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

FEB 08 2007

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of Product Characterization and Human Health Data for Plant- Incorporated Protectant *Bacillus thuringiensis* (*Bt*) Vip3Aa20 insect control protein (EPA Reg. No. 67979-EUP-A) and the genetic material necessary for its production in Event MIR162 corn in support for a temporary exemption from tolerances and Experimental Use Permit (EUP), submitted by Syngenta Seeds, Inc. – Field Crops-NAFTA.

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

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

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

ACTION REQUESTED: To review product characterization, toxicological and allergenicity data, human health data submitted by Syngenta Seeds, Inc.-Field Crops – NAFTA, in support for an Experimental Use Permit and a temporary exemption from tolerances for *Bt* Vip3Aa20 insect control protein expressed in Event MIR162 corn.

CONCLUSION: The product characterization, toxicological and allergenicity data support the finding that there is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Vip3Aa20 protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, the product characterization and human health data submitted are sufficient to support an EUP and a temporary exemption from the requirement of a tolerance. There were several studies classified as "**SUPPLEMENTAL, but UPGRADEABLE**". These data discrepancies do not ultimately affect the findings of the safety assessment for the proposed EUP; however, these data should be submitted with the Sec. 3 Registration in order to complete the Vip3Aa20 database for Event MIR162.

THIS REVIEW DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

DATA REVIEW RECORD:

Active Ingredient:	*Bacillus thuringiensis* Vip3Aa20 insecticidal protein and the genetic material necessary for their production (via pNOV1300) in transgenic corn plants derived from Syngenta Seeds' transformation Event MIR162.
Product Name:	Event MIR162 Corn Plants Expressing Vip3Aa20 *Bacillus thuringiensis* Protein [EUP Reg. No. 67979-A]
Company Name:	Syngenta Seeds, Inc. – Field Corps- NAFTA
ID No:	67979
Chemical Number:	006599
Decision Number:	368367
DP Barcode:	332013

MRID No:

Product Characterization and Identity

468648-01	Molecular characterization of Event MIR162 maize
468648-02	Characterization of the Vip3A protein expressed in Event MIR162-derived maize (corn) and comparison with microbially produced and plant-derived Vip3A test substances
468648-03	Characterization of Vip3A protein test substance (VIP3A-0104) and certificate of analysis
468648-04	Characterization of Vip3A protein test substance (VIP3A-0204) and certificate of analysis
468648-05	Re-characterization of Vip3A protein test substance (VIP3A-0204) and certificate of analysis
468648-06	Characterization of VIP3A protein produced in Pacha-derived maize (corn) and comparison with VIP3A protein expressed in recombinant *E. coli*
468648-07	Analytical method for the detection of Vip3Aa20 protein in maize tissues from event MIR162
468808-01	The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-endotoxin

Human Toxicity and Allergenicity

468648-08	Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known toxins
468648-09	Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known allergens
468648-10	Phosphomannose isomerase protein: Assessment of amino acid sequence homology with known toxins
468648-11	Phosphomannose isomerase: Assessment of amino acid sequence homology with known allergens

BACKGROUND:

Vip3A is a vegetative insecticidal protein (Vip) from *Bacillus thuringiensis* (*Bt*), a gram positive bacterium commonly found in soil. Vip proteins are produced during the vegetative stage of bacterial growth and are active against the following lepidopteran pests of corn: *Spodoptera*

frugiperda (fall armyworm), *Pseudaletia unipunctata* (armyworm), *Spodoptera exigua* (beet armyworm), *Helicoverpa zea* (corn earworm / cotton bollworm), *Agrotis ipsilon* (black cutworm), and *Striacosta albicosta* (western bean cutworm).

The native Vip protein, Vip3Aa1, was isolated from *Bt* strain AB88 and characterized by Estruch *et al.* in 1996. Syngenta has engineered a variant of the native gene for incorporation into corn. This engineered gene has been designated, *vip3Aa20,* and it has been stably incorporated (*via* pNOV1300 vector) into the genome of Event MIR162 corn by *Agrobacterium*-mediated transformation. The Vip3Aa20 protein encoded by this gene is approximately 89 kDa molecular weight and 789 amino acids in length differing by two amino acids from the native Vip3A. The sequence differences occur at positions 129 and 284 (M1291, K284Q). Another variant of Vip3Aa is also present as a PIP in Syngenta Event COT102 cotton [EPA Reg. No. 67979-O] and Event Pacha corn; this variant has been assigned the designation Vip3Aa19. Vip3Aa19 differs from the native Vip3Aa1 sequence by one amino acid at position 284, while differing from the Vip3Aa20 by one amino acid at position 129 (Crickmore *et al.* 2005). These substitutions are conservative and do not materially impact insecticidal activity. In fact, Vip3Aa20 shares >99.7% sequence homology with the native protein (Vip3Aa1) and Vip3Aa19. Table 1 compares selected sequence information for different sources of Vip3Aa discussed in this report.

Table 1. Comparison of amino acid residues at positions 129 and 284 in different variants of Vip3Aa protein

Source of Vip3Aa Proteins	Toxin Designation	Amino Acids	Position 129 *	Position 284 *
Bacillus thuringiensis strain AB88	Vip3Aa1	789	M	K
COT102 cotton	Vip3Aa19	789	M	Q
Pacha Corn	Vip3Aa19	789	M	Q
MIR162 corn	Vip3Aa20	789	I	Q

* M = methionine, I = isoleucine, k = lysine, Q = glutamine

Event MIR162 corn also contains the *pmi* gene, which was introduced along with the Vip3Aa20 protein via the same pNOV1300 transformation vector. The gene represents the *manA* gene from *Escherichia coli* and encodes the enzyme phosphomannose isomerase (PMI), which was employed as a selectable marker during the process of regenerating plant material following transformation (Negrotto, *et al.*, 2000).

Syngenta Seeds, Inc. is applying for an experimental use permit (EUP) to conduct field tests on Event MIR162 corn. The EUP program for MIR162 corn will also involve the production and evaluation of combined trait hybrids. MIR162 will be crossed with corn Event Bt11 and with corn Event MIR604 to produce combined trait hybrids that offer a broader insect control spectrum than MIR162 alone. Bt11 maize expressed the insecticidal protein Cry1Ab for control of *Ostrinia nubilalis* (European cornborer); MIR604 expresses the modified Cry3A (mCry3A) insecticidal protein for control of certain species of *Diabrotica* (corn rootworms). Data have been previously submitted to the Agency demonstrating mammalian safety of Cry1Ab produced in Bt11 maize (EPA, 2001) and mCry3A produced in MIR604 maize (EPA, 2006).

The proposed planting under the EUP will take place in 22 states and Puerto Rico for a total of 3,099 total acres of MIR162, hybrids thereof, and non-PIP corn. The objectives of the experimental field program are to introgress Event MIR162 into elite inbred lines of corn, to evaluate the insecticidal efficacy of the MIR162 corn hybrids, as well as combined insecticidal trait hybrids (Bt11 x MIR162 and Bt11 x MIR162 x MIR604) and to evaluate their agronomic performance, to conduct field IRM studies, and to conduct field studies supporting regulatory applications. The proposed experimental program and protocols were reviewed and found acceptable by the Agency [see memorandum- from T. Milofsky, M.S., through M. Hunter, to A. Reynolds, M.S., dated 12/06/2006].

Studies have been conducted and previously submitted to EPA to evaluate the safety of Vip3A proteins. Since the registrant is bridging this data in support for an EUP for Vip3Aa20, a table summarizing the submitted study titles, conclusions, and their MRID numbers (see Table 2) as well as a table demonstrating the protein equivalence for all Vip3A variants according to varying data parameters (see Table 3) are provided in this report as well. To satisfy the remaining data requirements specific for the EUP for Vip3Aa20, Syngenta Seeds, Inc. has submitted product characterization, toxicity, and allergenicity data, which are reviewed in this report. The registrant also submitted additional data for PMI to be reviewed in this report- specifically an updated amino acid homology assessment of PMI to known toxins and allergens, which will supersede the previously reviewed PMI data.

Temporary Exemption for the Requirement of a Tolerance

In the Federal Register of April 26, 2006 (71 FR 24582), the Agency established a temporary exemption from the requirement of a tolerance for *Bacillus thuringiensis* VIP3A insect control protein and the genetic material necessary for their production in cotton [see 40 CFR 174.452], which will expire May 1, 2007. An exemption from the requirement of a tolerance has been established for PMI in all crops when used as a plant-incorporated protectant inert ingredient (see 40 CFR 180.1252, effective May 14, 2004).

In conjunction with the EUP, Syngenta Seeds, Inc. – Field Crops- NAFTA has submitted a petition for a temporary exemption from the requirement of a tolerance pursuant to section 408(d)(1) of the Federal Food, Drug, and Cosmetic Act with respect to the plant-incorporated protectant Vip3Aa20 *Bacillus thuringiensis* insect control protein and the genetic material necessary for its production in all field corn, sweet corn, and popcorn.

Preliminary Safety Assessment

Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of the FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Section 408(b)(2)(C) of the FFDCA requires EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue"... Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider "available

information concerning the cumulative effects of a particular pesticide's residues and other substances that have a common mechanism of toxicity." EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

Product Characterization Profile

A Vip3Aa20 *Bacillus thuringiensis* (*Bt*) insect control protein is produced in transgenic corn plants derived from transformation Event MIR162. A *vip3Aa20* gene was synthetically created to optimize for expression in corn with activity against several major lepidopeteran corn pests. Introduced *via* transformation vector pNOV1300, a *vip3Aa20* specific probe, consisting of 2370 base pairs (bp), was incorporated between a promoter region from the *Zea mays* polyubiquitin gene (ZmUbInt (1993 bp)) and a terminator sequence from the 35S RNA from the cauliflower mosaic virus genome. An *Escherichia coli manA* gene encoding a phosphomannose isomerase *pmi* gene (1176 bp) was incorporated between the same promoter region from the *Z. mays* polyubiquitin gene (1993 bp) and a terminator sequence from the nopaline synthase gene (NOS (253 bp)) of *Agrobacterium tumefaciens*, which was used to provide a polyadenylation site. The *pmi* gene, which was introduced along with the Vip3Aa20 protein via the same pNOV1300 transformation vector, encodes the enzyme phosphomannose isomerase (PMI), which is employed as a selectable marker during the process of regenerating plant material following transformation. The PMI protein is a common enzyme involved in carbohydrate metabolism to allow for selection of transformants in cell culture, by only allowing transformed corn cells to utilize mannose as a sole carbon source, while corn cells lacking the *pmi* gene fail to grow.

Southern blot analyses and DNA sequencing indicate that one full length copy of each of the *vip3Aa20* and *pmi* genes were integrated into the maize genome, without the backbone sequences from transformation plasmid pNOV1300. Therefore, the overall integrity of the insert and the contiguousness of the functional elements were confirmed.

The native Vip protein, Vip3Aa1, was isolated from *Bt* strain AB88 and characterized by Estruch *et al.* in 1996. Syngenta has engineered a variant of the native gene for incorporation into corn. This engineered gene has been designated, *vip3Aa20,* and it has been stably incorporated (*via* pNOV1300 vector) into the genome of Event MIR162 corn by *Agrobacterium*-mediated transformation. The Vip3Aa20 protein encoded by this gene is approximately 89 kDa molecular weight and 789 amino acids in length differing by two amino acids from the native Vip3A. The sequence differences occur at positions 129 and 284 (M1291, K284Q). Another variant of Vip3Aa is also present as a PIP in Syngenta Event COT102 cotton and Event Pacha corn; this variant has been assigned the designation Vip3Aa19. Vip3Aa19 differs from the native sequence by one amino acid at position 284, while differing from the Vip3Aa20 by one amino acid at position 129. These substitutions do not appear to materially impact insecticidal activity. Vip3Aa20 shares >99.7% sequence homology with the native protein, Vip3Aa1 and Vip3Aa19.

Data have been submitted demonstrating the protein equivalency among the Vip3Aa1, Vip3Aa19, and Vip3Aa20 proteins and their respective protein test substances, expressed in recombinant *E. coli* (VIP3A-0199, VIP3A-0100, VIP3A-0104, and VIP3A-0204) or maize (LPPACHA-0199, LPMIR162-0105, and IAPMIR162-0105) for use as a surrogate in toxicity experiments (see MRID

No. 458358-12 and 468648-03, -04, -05, and -06). Since equivalency has been established for the Vip3A protein variants, all previous submitted data from Vip3Aa1 and Vip3Aa19 can be bridged to Vip3Aa20.

Toxicological Profile

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

Data have been submitted demonstrating the lack of mammalian toxicity at high levels of exposure to the pure Vip3A-0100 protein. These data demonstrate the safety of the products at levels well above maximum possible exposure levels that are reasonably anticipated in the crops. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.740(b)(2)(i)). For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study, to verify the observed effects and clarify the source of these effects (Tiers II and III).

An acute oral toxicity study was submitted for the Vip3Aa19 protein. Male and female mice (16 of each) were dosed with 3,675 milligrams/kilograms bodyweight (mg/kg bwt) of Vip3Aa19 protein. All mice survived the study, gained weight, had no test material-related clinical signs, and had no test material-related findings at necropsy. The acute oral toxicity data submitted also supports the prediction that the Vip3Aa20 protein would be non-toxic to humans.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. 1992). Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Vip3Aa20 protein is not considered toxic. Amino acid sequence comparisons showed no similarity between the Vip3Aa20 protein to known toxic proteins available in public protein data bases. According to the Codex Alimintarius guidelines, the assessment of potential toxicity also includes stability to heat (FAO/WH O Standards Programme, 2001). A heat lability study demonstrated that Vip3Aa19 is inactivated against FAW, when heated to 55 °C for 30 minutes.

Since Vip3Aa20 is a protein, allergenic sensitivities were considered. Currently, no definitive tests exist for determining the allergenic potential of novel proteins. Therefore, EPA uses a weight of the evidence approach where the following factors are considered: source of the trait; amino acid sequence similarity with known allergens; prevalence in food; and biochemical properties of the protein, including *in vitro* digestibility in simulated gastric fluid (SGF), and glycosylation. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation acid and proteases; may be glycosylated; and present at high concentrations in the food.

Data have been submitted that demonstrate that the Vip3A from recombinant maize (LPPACHA-0199) and *E. coli* (VIP3A-0100) proteins are rapidly degraded by gastric fluid *in vitro*. In a solution

of simulated gastric fluid (containing pepsin) and either 80 µL of LPPACHA-0199 or 320 µL of VIP3A-0100 test protein, both were shown to be susceptible to pepsin degradation. These data support the conclusion that Vip3Aa proteins expressed transgenic plants will be readily digested as conventional dietary protein under typical mammalian gastric conditions. Further data demonstrate that Vip3Aa20 is not glycoslylated and a comparison of amino acid sequences of known allergens uncovered no evidence of any homology with Vip3Aa20, even at the level of 8 contiguous amino acids residues. Preliminary data of the quantification of Vip3Aa20 protein in various maize tissues were also submitted. The data demonstrated that mean Vip3Aa20 concentrations in kernels ranged from *ca.* 24.6 - 40.3 µg Vip3Aa20/ g dry weight, representing *ca.* 0.003% of the total protein in grain (assuming that corn grain contains 10% total protein by weight). Therefore, Vip3Aa20 is present in low levels in corn tissue.

Therefore, the potential for the Vip3Aa20 protein to be a food allergen is minimal. As noted above, toxic proteins typically act as acute toxins with low dose levels. Therefore, since no effects were shown to be caused by the plant-incorporated protectant, even at relatively high dose levels, the Vip3Aa20 protein is not considered toxic.

Aggregate Exposures

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

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectant chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. The amino acid homology assessment included similarity to known aeroallergens. It has been demonstrated that there is no evidence of occupationally related respiratory symptoms, based on a health survey on migrant workers after exposure to *Bt* pesticides (Berstein et al. 1999). Exposure via residential or lawn use to infants and children is also not expected because the use sites for the Vip3Aa20 protein are all agricultural for control of insects. Oral exposure, at very low levels may occur from ingestion of processed corn products and, potentially, drinking water.

However, oral toxicity testing done at a dose in excess of 3 gm/kg showed no adverse effects. Furthermore, the expected dietary exposure from both cotton and corn are several orders of magnitude lower than the amounts of Vip3Aa protein shown to have no toxicity. Therefore, even if negligible aggregate exposure should occur, the Agency concludes that such exposure would present no harm due to the lack of mammalian toxicity and the rapid digestibility demonstrated for the Vip3Aa proteins.

Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity, resulting from the plant-incorporated protectant, we conclude that there are no cumulative effects for the Vip3Aa20 protein.

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

A. Toxicity and Allergenicity Conclusions
The data submitted and cited regarding potential health effects for the Vip3Aa20 protein include the characterization of the expressed Vip3Aa20 protein in corn, as well as the acute oral toxicity, heat stability, and *in vitro* digestibility of the proteins. The results of these studies were determined applicable to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were considered.

Adequate information was submitted to show that the Vip3Aa20 protein test material derived from microbial cultures was biochemically and functionally similar to the protein produced by the plant-incorporated protectant ingredients in corn. Microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the prediction that the Vip3Aa20 protein would be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al. 1992). Since no effects were shown to be caused by Vip3Aa20 protein, even at relatively high dose levels (3,675 mg Vip3Aa19/kg bwt), the Vip3Aa20 protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. (See 40 CFR 158.740(b)(2)(i)). Moreover, Vip3Aa20 showed no sequence similarity to any known toxin.

Protein residue chemistry data for Vip3Aa20 were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. However, preliminary data (that was submitted with administrative materials in the EUP application) demonstrated low levels of Vip3Aa20 in corn tissues with less than 40 micrograms Vip3Aa20 protein/gram dry weight in kernels and less than 75 micrograms Vip3Aa20 protein/gram dry weight of whole corn plant.

Since Vip3Aa20 is a protein, its potential allergenicity is also considered as part of the toxicity assessment. Data considered as part of the allergenicity assessment include that the Vip3Aa20 protein came from *Bacillus thuringiensis* which is not a known allergenic source, showed no sequence similarity to known allergens, was readily degraded by pepsin, and was not glycosylated when expressed in the plant. Therefore, there is a reasonable certainty that Vip3Aa20 protein will not be an allergen.

Neither available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children); nor safety factors that are generally recognized as appropriate for the use of animal experimentation data were evaluated. The lack of mammalian toxicity at high levels of exposure to the Vip3Aa20 protein, as well as the minimal potential to be a food allergen demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated in the crop.
The genetic material necessary for the production of the plant-incorporated protectant active ingredients are the nucleic acids (DNA, RNA) which comprise genetic material encoding these proteins and their regulatory regions. The genetic material (DNA, RNA) necessary for the production of Vip3Aa20 protein has been exempted under the blanket exemption for all nucleic acids (40 CFR 174.475).

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

In addition, FFDCA section 408(bf12)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the data base unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for the Vip3Aa20 protein and the genetic material necessary for their production. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

C. Overall Safety Conclusion
There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Vip3Aa20 protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as previously discussed, no toxicity to mammals has been observed, nor has there been any indication of allergenicity potential for the plant-incorporated protectant.

Other Considerations

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

B. Analytical Method(s)
A method for extraction and ELISA analysis of Vip3Aa20 protein in corn has been submitted and is under review by the Agency.

C. Codex Maximum Residue Level
No Codex maximum residue levels exist for the plant-incorporated protectant *Bacillus thuringiensis* Vip3Aa20 protein and the genetic material necessary for its production in corn.

RECOMMENDATION: As previously noted, there is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Vip3Aa20 protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, the product characterization and human health data submitted are sufficient to support an Experimental Use Permit and a temporary exemption from the requirement of a tolerance. However, there were several studies classified as **"SUPPLEMENTAL, but UPGRADEABLE"** with either clarifications of existing data or **"UNACCEPTABLE",** pending submission of new data. These include:

MRID No. 468648-01

- The registrant should submit clarification of which specific vector DNA sequences resulted in non-specific sequence binding in the Southern blot analyses (Figure 6, lanes 9 and 11). In addition, visual verification is also required by submitting a Southern blot containing genomic DNA from MIR162, a negative control, the plasmid control pNOV1300 hybridized with the *pmi*-specific probe, and use of a different molecular weight marker (to avoid non-specific sequence binding).

MRID No. 468648-06

- In Figure 1, the smaller protein bands that the study author attributed to VIP3A degradation products were not visible in determining the molecular weight via SDS-PAGE. The identity of the prominent band in the *E. coli*- produced VIP3A sample (lane 7) was also not addressed. There was a similar data discrepancy in Figure 2, where smaller bands were referred to in the text, but not present in the Western blot. Therefore, explanations for unidentified bands and better reproductions of the gels should be provided by the registrant.

- In the insect bioassay, the following data discrepancies should be addressed by the registrant: why the % mortality was not evaluated for all of the test substances and whether the experimental results were corrected for control mortality.

MRID No. 468648-07

- A new residue analytical method should be submitted (concurrently with the Sec. 3 registration of Event MIR162) and specifically conducted on the MIR162 transgenic grain (single seed and composite) in order for the ELISA to be verified as a suitable analytical method. This experiment should also be validated by an independent third party laboratory according to OPPTS 860.1340(c)(6) and PR Notice 96-1 with GLP compliance. The report should also include the following:

  1) Qualitative data to represent positive vs. negative transgenic specific event results with percent accuracy;

2) Utilization of a negative control (non- transgenic convention corn line) and positive control (confirmed transgenic corn line);
3) Testing of cross-reactivity against other transgenic events and other proteins; and
4) The intra- and inter- assay coefficient of variation should be reported.

