choices and generate economic benefits.

Table 23. Seed Cotton per Acre generated for control and transgenic cotton plants during 2006

Location	**Cocker 312, Seed cotton/acre**	**COT 102 x 67B, Seed cotton/acre**
Belle Mina, AL	1387	1330
College Station, TX	1958	2036
Estill, SC	1833	1713
Hartsville, SC	2467	2222
Haskell, TX	2254	1999
Portageville, MO	2014	2306
Red Springs, NC	2300	2072
Tifton, GA	2879	3303
Winterville, MS	3060	3339
Variety x Location ($p< 0.05$)	0.1031	
	Cocker 312, Avg. seed cotton/acre	**COT 102 x 67B, Avg. seed cotton/acre**
	2239	2257
Variety ($p< 0.05$)	0.7729	

Concerning fiber quality, lint samples of the component events COT102 and COT67B (but not VipCot) have been analyzed for fiber characteristics and quality cotton varieties. In work conducted to date, there were no significant differences between both events and non-transgenic genotypes for fiber micronaire, length, or uniformity and thus, none are expected when these events are combined as VipCot.

ii) Economic Benefits

Syngenta considers the major benefits resulting from the introduction of VipCot will be additional grower choice, increased competition, and extended useful life of Bt cotton technology generally (resulting from the unique mode of action of the Vip3A protein expressed by VipCot) rather than a major shift from chemical insecticide treated acres to new Bt planted acres. Syngenta projects additional economic benefits of $83 million will accrue from the regulatory approval and use of VipCot (report by Dr. Wailes submitted as Appendix 5 in PID, MRID 470347-01). The $83 million dollar value is the product of conservative estimates to predict the net present value of VipCot and additional value that VipCot will bring to the market in terms of added competition and grower choice for the first eight years of sales. Furthermore, Dr. Wailes estimates the regional distribution of these benefits based on existing adoption levels of transgenic cotton varieties (Appendix 5 in PID).

b) BPPD's response

VipCot cotton has similar general grower benefits as previously-registered cotton PIPs (i.e. yield, lint quality) as described by the Agency in the 2001 Bt crop reassessment. The general benefits for Bt cotton are summarized in U.S. EPA 2001 (http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm).

BPPD notes that the novel mode of action of Vip3A and lack of cross-resistance observed in Cry1Ac-resistant lab colonies of CBW and TBW may provide mechanisms by which the useful life of Bt technology could be extended.

Syngenta states that preliminary field testing results suggest that Vip3A proteins may have superior efficacy than Widestrike (registered by Dow AgroSciences) against caterpillar pests and equal or superior efficacy to Bollgard II (registered by Monsanto). No comparative data were submitted to the Agency to back this claim. At this point, BPPD will not consider this proposed benefit.

BPPD focused on the benefits from VipCot only and not on the potential agronomic and economic benefits (i.e. additional economic benefits of $83 million dollars as cited in Dr. Wailes analysis, Appendix 5 of PID, MRID 470347-01) of VipCot/RR Flex (herbicide tolerant trait). It may be that additional benefits are derived from an herbicide tolerant trait in VipCot. On the other hand, such a trait could also increase the risk of weed resistance. Nonetheless, BPPD would like to note the following regarding Dr. Wailes' analysis of expansion in Syngenta's share market. The analysis is based on two major assumptions as stated in the report: 1) increased productivity based on higher yields relative to conventional cotton and potentially other transgenic varieties and 2) increased productivity based on cost efficiencies available through more competitive technology fees and services provided relative to other transgenic varieties. As mentioned throughout BPPD's review of Syngenta's PID, the higher yield assumption for VipCot as compared to other transgenic varieties remains unsubstantiated at this time, and therefore, should not be considered as an input in the model. Furthermore, the 'new value extraction model of shared risk price structures' described in

the PID proposes to use the lower technology fee approach for non-irrigated cotton regions as well as growers who suffer reduced yield; however, the model is still awaiting final approval by the Joint Syngenta/D & PL License Management Committee. Therefore, the share market analysis predicting expansion in the marketplace is based on two major assumptions that contain some hypothetical (greater or equal yield than Bollgard II and greater yield than WideStrike) and unverified information (lower technology fees still awaiting approval). Once one of these assumptions or both are verified, the benefit claims of Syngenta's share market analysis may be warranted.