Once the recommended report has been submitted and found acceptable, EPA's Analytical Method Laboratory located in Fort Meade (Maryland) will have to independently validate Syngenta's ELISA protocol for accuracy, precision, and sensitivity.

- In regards to establishing field protein expression levels in MIR162 corn tissues and plants, this study is supplemental because it provides useful information for tissue expression levels to determine exposure for non-target organisms, for IRM dose levels, and dietary exposure estimates. However, it does not include quantification of Vip3Aa20 protein levels expressed in various plant tissues and the whole corn plant. A full report determining the protein concentrations of Vip3Aa20 and PMI at different stages of plant development should be submitted (including: the mean, range, and standard deviations) and reported on a dry weight basis (µg protein/g tissue) with GLP compliance. This data requirement can be addressed in the Sec. 3 Registration of Vip3Aa20. The study should also include the following:
  1) Standard curve data for the ELISA;
  2) The calculation method for determining the dry weight conversion factor from the fresh weight tissue samples; and
  3) Identification of the specific seed line and lot utilized as the test material with number of field sites and replicates.

These data discrepancies do not ultimately affect the findings of the safety assessment for the proposed EUP; however, these data should be submitted with the Sec. 3 Registration in order to complete the Vip3Aa20 database for Event MIR162.

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

Table 2. Summary of previously submitted product characterization, toxicity, and allergenicity studies for Vip3Aa protein variants

MRID	Title	Summary
457665-01	Characteristics of *Bacillus thuringiensis* VIP3A Protein and VIP3A Cotton Plants Derived from Event COT102	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 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**
458358-12	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*	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. Amino acid sequences corresponded identical to the predicted amino acid sequence of VIP3A and no evidence of any post-translational modification of VIP3A was observed. Peptides representing *ca.* 85% (673/789) of the complete VIP3A amino acid sequence were identified by mass spectral analysis of cotton produced VIP3A protein. Comparisons of bioactivity of *E. coli*-expressed and cotton-expressed VIP3A protein in larvae of four lepidopteran species demonstrated comparable activities. These data indicate that VIP3A proteins from recombinant *E. coli*, Pacha-derived maize and event COT102-derived cotton are substantially equivalent. **Classification: Acceptable**
457665-02	Summary of Mammalian Toxicology Data for VIP3A Proteins Produced by VIP3A Cotton Event COT102	Acute oral toxicity in male and female mice was not observed 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. **Classification: Acceptable**

457665-05	Acute Oral Toxicity Study with Test Substance VIP3A-0100 Protein in Mice	The test animals (male and female Crl-1® (ICR) BR mice, 16 each) were quarantined for 9 days and fasted approximately 4 hours prior to dosing. 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. All animals sacrificed on day 15 had normal body weight gains. Necropsy findings showed no test material related macroscopic alterations. In addition, 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 > 3675 mg VIP3A protein/kg body weight). **Classification: Acceptable**
458358-05	*In vitro* Digestibility of VIP3A Protein Under Simulated Mammalian Gastric Conditions	VIP3A from recombinant maize (field corn) plants was prepared as sample LPPACHA-0199 by extracting the leaves of recombinant corn plants and 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 *via* 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. Therefore, 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**
458358-04	Summary of Mammalian Toxicology Data for the VIP3A and APH4 Proteins Produced by Transgenic VIP3A Cotton Event COT102; Supplement to MRID No. 45766502	The study report is a summary of the results reported in the various reports submitted for consideration of a Section 5 EUP and a Section 3 registration. This volume does not constitute a study in the sense of data collection, but rather a compilation of data and concepts related to the risk assessment for the VIP3A protein. The data and information contained in this volume supplement information previously submitted to the Agency in a summary volume titled "Summary of Mammalian Toxicology Data for the VIP3A and APH4 Proteins Produced by Transgenic VIP3A Cotton Event COT102" (MRID No. 45766502; Vlachos, 2002; submitted September 24, 2002). Briefly, the VIP3A protein, as found in COT102, is non-toxic to mammals at the dose given (LD_{50} > 3675 mg VIP3A/kg body weight), susceptible to degradation in a simulated gastric fluid assay, heat labile, and contains no homology to allergens or toxins when compared to a database of known allergens and toxins. **Classification: Not Applicable, summary of previously reviewed data.**

SUMMARY OF DATA SUBMITTED:

Summaries and discussion of each study on the product characterization, human toxicity, and allergenicity of this product reviewed in this memorandum are provided below.

MRID No. 468648-01 Molecular characterization of Event MIR162 maize

The purpose of this study was to present molecular characterization data of the T-DNA insert and the genetic material required for its production (*via* pNOV1300) in MIR162 plants. Southern blot analysis and DNA sequencing showed that Event MIR162 maize contains single copies of the *vip3A* and phosphomannose isomerase (*pmi*) genes, but no backbone sequences from the transformation plasmid pNOV1300. Event MIR162 DNA had two single nucleotide changes in the *Vip3Aa* coding sequence compared to the *Vip3Aa* in pNOV1300, and was designated *vip3Aa20*. The substitution of thymine for guanine at base 387 changed the methionine at position 129 to isoleucine (M129I), but the substitution of cytosine for guanine at base 1683 was a silent mutation. The *pmi* coding sequence in Event MIR162 was identical to that in pNOV1300. The stability of the transgenic locus was shown by statistical analysis of the Event MIR162 segregation patterns over four generations, which confirmed the expected Mendelian inheritance ratio for both the *vip3Aa20* and *pmi* genes.

CLASSIFICATION: SUPPLEMENTAL- but UPGRADEABLE- pending submission of an additional Southern blot containing genomic DNA from MIR162, a negative control, the plasmid control pNOV1300 hybridized with the *pmi*-specific probe, and use a different molecular weight marker to avoid non-specific sequence binding.

MRID No. 468648-02 Characterization of the Vip3A protein expressed in Event MIR162-derived maize (corn) and comparison with microbially produced and plant-derived Vip3A test substances

The purpose of this study was to determine if Vip3Aa20 expressed in maize plants derived from transformation Event MIR162 is substantially equivalent to Vip3Aa19 or Vip3Aa1 present in various test substances previously used in toxicity and/or test substance characterization studies. Vip3Aa produced in recombinant *E. coli*, MIR162 maize, and Pacha maize were shown to be substantially equivalent based on the finding that (1) Vip3Aa20 from MIR162 maize (test material LPMIR162-0105 and IAPMIR162-0105), Vip3Aa19 from Pacha maize (LPPACHA-0199), Vip3Aa19 from several of *E. coli*-derived samples (VIP3A-0204, VIP3A-0104, VIP3A-0100), and Vip3Aa1 from *E. coli*-produced VIP3A-0199 each had the expected predicted molecular weight of ~89 kDa, and were immunoreactive with the same anti-Vip3Aa antibody on western blots, and (2) VIP3A-0204 and LPMIR162-0105 had comparable insecticidal activity against FAW (137 ng Vip3Aa19/cm^2 vs. 154 ng Vip3Aa20 ng/cm^2 diet surface). Additionally, there was no evidence of post-translational glycosylation of Vip3Aa from LPMIR162-0105 or VIP3A-0204. Therefore, the *E. coli* -produced Vip3A is considered an appropriate substitute for Vip3Aa20 expressed in MIR162 maize in toxicity and/or protein characterization studies. It was also be noted that the VIP3A-0204

Vip3Aa19 protein N-terminal amino acid sequence matched the predicted sequence however, the plant-expressed Vip3A was not determined due to technical difficulties.

CLASSIFICATION: ACCEPTABLE

MRID No. 468648-03 Characterization of Vip3A protein test substance (VIP3A-0104) and certificate of analysis

The purpose of this study was to characterize test substance VIP3A-0104 containing the vegetative insecticidal protein VIP3A encoded by the synthetic *vip3A(a)* gene. VIP3A-0104 test material (Vip3Aa19 protein) produced from the synthetic *vip3A(a)* gene in an *E. coli* over-expression system was purified by ammonium sulfate precipitation, phenyl sepharose interaction chromatography, and DEAE anion exchange chromatography. The VIP3A-0104 samples were determined to be 63.1% pure by SDS-PAGE in conjunction with Coomassie blue staining and densitometric analysis, and were shown to contain ~ 2 µg endotoxin /g VIP3A-0104 by lipopolysaccharide analysis. Western blots using goat anti-VIP3A polyclonal primary antibody and donkey anti-goat alkaline phosphatase-linked secondary antibody revealed a dominant immunoreactive band at the predicted molecular weight of ~89,800 Da. VIP3A-0104 had insecticidal activity against first-instar fall army worm (FAW) larvae in insect feeding assays, with an LC_{50} of 272 ng VIP3A/cm^2 diet surface (95% confidence interval of 184 - 384 ng VIP3A/cm^2) after 168 hours.

CLASSIFICATION: ACCEPTABLE

MRID No. 468648-04 Characterization of Vip3A protein test substance (VIP3A-0204) and certificate of analysis

The purpose of this study was to characterize test substance VIP3A-0204 containing the vegetative insecticidal protein VIP3A encoded by the synthetic *vip3A(a)* gene. VIP3A-0204 test material (Vip3Aa19 protein) produced from the synthetic *vip3A(a)* gene in an *E. coli* over-expression system was purified by Q Sepharose FF anion exchange chromatography. The VIP3A-0204 test material was determined to be soluble in aqueous solution at 50 mg/mL, to be 89.7% pure by SDS-PAGE in conjunction with Coomassie blue staining and densitometric analysis, and to contain ~ 62 µg endotoxin/g VIP3A-0204 by lipopolysaccharide analysis. Western blots using goat anti-VIP3A polyclonal primary antibody and donkey anti-goat alkaline phosphatase-linked secondary antibody revealed a dominant immunoreactive band at the predicted molecular weight of ~89,800 Da. VIP3A-0204 had insecticidal activity against first-instar fall army worm (FAW) larvae in insect feeding assays, with an LC_{50} of 45.1 ng VIP3A/cm^2 diet surface (95% confidence interval of 24.5 – 71.0 ng VIP3A/cm^2 diet surface) after 120 hours.

CLASSIFICATION: ACCEPTABLE

MRID No. 468648-05 Re-characterization of Vip3A protein test substance (VIP3A-0204) and certificate of analysis

The purpose of this study was to re-characterize test substance VIP3A-0204, containing the vegetative insecticidal protein VIP3A encoded by the synthetic *vip3A(a)* gene. VIP3A-0204 test material (Vip3Aa19 protein) produced from the synthetic *vip3A(a)* gene in an *E. coli* over-expression system was previously purified and characterized (MRID 46864804). It was shown to be ~89,800 Da and 89.7% pure (SDS-PAGE with Coomassie blue staining and densitometric analysis), immunoreactive with anti-VIP3A antibody (western blots), and to have insecticidal activity against first-instar fall army worm (FAW) larvae (LC_{50} of 45.1 ng VIP3A/cm^2 diet surface after 120 hours). In the present study, this VIP3A-0204 sample was similarly re-characterized after seven months storage lyophilized at -20°C, and shown to have retained its integrity and bioactivity. SDS-PAGE and western analysis determined a molecular weight of ~89,800 Da and a purity of 91.8%, and insecticidal activity assays with FAW larvae found an LC_{50} of 38.1 ng VIP3A/cm^2 diet surface after 120 hours. Therefore, it can be concluded that the test substance was stable when stored at -20°C, over *ca.* seven months.

CLASSIFICATION: ACCEPTABLE

MRID No. 468648-06 Characterization of VIP3A protein produced in Pacha-derived maize (corn) and comparison with VIP3A protein expressed in recombinant *E. coli*

The purpose of this study was to demonstrate the equivalency of the VIP3A protein as expressed in recombinant bacteria and transgenic maize plants derived from the Pacha VIP3A Event. Functional and biochemical parameters were evaluated and compared in order to justify the use of the microbially produced VIP3A test substance as a surrogate for maize-expressed VIP3A protein in safety evaluations. Comparisons indicated that VIP3A protein produced by Pacha-derived maize (LPPACHA-0199 sample; Vip3Aa19 protein) and by *E. coli* (VIP3A-0199 sample; Vip3Aa1 protein) was substantially equivalent. SDS-PAGE and western blot analysis showed that both proteins had a MW of ~89,000 and were immunoreactive against the same anti-VIP3A antibody. Edman degradation was used to determine that the N-terminus of *E. coli* VIP3A was MNKN, beginning with methionine-1, and of maize VIP3A was KNNXKL, beginning with lysine-3 (X indicates that a definitive amino acid could not be assigned). The lack of two predicted amino acids at the N-terminus of maize VIP3A was likely due to proteolytic degradation *in planta* or *in vitro*. The two VIP3A proteins had a similar insecticidal activity profile against first-instar larvae, the *E. coli*-derived protein being slightly more active. Both were the most active against black cutworm, with estimated 96-hour LC_{50} values of 70.4 and 88.5 ng VIP3A/cm^2 diet surface, respectively. Both were slightly less active against corn earworm and fall armyworm, and as expected, were inactive against the European corn borer and diamondback moth. Mass spectral (MS) analysis of VIP3A proteolytic digests confirmed the predicted complete sequence of ~95% of the *E. coli* and ~93% of the maize VIP3A protein. Neither MS nor an independent glycosylation analysis (DIG Glycan) showed any evidence of post-translational glycosylation of either the microbially-derived or maize-derived VIP3A.

CLASSIFICATION: SUPPLEMENTAL, but UPGRADEABLE- pending submission of better reproductions of the SDS-PAGE and Western Blots of the *E. coli*- and maize-derived VIP3A test proteins; as well as, clarifications to data discrepancies noted in the insect bioassay.

MRID No. 468648-07 **Analytical method for the detection of Vip3Aa20 protein in maize tissues from event MIR162**

The purpose of this study was to determine the Vip3Aa20 extraction efficiency from maize plant tissues in MIP162 maize, using an ELISA assay (Tijssen, 1985) to measure Vip3Aa20 levels. An ELISA procedure was used to determine Vip3Aa20 levels in tissues of Event MIP162 maize and the Vip3Aa20 protein tissue extraction efficiency. The ELISA method used 96-well plates, purified rabbit anti-Vip3A polyclonal primary antibody, donkey anti-rabbit alkaline phosphatase conjugated secondary antibody, and phosphatase substrate. Each plate included the standard test substance (MIR162-VIP3A-0106 or VIP3A-0104) that was used to generate a standard curve, but this data was not shown. The LOQ and LOD for Vip3Aa20 ranged from, respectively, 0.04-0.25 and 0.003-0.032 µg Vip3Aa20/gram fresh weight; and 0.21-0.35 µg and 0.029-0.045 Vip3Aa20/gram dry weight. The average extraction efficiency of Vip3Aa20 was 82.7% in leaves, 81.0% in roots, 79.5% in pith, 88.3% in silk, 79.7% in kernels, >98% in pollen, and 78.9% in whole plants at maturity.

CLASSIFICATION: This data packet is classified as **UNACCEPTABLE** for residue analytical method. A new study should be submitted (concurrently with the Sec. 3 registration of Event MIR162) and specifically conducted on the MIR162 transgenic grain (single seed and composite) in order to be verified as a suitable analytical method. This experiment should also be validated by an independent third party laboratory according to OPPTS 860.1340(c)(6) and PR Notice 96-1 with GLP compliance. The report should also include the following:

1) Qualitative data to represent positive vs. negative transgenic specific event results with percent accuracy;
2) Utilization of a negative control (non- transgenic convention corn line) and positive control (confirmed transgenic corn line);
3) Testing of cross-reactivity against other transgenic events and other proteins; and
4) The intra- and inter- assay coefficient of variation should be reported.

Once the recommended report has been submitted and found acceptable, EPA's Analytical Method Laboratory located in Fort Meade (Maryland) will have to independently validate Syngenta's ELISA protocol for accuracy, precision, and sensitivity.

In regards to establishing field protein expression levels in MIR162 corn tissues and plants, the study is supplemental. It does provide useful information for tissue expression levels to determine exposure for non-target organisms, for IRM dose levels, and dietary exposure estimates. However, it does not include quantification of Vip3Aa20 protein levels expressed in various plant tissues and the whole corn plant. A full report determining the protein concentrations of Vip3Aa20 and PMI at different stages of plant development should be submitted (including: the mean, range, and standard deviations) and reported on a dry weight basis (µg protein/g tissue) with GLP compliance. This data requirement can be addressed in the Sec. 3 Registration of Vip3Aa20. The study should also include the following:

1) Standard curve data for the ELISA;
2) The calculation method for determining the dry weight conversion factor from the fresh weight tissue samples; and

3) Identification of the specific seed line and lot utilized as the test material with number of field sites and replicates

MRID No. 468808-01 The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-endotoxin

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

MRID No. 468648-08 Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known toxins

The purpose of the study was to determine if Event MIR162 Vip3A protein had any significant amino acid sequence homology to known or putative protein toxins. The database identified 32 entries with E values below 6×10^{-6}, of which 30 were vegetative insecticidal proteins of *B. thuringiensis* and had E values of 0.0 to 1×10^{-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

MRID No. 468648-09 Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known allergens

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

MRID No. 468648-10 Phosphomannose isomerase protein: Assessment of amino acid sequence homology with known toxins

The purpose of this study was to determine if phosphomannose isomerase (PMI) derived from *Escherichia coli* had significant amino acid sequence homology to known protein toxins. The database identified 186 entries with E values below 0.087. All of these entries were known or putative PMI enzymes (including mannose-6-phosphate isomerase (MPI) and ManA) with no known toxic activity from 126 species, with E values of 0.0 – 0.067. Therefore, no relevant similarities were found between the *E. coli* PMI query sequence and known protein toxins.

CLASSIFICATION: ACCEPTABLE

MRID No. 468648-11 Phosphomannose isomerase: Assessment of amino acid sequence homology with known allergens

The purpose of this study was to determine if phosphomannose isomerase (PMI) protein derived from *E. coli* had any significant amino acid sequence homology to known or putative protein allergens. No significant sequence homology was found between any sequential PMI 80-amino acid peptides and any entry in the SBI Allergen Database. Screening of PMI amino acid sequence for matches of eight or more contiguous amino acids with the allergen database revealed one alignment, that with the allergen α-parvalbumin from *Rana species* CH2001. Hilger et al. (2002) identified α-parvalbumin as an allergen in an individual who had severe anaphylaxis after eating frog legs of Indonesian origin. This patient's serum was not cross-reactive to related parvalbumins from the common edible frog (*Rana esculenta*). The common amino acid sequence of DLSDKETT occurred at positions 327-334 in PMI, and at positions 77-84 in α-parvalbumin. In order to determine if the IgE antibodies present in this patient's serum recognized PMI, serum obtained from the one person with IgE-mediated allergy to α-parvalbumin was not cross-reactive with PMI overexpressed in *E. coli* (PMI-098; containing 61% w/w PMI protein and having PMI enzymatic activity). Therefore it is concluded that this 8-amino acid sequence identity with α-parvalbumin was not biologically relevant, and that there is no evidence that *E. coli*-derived PMI has significant amino acid sequence homology to any known or putative allergenic proteins. EPA previously reviewed this study and concurred with the study author's conclusion (see MRID No. 464252-01 and EPA 2005 a, b, and c).

CLASSIFICATION: ACCEPTABLE

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DATA EVALUATION RECORD

Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Product Characterization
MRID NO:	468648-01
TEST MATERIAL:	Vip3Aa20 protein from Event MIR162 maize
STUDY NO:	SSB-127-06
SPONSOR:	Syngenta Seeds, Inc. Product Registration Group P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Molecular characterization of Event MIR162 maize
AUTHORS:	Nykoll Long and Derrick Pulliam
STUDY COMPLETED:	June 1, 2006
STUDY SUMMARY:	Southern blot analysis and DNA sequencing showed that Event MIR162 maize contains single copies of the *vip3A* and phosphomannose isomerase (*pmi*) genes, but no backbone sequences from the transformation plasmid pNOV1300. Event MIR162 DNA had two single nucleotide changes in the *Vip3Aa* coding sequence compared to the *Vip3Aa* in pNOV1300, and was designated *vip3Aa20*. The substitution of thymine for guanine at base 387 changed the methionine at position 129 to isoleucine (M129I), but the substitution of cytosine for guanine at base 1683 was a silent mutation. The *pmi* coding sequence in Event MIR162 was identical to that in pNOV1300. The stability of the transgenic locus was shown by statistical analysis of the Event MIR162 segregation patterns over four generations, which confirmed the expected Mendelian inheritance ratio for both the *vip3Aa20* and *pmi* genes.
CLASSIFICATION:	**SUPPLEMENTAL- but UPGRADEABLE-** pending submission of an additional Southern blot containing genomic DNA from MIR162, a negative control, the plasmid control pNOV1300 hybridized with the *pmi*-specific probe, and use a different molecular weight marker to avoid non-specific sequence binding.
GOOD LABORATORY PRACTICE:	Not GLP-compliant, but study conducted using accepted scientific methods; and the data and study records have been retained.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. **STUDY DESIGN:** The purpose of this study was to present molecular characterization data of the T-DNA insert and the genetic material required for its production (*via* pNOV1300) in MIR162 plants, which includes Southern analyses, DNA sequencing, and statistical analysis of segregation patterns over several generations.

II. MATERIALS AND METHODS:

A. Test Materials:

1. Donor genes and regulatory sequences:

Active ingredient cassette	Selectable marker cassette	Vector backbone components
ZmUbiInt (1993 bp): Promoter region from *Zea mays* polyubiquitin gene that contains the first intron (GenBank Acc. No. S94464) and confers constitutive expression in monocots. ***vip3Aa19* (2370 bp):** Synthetic maize-optimized *vip3Aa* gene used in plant transformations. Encodes Vip3Aa19 protein, which differs from the native *vip3Aa1* gene product from *Bacillus thuringiensis* strain AB88 in that it encodes glutamine at amino acid position 284, whereas the native gene encodes lysine. Vip3Aa19 also confers resistance to several lepidopteran insects. **iPEPC9 (108 bp):** Intron #9 from the phosphoenolpyruvate carboxylase gene (GenBank Acc. No. X15239) from *Zea mays*. **35S terminator (70 bp):** Terminator sequence from the 35S RNA from the cauliflower mosaic virus genome (similar to GenBank Acc. No. AF140604), which provides a polyadenylation site.	**ZmUbiInt (1993 bp):** Promoter region from *Zea mays* polyubiquitin gene that contains the first intron (GenBank Acc. No. S94464) and confers constitutive expression in monocots. ***pmi* (1176 bp):** *E. coli pmi* gene encoding phosphomannose isomerase (PMI) (GenBank Acc. No. M15380; also known as *manA*). Catalyzes isomerization of mannose-6-phosphate to fructose-6-phosphate. **NOS (253 bp):** Terminator sequence from the nopaline synthase gene of *Agrobacterium tumefaciens* (GenBank Acc. No. V00087), which provides a polyadenylation site.	**Spec (789 bp):** Streptomycin adenylyltransferase, *aadA* gene from *E. coli* Tn7 (GenBank Acc. No. X03043). Bacterial selectable marker that confers resistance to erythromycin, streptomycin, and spectinomycin. **VS1ori (405 bp):** Origin of replication in *A. tumefaciens* host, is the consensus sequence for the origin of replication and partitioning region from plasmid pVS1 of *Pseudomonas* (similar to GenBank Acc. No. U10487). **ColE1ori (807 bp):** Origin of replication allowing replication of plasmid in *E. coli*. (similar to GenBank Acc. No. V00268). **LB (25 bp):** Left border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (GenBank Acc. No. J01825) required for the transfer of the T-DNA into the plant cell. **RB (25 bp):** Right border region of T-DNA from *A. tumefaciens* nopaline Ti-plasmid (GenBank Acc. No. J01826) required for transfer of T-DNA into plant cell. ***virG* (726 bp):** Part of regulatory system for the vir regulon in *Agrobacterium* from pAD1289 (similar to GenBank Acc. No. AF242881). The N54D substitution results in a constitutive *virG* phenotype (VirGN54D). ***repA* (1074 bp):** pVS1 replication protein from *Pseudomonas* (GenBank Acc. No. AF133831). This is part of the minimal pVS1 replicon functional in gram-negative plant-associated bacteria.

(Data from pp. 9-10 of MRID 46864801)

B. Test Methods:

1. **Transformation system and method:** Maize Event MIR162 was generated by *Agrobacterium*-mediated transformation of immature *Zea mays* embryos from 8-12 day old ears (proprietary line; Negrotto *et al.*, 2000). The embryos were mixed, vortexed, and then incubated with a suspension of *Agrobacterium* cells containing transformation plasmid pNOV1300 (Figure 1) for five minutes. Embryos were then moved to non-selective plates and cultured with *Agrobacterium* at 22°C for 2-3 days in the dark. The embryos were transferred to culture medium containing 100 mg/mL ticarcillin and 1.6 mg/L silver nitrate and incubated in the dark for ten days, and embryos producing a callus were transferred to medium containing mannose. The phosphomannose isomerase gene (*pmi)* was used as a transformation selectable marker. After initial incubation with *Agrobacterium*, transformed tissue was moved to, and grown for four months on, selective medium containing 500 mg/L cefotaxime (antibiotic) to remove residual *Agrobacterium.* The resulting plantlets were tested for the presence of the *pmi* and *vip3Aa20* genes and for the absence of spectinomycin antibiotic resistance by TaqMan® PCR analysis (Ingham, *et al.*, 2001), and the transformants further propagated in a greenhouse.

2. **Sequencing of *vip3Aa* and *pmi* genes:** The nucleotide sequence of the *vip3Aa* and *pmi* genes in the T-DNA insert in Event MIR162 was determined using DNA from the BC4 generation of maize (Figure 2). The genes were amplified using either the Expand High-Fidelity PCR system (Roche) or *Pfu*Ultra™ Hotstart High-Fidelity DNA polymerase (Stratagene), and the PCR products were individually cloned into a vector (pCR®-XL-TOPO or pCR®-BluntII-TOPO vector from Invitrogen). Three separate clones were identified for each PCR product, and were sequenced using the ABI3730XL analyzer and ABI BigDye® 1.1 or Big Dye 3.1 dGTP (for GC-rich templates) chemistry. Sequence analysis was performed using the Phred, Phrap, and Consed package (University of Washington) at an error rate of less than 1 in 10,000 bases (Ewing and Green, 1998). Sequence data from the three clones were combined to generate the final consensus sequence for each gene, and sequences were aligned using the ClustalW program (Thompson *et al*, 1994) with scoring matrix blosum 55, gap opening penalty 15, and gap extension penalty 6.66.

3. **Southern analyses to determine copy number of *vip3Aa* and *pmi* genes:** Southern analysis was conducted using standard methods (Chomczynski, 1992) on genomic DNA isolated from pooled leaf tissue of ten plants representing the backcross four (BC4) generation of Event MIR162. Each plant was confirmed to contain the *vip3Aa20* and *pmi* genes by TaqMan®PCR. Negative control DNA was prepared from pooled leaf tissue of 10 Event MIR162 negative segregant BC4 generation plants, shown by PCR to lack *vip3Aa20* and *pmi*. All plants contained the maize internal control *adh* 1 gene (maize alcohol dehyrdrogenase gene). The number of copies of each element (*vip3Aa20* and *pmi* genes, backbone vector DNA) in the genomic DNA was determined by digestion (7.5 µg DNA /reaction) with restriction enzymes that cut within the Event MIR162 insert from plasmid pNOV1300 (Figure 1), but not within the functional element to which the respective DNA probe binds. This produces one hybridization band per copy of the element. The Event MIR162 insert was also digested with restriction enzymes that release a fragment of known size from the insert, which confirmed the presence of a single copy of each functional element in Event MIR162, and could detect closely linked partial copies of the insert. The DNA digests were subjected to agarose gel electrophoresis and alkaline transfer to a Zeta-Probe® GT membrane (Bio-Rad), and hybridized at 65°C to element-specific full-length PCR generated probes ^{32}P-labeled by random priming using the MegaPrime™ system (Amersham

Biosciences). The location of the restriction sites and position of the probes for the digests are shown in Figures 3, 5, and 7. The membranes were washed several times in 2X SSC, 0.1% SDS, then with 0.1X SSC and 0.1% SDS, and autoradiographed.

Each Southern blot included (1) negative control *Zea mays* DNA, to identify endogenous cross-hybridizing sequences, (2) negative control *Zea mays* DNA spiked with digested pNOV1300 equal to one copy/genome based[1] on plasmid size, to show the system sensitivity, and (3) a positive hybridization control digested pNOV1300 plasmid, equal to one copy/genome based on plasmid size.

4. **Inheritance pattern of the T-DNA insert:** The breeding pedigree of Event MIR162 maize is shown in Figure 2. The presence of the *vip3Aa20* and *pmi* genes was evaluated in individual plants by TaqMan® PCR. The initial Event MIR162 plant was crossed with maize inbred line NPH8431, the NPH8431(MIR162) F_1 plants were crossed to the inbred line NP2161 to yield NP2161(MIR162) F_1 plants, and these were backcrossed to NP2161 to yield the NP2161(MIR162) BC1F_1 generation. The latter were crossed to the inbred line B9620 to yield B9620 (MIR162) F_1 plants, which were backcrossed to B9620 for three subsequent generations to yield B9620(MIR162) BC1F_1, B9620(MIR162) BC2F_1, B9620(MIR162) BC3F_1, and B9620(MIR162) BC4F_1 plants. The B9620(MIR162) generation BC1, BC2 and BC4 plants were evaluated for Mendelian inheritance. For a hemizygous trait (i.e one copy per genome), the expected Mendelian inheritance ratio of positive and negative plants is 1:1. The goodness-of-fit of the observed to the expected genotypic ratio was assessed by Chi Square (X^2) analysis[2] with the Yates correction factor (Strickberger, 1976).

III. RESULTS:

1. **Sequence of the *vip3Aa* and *pmi* genes:** The *vip3Aa* coding sequence in Event MIR162 had two single nucleotide changes from the transformation plasmid pNOV1300. The substitution of a thymine for a guanine at base 387 of Vip3Aa19 changed the methionine at position 129 to isoleucine (M129I). The second nucleotide change, a substitution of cytosine for guanine at base 1683, did not result in an amino acid change but was a silent mutation. The *vip3Aa* gene in Event MIR162 was designated *vip3Aa20*. The *pmi* coding sequence in Event MIR162 was identical to that in pNOV1300.

2. **Southern analyses to determine copy number of inserted elements:** Southern analyses showed that Event MIR162 contains a single copy of the *vip3Aa20* and *pmi* genes, but no backbone sequence (Figures 4, 6 and 8). The *vip3Aa19*-specific probe used for *vip3Aa20* Southern analysis (Figure 3) hybridized to the *vip3Aa20* sequence present in MIR162 since the nucleotide sequences of *vip3Aa19* and *vip3Aa20* are 99.9 % identical. The *vip3Aa19*-specific probe detected single hybridization bands at approximately 8 kb and 13 kb, respectively, for the *Kpn*I and *Eco*RV digests, and a single 4.6 kb band, as expected, for the *Nco*I double digest (Figure 4).

---

[1] The formula used to determine one copy equivalent based on plasmid size was: ((Plasmid size/(Genome size*Ploidy))*µg loaded)*1.00E+06. This equaled 20.2 pg for 1 copy pNOV1300 if the *Zea mays* genome size is 2.67E+09 bp, ploidy = 2, the pNOV1300 plasmid is 14405 bp, and 7.5 µg Event MIR162 digested DNA was loaded per gel lane.

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

The *pmi*-specific probe (Figure 5) detected single hybridization bands at ~4 kb and 6 kb, respectively, in the *Acc*65I and *Bam*HI digests, as well as the expected 8.1 kb band in the *Xma*I + *Hin*dIII double digest (Figure 6). The pNOV1300 positive control also yielded the expected 8.1 kb *Xma*I + *Hin*dIII band, and hybridized with the *vip3Aa19*-specific and *pmi*-specific probes. The DNA ladder probe cross-hybridized with the plasmid control sequences (Lanes 9 and 11 of Figure 6) - which the study author considered irrelevant to the study conclusion.

> ***EPA Reviewer's Comment:*** *It should be noted that there were no literature citations or documentation provided from the MW ladder supplier to support the study author's conclusion of the non-specific sequence binding. Syngenta Seeds, Inc. has previously observed this phenomenon in another Southern analysis (MRID No. 467965-01). In response, EPA asked the registrant for clarification of which specific vector DNA sequences may result in non-specific sequence binding as well as required visual verification to confirm the lack of any unexpected hybridization bands in the plasmid control lanes (for Southern blots hybridized with a specific gene probe). Therefore, in the case of Event MIR162, the registrant is requested to submit the same data clarifications and visual verification.*

Lastly, the pNOV1300 backbone-specific probe (Figure 7) did not hybridize to any Event MIR162 sample (Figure 8), indicating that no pNOV1300 vector backbone sequence was incorporated into Event MIR162.

3. **Mendelian inheritance of the T-DNA insert:** The hypothesis that the genetic trait (T-DNA insert) is segregating in a Mendelian fashion was accepted because the Chi squared value for all tested generations was less than the critical value needed to reject the hypothesis (3.84 at the 5% level). Results of the analysis are showed in Tables 1 and 2.

IV. CONCLUSION:

The Southern analyses and DNA sequencing showed that single copies of the *vip3Aa20* gene and *pmi* gene, but no backbone sequences from the transformation plasmid pNOV1300, are present in Event MIR162 maize. The *vip3Aa* coding sequence in Event MIR162 had two single nucleotide changes from the transformation plasmid, only one of which encoded an amino acid change (methionine at position 129 was substituted by isoleucine). The expected Mendelian inheritance ratio (1:1) was found for the *vip3Aa20* and *pmi* genes by statistical analysis over several generations of Event MIR162 plants, demonstrating the stability of the transgenic locus.

V. CLASSIFICATION:

This study packet is classified as **SUPPLEMENTAL- but UPGRADEABLE-** pending submission of an additional Southern blot containing genomic DNA from MIR162, a negative control, the plasmid control pNOV1300 hybridized with the *pmi*-specific probe, and use a different molecular weight marker to avoid non-specific sequence binding.

VI. REFERENCES:

Chomczynski, P. (1992). One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Analytical Biochemistry, 201: 34-139.

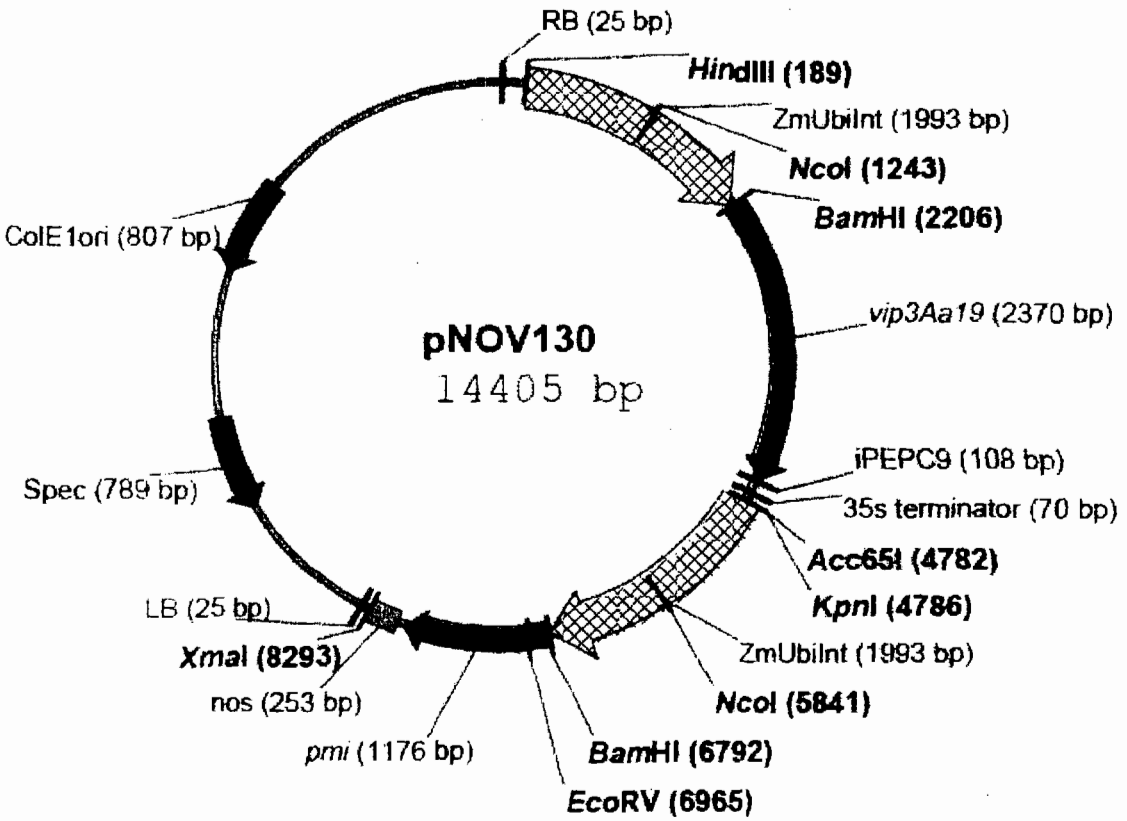
Ingham D.J., Beer S., Money S. and Hansen G. (2001). Quantitative RT-PCR assay for determining transgene copy number in transformed plants. Biotechniques, 31: 136-140.

Negrotto, D., Jolley, M., Beer, S., Wenck, A.R., and Hansen, G. (2000). The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays L.*) via *Agrobacterium* transformation. Plant Cell Reports, 19: 798-803.

Strickberger, M. W. (1976). Probability and statistical testing. Genetics. (2nd ed., pp. 140-163). New York: Macmillan Publishing Company.

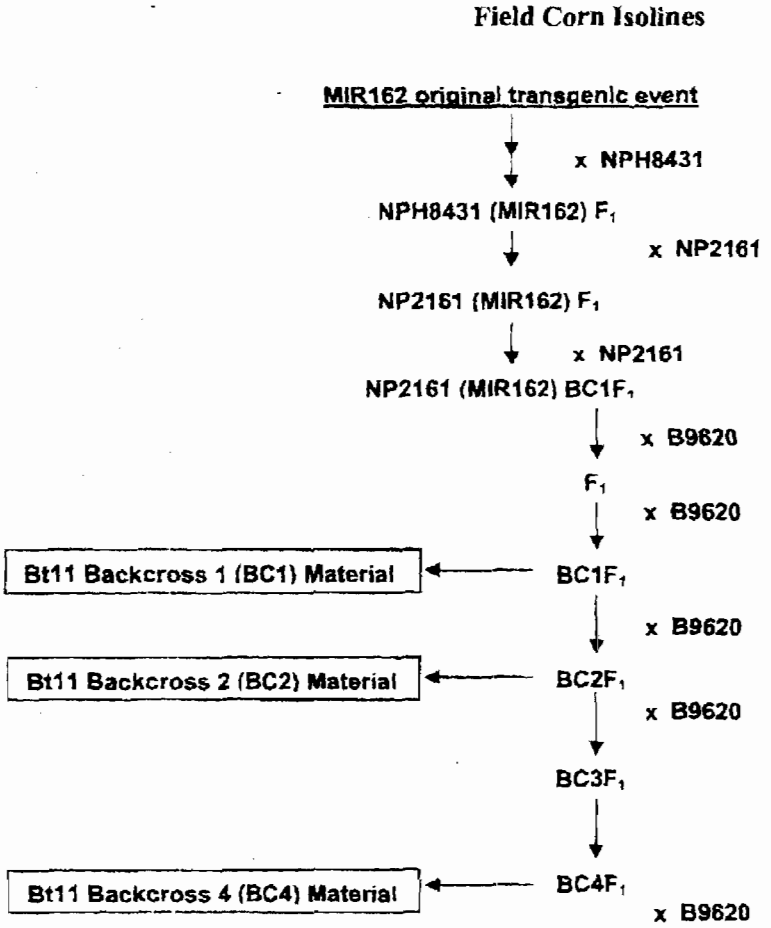
Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 2673-4680.

Figure 1. Plasmid map of pNOV1300 indicating the restriction sites used for Southern analysis.



(Data from p. 17 of MRID 468648-01)

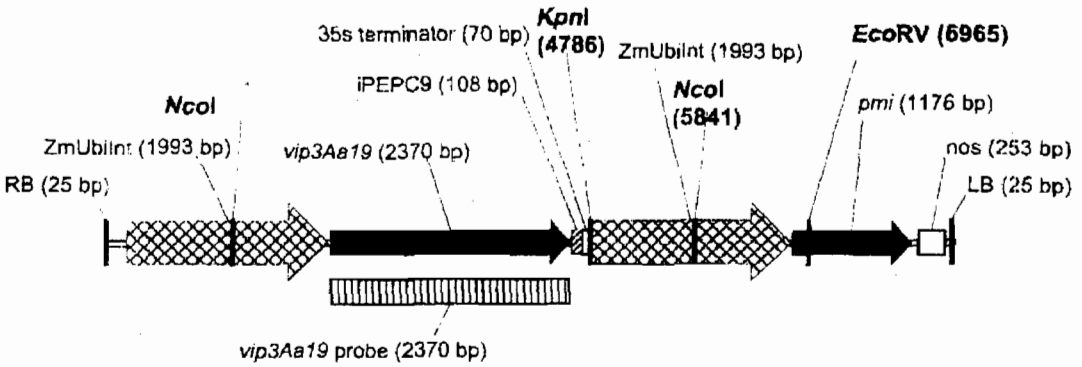
Figure 2. Breeding pedigree indicating the generations tested in the molecular analysis of Event MIR162 maize.
Plant material from BC1, BC2 and BC4 were used for the Mendelian Inheritance study. BC4 material was used for the Southern Analyses to Determine Copy Number of Functional Elements and Gene Sequencing. The "Negative Control" was 10 negative segregant plants from the BC4 generation.

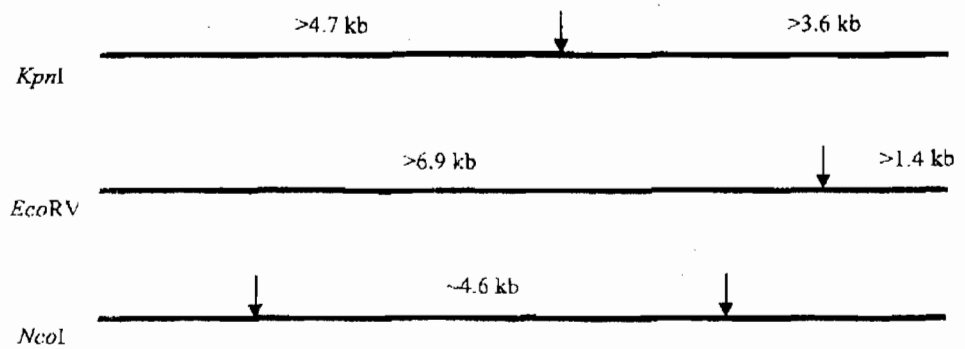


(Data from p. 18 of MRID 468648-01)

Figure 3. Location of *Kpn*I, *Eco*RV, *Nco*I restriction sites and position of *vip3Aa19*-specific probe in the T-DNA region of transformation vector pNOV1300 introduced into Event MIR162.