With respect to the seed yield data and analysis submitted, BPPD notes that the 'Variety x Location' interaction value is 0.1031 (variety meaning Cocker 312 and VipCot). This value seems to indicate that there is potential for interaction despite it not being statistically significant at the 5% level. Nonetheless, it may be important for farmers to realize that when planting VipCot across states in the cotton belt, such as tested in the study (MS, AL, TX, SC, MO, NC, GA), 90 times out of 100 times, they may see an effect on seed yield due to geographic/environmental differences. BPPD notes that this frequency of 'Location x Variety' interaction is rather high and should be taken into consideration when thinking about a variety development program even if Syngenta concludes, based on statistical significance at the 5% level, that no negative characteristics are exhibited.

4. Human Health and Environmental Benefits

a) Summary of Syngenta's Submission (MRID 470347-01):

EPA has consistently found that the registration of Bt PIPs is in the public interest. These findings have largely been based on a determination that Bt PIPs present less risk than conventional pesticide alternatives. The Agency's view concerning Bt PIPs is well accepted and supported by the work of others. Brookes and Barfoot (2005) presented findings that the global introduction of genetically modified crops resulted in the reduction in use of chemical pesticides by 172 million kilograms, and reduced the environmental footprint associated with pesticide use by 14% during the period 1996 to 2004.

Neither the Vip3A, nor the FLCry1Ab protein is likely to be a food allergen, and toxicity studies indicate no hazard concern. The same profile holds for the marker protein, hygromcyin B phosphotransferase, which is exempt from the requirement for a tolerance. Syngenta studies also provide the same solid evidence for the environmental safety of the proteins expressed in VipCot. Extensive testing shows no real risk concern.

b) BPPD's response

EPA reviewed product characterization, human health safety, and aquatic and terrestrial wildlife studies submitted by Syngenta and agrees with Syngenta's conclusion. There is no human health concern with respect to toxicity or allergenicity and no environmental concern with respect to

toxicity of the two proteins expressed in VipCot, Vip3Aa and FLCry1Ab. VipCot cotton has similar general human health and environmental benefits of already registered cotton PIPs. For further information regarding the Agency's summary of these benefits, refer to the 2001 Bt crop reassessment (U.S. EPA 2001, http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm).

5. Resistance Management Benefits

a) Summary of Syngenta's Benefits Submission (MRID 470347-01) and IRM Submission (470176-34):

While COT102 alone does not express a high-dose against any of the three key pests, it is active against CBW, TBW, and PBW at greater than 25-fold dilutions. COT67B alone expresses a high-dose for all three pests, and both proteins together, as expressed in VipCot cotton, result in a high-dose for CBW, TBW, and PBW.

Supporting information (i.e. lack of sequence homology between Vip3A and Cry proteins, no indication of similar domain structure between Vip3A and Bt Cry proteins, secretion of Vip and Bt Cry proteins at different stage of bacterial growth, different binding sites) and study results demonstrate the lack of cross-resistance between Vip3A and other Bt Cry proteins including FLCry1Ab protein in VipCot as well as other Cry proteins in currently sold Bt cotton PIPs.

Resistance risk assessment modeling by Dr. Caprio predicts that the risk of resistance developing to VipCot is very low, and further, modeling predicts that the use of VipCot can delay the development of resistance to cotton varieties expressing Cry toxins.

b) BPPD's response

BPPD concurs with Syngenta's conclusion that VipCot has the following benefits: lack of cross-resistance and potential to delay development of resistance in other cotton varieties expressing Cry toxins (based on i.e. unique mode of action of Vip3Aa and cross-resistance studies done on Cry1Ac resistant lab colonies of CBW and TBW). As a unique mode of action, VipCot will have similar general IRM benefits as other cotton PIP products, which are thoroughly discussed and summarized in the Agency's 2001 Bt crop reassessment (U.S. EPA 2001, http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm).

6. VipCot Cotton Marketing Plan

Syngenta submitted a marketing plan for VipCot cotton as part of the benefits submission. This plan included information on VipCot stacking with herbicide tolerance traits, potential market share, pricing, and models to determine marketing strategy. This portion of the benefits submission was classified as Confidential Business Information and is not included or summarized in this document.

BPPD notes that benefits from Syngenta's marketing plan seem numerous, especially if the pricing models will get adopted as proposed in the PID. Furthermore, the introduction of VipCot is expected to increase competition and make the demand curve more elastic. Thus, VipCot may lead to an increase in Bt use and a reduction in price of other registered Bt cotton products. These are examples of benefits to growers, the public, and environment. However, benefits based on modeling are still hypothetical until the model has been vetted by management of Syngenta and partners.