The 8.4 kb insert of pNOV1300 used to create transformation Event MIR162 maize is shown. The positions of the recognition sequences for the *Kpn*I, *Eco*RV, and *Nco*I restriction enzymes used in the Southern blot analysis with the *vip3Aa19* probe are indicated. The arrow indicates the site of restriction digestion. Sizes of the predicted restriction fragments, calculated from the size of the pNOV1300 linear map, are indicated.

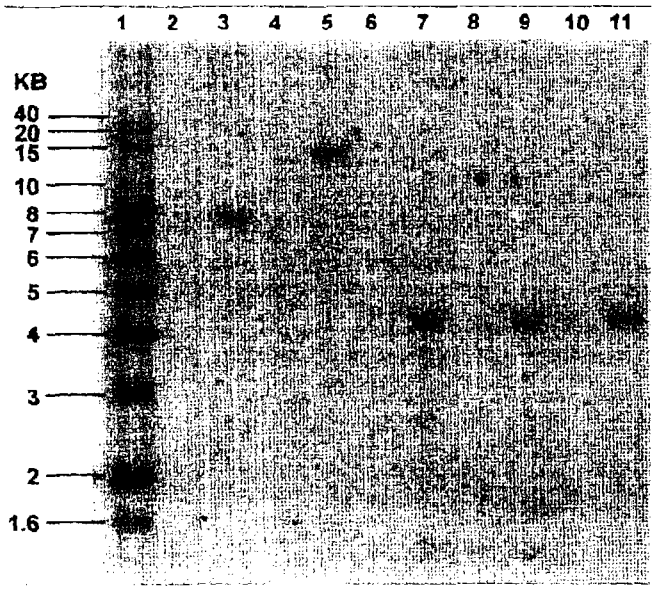




(Data from p. 19 of MRID 468648-01)

Figure 4. Southern analysis of Event MIR162 with a *vip3Aa19*-specific probe. Maize genomic DNA (7.5µg) was digested with *Kpn*I, *Eco*RV, and *Nco*I restriction enzymes and, following electrophoresis and transfer to a Zeta-Probe® GT membrane, hybridized to a *vip3Aa19*-specific probe (2370 bp).

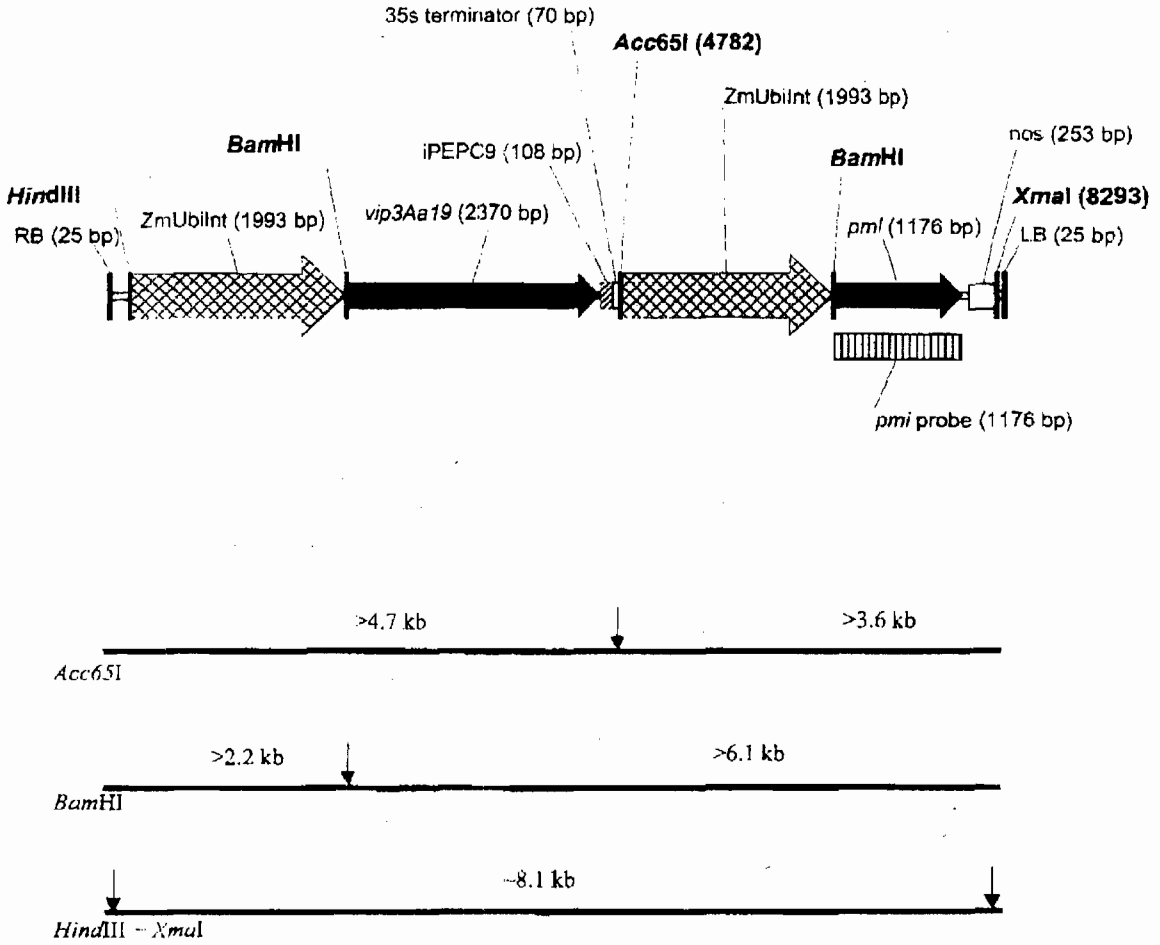
Lane 1: Molecular Weight Marker (1Kb Extension Ladder, Invitrogen. Cat. No. 10511.012);
Lane 2: Blank
Lane 3: BC4 generation of Event MIR162 digested with *Kpn*I;
Lane 4: Negative control from BC4 generation of Event MIR162 digested with *Kpn*I;
Lane 5: BC4 generation of Event MIR162 digested with *Eco*RV;
Lane 6: Negative control from BC4 generation of Event MIR162 digested with *Eco*RV;
Lane 7: BC4 generation of MIR162 digested with *Nco*I;
Lane 8: Negative control from BC4 generation of Event MIR162 digested with *Nco*I;
Lane 9: Negative control from BC4 generation of Event MIR162 digested with *Nco*I plus 20.2 pg of *Nco*I digested pNOV1300 plasmid;
Lane 10: Blank;
Lane 11: 20.2 pg of *Nco*I digested pNOV1300 plasmid.



(Data from p. 20 of MRID 468648-01)

Figure 5. Location of *Acc*65I, *Bam*HI, *Hind*III and *Xma*I restriction sites and position of *pmi*-specific probe in the T-DNA region of transformation vector pNOV1300 introduced into Event MIR162.

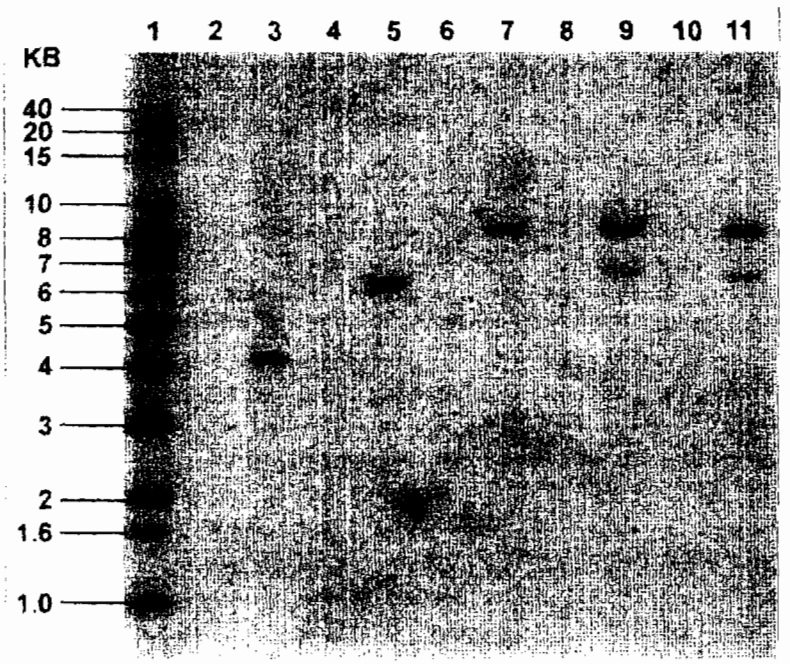
The 8.4 kb insert of pNOV1300 used to create transformation Event MIR162 is shown. The position of the recognition sequence for the *Acc65*I, *Bam*III, *Hind*III, and *Xma*I restriction enzymes used in the Southern blot analysis with the *pmi* probe are indicated. The arrow indicates the site of restriction digestion. Sizes of the predicted restriction fragments, calculated from the size of the pNOV1300 linear map, are indicated.



(Data from p. 21 of MRID 468648-01)

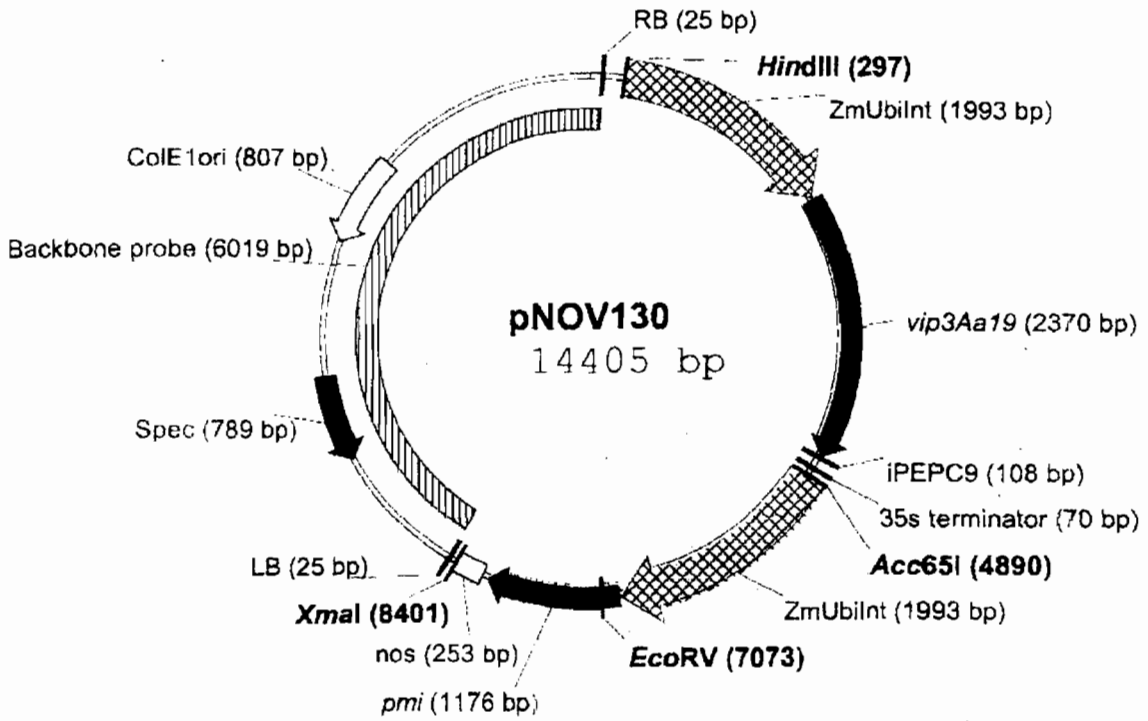
Figure 6. Southern analysis of Event MIR162 with *pmi*-specific probe. Maize genomic DNA (7.5 µg) was digested with *Acc*65I, *Bam*HI, *Hin*dIII and *Xma*I restriction enzymes and, following electrophoresis and transfer to a Nytran membrane, hybridized to a *pmi*-specific probe (1176 bp).

Lane 1: Molecular Weight Marker (1Kb Extension Ladder, Invitrogen. Cat. No. 10511.012);
Lane 2: Blank.
Lane 3: BC4 generation of Event MIR162 digested with *Acc*65I;
Lane 4: Negative control from BC4 generation of Event MIR162 digested with *Acc*65I;
Lane 5: BC4 generation of Event MIR162 digested with *Bam*HI;
Lane 6: Negative control from BC4 generation of Event MIR162 digested with *Bam*HI;
Lane 7: BC4 generation of Event MIR162 digested with *Hin*dIII and *Xma*I;
Lane 8: Negative control from BC4 generation of Event MIR162 digested with *Hin*dIII x *Xma*I;
Lane 9: Negative control from BC4 generation of Event MIR162 digested with *Hin*dIII x *Xma*I plus 20.2 pg of *Hin*dIII x *Xma*I digested pNOV1300 plasmid;
Lane 10: Blank
Lane 11: 20.2 pg of *Hin*dIII x *Xma*I digested pNOV1300 plasmid.



(Data from p. 22 of MRID 468648-01)

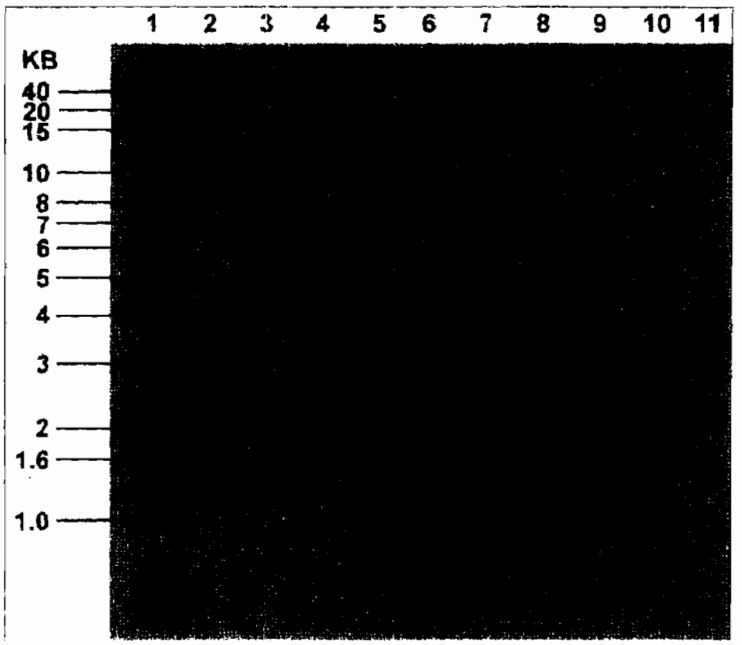
Figure 7. Location of *Xma*I and *Hind*III restriction sites and the position of backbone-specific probe in the transformation vector pNOV1300.



(Data from p. 23 of MRID 468648-01)

Figure 8. Southern analysis of Event MIR162 with pNOV1300 backbone-specific probe. Maize genomic DNA (7.5µg) was digested with *Acc*65I, *Eco*RV, *Hin*dIII and *Xma*I restriction enzymes and, following electrophoresis and transfer to a Nytran membrane, hybridized to a backbone-specific probe (6019 bp).

Lane 1: Molecular Weight Marker (1Kb Extension Ladder, Invitrogen. Cat. No.10511.012);
Lane 2: Blank;
Lane 3: BC4 generation of Event MIR162 digested with *Acc*65I;
Lane 4: Negative control from BC4 generation of Event MIR162 digested with *Acc*65I;
Lane 5: BC4 generation of Event MIR162 digested with *Eco*RV;
Lane 6: Negative control from BC4 generation of Event MIR162 digested with *Eco*RV;
Lane 7: BC4 generation of Event MIR162 digested with *Hin*dIII and *Xma*I;
Lane 8: Negative control from BC4 generation of Event MIR162 digested with *Hin*dIII x *Xma*I;
Lane 9: Negative control from BC4 generation of Event MIR162 digested with *Hin*dIII x *Xma*I plus 20.2 pg of *Hin*dIII x *Xma*I digested pNOV1300 plasmid;
Lane 10: Blank;
Lane 11. 20.2 pg of *Hin*dIII x *Xma*I digested pNOV1300 plasmid.



(Data from p. 24 of MRID 468648-01)

TABLE 1. Observed vs. expected genotype for *vip3Aa20* for multiple Event MIR162 generations as determined by TaqMan® PCR analysis						
Trait	BC1		BC2		BC4	
	O*	E*	O*	E*	O*	E*
Positive	21	20.5	45	48.5	148	143.5
Negative	20	20.5	52	48.5	139	143.5
Total	41	41.0	97	97.0	287	287.0
X^2 value*	0.000		0.371		0.223	

*O = Observed values
*E = Expected values
*$X^2 = \sum$ [Observed - expected - 0.5]^2 / expected

(data from p. 31 of MRID 468648-01)

TABLE 2. Observed vs. expected genotype for *pmi* for multiple Event MIR162generations as determined by TaqMan® PCR analysis						
Trait	BC1		BC2		BC4	
	O*	E*	O*	E*	O*	E*
Positive	21	20.5	45	48.5	148	143.5
Negative	20	20.5	52	48.5	139	143.5
Total	41	41.0	97	97.0	287	287.0
X^2 value*	0.000		0.371		0.223	

*O = Observed values
*E = Expected values
*$X^2 = \sum$ [Observed - expected - 0.5]^2 / expected

(data from p. 31 of MRID 468648-01)

DATA EVALUATION RECORD

Bacillus thuringiensis VIP3A Protein Expressed in Event MIR162 Maize (Vip3Aa20)

STUDY TYPE: Molecular Characterization of PIP

MRID 468648-01

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T.

Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S.

Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader

Signature: _____
Date: _____

Quality Assurance:
Angela M. Edmonds, B.S.

Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

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DATA EVALUATION RECORD

Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Product Characterization
MRID NO:	468648-02
TEST MATERIAL:	*Bacillus thuringiensis* VIP3A protein expressed in *E. coli*, Event MIR162 Maize, and Pacha-derived maize
STUDY NO:	SSB-017-06; MIR162-05-03
SPONSOR:	Syngenta Biotechnology, Inc. Regulatory Science P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Seeds, Inc. – Field Crops – NAFTA P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Characterization of the Vip3A protein expressed in Event MIR162-derived maize (corn) and comparison with microbially produced and plant-derived Vip3A test substances.
AUTHORS:	Gerson Graser and Cheryl Stacy
STUDY COMPLETED:	May 25, 2006
STUDY SUMMARY:	Vip3Aa produced in recombinant *E. coli*, MIR162 maize, and Pacha maize were shown to be substantially equivalent based on the finding that (1) Vip3Aa20 from MIR162 maize (test material LPMIR162-0105 and IAPMIR162-0105), Vip3Aa19 from Pacha maize (LPPACHA-0199), Vip3Aa19 from several of *E. coli*-derived samples (VIP3A-0204, VIP3A-0104, VIP3A-0100), and Vip3Aa1 from *E. coli*-produced VIP3A-0199 each had the expected predicted molecular weight of ~89 kDa, and were immunoreactive with the same anti-Vip3Aa antibody on western blots, and (2) VIP3A-0204 and LPMIR162-0105 had comparable insecticidal activity against FAW (137 ng Vip3Aa19/cm^2 vs. 154 ng Vip3Aa20 ng/cm^2 diet surface). Additionally, there was no evidence of post-translational glycosylation of Vip3Aa from LPMIR162 VIP3A-0105 or VIP3A-0204. Therefore, the *E. coli* - produced Vip3A is considered an appropriate substitute for Vip3Aa20 expressed in MIR162 maize in toxicity and/or protein characterization studies. It was also be noted that the VIP3A-0204 Vip3Aa19 protein N-terminal amino acid sequence matched the predicted sequence however, the plant-expressed Vip3A was not determined due to technical difficulties.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	The signed GLP statement indicated the study was conducted in compliance with 40 CFR Part 160 except that the N-terminal sequencing was not conducted under GLP.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN: The purpose of this study was to determine if Vip3Aa20 expressed in maize plants derived from transformation Event MIR162 is substantially equivalent to Vip3Aa19 or Vip3Aa1 present in various test substances previously used in toxicity and/or test substance characterization studies.

II. MATERIALS AND METHODS:

A. TEST MATERIALS:

The amino acid differences of the various Vip3Aa proteins addressed in the present study are summarized in Table 1.

TABLE 1. Comparison of the amino acid sequence differences of Vip3Aa proteins examined in this study (data from p. 18 of MRID 46864802)

Vip3Aa Sample	Source	Amino acid at position 129	Amino acid at position 284	Crickmore ID[a]
native Vip3Aa	*B. thuringiensis*	M	K	Vip3Aa1
VIP3A-0199	Recombinant *E. coli*	M	K	Vip3Aa1
LPPACHA-0199	Pacha maize	M	Q	Vip3Aa19
VIP3A-0100	Recombinant *E. coli*	M	Q	Vip3Aa19
VIP3A-0104	Recombinant *E. coli*	M	Q	Vip3Aa19
VIP3A-0204	Recombinant *E. coli*	M	Q	Vip3Aa19
LPMIR162-0105	MIR162 maize	I	Q	Vip3Aa20
IAPMIR162-0105	MIR162 maize	I	Q	Vip3Aa20

[a] http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/, accessed May 17, 2006.

1. **Test substance LPMIR162-0105 (leaf-derived):** Vip3Aa20-enriched protein sample was prepared using leaves from greenhouse-grown Event MIR162 maize. Leaf protein extract was precipitated with 40% ammonium sulfate per Syngenta SOPs 2.72 and SOP 2.46 and the precipitate resuspended in buffer and dialyzed. The dialyzed extract was lyophilized, stored at -20°C, and designated as test substance LPMIR162-0105 (for use in activity bioassays and Western blots). Vip3Aa20 encoded in Event MIR162 maize differs from the protein encoded by native *vip3Aa1* from *Bacillus thuringiensis* at two amino acids: lysine-284 is replaced by glutamine, and methionine-129 is replaced by isoleucine (Pulliam & Long, 2006).

2. **Test substance IAPMIR162-0105 (leaf-derived):** Vip3Aa20 was immunoaffinity-purified from the dialyzed ammonium sulfate precipitate prepared from maize leaf extract for test substance LPMIR162-0105 (Syngenta SOP 2.23). The immunoaffinity column matrix contained bound rabbit anti-Vip3Aa antibody dialyzed and Vip3Aa20 was eluted with a pH gradient, concentrated with ultra-filtration and stored at 20°C. The immunoaffinity-purified Vip3Aa20 sample was designated test substance IAPMIR162-0105 (used to evaluate molecular weight, glycosylation, and immunoreactivity).

3. **Control substance LPMIR162-0105C (leaf-derived):** Prepared similarly to LPMIR162-0105 using leaves from a corresponding near-isogenic, nontransgenic maize line, and stored at -20°C.

4. **Test substance LPPACHA-0199 (leaf-derived):** Vip3Aa19-enriched leaf protein sample isolated from field-grown Event Pacha-derived maize (Privalle, 2001). The sample contains ~0.36% Vip3Aa19 by weight, is bioactive against Vip3Aa-sensitive lepidopteran larvae, and is stored at -20°C. The leaf Vip3Aa19 has the same amino acid sequence as Vip3Aa19 from the *E. coli*-derived test substances VIP3A-0204, VIP3A-0104 and VIP3A-0100.

5. **Test substances VIP3A-0204, VIP3A-0104, VIP3A-0100 and VIP3A-0199 (*E. coli*-derived):** All four substances were produced in an *E. coli* over-expression system. VIP3A-0204, VIP3A-0104 and VIP3A-0100 contain the identical synthetic *vip3Aa19* gene that was used to transform plants, and were characterization previously (Graser, 2004a; Graser, 2004b; and Privalle, 2002a). The synthetic Vip3Aa19 protein differs from the native *B. thuringiensis* Vip3Aa1 protein by a having a glutamine instead of a lysine at position 284. This is a conservative substitution (both amino acids are polar and have a molecular weight of 146) and had no apparent effect on the Vip3Aa19 insecticidal activity against sensitive lepidopteran pests (Privalle, 2002b). Test substance VIP3A-0199 was produced by *E. coli* and expresses the native *vip3Aa1* gene from *B. thuringiensis* strain AB88 (Estruch et al., 1996 and Yuetal et al., 1997) and was previously described (Privalle, 1999). Initial characterization of VIP3A-0204, VIP3A-0104, VIP3A-0100 and VIP3A-0199 determined their Vip3Aa content as ~90, 63, 74, and 54% by weight, respectively, and demonstrated their insecticidal activity against sensitive lepidopteran larvae. All four test substances were stored at -20°C.

B. TEST METHODS:

1. **Quantitation of VIP3A protein:** VIP3A content was determined by enzyme linked immunosorbent assay (ELISA- Tijssen, 1985), using immunoaffinity-purified VIP3A-specific polyclonal goat and protein A-purified rabbit antibodies (Syngenta SOP 2.38).

2. **Total protein quatititation:** Test substance LPMIR162-0105 and control substance LPMIR162-0105C total protein was quantified with the BCA™ method (Hill and Straka, 1988) per Syngenta SOP 2.16, using bovine serum albumin (BSA) as the standard.

3. **Determination of molecular weight and immunoreactivity:** The integrity and immunoreactivity of VIP3A produced by *E. coli* and by maize (LPMIR162-0105, IAPMIR162-0105, VIP3A-0204, VIP3A-0104, VIP3A-0100, VIP3A-0199, LPPACHA-0199, and) were evaluated by western blot analysis (Syngenta SOP 2.3). Samples of ~10 ng Vip and an equivalent amount of negative control protein (LPMIR162-0105C) were diluted in NuPAGE® sample buffer and run on NuPAGE® 4-12% gradient SDS-PAGE gels (Syngenta SOP 2.4) with morpholinoethanesulfonic acid (MES) buffer. Each gel also contained SeeBlue Plus2® molecular weight standards (Invitrogen; San Diego, CA). After electrophoresis, the proteins were electroblotted and the membrane probed with immunoaffinity-purified VIP3A-specific goat polyclonal antiserum, followed by a 1:3,000 dilution of donkey anti-goat IgG linked to alkaline phosphatase (Jackson; West Grove, PA). Immunoreactive proteins were visualized by development with alkaline phosphatase substrate solution.

4. **Glycosylation analysis:** To determine if the VIP3A in test substances IAPMIR162-0105 and VIP3A-0204 was post-translationally glycosylated, 1 µg and 2 µg samples were analyzed with the DIG Glycan Detection Kit (Roche Diagnostics GmbH, Mannheim,

Germany) per the manufacturer's directions. The method details were not provided, but it was stated that glycan moieties are oxidized with periodate, labeled with digoxigenin, and detected with anti-digoxigenin antibody coupled to alkaline phosphatase. A negative control non-glycosylated protein (creatinase) and a positive control glycosylated protein (transferrin) were included in the assay.

5. **N-terminal amino acid sequencing:** The N-terminal sequence of VIP3Aa19 in test substance VIP3A-0204 was determined by automated Edman degradation. Prior to sequencing, the VIP3A-0204 was subjected to SDS-PAGE, electroblotted to a PVDF membrane (Invitrogen; San Diego, CA), and stained with Amido black. The Vip3Aa19 protein band was excised and sent to Proseq Inc. Protein Sequencing Services (Boxford, MA, USA) for N-terminal amino acid sequence analysis using non-GLP proprietary methods developed specifically for proteins immobilized on PVDF membranes and automated Edman-based chemistry (Brauer et al, 1982).

6. **Insecticidal activity assays:** The VIP3A-0204 and LPMIR162-0105 insecticidal activity was assessed in insect feeding assays using freshly hatched first-instar fall army worm larvae (FAW; *Spodoptera frugiperda*), per Syngenta SOP 2.70. Each well of a Costar 24-well plate (Fisher Scientific) was filled with 800 µL insect diet (General Purpose Insect Diet from Bio-Serv, Inc.; Frenchtown, NJ) overlaid with 50 µL VIP3A-0204 test solution in 50 mM Tris-HCl (pH 9.5) buffer containing 2 mM EDTA. Concentrations tested were 7.5-825 ng VIP3A/cm^2 diet surface. Each assay consisted of 24 replicate wells of one FAW larva per well, and included concurrent negative controls of untreated insect diets, diets treated with buffer, and diets treated with LPMIR162-0105C control substance (~1797 µg protein, which is equivalent to the total protein applied in the highest test concentration of LPMIR162-0105). The wells were covered with silicone stoppers, kept at room temperature under ambient conditions, and mortality was assessed after 120 hours. Mortality was reported for each treatment as the percent mortality among 24 larvae. Statistical analyses for the LC_{50} values determined for the FAW bioassay were calculated using EPA Probit Analysis Program, Version 1.5.

III. RESULTS:

1. **Immunoreactivity and molecular weight:** An immunoreactive band at *ca.* 89 kDa was produced on the Western blot for all test materials expressing native Vip3Aa19 protein (*E. coli*-derived VIP3A-0199, VIP3A-0100, VIP3A-0104 and VIP3A-0204, and plant-derived LPPACHA-0199), as well as plant samples expressing the synthetic Vip3Aa20 protein (LPMIR162-0105 and IAPMIR162-0105) (Figure 1). As expected, control LPMIR162-0105C had no immunoreactivity with anti-Vip3Aa antibody on the Western blot (Figure 1, lane 9).

2. **Insecticidal activity:** VIP3A-0204 and LPMIR162-0105 had similar insecticidal activity against *S. frugiperda* after 120 hours (Table 2). By comparison, the negative controls (insect diet alone, diet treated with buffer, and substance LPMIR162-0105C) caused much lower mortality (0%, 12%, and 4%, respectively). Results are shown in Table 3.

Table 2. Comparative Bioactivity of Vip3Aa19 from recombinant *E. coli* (test substance Vip3A-0204) and Vip3Aa20 from Event MIR162 Maize (test substance LPMIR162-0105) in Diet Surface bioassay with first-instar *S. frugiperda* larvae.	
Test Substance	**LC_{50} [ng Vip3Aa/cm^2] (95% confidence intervals)**
VIP3A-0204	137 ng/cm^2 (82-199)
LPMIR162-0105	154 ng/cm^2 (94-222)

Table 3. Relative bioactivity of Vip3Aa19 from recombinant *E. coli* (test substance VIP3A-0204) and Vip3Aa20 from Event MIR162 maize (test substance LPMIR162-0105) in diet surface bioassay with first-instar *S. frugiperda* larvae.		
Sample	**Mortality at 120 hours [%]**	
[ng Vip3Aa/cm^2] [1]	**VIP3A-0204**	**LPMIR162-0105**
7.5	8	8
15	4	0
30	17	21
60	38	38
120	54	46
275	75	58
550	83	92
825	92	92
Water control [2]	0	
Buffer control [3]	12	
LPMIR162-0105C [4]	4	

[1] Insect diet treated with Vip3Aa19 from test substance VIP3A-0204 or Vip3Aa20 from test substance LPMIR162-0105 dissolved in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (50 µl/dish)
[2] Water control diet: Insect diet treated with water (50 µl/dish)
[3] Buffer control diet: Insect diet treated with 50 mM Tris-HCl (pH 9.5), 2 mM EDTA (50 µl/dish)
[4] Insect diet treated with control substance LPMIR162-0105C in 50 mM Tris-HCl (pH 9.5), 2 mM EDTA.

3. **Glycosylation analysis:** DIG Glycan analysis showed no evidence of glycosylation of either the microbial or maize VIP3A protein (loaded 1000-2000 ng/lane), as shown in Figure 2. The response of the positive control transferrin, also loaded on the gel, indicated that the limit of detection for the system was 1.25 ng glycan (calculated based on the transferrin molecular weight of ~80,000 and ~5% glycan content by weight). This detection limit corresponds to ~ 0.0628% by weight (1.25 ng/2000 ng) or ~0.34 glucose equivalents per Vip3Aa molecule, indicating that either Vip3Aa is not glycosylated, or that glycan moieties occur at a frequency of less than one glucose equivalent per molecule of Vip3Aa.

4. **N-terminal amino acid sequencing:** The VIP3A-0204 Vip3Aa19 protein N-terminal amino acid sequence matched exactly the predicted sequence (MNKNNTKLSTRA). The plant-expressed Vip3A was not determined due to technical difficulties. The study author attributed this finding to N-terminal blockage of the protein, but concluded that this had no adverse impact on the outcome of the study.

EPA Reviewer's Comment: *It is plausible to agree with the study author for the rationale provided for the technical difficulties noted in the N-terminal amino acid sequencing for the plant-expressed Vip3A. Moreover, another plant-expressed Vip3A variant (IAPACHA-0100) N-terminal amino acid sequence was determined in another report and closely matched the predicted sequence (see MRID No. 46848-06).*

IV. CONCLUSION:

The Vip3Aa produced in recombinant *E. coli,* MIR162 maize, and Pacha maize are substantially equivalent. Therefore, the bacterially-produced Vip3A is an appropriate substitute for Vip3Aa20 expressed in MIR162 maize in toxicity and/or protein characterization studies. This conclusion was based on the finding that (1) western blots showed that Vip3Aa20 from MIR162 maize (LPMIR162-0105 and IAPMIR162-0105), Vip3Aa19 from Pacha maize (LPPACHA-0199), Vip3Aa19 from a number of *E. coli*-derived samples (VIP3A-0204, VIP3A-0104, VIP3A-0100), and Vip3Aa1 from microbially-produced VIP3A-0199 each had the expected predicted molecular weight of ~89 kDa and were immunoreactive with the same anti-Vip3Aa antibody, (2) VIP3A-0204 and LPMIR162-0105 had comparable insecticidal activity against FAW, and (3) there was no evidence of post-translational glycosylation of Vip3Aa from LPMIR162 VIP3A-0105 or VIP3A-0204. It should also be noted that the VIP3A-0204 Vip3Aa19 protein N-terminal amino acid sequence matched the predicted sequence however, the plant-expressed Vip3A was not determined due to technical difficulties.

V. CLASSIFICATION: ACCEPTABLE

VI. REFERENCES:

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Hill, H.D. and Straka, J.G. (1988) Protein determination using Bicinchoninic acid in the presence of sulfhydryl reagents. *Anal. Biochem.* 170: 203-208.

Privalle, L. (1999) Characterization of test substance VIP3A-0199. Test substance characterization report and certificate of analysis. Novartis Seeds Biotechnology Report #NSB-004-99. This report appears as an appendix to the report 'Single Dose Oral Toxicity Study with LPPACHA-0199 in Mice', Glaza, S.M. (2002). (MRID 45766504)

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Privalle, L. (2002b) Characterization of VIP3A Protein Produced in Pacha-Derived Maize (Corn) and Comparison with VIP3A Protein Expressed in Recombinant *Escherichia coli*. Syngenta Seeds Biotechnology Report No. SSB-004-00.

Pulliam, D., and N. Long. (2006) Molecular Characterization of Event MIR162 Maize. Syngenta Seeds Biotechnology Report No. SSB-113-06.

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immuoassays. *In practice and theory of enzyme immunoassays*. (Laboratory techniques in Biochemistry and Molecular Biology, V. 15) Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421

Yu, C-G., Mullins, M.A., Warren, G.W., Koziel, M.G. and Estruch, J.J. (1997) The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. *Appl. Environ. Microbiol.* 63: 532-536.

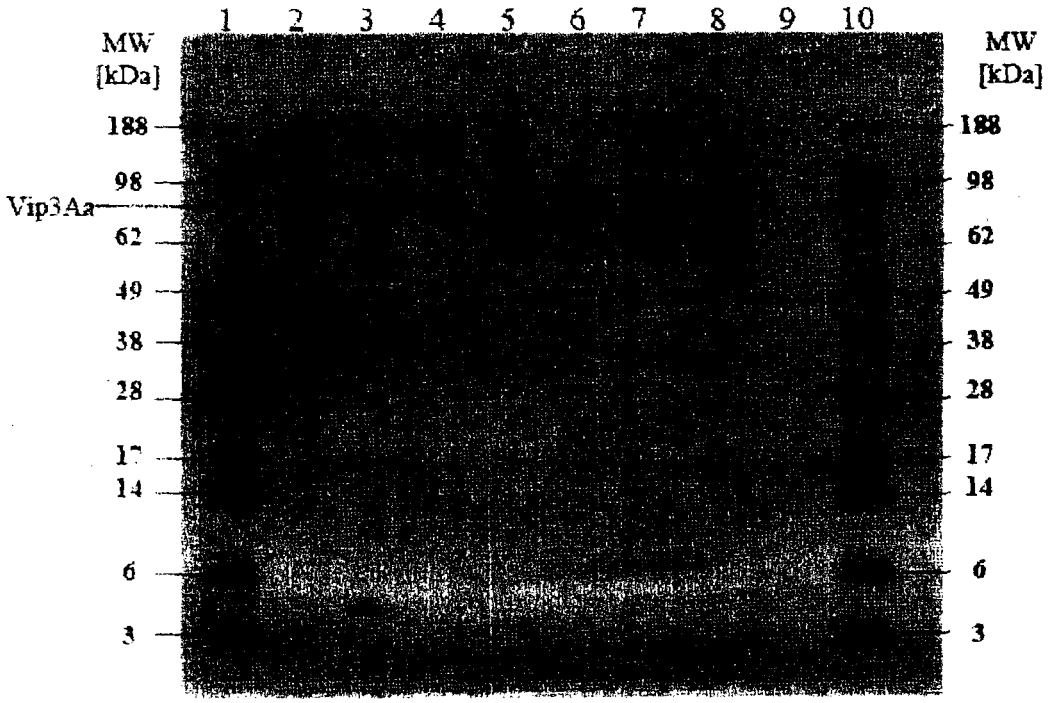
Reference SOP:

SOP 2.3 Western Blot analysis
SOP 2.4 SDS-Polyacrylamide Gel Electrophoresis
SOP 2.16 BCA™ Microtiter Protein Determination
SOP 2.23 Immunoaffinity Chromatography
SOP 2.38 Quantitative Analysis for VIP3A by ELISA
SOP 2.46 VIP3A Enrichment from VIP3 Transgenic maize leaves
SOP 2.70 Diet Surface Bioassay of Fall Armyworm (*Spodoptera frugiperda*)
SOP 2.72 Tissue Preparation for Extraction

Figure 1. Immunoreactivity of Vip3Aa in Test Substances from Event MIR162 (LPMIR162-0105 and IAPMIR162-0105), Event Pacha Maize (LPPACHA-0199) and Recombinant *E. coli* (VIP3A-0199, VIP3A-0100, VIP3A-0104 and VIP3A-0204)

Lanes 1 and 10: Molecular weight standard SeeBlue Plus2® (Invitrogen, CA, USA)
Lane 2: 10 ng Vip3Aa1, from VIP3A-0199
Lane 3: 10 ng Vip3Aa19, from VIP3A-0100
Lane 4: 10 ng Vip3Aa19, from VIP3A-0104
Lane 5: 10 ng Vip3Aa19, from LPPACHA-0199
Lane 6: 10 ng Vip3Aa19, from VIP3A-0204
Lane 7: 10 ng Vip3Aa20, from LPMIR162-0105
Lane 8: 10 ng Vip3Aa20, from IAPMIR162-0105
Lane 9: *ca.* 676 ng total protein, LPMIR162-0105C

The molecular weight of Vip3Aa corresponds to *ca.* 89,000 Da.



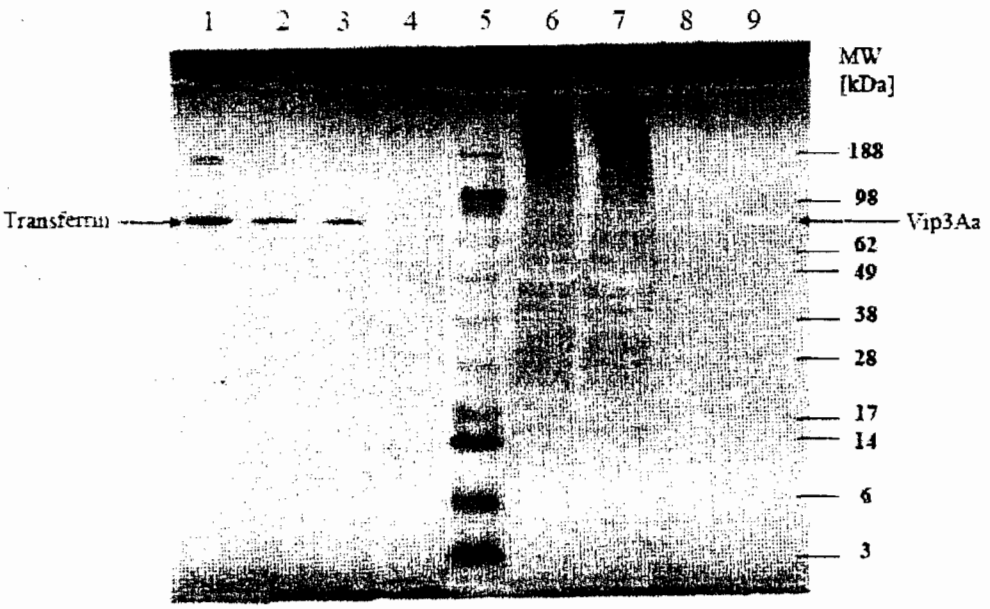
(data from p. 20 of MRID 46864802)

Figure 2. Glycosylation Analysis of Vip3Aa Expressed in Event MIR162 Maize (Test Substance IAPMIR162-0105) and Recombinant *E. coli* (Test Substance VIP3A-0204)

Immunoaffinity-purified Vip3Aa20 from MIR162 maize (test substance IAPMIR162-0105) and Vip3Aa19 from *E. coli* (test substance VIP3A-0204), were analyzed for the presence of glycosyl residues using the DIG Glycan Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany).

Lanes 1, 2 and 3: 100, 50, 25 ng transferrin (positive control), respectively
Lane 4: 2 µg creatinase (negative control)
Lane 5: Molecular weight standard SeeBlue® Plus2 (Invitrogen, CA, USA)
Lanes 6 and 7: 1 and 2 µg Vip3Aa20, respectively, from IAPMIR162-0105
Lanes 8 and 9: 1 and 2 µg Vip3Aa19, respectively, from VIP3A-0204

The molecular weight of Vip3Aa corresponds to *ca.* 89,000 Da.