BPPD's comments regarding VipCot/herbicide tolerant trait are first listed in section 3 (Grower's Benefits) and reiterated here. BPPD focuses only on the benefits from VipCot and not on the potential agronomic and economic benefits of VipCot/RR Flex. It may be that additional benefits are derived from an herbicide tolerant trait in VipCot. On the other hand, such a trait could also increase the risk of weed resistance.

7. References

BPPD, 2003. EPA preliminary review of public interest document submitted by Syngenta Seeds, Inc. for VIP3A cotton (event COT102) in support of a conditional registration under FIFRA Section 3(c)(7)(C). E. Brandt and S. Matten memorandum to L. Cole; Nov 13, 2003.

Kurtz, R., McCaffery, A., O'Reilly, D., Stone, T. 2006. Insect resistance management considerations for VipCot™ Cotton. Report submitted from Syngenta Biotechnology, Inc. MRID 470176-34.

O'Reilly, D., Dickerson, D., Negretto, D., Minton, B., Martin, S. 2006. Field Efficacy Evaluation of component events COT102 and COT67B and stacked COT102 x COT67B cotton (VipCot™ Cotton) in 2005 and 2006. Report submitted from Syngenta Biotechnology, Inc. MRID 470176-33.

Schnepf, E., Crickmore, N., Van Rie, D. Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews, Vol. 62 (3): 775-806.

Stone, T., Martin, T., and Tinsworth, E.F. 2006. Public Interest Document in Support of Registration of the Plant Incorporated Protectant Proteins Vip3A and FLCry1Ab as Expressed in the Combined

Trait COT102 x COT67B (VipCot™ Cotton). Report submitted from Syngenta Biotechnology, Inc. MRID 470347-01.

US EPA, 1998. FIFRA Scientific Advisory Panel Subpanel on *Bacillus thuringiensis (Bt)* Plant-Pesticides and Resistance Management, February 9 and 10, 1998.

US EPA, 2001. Biopesticides Registration Action Document – *Bacillus thuringiensis* Plant Incorporated Protectants, http://www.epa.gov/oppbppd1/biopesticides/pips/bt_brad.htm.

III. Terms and Conditions of the Registration

The following terms and/or conditions are required for the section 3(c)(7)(C) registration of VipCot cotton:

1) The registration will automatically expire on midnight September 30, 2011.

2) All data required for registration of the product under FIFRA § 3(c)(5) must be submitted when the Agency requires all registrants of similar products to submit such data.

3) The following restrictions regarding gene flow are required:

The following information regarding commercial production must be included in the grower guide for VipCot Cotton:

a) No planting of VipCot cotton is permitted south of Route 60 (near Tampa) in Florida.
b) Commercial culture of VipCot cotton is prohibited in Hawaii, Puerto Rico, and the U.S. Virgin Islands.

The following information regarding test plots and seed production must be included on bags of VipCot cotton intended for these purposes:

a) Test plots or breeding nurseries, regardless of the plot size, established in Hawaii must not be planted within 3 miles of *Gossypium tomentosum*;

b) Experimental plots and breeding nurseries of Bt cotton are prohibited on the U.S. Virgin Islands; and

c) Test plots or breeding nurseries, regardless of the plot size, established on the island of Puerto Rico must not be planted within 3 miles of feral cotton plants.

4) The following restrictions regarding Insect Resistance Management are required:

VipCot Bt cotton is not permitted to be planted in the following counties of the Texas panhandle: Dallam, Sherman, Hansford, Ochiltree, Lipscomb, Hartley, Moore, Hutchinson, Roberts, and Carson.

5) The following data and/or information must be submitted in the time frames listed:

Study Type	Required Data	Due Date
Residue Analytical Method - Plants (OPPTS 860.1340)	An independent lab validation of the "SeedChek" analytical method for the detection of Vip3Aa19 and modified Cry1Ab. You must also agree to provide to the EPA laboratory (Ft. Meade, MD) methodology and/or reagents necessary for validation of such analytical method within 6 months from the date that the Agency requests them.	May 1, 2009
Aquatic Invertebrate Toxicity (OPPTS 885.4240)	A 7-14 day *Daphnia* study as per the OPPTS 885.4240 guideline must be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the 7-14 day *Daphnia* study. **Separate studies for Vip3Aa (COT102) and modified Cry1Ab (COT67B) must be preformed.**	May 1, 2009
Insect Resistance Management - Resistance Monitoring	A detailed resistance monitoring plan for the major pests of VipCot cotton: tobacco budworm, cotton bollworm, and pink bollworm.	Within 90 days of the date of registration
Insect Resistance Management - Resistance Monitoring	Baseline susceptibility and diagnostic concentration determinations for tobacco budworm, cotton bollworm, and pink bollworm to Vip3Aa19 and modified Cry1Ab.	January 31, 2009
Insect Resistance Management - Compliance	A compliance assurance program (CAP) for VipCot must be submitted and must include a "phased compliance approach" that outlines instances of non-compliance to the IRM requirements and options of responding to non-compliant growers.	Within 90 days of the date of registration
Insect Resistance Management - Compliance	A copy of the grower agreement/stewardship documents and written description of a system assuring that growers will sign grower agreement must be submitted.	Within 90 days of the date of registration
Insect Resistance Management – Remedial Action Plans	A final remedial action plan for tobacco budworm and cotton bollworm. The remedial action plan must include definitions of "suspected" and "confirmed" resistance and steps to take in the event of confirmed resistance.	Within 90 days of the date of registration