(data from p. 21 of MRID 46864802)

DATA EVALUATION RECORD

VIP3A Protein Expressed in *E. coli*, Event MIR162 Maize, and Pacha-derived Maize

STUDY TYPE: Product Characterization and Protein Equivalency

MRID 468648-02

Prepared for
Biopesticides and Pollution Prevention Division
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Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

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DATA EVALUATION RECORD	
Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Product characterization
MRID NO:	468648-03
TEST MATERIAL:	*Bacillus thuringiensis* (*Bt*) VIP3A-0104 test material (Vip3Aa19 protein) expressed in *E. coli*
STUDY NO:	SSB-026-04; COT200-04-02
SPONSOR:	Syngenta Seeds, Inc. – Field Crops – NAFTA 3054 East Cornwallis Road; P.O. Box 12257 Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science Laboratory 3054 East Cornwallis Road; P.O. Box 12257 Research Triangle Park, NC 27709
TITLE OF REPORT:	Characterization of Vip3A protein test substance (VIP3A-0104) and certificate of analysis
AUTHOR:	Gerson Graser
STUDY COMPLETED:	September 9, 2004
STUDY SUMMARY:	VIP3A-0104 test material (Vip3Aa19 protein) produced from the synthetic *vip3A(a)* gene in an *E. coli* over-expression system was purified by ammonium sulfate precipitation, phenyl sepharose interaction chromatography, and DEAE anion exchange chromatography. The VIP3A-0104 samples were determined to be 63.1% pure by SDS-PAGE in conjunction with Coomassie blue staining and densitometric analysis, and were shown to contain ~ 2 µg endotoxin /g VIP3A-0104 by lipopolysaccharide analysis. Western blots using goat anti-VIP3A polyclonal primary antibody and donkey anti-goat alkaline phosphatase-linked secondary antibody revealed a dominant immunoreactive band at the predicted molecular weight of ~89,800 Da. VIP3A-0104 had insecticidal activity against first-instar fall army worm (FAW) larvae in insect feeding assays, with an LC_{50} of 272 ng VIP3A/cm^2 diet surface (95% confidence interval of 184 - 384 ng VIP3A/cm^2) after 168 hours.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating the study was conducted in compliance with 40 CFR Part 160.

THIS REPORT <u>DOES NOT</u> CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

The purpose of this study was to characterize test substance VIP3A-0104 containing the vegetative insecticidal protein VIP3A encoded by the synthetic *vip3A(a)* gene, via evaluation of the total protein concentration, bioactivity, immunoreactivity, and lipopolysaccharide (LPS) concentration.

II. MATERIALS AND METHODS:

1. **Production of VIP3A-0104 test material:** VIP3A-0104 test material (Vip3Aa19 protein) was produced by cloning the synthetic *vip3A(a)* gene into the inducible, over-expression vector pET3a® (Novagen; Madison, WI) in *E. coli* strain BL21DE3pLysS. The synthetic VIP3A protein differs from the native VIP3A protein from *Bacillus thuringiensis* by a single conservative substitution at amino acid position 284, where lysine has been changed to glutamine. VIP3A-0104 protein was purified from three batches of *E. coli* cell paste by Apex Bioscience, Inc. (Research Triangle Park, NC), as described in detail in the Apex Bioscience reports "Final Report Summary for the Purification Of V-Protein", dated April 19, July 10 and August 28, 2001. *E. coli* cell pellets were ruptured by grinding, the cell debris removed by centrifugation, and the supernatant precipitated with 20% ammonium sulfate. The resulting supernatant was subjected to phenyl sepharose interaction chromatography, where VIP3A protein was eluted by a step gradient of decreasing ammonium sulfate concentration. The VIP3A-containing fractions were applied to Toyopearl™ DEAE anion exchange chromatography, and VP3A was eluted with an increasing sodium chloride step gradient. Fractions containing VIP3A protein were provided to Syngenta where they were dialyzed, lyophilized, pooled, designated as sample VIP3A-0104, and stored at -20°C.

2. **Total protein quantitation:** VIP3A-0104 total protein was quantified with the BCA™ method per Syngenta SOP 2.16, using bovine serum albumin (BSA) as the standard.

3. **VIP3A-0104 densitometry and sample purity:** The purity of VIP3A-0104 samples was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an 8-16% polyacrylamide gradient gel (Syngenta SOP 2.4). The gels contained 3.2 to 7.6 µg VIP3A per lane, and Mark 12™ molecular weight standards (15 µL, Invitrogen, San Diego, CA). Protein bands were stained with Coomassie blue per Syngenta SOP 2.4, and their intensity estimated by densitometric analysis (Syngenta SOP 2.6, Gelbase/GelBlot, Version 2.1). The purity of VIP3A protein in the test material was calculated from the sample total weight, total protein content (by BCA assay), and the densitometry data.

4. **Lipopolysaccharide (LPS) quantitation:** *E. coli* protein preparations are often contaminated with LPS, also known as endotoxin. LPS levels in the VIP3A-0104 samples were estimated using the PYROGENT® Plus Gel Clot LAL test (Cambrex, Walkersville, MD), per the manufacturer's instructions.

5. **Immunoreactivity and molecular weight determination:** VIP3A-0104 samples were subjected to western blot analysis (per Syngenta SOP 2.3) to determine the molecular weight and immunoreactivity of the VIP3A-0104 protein. Each gel contained molecular weight standards (15 µL SeeBlue® Plus; Invitrogen, San Diego, CA) and test substance VIP3A-0199 as a positive control (characterized in report Novartis Seeds, 1999). Dilutions of 5, 15 and 30 ng VP3A-0104 and VIP3A-0199 (by densitometry) were made in SDS-PAGE buffer and subjected to SDS-PAGE using an 8-16% polyacrylamide gradient gel (Syngenta SOP 2.4).

The gel was electroblotted and the membrane probed with immunoaffinity-purified goat anti-VIP3A polyclonal antibody. The secondary antibody was donkey anti-goat IgG linked to alkaline phosphatase (Jackson; West Grove, PA) diluted 1:3,000 in blocking buffer. Proteins were visualized by development with alkaline phosphatase substrate solution, and examined for the presence of intact VIP3A (~89,000 molecular weight) and other immunoreactive VIP3A polypeptides.

6. **Insecticidal activity:** The VIP3A-0104 insecticidal activity was assessed in insect feeding assays using first-instar fall army worm (FAW; *Spodoptera frugiperda*) larvae. FAW eggs (Crop Chacteristics, Inc.; Fannington, MN) were transferred into 16 oz. deli cups with lids, placed on Gelman Absorbent Pads (Fisher Scientific) slightly moistened with deionized water, and incubated at 24°C until hatching (~ 3 - 4 days later). Freshly hatched FAW larvae were assayed for bioactivity in Costar 24-well plates (Fisher Scientific) per Syngenta SOP 2.70. Each well contained 750 µL insect diet (General Purpose Insect Diet from Bio-Serv, Inc.; Frenchtown, NJ) overlaid with 50 µL VIP3A-0104 test solution in 50 mM Tris-HCl (pH 9.5) buffer containing 2 mM EDTA, at concentrations of 5-1000 ng VIP3A/cm^2 diet surface. Each assay consisted of 24 replicate wells of one FAW larva per well, and concurrent negative controls: untreated insect diets, or diets treated with buffer. The wells were covered with silicone stoppers, kept at room temperature under ambient conditions, and mortality was assessed after 168 hours.

III. RESULTS:

1. **VIP3A-0104 quantitation and purity:** The purified VIP3A-0104 sample consisted of 97.5% total protein (BCA™ method), of which VIP3A represented *ca.* 64.7% (densitometric analysis: see Figure 1). Thus, the overall purity of VIP3A in the test material was 63.1%.

2. **Immunoreactivity and molecular weight:** Western blot analysis revealed a dominant immunoreactive band at the predicted molecular weight of VIP3A (~89,800 Da; Figure 2) for both VIP3A-0104 and the positive control VIP3A-0199 samples.

3. **Lipopolysaccharide measurement:** Test substance VIP3A-0104 contained ~0.02 endotoxin units/µg, which corresponds to approximately 2 µg endotoxin /g VIP3A-0104.

4. **Insecticidal activity:** The results of the FAW bioassay indicate that VIP3A-0104 was bioactive, with an LC_{50} of 272 ng VIP3A/cm^2 diet surface (95% confidence interval of 184 - 384 ng VIP3A/cm^2) after 168 h (Tables 1 and 2). The negative control of diet alone or diet treated with buffer had the expected much lower mortality (4.2% and 8.3%, respectively).

IV. CONCLUSION:

The VIP3A protein in test substance VIP3A-0104 had the predicted molecular weight of *ca.* 89,000 Da, was immunologically cross-reactive with the anti-VIP3A antibody, and had insecticidal activity against sensitive test species FAW larvae.

V. CLASSIFICATION: ACCEPTABLE

VI. REFERENCES:

Novartis Seeds, Inc. (1999). Characterization of test substance VIP3A-0199. Test substance characterization report and certificate of analysis. Novartis Seeds Biotechnology Report No. NSB-004-99 (L. Privalle, author).

Reference SOP:

SOP 2.3 Western Blot analysis
SOP 2.4 SDS-Polyacrylamide Gel Electrophoresis
SOP 2.6 Quantification of Proteins by Densitometry
SOP 2.16 BCA™ Microtiter Protein Determination
SOP 2.70 Diet Surface Bioassay of Fall Armyworm (*Spodoptera frugiperda*)

Table 1. Characterization of VIP3A in Sample VIP3A-0104		
Densitometric Analysis [% VIP3A/total protein]	**Purity [% VIP3A/VIP3A-0104]**	**168 hour FAW LC_{50} [ng VIP3A/cm^2 diet surface] (95% Confidence Interval)**
64.7[a]	63.1	272 (184 – 384)

[a] The total protein concentration of test substance VIP3A-0104 was 0.975 g protein/g sample (97.5% purity)

Data from p. 12 of MRID 46864803.

Table 2. Bioactivity of VIP3A in Test Substance VIP3A-0104	
Sample VIP3A-0104[a] [ng VIP3A/cm^2]	**Fall Armyworm Mortality at 168 h [%]**
5	4.2
25	4.2
50	12.5
100	25.0
250	50.0
500	58.3
1000	95.8
Untreated control [b]	4.2
Buffer control [c]	8.3

[a] FAW diet treated with VIP3A from test substance VIP3A-0104

[b] Untreated Control Diet: FAW stock diet without any treatment

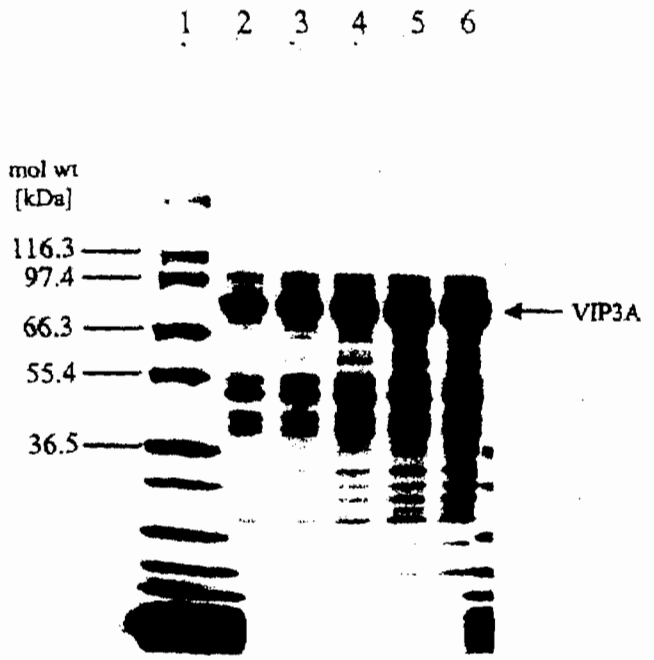
[c] Buffer Control Diet: FAW stock diet treated with 50mM Tris-HCL (pH 9.5), 2 mM EDTA (50 µL/well)

Data from p. 13 of MRID 46864803

Figure 1. Coomassie Blue Stained SDS-Polyacrylamide Gel of Test Substance VIP3A-0104 used for Purity Determination of VIP3A

Lane 1: Molecular weight standard Mark 12™ (Invitrogen; CA, USA)
Lanes 2 to 6: 3.2, 3.8, 5.0, 6.3 and 7.6 µg VIP3A, respectively, from VIP3A-0104

The molecular weight of VIP3A corresponds to *ca.* 89,000 Da.



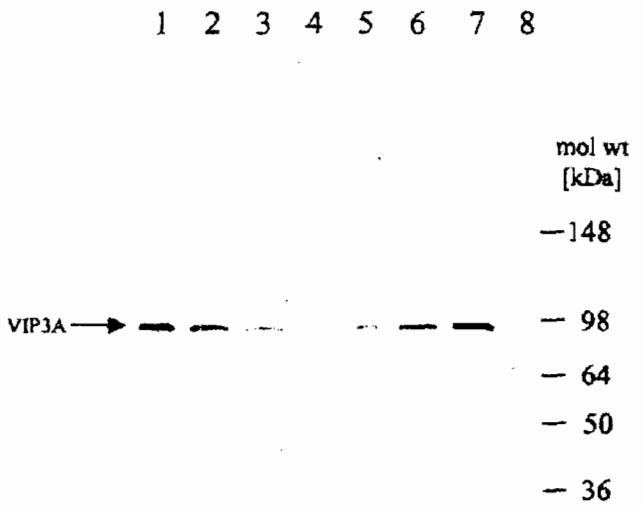
Data from p. 14 of MRID 46864803.

Figure 2. Immunoreactivity of VIP3A Protein in Test Substances VIP3A-0104 and VIP3A-0199

Western blot analysis:

Lanes 1, 2 and 3: 30, 15 and 5 ng VIP3A, respectively, from VIP3A-0199
Lane 4 and 8: Molecular weight standard SeeBlue®Plus2 (Invitrogen; CA, USA)
Lanes 5, 6 and 7: 5, 15 and 30 ng VIP3A, respectively, from VIP3A-0104

The molecular weight of VIP3A corresponds to *ca*. 89,000 Da.



Data from p. 15 of MRID 46864803.

DATA EVALUATION RECORD

Bacillus thuringiensis VIP3A-0104 Test Material (Vip3Aa19 Protein) Expressed in *E. coli*

STUDY TYPE: Product Characterization

MRID 468648-03

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T. Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S. Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader Signature: _____
Date: _____

Quality Assurance:
Angela M. Edmonds, B.S. Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

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DATA EVALUATION RECORD

Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Product characterization
MRID NO:	468648-04
TEST MATERIAL:	*Bacillus thuringiensis* (*Bt*) VIP3A-0204 test material (Vip3Aa19 protein) expressed in *E. coli*
STUDY NO:	SSB-029-04; COT200-04-03
SPONSOR:	Syngenta Seeds, Inc. – Field Crops NAFTA 3054 East Cornwallis Road P.O. Box 12257 Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science Laboratory 3054 East Cornwallis Road P.O. Box 12257 Research Triangle Park, NC 27709
TITLE OF REPORT:	Characterization of Vip3A protein test substance (VIP3A-0204) and certificate of analysis
AUTHOR:	Gerson Graser
STUDY COMPLETED:	November 29, 2004
STUDY SUMMARY:	VIP3A-0204 test material (Vip3Aa19 protein) produced from the synthetic *vip3A(a)* gene in an *E. coli* over-expression system was purified by Q Sepharose FF anion exchange chromatography. The VIP3A-0204 test material was determined to be soluble in aqueous solution at 50 mg/mL, to be 89.7% pure by SDS-PAGE in conjunction with Coomassie blue staining and densitometric analysis, and to contain ~ 62 µg endotoxin/g VIP3A-0204 by lipopolysaccharide analysis. Western blots using goat anti-VIP3A polyclonal primary antibody and donkey anti-goat alkaline phosphatase-linked secondary antibody revealed a dominant immunoreactive band at the predicted molecular weight of ~89,800 Da. VIP3A-0204 had insecticidal activity against first-instar fall army worm (FAW) larvae in insect feeding assays, with an LC_{50} of 45.1 ng VIP3A/cm^2 diet surface (95% confidence interval of 24.5 – 71.0 ng VIP3A/cm^2 diet surface) after 120 hours.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating the study was conducted in compliance with 40 CFR Part 160.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

The purpose of this study was to characterize test substance VIP3A-0204 containing the vegetative insecticidal protein VIP3A encoded by the synthetic *vip3A(a)* gene, via evaluation of the total protein concentration, solubility, bioactivity, immunoreactivity, and lipopolysaccharide (LPS) concentration.

II. MATERIALS AND METHODS:

1. **Production of VIP3A-0204:** VIP3A-0204 test material (Vip3Aa19 protein) was produced by cloning the synthetic *vip3A(a)* gene into the inducible, over-expression vector pET3a® (Novagen, Madison, WI) in *E. coli* strain BL21DE3pLysS. The synthetic VIP3A protein differs from the native VIP3A protein from *Bacillus thuringiensis* by a single conservative substitution at amino acid position 284, where lysine has been changed to glutamine. VIP3A-0204 protein was purified from pooled batches of *E. coli* cell paste by Syngenta Ltd. (Jealott's Hill, Brachell, Berkshire, UK), as described previously in the 2004 Syngenta Ltd. study report (Syngenta 2004; author S. Attenborough). *E.* coli cell pellets were ruptured, the cell debris removed by centrifugation, and VIP3A in the supernatant was further purified by anion exchange chromatography using a Q Sepharose FF column. VIP3A was eluted with a gradient of increasing sodium chloride concentration, and the VIP3A-containing fractions were dialyzed in 20 mM ammonium bicarbonate buffer (pH 10), frozen and lyophilized. The lyophilized VIP3A fractions were pooled, designated as sample VIP3A-0204, and sent to the Syngenta Regulatory Science & Product Support Laboratory where the lyophilized material was stored at -20°C.

2. **Solubility determination:** The solubility of the VIP3A-0204 sample in aqueous solution [50 mM Tris-HCl, 2 mM EDTA, pH 9.5 buffer or water purified by a Milli-Q® system (Millipore Corp.)] was determined per Syngenta Biotechnology, Inc. SOP 2.36.

3. **Protein Quantitation:** VIP3A-0204 total protein was quantified spectrophotometrically by determining the absorption at 280 nm (Syngenta SOP 2.75).

4. **Densitometry and sample purity:** The purity of VIP3A-0204 samples was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE® 4-12% Bis-Tris gel (Syngenta SOP 2.4). The gels contained 0.85 to 12.7 µg VIP3A per lane, and 8 µL SeeBlue® Plus molecular weight standards (Invitrogen, San Diego, CA). Protein bands were stained with Coomassie blue per Syngenta SOP 2.4, and their intensity estimated by densitometric analysis (Syngenta SOP 2.6, Gelbase/GelBlot, Version 2.1). The purity of VIP3A protein in the test material was calculated from the sample total weight, total protein content (A_{280} method), and the densitometry data.

5. **Lipopolysaccharide (LPS) Quantitation:** *E. coli* protein preparations are often contaminated with LPS, also known as endotoxin. LPS levels in the VIP3A-0204 samples were estimated using the PYROGENT® Plus Gel Clot LAL test (Cambrex, Walkersville, MD), per the manufacturer's instructions.

6. **Immunoreactivity and molecular weight determination:** VIP3A-0204 samples were subjected to western blot analysis (per Syngenta SOP 2.3) to determine the molecular weight and immunoreactivity of the VIP3A-0204 protein. Each gel contained molecular weight standards (8 µL SeeBlue® Plus) and test substance VIP3A-0104 (Vip3Aa19 protein) as a positive control (characterized in report Syngenta Seeds, 2004). Dilutions of 4.3, 12.9 and

25.8 ng VIP3A-0204 and 3.2, 9.5, and 18.9 ng VIP3A-0104 in NuPAGE sample buffer (determined by densitometry) were subjected to SDS-PAGE using NuPAGE 4-12% Bis-Tris gel (Syngenta SOP 2.4). The gel was electroblotted and the membrane probed with immunoaffinity-purified goat anti-VIP3A polyclonal antibody. The secondary antibody was donkey anti-goat IgG linked to alkaline phosphatase (Jackson; West Grove, PA) diluted 1:3,000 in blocking buffer. Proteins were visualized by development with alkaline phosphatase substrate solution, and examined for the presence of intact VIP3A (~89,000 molecular weight) and other immunoreactive VIP3A polypeptides.

7. **Insecticidal activity:** The VIP3A-0204 insecticidal activity was assessed in insect feeding assays using first-instar fall army worm (FAW; *Spodoptera frugiperda*) larvae. FAW eggs (Crop Chacteristics, Inc.; Fannington, MN) were transferred into 16 oz. deli cups with lids, placed on Gelman Absorbent Pads (Fisher Scientific) slightly moistened with deionized water, and incubated at 24°C until hatching (~ 3 - 4 days later). Freshly hatched FAW larvae were assayed for bioactivity in Costar 24-well plates (Fisher Scientific) per Syngenta SOP 2.70. Each well contained 800 µL insect diet (General Purpose Insect Diet from Bio-Serv, Inc.; Frenchtown, NJ) overlaid with 50 µL VIP3A-0204 test solution in 50 mM Tris-HCl (pH 9.5) buffer containing 2 mM EDTA, at concentrations of 4.4-879.0 ng VIP3A/cm^2 diet surface. Each assay consisted of 24 replicate wells of one FAW larva per well, and concurrent negative controls: untreated insect diets, or diets treated with buffer. The wells were covered with silicone stoppers, kept at room temperature under ambient conditions, and mortality was assessed after 120 hours. Statistical analyses for the LC_{50} values determined for the FAW bioassay were calculated using EPA Probit Analysis Program, Version 1.5.

II. RESULTS:

1. **Solubility determination:** VIP3A-0204 solubility in aqueous solutions (in 50 mM Tris-HCl, 2 mM EDTA, pH 9.5 buffer or purified water) was determined to be 50 mg/mL.

2. **VIP3A quantitation and purity determination:** The purified VIP3A-0204 sample consisted of 98.1% total protein, as measured by absorption at 280 nm. VIP3A represented *ca.* 91.4% of the total protein by densitometric analysis (Figure 1), thus, the overall purity of VIP3A in the test material was 89.7%.