6) The following Insect Resistance Management Program is required for VipCot:

a) The required IRM program for VipCot Bt cotton must have the following elements:

- Requirements relating to creation of a non-Bt cotton refuge in conjunction with the planting of any acreage of VipCot Bt cotton.
- Requirements for Syngenta Seeds to prepare and require VipCot Bt cotton users to sign "grower agreements" which impose binding contractual obligations on the grower to comply with the refuge requirements;

- Requirements for Syngenta Seeds to develop, implement, and report to EPA on programs to educate growers about IRM requirements;
- Requirements for Syngenta Seeds to develop, implement, and report to EPA on programs to evaluate and promote growers' compliance with IRM requirements;
- Requirements for Syngenta Seeds to develop, implement, and report to EPA on programs to evaluate whether there are statistically significant and biologically relevant changes in susceptibility to the Vip3Aa19 and modified Cry1Ab proteins in the target insects;
- Requirements for Syngenta Seeds to develop, and if triggered, to implement a "remedial action plan" which would contain measures Syngenta Seeds would take in the event that any insect resistance was detected as well as to report on activity under the plan to EPA;
- Annual reports on or before January 31st each year.

b) Refuge Requirements

All growers of VipCot cotton must employ one of the following structured refuge options:

1) External, Unsprayed Refuge

Ensure that at least 5 acres of non-Bt cotton (refuge cotton) is planted for every 95 acres of VipCot cotton. The size of the refuge must be at least 150 feet wide, but preferably 300 feet wide. This refuge may not be treated with sterile insects, pheromone, or any insecticide (except listed below) labeled for the control of tobacco budworm, cotton bollworm, or pink bollworm. At the pre-squaring cotton stage only, the refuge may be treated with any lepidopteran insecticide to control foliage feeding caterpillars. The refuge may be treated with acephate or methyl parathion at rates which will not control tobacco budworm or the cotton bollworm (equal to or less than 0.5 lbs active ingredient per acre). The variety of cotton planted in the refuge must be comparable to VipCot cotton, especially in the maturity date, and the refuge must be managed (e.g., planting time, use of fertilizer, weed control, irrigation, termination, and management of other pests) similarly to VipCot cotton. Ensure that a non-Bt cotton refuge is maintained within at least ½ linear mile (preferably adjacent to or within 1/4 mile or closer) from the *Bt* cotton fields.

2) External, Sprayed Refuge

Ensure that at least 20 acres of non-Bt cotton are planted as a refuge for every 80 acres of VipCot cotton (total of 100A). The variety of cotton planted in the refuge must be comparable to Bt cotton, especially in the maturity date, and the refuge must be managed (e.g., planting time, use of fertilizer, weed control, irrigation, termination, and management of other pests) similarly to VipCot cotton. The non-Bt cotton may be treated with sterile insects, insecticides (excluding foliar Bt kurstaki products), or pheromones labeled for control of the tobacco budworm, cotton bollworm, or pink bollworm. Ensure that a non-Bt refuge is maintained within at least 1 linear mile (preferably within ½ mile or closer) from the Bt cotton fields.

3) Embedded Refuge

Ensure that at least 5 acres of non-Bt cotton (refuge cotton) are planted for every 95 acres of VipCot cotton (total of 100A). The refuge cotton must be embedded as a contiguous block within the VipCot field, but not at one edge of the field (i.e., refuge block(s) surrounded by Bt cotton). For very large fields, multiple blocks around the field may be used. For small or irregularly shaped fields, neighboring fields farmed by the same grower can be grouped into blocks to represent a larger field unit, provided the block exists within one mile squared of the Bt cotton and the block is at least 150 feet wide, but preferably 300 feet wide. Within the larger field unit, one of the smaller fields planted to non-Bt cotton may be utilized as the embedded refuge. The variety of cotton planted in the refuge must be comparable to Bt cotton, especially in the maturity date, and the refuge must be managed (e.g., planting time, use of fertilizer, weed control, irrigation, termination, and management of other pests) similarly to VipCot cotton. The non-Bt cotton may be treated with sterile insects, insecticides (excluding foliar Bt kurstaki products), or pheromones labeled for control of the tobacco budworm, cotton bollworm, or pink bollworm whenever the entire field is treated. The refuge may not be treated independently of the surrounding VipCot field in which it is embedded (or fields within a field unit).

4) Embedded Refuge (for pink bollworm only)

Refuge cotton must be planted as at least one single non-Bt cotton row for every six to ten rows of VipCot cotton. The refuge may be treated with sterile insects, any insecticide (excluding foliar Bt kurstaki products), or pheromone labeled for the control of pink bollworm whenever the entire field is treated. The in-field refuge rows may not be treated independently of the surrounding Bt cotton field in which it is embedded. The refuge must be managed

(fertilizer, weed control, etc.) identically to the VipCot cotton. There is no field unit option.

5) Community Refuge Option

This option allows for multiple growers to manage refuge for external, unsprayed and external, sprayed refuge options or both. This option is not allowed for the embedded/in-field refuge options. The community refuge for insect resistance management must meet the requirements of the 5% external, unsprayed and/or 20% sprayed option, or an appropriate combination of the two options. The community refuge program must consist of the following:

There will be a community refuge coordinator for each community. Each community refuge coordinator must submit a signed community refuge form listing all of the participants in the community to Syngenta Seeds by July 1st annually. Syngenta Seeds must provide EPA, if requested, with a copy of the signed community refuge form. The community refuge coordinator will maintain a copy of the field map (to scale) or suitable scalar representation of the community refuge for review by Syngenta Seeds or EPA as part of the compliance program.

On an annual basis, Syngenta Seeds must conduct at least one telephone audit of a statistically representative sample of community refuge coordinators from communities in all states participating in the community refuge. EPA shall review the questions annually prior to the start of the growing season.

The community refuge program users must be included in the telephone compliance survey and the on-farm visits to be conducted by Syngenta Seeds under section d. below.

Beginning January 31, 2010 and annually each January 31st, Syngenta Seeds must provide a written report to EPA annually on community refuge use and compliance. The community refuge report may be combined in a single report with other compliance activities.

On an annual basis, Syngenta Seeds must conduct a review of the community refuge program and submit that review to the Agency as to any proposed changes by January 31st. An appropriate amendment for any proposed changes must be submitted to the Agency.

c) Grower Agreements

The following provisions regarding grower agreements are required for VipCot:

1) Persons purchasing the VipCot cotton product must sign a grower agreement. The term "grower agreement" refers to any grower purchase contract, license agreement, or similar legal document.

2) The grower agreement and/or specific stewardship documents referenced in the grower agreement must clearly set forth the terms of the current IRM program. By signing the grower agreement, a grower must be contractually bound to comply with the requirements of the IRM program.

3) Syngenta Seeds must implement a system which is reasonably likely to assure that persons purchasing the Bt cotton product will affirm annually that they are contractually bound to comply with the requirements of the IRM program. A description of the system must be submitted to EPA within 90 days from the date of registration.

4) Syngenta Seeds must use an approved grower agreement and must submit to EPA within 90 days from the date of registration a copy of that agreement and any specific stewardship documents referenced in the grower agreement. If Syngenta Seeds wishes to change any part of the grower agreement that would affect either the content of the IRM program or the legal enforceability of the provisions of the agreement relating to the IRM program, thirty days prior to implementing a proposed change, Syngenta Seeds must submit to EPA the text of such changes to ensure the agreement is consistent with the terms and conditions of this amendment.

5) Syngenta Seeds must implement an approved system which is reasonably likely to assure that persons purchasing VipCot cotton sign grower agreement(s). A description of the system must be submitted to EPA within 90 days from the date of registration.

6) Syngenta Seeds shall maintain records of all VipCot cotton grower agreements for a period of three years from December 31 of the year in which the agreement was signed.

7) Beginning on January 31, 2010 and annually thereafter, Syngenta Seeds shall provide EPA with a report on the number of units of the VipCot cotton seed shipped and not returned and the number of such units that were sold to persons who have signed grower agreements. The report shall cover the time

frame of the twelve-month period covering the prior October through September.