3. **Immunoreactivity and molecular weight determination:** The western blot analysis revealed a dominant immunoreactive band at the predicted molecular weight of VIP3A (~89,800 Da: Figure 2) for both VP3A-0204 and the positive control VIP3A-0104 samples.

4. **Lipopolysaccharide measurement:** Test substance VIP3A-0204 contained ~0.62 endotoxin units/µg, which corresponds to approximately 62 µg endotoxin /g VIP3A-0204.

5. **Insecticidal activity:** The results of the FAW bioassay indicate that VIP3A-0204 had insecticidal activity against FAW larvae, with an LC_{50} of 45.1 ng VIP3A/cm^2 diet surface (95% confidence interval of 24.5 – 71.0 ng VIP3A/cm^2) after 120 hours (Tables 1 and 2). The negative control of diet alone or diet treated with buffer had the expected much lower mortality (4.2% each).

III. CONCLUSION:

The VIP3A protein in test substance VIP3A-0204 had the predicted molecular weight of *ca.* 89,000 Da, was immunologically cross-reactive with anti-VP3A antibody, and had insecticidal activity against the sensitive test species FAW larvae.

IV. CLASSIFICATION: ACCEPTABLE

V. REFERENCES:

Syngenta Seeds, Inc. (2004) Characterization of VIP3A protein test substance (VIP3A-0104) and certificate of analysis. Syngenta Seeds Biotechnology Report No. SSB-026-04 (G. Graser, author).

Reference SOP:

SOP 2.3 Western Blot analysis
SOP 2.4 SDS-Polyacrylamide Gel Electrophoresis
SOP 2.6 Quantification of Proteins by Densitometry
SOP 2.70 Diet Surface Bioassay of Fall Armyworm (*Spodoptera frugiperda*)
SOP 2.16 BCA™ Microtiter Protein Determination
SOP 2.75 Quantitation of Protein with the Ultraviolet Absorption Method

Table 1. Characterization of VIP3A in Sample VIP3A-0204		
Densitometric Analysis [% VIP3A/total protein]	**Purity [% VIP3A/VIP3A-0204]**	**120 hour FAW LC_{50} [ng VIP3A/cm^2 diet surface] (95% Confidence Interval)**
91.4 [a]	89.7	45.1 (24.5 – 71.0)

[a] The total protein concentration of test substance VIP3A-0204 was 0.981 g protein/g sample (98.1% purity)

Data from p. 12 of MRID 46864804.

Table 2. Bioactivity of VIP3A in Test Substance VIP3A-0204	
Sample VIP3A-0204[a] [ng VIP3A/cm^2]	**Fall Armyworm Mortality at 120 h [%]**
4.4	12.5
22.0	33.3
44.0	45.8
87.9	79.2
219.8	83.3
439.5	87.5
879.0	95.8
Untreated control [b]	4.2
Buffer control [c]	4.2

[a] FAW diet treated with VIP3A from test substance VIP3A-0204

[b] Untreated Control Diet: FAW stock diet without any treatment

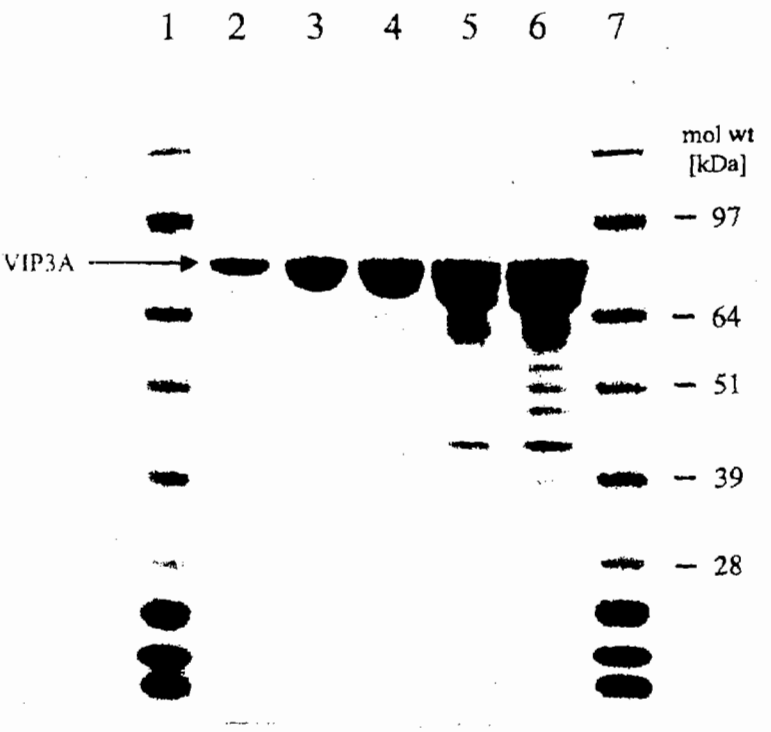
[c] Buffer Control Diet: FAW stock diet treated with 50mM Tris-HCL (pH 9.5), 2 mM EDTA (50 µL/well)

Data from p. 13 of MRID 46864804

Figure 1. Coomassie Blue Stained SDS-Polyacrylamide Gel of Test Substance VIP3A-0204 used for Purity Determination of VIP3A

Lanes 1 and 7: Molecular weight standard SeeBlue® Plus2 (Invitrogen; CA, USA)
Lanes 2 to 6: 0.9, 2.5, 4.2, 8.5 and 12.7 µg VIP3A, respectively, from VIP3A-0204

The molecular weight of VIP3A corresponds to *ca.* 89,000 Da.



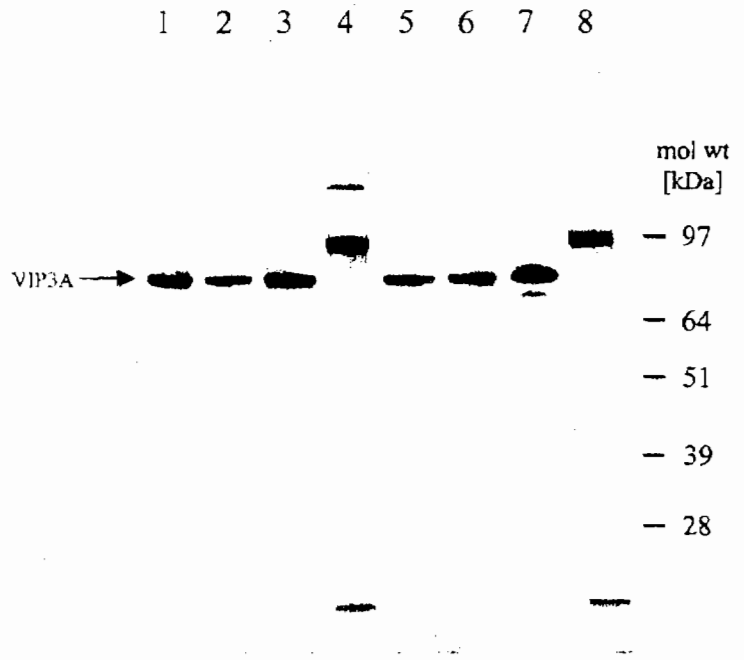
Data from p. 14 of MRID 46864804.

Figure 2. Immunoreactivity of VIP3A Protein in Test Substances VIP3A-0204 and VIP3A-0104

Western blot analysis:

Lanes 1, 2 and 3: 3.2, 9.5 and 18.9 ng VIP3A, respectively, from VIP3A-0104
Lane 4 and 8: Molecular weight standard SeeBlue® Plus2 (Invitrogen; CA, USA)
Lanes 5, 6 and 7: 4.3, 12.9 and 25.8 ng VIP3A, respectively, from VIP3A-0204

The molecular weight of VIP3A corresponds to *ca.* 89,000 Da.



Data from p. 15 of MRID 46864804.

DATA EVALUATION RECORD

Bacillus thuringiensis VIP3A-0204 Test Material (Vip3Aa19 protein) Expressed in *E. coli*

STUDY TYPE: Product Characterization

MRID 468648-04

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T.
Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S.
Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader
Signature: _____
Date: _____

Quality Assurance:
Angela M. Edmonds, B.S.
Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory managed and operated by UT-Battelle, LLC., for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Product characterization- Storage Stability
MRID NO:	468648-05
TEST MATERIAL:	*Bacillus thuringiensis* (*Bt*) VIP3A-0204 test material (Vip3Aa19 protein) expressed in *E. coli*
STUDY NO:	SSB-023-05; COT200-05-02
SPONSOR:	Syngenta Seeds, Inc. – Field Crops NAFTA P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science & Product Support P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Re-characterization of Vip3A protein test substance (VIP3A-0204) and certificate of analysis
AUTHOR:	Gerson Graser
STUDY COMPLETED:	July 11, 2005
STUDY SUMMARY:	VIP3A-0204 test material (Vip3Aa19 protein) produced from the synthetic *vip3A(a)* gene in an *E. coli* over-expression system was previously purified and characterized (MRID 46864804). It was shown to be ~89,800 Da and 89.7% pure (SDS-PAGE with Coomassie blue staining and densitometric analysis), immunoreactive with anti-VIP3A antibody (western blots), and to have insecticidal activity against first-instar fall army worm (FAW) larvae (LC_{50} of 45.1 ng VIP3A/cm^2 diet surface after 120 hours). In the present study, this VIP3A-0204 sample was similarly re-characterized after seven months storage lyophilized at -20°C, and shown to have retained its integrity and bioactivity. SDS-PAGE and western analysis determined a molecular weight of ~89,800 Da and a purity of 91.8%, and insecticidal activity assays with FAW larvae found an LC_{50} of 38.1 ng VIP3A/cm^2 diet surface after 120 hours. Therefore, it can be concluded that the test substance was stable when stored at -20°C, over *ca.* seven months.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating the study was conducted in compliance with 40 CFR Part 160.

THIS REPORT <u>DOES NOT</u> CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN: The purpose of this study was to re-characterize test substance VIP3A-0204, containing the vegetative insecticidal protein VIP3A encoded by the synthetic *vip3A(a)* gene by determining the purity, intactness and bioactivity compared to results with the previous analyses after storage (desiccated and at -20°C).

II. MATERIALS AND METHODS:

1. **Production of VIP3A-0204 protein:** VIP3A-0204 test material (Vip3Aa19 protein) was produced by cloning the synthetic *vip3A(a)* gene into the inducible, over-expression vector pET3a® (Novagen; Madison, WI) in *E. coli* strain BL21(DE3)pLysS. The synthetic VIP3A protein differs from the native VIP3A protein from *Bacillus thuringiensis* by a single conservative substitution at amino acid position 284, where lysine has been changed to glutamine. VIP3A-0204 protein was purified from pooled batches of *E. coli* cell paste by Syngenta Ltd. (Jealott's Hill, Brachell, Berkshire, UK), as described previously in the 2004 Syngenta Ltd. study report (Attenborough, 2004). *E. coli* cell pellets were ruptured, the cell debris removed by centrifugation, and VIP3A in the supernatant was further purified by anion exchange chromatography using a Q Sepharose FF column. VIP3A protein was eluted with a sodium chloride gradient, and the VIP3A-containing fractions were pooled and dialyzed in 20 mM ammonium bicarbonate buffer (pH 10), frozen, lyophilized, and designated as sample VIP3A-0204, and sent to the Syngenta Regulatory Science & Product Support Laboratory where the lyophilized material was stored at -20°C.

2. **Protein quantitation:** VIP3A-0204 total protein was quantified spectrophotometrically by determining the absorption at 280 nm of the aromatic amino acids tryptophan and tyrosine (Syngenta SOP 2.75). The instrument used was a Genesys 6 spectrophotometer (Thermo Electron Corporation, Madison WI), and Vector NTI software (Invitrogen; San Diego, CA) was used to calculate the VIP3A protein extinction coefficient at 280 nm. An approximate total protein concentration was obtained by multiplying the absorbance at 280 by the correlation factor for the extinction coefficient.

3. **Densitometry and sample purity:** The purity of VIP3A-0204 samples was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE® 4-12% Bis-Tris gel (Syngenta SOP 2.4). The gels lanes contained 0.9 to 2.3 µg VIP3A per lane, and 8 µL SeeBlue® Plus molecular weight standards (Invitrogen, San Diego, CA). After electrophoresis, the protein bands were stained with Coomassie blue, and their intensity estimated by densitometric analysis (Syngenta SOP 2.6, Gelbase/GelBlot, Version 2.1). The purity of VIP3A protein in the test material was calculated from the sample total weight, total protein content (A_{280} method), and the densitometry data.

4. **Immunoreactivity and molecular weight determination:** VIP3A-0204 samples were subjected to western blot analysis (Syngenta SOP 2.3) to determine the molecular weight and immunoreactivity of the VIP3A-0204 protein. Each gel contained molecular weight standards (8 µL SeeBlue® Plus). Dilutions of 5, 10 and 25 ng VIP3A-0204 in Laemmli sample buffer (determined by densitometry) were subjected to SDS-PAGE using NuPAGE 4-12% Bis-Tris gel (Syngenta SOP 2.4). The gel was electroblotted and the membrane probed with immunoaffinity-purified goat anti-VIP3A polyclonal antibody. The secondary antibody was donkey anti-goat IgG linked to alkaline phosphatase (Jackson; West Grove, PA) diluted 1:5,000 in TBST buffer (Tween in Tris Buffered Saline). Proteins were visualized by development with alkaline phosphatase substrate solution, and examined for the presence of intact VIP3A protein (~89,000 Da) and other immunoreactive polypeptides.

5. **Insecticidal activity:** The VIP3A-0204 insecticidal activity was assessed in insect feeding assays using freshly hatched first-instar fall army worm (FAW) larvae (*Spodoptera frugiperda*) per Syngenta SOP 2.70. Each well of a Costar 24-well plate (Fisher Scientific) was filled with 800 µL insect diet (General Purpose Insect Diet from Bio-Serv, Inc., Frenchtown, NJ) overlaid with 50 µL VIP3A-0204 test solution in 50 mM Tris-HCl (pH 9.5) buffer containing 4 mM EDTA. Concentrations tested were 5-1000 ng VIP3A/cm^2 diet surface. Each assay consisted of 24 replicate wells of one FAW larva per well, and included concurrent negative controls of untreated insect diets, and diets treated with buffer. The wells were covered with silicone stoppers, kept at room temperature under ambient conditions, and mortality was assessed after 120 hours.

III. RESULTS:

1. **VIP3A quantitation and purity determination:** The purified VIP3A-0204 sample consisted of 100% total protein, as measured by absorption at 280 nm. VIP3A represented *ca.* 91.8% of the total protein by densitometric analysis (Figure 1), thus, the overall purity of VIP3A in the test material was 91.8%.

2. **Immunoreactivity and molecular weight determination:** The western blot analysis revealed a dominant immunoreactive band at the predicted molecular weight of VIP3A (~89,800 Da; Figure 2) for VIP3A-0204. A number of faint, lower molecular weight bands were also seen that were likely VIP3A degradation products.

3. **Insecticidal activity:** The results of the FAW bioassay indicate that VIP3A-0204 had insecticidal activity against FAW larvae, with an LC_{50} of 38.1 ng VIP3A/cm^2 diet surface (95% confidence interval of 19.9 – 60.5 ng VIP3A/cm^2) after 120 hours (Tables 1 and 2). The negative control of diet alone or diet treated with buffer had the expected much lower mortality (4.2% and 0%, respectively).

4. **Sample stability:** The purity of VIP3A-0204 test material was initially determined to be *ca.* 89.7% VIP3A, based on total protein and Coomassie Blue-stained SDS-PAGE conducted on October 19 and on September 24, 2004, respectively, and reported previously (Graser, 2004). Re-analysis of the VIP3A-0204 test material after seven months of frozen storage determined its purity to be *ca.* 91.8 % (total protein and Coomassie Blue-stained SDS-PAGE conducted April 26 and April 28, 2005, respectively). The most recent analysis revealed no significant changes in the profile of the test substance on the Coomassie® blue stained gel (Fig. 1B) or on a western blot (conducted May 10, 2005) since its initial characterization on October 12, 2004 (Fig. 2). Re-evaluation of the test substance insecticidal activity after seven months also showed little change, the LC_{50} of 38.1 ng VIP3A/cm^2 diet surface being similar to the initially determined LC_{50} of 45.1 ng VIP3A/cm^2 diet surface after 120 hours.

IV. CONCLUSION:

It wad determined that test substance VIP3A-0204 was substantially stable from the time it was initially characterized to approximately seven months later, under the conditions of storage in this study (desiccated and at -20°C).

V. CLASSIFICATION: ACCEPTABLE

VI. REFERENCES:

Attenborough, S. (2004) Purification of Vip3A from an E. coli expression system. Syngenta protein Science Report No. PS-2004-001.

Graser, G. (2004) Characterization of VIP3A protein test substance (VIP3A-0204) and certificate of analysis. Syngenta Seeds Biotechnology Report No. SSB-029-04 (G. Graser, author). MRID 46864804.

Reference SOP:

SOP 2.3 Western Blot analysis
SOP 2.4 SDS-Polyacrylamide Gel Electrophoresis
SOP 2.6 Quantification of Proteins by Densitometry
SOP 2.70 Diet Surface Bioassay of Fall Armyworm (*Spodoptera frugiperda*)
SOP 2.75 Quantitation of Protein with the Ultraviolet Absorption Method (280nm)

TABLE 1. Re-analysis of VIP3A in test substance VIP3A-0204 and comparison to initial analysis[1]				
Analysis	**Total Protein** [g protein/g VIP3A-0204]	**Densitometric Analysis** [% VIP3A/total protein]	**Purity** [% VIP3A/ VIP3A-0204]	**120-hour FAW LC_{50}** [ng VIP3A/cm^2 diet surface] (95% Confidence Interval)
Sept - Oct. 2004	0.98	91.4	89.7	45.1 (24.5 - 71.0)
April - May 2005	1.0	91.8	91.8	38.1 (19.9 – 60.5)

[1] Graser, 2004

(data from p. 13 of MRID 46864805)

Table 2. Bioactivity of VIP3A in test substance VIP3A-0204 against first-instar fall armyworm larvae and comparison to initial analysis [1]		
Sample	**Fall armyworm mortality at 120 hours [%]**	
VIP3A-0204[1] [ng VIP3A/cm^2] First / second analysis	**First Analysis on October 7, 2004**	**Second Analysis on May 18, 2005**
4.4 / 5	12.5	20.8
22.0 / 25	33.3	37.5
44.0 / 50	45.8	41.7
87.9 / 100	79.2	91.7
219.8 / 250	83.3	87.5
439.5 / 500	87.5	95.8
879.0 / 1000	95.8	95.8
Untreated control [2]	4.2	4.2
Buffer control [3]	4.2	0

[1] FAW diet treated with test substance VIP3A-0204 dissolved in 50mM Tris-HCl (pH 9.5), 4 mM EDTA
[2] Untreated control diet: FAW stock diet without added VIP3A-0204 or buffer
[3] Buffer control diet: FAW stock diet treated with 50mM Tris-HCl (pH 9.5), 4 mM EDTA (50 µL/dish)

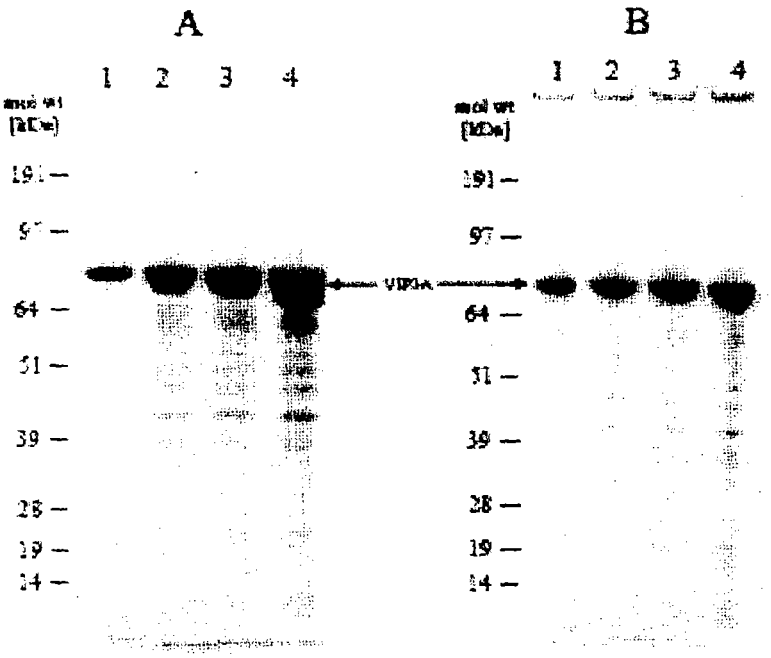
(data from p. 14 of MRID 46864805)

Figure 1. Coomassie® blue stained SDS-polyacrylamide gel of test substance VIP3A-0204 used for purity determination and comparison to initial analysis[1]

A. Initial analysis done on September 24, 2004
Lane 1: 0.9 µg VIP3A from VIP3A-0204
Lane 2: 2.5 µg VIP3A from VIP3A-0204
Lane 3: 4.2 µg VIP3A from VIP3A-0204
Lane 4: 8.5 µg VIP3A from VIP3A-0204

B. Re-analysis done on April 28, 2005
Lane 1: 0.9 µg VIP3A from VIP3A-0204
Lane 2: 1.4 µg VIP3A from VIP3A-0204
Lane 3: 1.8 µg VIP3A from VIP3A-0204
Lane 4: 2.3 µg VIP3A from VIP3A-0204

Molecular weight standard SeeBlue® Plus2; sizes indicated on the left side of the gels. The molecular weight of VIP3A corresponds to ca. 89,000 Da.