8) Syngenta Seeds must allow a review of the grower agreements and grower agreement records by EPA or by a State pesticide regulatory agency if the State agency can demonstrate that the names, personal information, and grower license number will be kept as confidential business information.

d) IRM Education and IRM Compliance Monitoring Programs

The following IRM education and compliance monitoring programs must be implemented for VipCot:

1) Syngenta Seeds must design and implement a comprehensive, ongoing IRM education program designed to convey to VipCot cotton users the importance of complying with the IRM program. The program shall include information encouraging Bt cotton users to pursue optional elements of the IRM program relating to refuge configuration and proximity to Bt cotton fields. The education program shall involve the use of multiple media, e.g. face-to-face meetings, mailing written materials, and electronic communications such as by internet or television commercials. Copies of the materials, including the Grower Guide or other technical bulletins, must be submitted to EPA for their records. The program shall involve at least one written communication annually to each VipCot cotton grower separate from the grower agreement. Syngenta Seeds shall coordinate its education program with educational efforts of other organizations, such as the National Cotton Council and state extension programs.

2) Annually, Syngenta Seeds shall revise, and expand as necessary, its education program to take into account the information collected through the compliance survey required under paragraph 6 below and from other sources. The changes shall address aspects of grower compliance that are not sufficiently high.

3) Beginning January 31, 2009 and annually thereafter, Syngenta Seeds shall provide a report to EPA summarizing the activities it carried out under its education program for the prior year and its plans for its education program during the current year.

4) Syngenta Seeds shall design and implement an IRM compliance assurance program designed to evaluate the extent to which growers are complying with the IRM program and that takes such actions as are reasonably needed to

assure that growers who have not complied with the program either do so in the future or lose their access to VipCot cotton. Syngenta Seeds must prepare and submit within 90 days of the date of registration a written description of the compliance assurance program. Other required features of the program are described in paragraphs 5 - 12 below.

5) Syngenta Seeds shall establish and publicize a "phased compliance approach," i.e., a guidance document that indicates how Syngenta Seeds will address instances of non-compliance with the terms of the IRM program and general criteria for choosing among options for responding to any non-compliant growers. The options shall include withdrawal of the right to purchase VipCot cotton for an individual grower or for all growers in a specific region. An individual grower found to be significantly out of compliance two years in a row would be denied sales of the product the next year.

6) The IRM compliance assurance program shall include an annual survey of a statistically representative sample of VipCot cotton growers conducted by an independent third party. The survey shall measure the degree of compliance with the IRM program by growers in different regions of the country and consider the potential impact of non-response. Syngenta Seeds shall provide a written summary of the results of the prior year's survey to EPA by January 31st of each year. Syngenta Seeds shall confer with EPA on the design and content of the survey prior to its implementation.

7) Annually, Syngenta Seeds shall revise, and expand as necessary, its compliance assurance program to take into account the information collected through the compliance survey (required under paragraph 6) and from other sources. The changes shall address aspects of grower compliance that are not sufficiently high. Syngenta Seeds will confer with EPA prior to adopting any changes.

8) Syngenta Seeds must conduct an annual on-farm assessment program. Syngenta Seeds shall train its representatives who make on-farm visits with VipCot cotton growers to perform assessments of compliance with IRM requirements. In the event that any of these visits results in the identification of a grower who is not in compliance with the IRM program, Syngenta Seeds shall take appropriate action, consistent with its "phased compliance approach," to promote compliance.

9) Syngenta Seeds shall carry out a program for investigating "tips and complaints" that an individual grower or growers is/are not in compliance

with the IRM program. Whenever an investigation results in the identification of a grower who is not in compliance with the IRM program, Syngenta Seeds shall take appropriate action, consistent with its "phased compliance approach."

10) If a grower, who purchases VipCot cotton for planting, was specifically identified as not being in compliance during the previous year, Syngenta Seeds shall visit the grower and evaluate whether that the grower is in compliance with the IRM program for the current year.

11) Beginning January 31, 2010 and annually thereafter, Syngenta Seeds shall provide a report to EPA summarizing the activities it carried out under its compliance assurance program for the prior year and its plans for its compliance assurance program during the current year. Included in that report will be the percent of growers using each refuge option (or combination of options) by region, the approximate number or percent of growers visited on farm by Syngenta Seeds and the results of these visits the number of tips investigated, the percent of growers not in compliance with each refuge option (both size and distance), and the follow-up actions taken.

12) Syngenta Seeds must allow a review of the compliance records by EPA or by a State pesticide regulatory agency if the State agency can demonstrate that the names, personal information, and grower license number of the growers will be kept as confidential business information.

e. Insect Resistance Monitoring.

The registration of Vip3Aa19 and modified Cry1Ab PIPs expressed in VipCot cotton is conditioned on Syngenta Seeds carrying out appropriate programs to detect the emergence of insect resistance as early as possible. Resistance monitoring programs include surveying insects for potential resistance and collection of information from growers about events that may indicate resistance. Syngenta Seeds should coordinate its monitoring efforts VipCot with the current resistance monitoring programs for other registered Bt cotton products. The following resistance monitoring terms are required for VipCot:

1) Syngenta Seeds must submit a VipCot cotton (Vip3Aa19 and modified Cry1Ab toxins) resistance monitoring plan for *Heliothis virescens* (tobacco budworm), *Helicoverpa zea* (cotton bollworm), and *Pectinophora gossypiella* (pink bollworm) to EPA within 90 days of the date of registration. The monitoring program description must include sampling (number of locations and samples per location), sampling methodology, bioassay methodology,

standardization procedures, detection technique and sensitivity, and the statistical analysis of the probability of detecting resistance. Collection sites must be focused in areas of high adoption of VipCot for tobacco budworm, cotton bollworm, and pink bollworm. Syngenta Seeds shall provide baseline susceptibility and diagnostic concentration determinations for tobacco budworm, cotton bollworm, and pink bollworm to Vip3Aa19 and modified Cry1Ab by January 31, 2009.

2) The following testing scheme for survivors of the diagnostic or discriminating concentrations (or identified survivors of any resistance detection method) must be implemented: 1) Determine if the observed effect is heritable; 2) Determine if the increased tolerance can be observed in the field (i.e., survive on VipCot cotton plants); 3) Determine if the effect is due to resistance, 4) Determine the nature of resistance (dominant, recessive), 5) Determine the resistance allele frequency, 6) Determine, in subsequent years, whether the resistance allele frequency is increasing, and 7) Determine the geographic extent of the resistance allele (or alleles) distribution. Should the resistance allele frequency be increasing and spreading, a specific remedial action plan should be designed to mitigate the extent of Bt resistance. See section f ("Remedial Action Plans") below.

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3) Syngenta Seeds must also follow up on grower, extension specialist or consultant reports of less than expected results or control failures (such as increases in damaged squares or bolls) for the target lepidopteran pests (*Heliothis virescens* (TBW) and *Helicoverpa zea* (CBW), *Pectinophora gossypiella* (PBW)) as well as for cabbage looper, soybean looper, saltmarsh caterpillar, black cutworm, fall armyworm, southern armyworm, and European corn borer. Syngenta Seeds will instruct its customers (growers and seed distributors) to contact them (e.g., via a toll-free customer service number) if incidents of unexpected levels of tobacco budworm, cotton bollworm, or pink bollworm damage occur. Syngenta Seeds will investigate all damage reports. See Remedial Action Plans (section f) below.

4) Syngenta Seeds must provide to EPA for review and approval any revisions to the tobacco budworm, cotton bollworm, and pink bollworm resistance monitoring plans prior to their implementation.

5) Beginning in 2009, a report on results of resistance monitoring and investigations of damage reports must be submitted to the Agency annually by September 1st each year for the duration of the conditional registration.

f. Remedial Action Plans

Specific remedial action plans are required for VipCot cotton for the purpose of containing resistance and perhaps eliminating resistance if it develops. One remedial action plan is for the areas where pink bollworm is the predominate pest and the other is for the areas where tobacco budworm and cotton bollworm are the predominate pests.

1) Remedial Action Plan for Pink Bollworm

If resistance involves the pink bollworm (*Pectinophora gossypiella*), Syngenta Seeds must implement the Arizona Bt Cotton Working Group's Remedial Action Plan. Syngenta Seeds must obtain approval from EPA before modifying the Arizona Bt Cotton Working Group's Remedial Action Strategy. The Arizona Bt Cotton Working Group's Remedial Action Plan can be found in Enclosure 1.

2) Remedial Action Plan for Tobacco Budworm and Cotton Bollworm

If resistance involves the tobacco budworm (*Heliothis virescens*) and/or the cotton bollworm (*Helicoverpa zea*), Syngenta Seeds must implement a Remedial Action Plan approved by EPA. Once approved, Syngenta Seeds must obtain approval from EPA before modifying the Remedial Action Plan for tobacco budworm and cotton bollworm. A final remedial action plan for tobacco budworm and cotton bollworm must be submitted within 90 days of the date of registration. This remedial action plan must include definitions of "suspected" and "confirmed" resistance and steps to take in the event of confirmed resistance. The plan should be based on the steps described in Syngenta Seed's IRM submission, including:

- Notification to the Agency within 30 days of resistance confirmation;
- Notification to affected customers and extension agents about confirmed resistance;
- Encourage affected customers and extension agents to employ alternative lepidopteran control measures;
- Cease sale and distribution of VipCot cotton in affected area;
- Devise long-term resistance management action plan according to characteristics of resistance event and local agronomic needs.

g. Annual Reporting

The annual reporting requirements for VipCot are as follows:

1) Annual Sales: reported and summed by state (county level data available by request), January 31st each year, beginning in 2010;

2) Grower Agreements: number of units of Bt corn seeds shipped or sold and not returned, and the number of such units that were sold to persons who have signed grower agreements, January 31st each year, beginning in 2010;

3) Grower Education: substantive changes to education program completed previous year, January 31st each year, beginning in 2009;

4) Compliance Assurance Plan: Compliance Assurance Program activities and results, January 31st each year, beginning in 2010;

5) Compliance Survey Results: to include annual survey results and plans for the next year; full report January 31st each year, beginning in 2010;

6) Insect Resistance Monitoring Results: results of monitoring and investigations of damage reports, September 1 each year, beginning in 2009.

IV. Regulatory Position for *Bacillus thuringiensis* modified Cry1Ab (OECD Unique Identifier SYN-IR67B-1) and Vip3Aa19 (OECD Unique Identifier SYN-IR102-7) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton (VipCot)

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

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

1) A 7-14 day Daphnia study as per the OPPTS 885.4240 guideline (Aquatic Invertebrate Testing) on Vip3Aa and modified Cry1Ab. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the 7-14 day Daphnia study.

2) An independent lab validation of the analytical method for the detection of Vip3Aa19 and modified Cry1Ab.

3) Insect resistant management data for Vip3Aa and modified Cry1Ab: a) development of baseline susceptibility and diagnostic concentrations for resistance monitoring of the major target pests; b) development of a compliance assurance program for refuge requirements; c) completion of a final remedial action plan in the event of pest resistance.

The applicants submitted or cited data sufficient for EPA to determine that conditional registration of Vip3Aa19 and modified Cry1Ab protein and the genetic material necessary for their production in COT102 X COT67B cotton (VipCot) under FIFRA 3(c)(7)(C) will not result in unreasonable adverse effects to the environment, as discussed above. The applicants submitted and/or cited satisfactory data pertaining to the proposed use. The human health effects data and non-target organism effects data are considered sufficient for the period of the conditional registration. These data demonstrate that no foreseeable human health hazards or ecological effects are likely to arise from the use of the product and that the risk of resistance developing to Vip3Aa19 and/or modified Cry1Ab proteins during the time of the conditional registration is not expected to be significant.

Registration of Vip3Aa19 and modified Cry1Ab proteins and the genetic material necessary for their production in COT102 X COT67B cotton (VipCot) is in the public interest because:

1. Efficacy trials have demonstrated effective control of the major cotton target pests of Vip3Aa19 and modified Cry1Ab: cotton bollworm, tobacco budworm, and pink bollworm.

2. Vip3Aa19 has a novel mode of action which may benefit insect resistance management for this and other cotton PIP products.

3. If COT102 X COT67B (VipCot) cotton is registered, it will be the fourth *Bt* cotton product on the market for control of cotton bollworm, tobacco budworm, and pink bollworm. The availability of multiple Bt cotton products will increase grower choice and price competition, likely resulting in lower seed prices for consumers and higher adoption rates.

4. The registration of VipCot cotton is expected to result in further reduction of chemical insecticide use by cotton growers. Lower insecticide use should result in benefits for both human health and the environment.

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In view of these minimal risks and the clear benefits related to Vip3Aa19 and modified Cry1Ab proteins and the genetic material necessary for their production in COT102 X COT67B cotton (VipCot), EPA believes that the use of the product during the limited period of the conditional registration will not cause any unreasonable adverse effects.

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

EPA has determined, as explained in section II.E., that the third criterion for a FIFRA 3(c)(7)(C) conditional registration has been fulfilled because the use of Vip3Aa19 and modified Cry1Ab proteins and the genetic material necessary for their production in COT102 X COT67B cotton (VipCot) under this registration is in the public interest.

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

The expiration date of the registration has been set to September 30, 2011.