[1] Graser, 2004

(data from p. 15 of MRID 46864805)

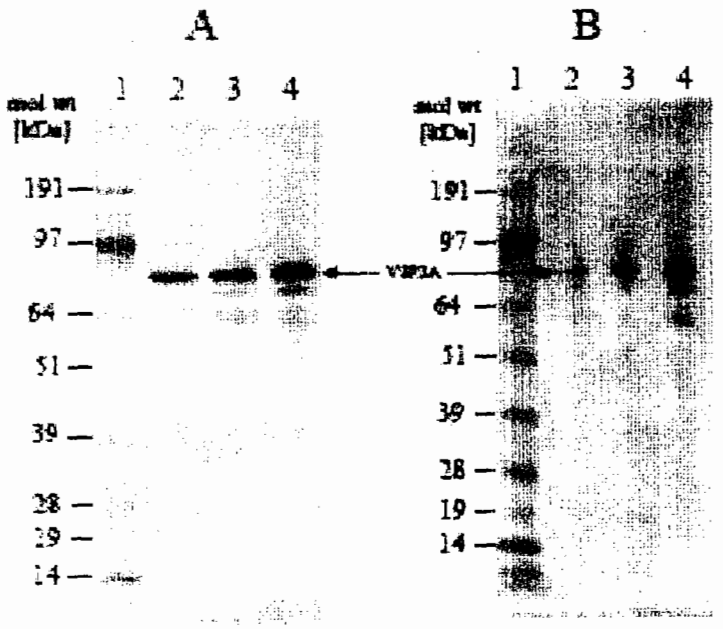
Figure 2. Immunoreactivity of VIP3A Protein in Test Substance VIP3A-0204 and Comparison to Initial Analysis[1]

Western blot analysis:

A. Initial analysis done on October 12, 2004
Lane 1: Molecular weight standard SeeBlue® Plus2
Lane 2: 4 ng VIP3A from VIP3A-0204
Lane 3: 13 ng VIP3A from VIP3A-0204
Lane 4: 26 ng VIP3A from VIP3A-0204

B. Re-analysis done on May 10, 2005
Lane 1: Molecular weight standard SeeBlue® Plus2
Lane 2: 5 ng VIP3A from VIP3A-0204
Lane 3: 10 ng VIP3A from VIP3A-0204
Lane 4: 25 ng VIP3A from VIP3A-0204

The molecular weight of VIP3A corresponds to ca. 89,000 Da.



[1] Graser, 2004

(data from p. 16 of MRID 46864805)

DATA EVALUATION RECORD

VIP3A-0204 Test Material (Vip3Aa19 Protein) Expressed in *E. coli*

STUDY TYPE: Product Characterization (Storage Stability)

MRID 468648-05

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T. Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S. Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader Signature: _____
Date: _____

Quality Assurance:
Angela M. Edmonds, B.S. Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

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	DATA EVALUATION RECORD
Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Product Characterization
MRID NO:	468648-06
TEST MATERIAL:	*Bacillus thuringiensis* (*Bt*) VIP3A protein expressed in *E. coli* (VIP3A-0199 sample; Vip3Aa1 protein), and *Bt* VIP3A protein expressed in corn (LPPACHA-0199 sample; Vip3Aa19 protein)
STUDY NO:	SSB-004-00; PACHA-99-03
SPONSOR:	Syngenta Seeds, Inc. Product Registration Group P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Seeds, Inc. – Field Crops – NAFTA P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Characterization of VIP3A protein produced in Pacha-derived maize (corn) and comparison with VIP3A protein expressed in recombinant *E. coli*.
AUTHORS:	Laura Privalle
STUDY COMPLETED:	August 15, 2002
STUDY SUMMARY:	Comparison of several functional and biochemical parameters indicated that VIP3A protein produced by Pacha-derived maize (LPPACHA-0199 sample; Vip3Aa19 protein) and by *E. coli* (VIP3A-0199 sample; Vip3Aa1 protein) was substantially equivalent. SDS-PAGE and western blot analysis showed that both proteins had a MW of ~89,000 and were immunoreactive against the same anti-VIP3A antibody. Edman degradation was used to determine that the N-terminus of *E. coli* VIP3A was MNKN, beginning with methionine-1, and of maize VIP3A was KNNXKL, beginning with lysine-3 (X indicates that a definitive amino acid could not be assigned). The lack of two predicted amino acids at the N-terminus of maize VIP3A was likely due to proteolytic degradation *in planta* or *in vitro*. The two VIP3A proteins had a similar insecticidal activity profile against first-instar larvae, the *E. coli*-derived protein being slightly more active. Both were the most active against black cutworm, with estimated 96-hour LC_{50} values of 70.4 and 88.5 ng VIP3A/cm^2 diet surface, respectively. Both were slightly less active against corn earworm and fall armyworm, and as expected, were

	inactive against the European corn borer and diamondback moth. Mass spectral (MS) analysis of VIP3A proteolytic digests confirmed the predicted complete sequence of ~95% of the *E. coli* and ~93% of the maize VIP3A protein. Neither MS nor an independent glycosylation analysis (DIG Glycan) showed any evidence of post-translational glycosylation of either the microbially-derived or maize-derived VIP3A.
CLASSIFICATION:	**SUPPLEMENTAL, but UPGRADEABLE**- pending submission of better reproductions of the SDS-PAGE and Western Blots of the *E. coli*- and maize-derived VIP3A test proteins; as well as, clarifications to data discrepancies noted in the insect bioassay.
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating the study was conducted in compliance with 40 CFR Part 160 with several exceptions (1) the N-terminal sequencing scientist did not receive FIFRA GLP training until after the sequencing, (2) the instrument used for N-terminal analysis was not maintained in strict compliance with GLP, (3) the sample was not maintained in a device that recorded the temperature just prior to N-terminal sequencing, (4) receipts were not kept for the eggs from which larvae hatched, (5) the pre-final sample weights and diluent volumes were not recorded, (6) mass spectral analysis was not conducted under GLP, and (7) results of probit analysis were not always initialed and dated prior to placement in the lab notebook. These GLP exceptions were not considered to have affected the integrity or outcome of the study.

THIS REPORT <u>DOES NOT</u> CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

The purpose of this study was to demonstrate the equivalency of the VIP3A protein as expressed in recombinant bacteria and transgenic maize plants derived from the Pacha VIP3A Event. Functional and biochemical parameters were evaluated in order to justify the use of the microbially produced VIP3A test substance as a surrogate for maize-expressed VIP3A protein in safety evaluations.

II. MATERIALS:

1. **<u>VIP3A-0199 (*E. coli*-produced native *Bt* VIP3A protein)</u>:** The VIP3A protein (Vip3Aa1) was produced in *E. coli* BL21DE3pLysS containing the pET-3d® vector (Novagen, Madison WI), which overexpresses the native *Bacillus thuringiensis* (*Bt*) AB88 *vip3A(a)* gene. The expressed VIP3A protein was purified, lyophilized, and stored desiccated at -20°C. VIP3A-0199 contained 54% by weight VIP3A protein, which was bioactive against black cutworm (BCW) larvae (characterized in Novartis Seeds Biotechnology report No. NSB-004-99). Working 5 mg/mL protein solutions were prepared in 50 mM Tris-HCl, 2 mM EDTA (pH 9.5) buffer. Total protein was quantitated using the BCA™ procedure and ovalbumin as standard. Mass spectroscopy (MS) was conducted using test material obtained prior to its lyophilization.

2. **VIP3A-0100 (*E. coli*-produced synthetic VIP3A protein):** The VIP3A protein (Vip3Aa19) was produced in *E. coli* BL21DE3pLysS containing the pET-3a® vector (Novagen, Madison WI), which overexpresses a synthetic *vip3A(a)* gene that was optimized for expression in maize. The synthetic and native VIP3A proteins differ at amino acid position 284, where the native protein contains lysine, and the synthetic protein contains glutamine. The synthetic protein has been introduced into maize plants, including the Pacha transformation event. The expressed VIP3A protein was purified, lyophilized, and stored desiccated at -20°C. VIP3A-0100 contained 74% by weight VIP3A protein, which was bioactive against BCW larvae (characterized in Syngenta Seeds Biotechnology Report No. SSB-008-00). Working 10 mg/mL protein solutions were prepared in 50 mM Tris-HCl, 2 mM EDTA (pH 9.5) buffer. Total protein was quantitated as for VIP3A-0199.

3. **LPPACHA-0199 (maize-produced VIP3A and isogenic control):** Protein (Vip3Aa19) was extracted from leaves of event Pacha-derived maize, the VIP3A protein was concentrated by ammonium sulfate precipitation, excess salt was removed by dialysis, and the material was lyophilized to yield a fine powder. The powder contained 0.36% w/w VIP3A, which was bioactive against fall armyworm (FAW) larvae (characterized in Syngenta Seeds Biotechnology Report No. SSB-002-00). An isogenic control sample, LPPACHA-0199c, was prepared similarly from corn lacking the VIP3A protein. The two test materials were stored frozen at -20°C, and used to prepare 10 mg/mL working solutions in buffer (as for VIP3A-0199) for the insect bioassay comparisons.

4. **IAPPACHA-0199 (1st immunopurified maize-produced VIP3A):** VIP3A protein (Vip3Aa19) was extracted from maize leaves and enriched as was LPPACHA-0199, except the final lyophilization step was replaced by immunoaffinity chromatography. The resulting liquid sample was stored frozen at -20°C, and its VIP3A content was evaluated by SDS-PAGE, western blot, glycosylation analysis, and MS. This sample was unacceptable for N-terminal amino acid sequencing or MS analysis, and a second immunopurified material was prepared (IAPPACHA-0100).

5. **IAPPACHA-0100 (2nd immunopurified maize-produced VIP3A):** VIP3A protein (Vip3Aa19) was prepared similarly to immunopurified sample IAPPACHA-0199, except that the procedure was performed more quickly (in two days) to reduce sample degradation. The resulting material was acceptable for N-terminal amino acid sequencing and MS analysis.

III. METHODS:

1. **Quantitation of VIP3A protein:** VIP3A content was determined by enzyme linked immunosorbent assay (ELISA), using immunoaffinity-purified VIP3A-specific polyclonal goat and protein A-purified rabbit antibodies.

2. **Determination of molecular weight (MW):** The MW of the maize-produced VIP3A was estimated by applying IAPPACHA-0199 sample (0.1 – 1 µg/lane) to an 8% SDS-PAGE gel along with MW markers and VIP3A-0199 produced by E. coli (0.1 – 1 µg/lane). The gel was stained with Coomassie blue.

3. **Immunoreactivity analysis:** The integrity and immunoreactivity of VIP3A produced by *E. coli* (VIP3A-0199; 12.5 and 25 ng VIP3A/lane) and maize (IAPPACHA-0199; 5-25 ng/lane) were evaluated by western blot analysis. Samples run on 8% SDS-PAGE gels were electroblotted and probed with VIP3A-specific goat polyclonal antiserum, followed by rabbit

anti-goat IgG linked to horseradish peroxidase (diluted 1:3000 in blocking buffer). VIP3A was visualized after reaction with the chromogen diaminobenzidine.

4. **N-terminal amino acid sequencing:** The N-terminal sequence of VIP3A produced by *E. coli* (VIP3A-0199) and maize (IAPPACHA-0199; IAPPACHA-0100) was determined by automated Edman degradation with resolution by HPLC fractionation. Prior to sequencing, the samples were subjected to SDS-PAGE, electroblotted, stained with Amido black, and the VIP3A bands were excised and subjected to phenylhydantoin coupling. Two maize samples were sequenced because the first sample (IAPPACHA-0199) was inconclusive.

5. **Glycosylation analysis:** To determine if the VIP3A produced by *E. coli* (VIP3A-0199) and maize (IAPPACHA-0199) was post-translationally glycosylated, samples were analyzed with the DIG Glycan Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN) per the manufacturer's directions. The method details were not provided, but it was stated that glycan moities are oxidized with periodate, labeled with digoxigenin, and detected with anti-digoxigenin antibody coupled to alkaline phosphatase.

6. **Insecticidal activity assays**: The meridic diet surface bioassay (method detailed in Syngenta SOP 2.33) was used to concurrently measure the insecticidal activity of *E. coli*-produced and maize-produced VIP3A against first-instar fall armyworm (FAW), black cutworm (BCW), and corn earworm (CEW). The concentrations tested were 85.3-341.3 ng VIP3A/cm^2 diet surface for VIP3A-0199, and 91.5-366.1 ng VIP3A/cm^2 diet surface for IAPPACHA-0199. The control maize leaf protein LPPACHA-0199C was similarly tested beginning a day later, the amount of test material corresponding to the maximum tested total protein level of LPPACHA-0199. Triplicate plates of 10 first instars/plate/concentration were treated at each test concentration. Mortality was evaluated after four days of treatment and LC_{50} and LC_{90} values for both VIP3A proteins were determined by probit analysis. The insecticidal activity of the two VIP3A proteins was then similarly tested concurrently against first-instar larvae of the VIP3A-insensitive European corn borer (ECB) and diamondback moth (DBM), and the control protein LPPACHA-0199C. The VIP3A test concentrations approximated the LC_{90} for CEW (872 and 1817 ng VIP3A/ cm^2 diet surface) and the control test material amount corresponded to the total protein of LPPACHA-0199 applied at 1817 ng VIP3A/ cm^2 diet surface

7. **Mass spectroscopy:** VIP3A protein was provided as a liquid sample (VIP3A-0199) or as a silver-stained band excised from an 8% SDS-PAGE gel (VIP3A-0100; IAPPACHA-0199; IAPPACHA-0100). The maize VIP3A 60kD degradation product band was also analyzed. The liquid VIP3A-0199 sample (5 µL) was diluted 1:10 in buffer prior to peptide digestion with 0.5 µg trypsin (Roche Molecular Biochemicals) [buffer: 5% acetonitrile, 1 mM dithiothreitol (DTT), 0.1 N ammonium bicarbonate, pH 8.0] or digestion with 1 µg Lys-C (Promega Corp., Madison WI) (buffer: 0.1 N ammonium bicarbonate, pH 8.0). The overnight digestion at room temperature was terminated with 5 µL glacial acetic acid, and 1 µL of the sample was analyzed.

   The excised silver-stained gel bands were chopped into ~1 mm cubes, rinsed with ammonium bicarbonate, dehydrated with 100% acetonitrile, vacuum-dried, and reduced for one hour at 56°C in 100 mM ammonium bicarbonate, 100 mM DTT, pH 7.8. The solution was cooled and replaced with 55 mM iodoacetamide in 100 mM ammonium bicarbonate, pH 7.8. After 45 minutes, the dehydration process was repeated. The dried gel pieces were swelled in 100 mM ammonium bicarbonate and digested for 45 minutes on ice with 12.5 ng

/µL trypsin or V8 protease (Roche Molecular Biochemicals). The liquid digest was then removed the gel pieces thrice alternately rinsed with ammonium bicarbonate and dehydrated with 50% acetonitrile/5% formic acid or 100% acetonitrile (final two times). The rinses were pooled, evaporated to 1-2 µL, and resuspended in 10 µL of 0.1% acetic acid for analysis.

The sample peptides' amino acid sequence was determined by nano-HPLC-micro-electrospray ionization mass spectrometry using a Thermo Finnigan (San Jose, CA) LCQ™ Deca ion trap mass spectrometer. Data were collected at 1000-1500 MS/MS spectra/hour for 1-2 hours. Data quality was enhanced by exclusion and filtration software, after which it was searched against an amino acid sequence database that included VIP3A and all publicly available *E. coli* sequence data using the Sequest® software program (Thermo Finnigan).

The measured molecular mass of each peptide was compared to its predicted mass based on its amino acid sequence to determine the presence of post-translational modification.

IV. RESULTS:

1. **Determination of VIP3A MW:** VIP3A produced by maize (IAPPACHA-0199) and *E. coli* (VIP3A-0199) co-migrated on the gel at a MW of approximately 89,000, as shown in Figure 1. The study author also noted that the samples had some smaller bands, perhaps due to VIP3A degradation products.

   EPA Reviewer's Comment: *The smaller bands that the study author attributed to VIP3A degradation products were not visible. The identity of the prominent band in the E. coli-produced VIP3A sample, with the protein concentration of 1.0 µg (lane 7) was not addressed. Better reproductions of the gels should be presented.*

2. **Immunoreactivity analysis:** The majority of the immunoreactive material from the maize and *E. coli* VIP3A samples co-migrated at a MW of ~89,000, as shown in Figure 2. Both samples also had a less intense band at a slightly lower MW. The maize sample had several additional bands in the 60,000-80,000 MW range, the most prominent band being 60,000 Da (not visible in Figure 2). The study author attributed these bands due to VIP3A degradation during storage at 4 °C.

   EPA Reviewer's Comment: *It would have been helpful if the study had included an evaluation of sample storage stability at 4°C to confirm that the smaller bands present in the gels were due to in vitro protein degradation. Additionally, better reproductions of the gels should have been presented in MRID No. 468648-06, as results (i.e. smaller bands) referred to in the text were not visible.*

3. **N-terminal amino acid sequencing:** The N-terminal sequence of VIP3A produced by *E. coli* (VIP3A-0199) was MNKN, beginning with methionine-1, and of the maize VIP3A (1APPACHA-0100) was KNNXKL (X indicates that a definitive amino acid could not be assigned), beginning with lysine-3. The lack of two predicted amino acids at the N-terminus of the maize-derived VIP3A was likely due to proteolytic degradation in the plant or in the *in vitro* sample.

4. **Glycosylation analysis:** DIG Glycan analysis showed no evidence of glycosylation of either the microbial or maize VIP3A protein, as shown in Figure 3. The positive control

transferring, also loaded on the gel, indicated that the limit of detection for the system was 2.5 ng glycan (calculated to be present in 50 ng transferrin).

5. **Insecticidal activity:** VIP3A produced by *E. coli* (VIP3A-0199) and maize (IAPPACHA-0199) had relatively similar insecticidal activity against first-instar FAW, BCW, CEW, the 96-hour LC_{50} values being somewhat higher for the maize-derived VIP3A (as shown in Table 1). The microbial and maize-derived VIP3A proteins were both the most active against BCW, with estimated 96-hour LC_{50} values of 70.4 ng VIP3A/cm^2 diet surface (95% CI 36.3-94.1) and 88.5 ng VIP3A/cm^2 diet surface (95% CI 33.2-126.9), respectively. The maize control protein (LPPACHA-0199C) caused minimal mortality. Both the plant and maize VIP3A lacked significant insecticidal activity against ECB and DBM (as shown in Table 2), when tested at the highest test substance concentration previously applied.

   EPA Reviewer's Comment: *The maize-derived protein appeared to be slightly less active than the E. coli-derived VIP3A. This may be due to the amino acid substitution at position 284 (lysine to glutamine). There were also several anomalous aspects of this experiment, which include: why the % mortality was not evaluated for all of the test substances; 2) why the estimated lethal dose at which 90% of the corn earworm population is expected to die of the LPPACHA-0199 sample had an unquantifiable result (this suggests that perhaps that test substance was not active enough to kill 90% of the population); 3) the relatively insensitive insects had very similar % mortality (although tested with different test substances with varying concentrations) and 4) it is unknown whether the experimental results were corrected for control mortality (such as Abbott's correction).*

6. **Mass spectroscopy:** Peptides were sequenced covering ~95% (750/789 amino acids) of the *E. coli*-produced VIP3A protein (pooled data from samples VIP3A-0199 and VIP3A-0100) and ~93% (731/789 amino acids) of the maize-derived VIP3A protein (pooled data from samples IAPPACHA-0199 and IAPPACHA-0100). Results are shown in Figure 4. The obtained amino acid sequences were exactly as predicted by the *vip3A* DNA sequence. No evidence of post-translational glycosylation was found in the microbially-derived VIP3A [sequenced 8/8 potential N-glycosylation sites and almost all O-glycosylation sites (56/58 serine; 61/62 threonine)] or in the maize-derived VIP3A [sequenced 7/8 potential N-glycosylation sites and almost all O-glycosylation sites (55/58 serine; 56/62 threonine)]. The eighth potential glycosylation site in the maize VIP3A, starting with asparagine-4 and ending with lysine-7, was missing from the N-terminus as its N-terminal amino acid was serine-9. Since Edman degradation showed that the N-terminus in the maize VIP3A was lysine-3, it appeared that additional degradation had occurred during sample processing.

   Mass Spectral analysis of the 60,000 Da band present on the immunoblot of maize-derived VIP3A (Figure 3) confirmed that this was a VIP3A degradation product. The peptide lacked a major portion of the VIP3A N-terminus, as the N-terminal amino acid was aspartate-207.

V. CONCLUSION:

The VIP3A produced by Pacha-derived maize (Vip3Aa19) and *E. coli* (Vip3Aa1) was substantially equivalent, based on the evaluated functional and biochemical parameters. Insecticidal activity was similar for the two VIP3A proteins, both being the most active against black cutworm, slightly less active against corn earworm and fall armyworm, and inactive against the European corn borer and diamondback moth. SDS-PAGE and Western

blots showed that both proteins had a MW of ~89,000 and were immunoreactive against the same anti-VIP3A antibody. N-terminal protein sequencing showed that both proteins had the same N-terminal amino acids, except the plant-derived VIP3A was missing the first two N-terminal amino acids possibly due to *in planta* or *in vitro* degradation. Mass spectral (MS) analysis of VIP3A proteolytic digests confirmed the predicted complete sequence of ~95% of the *E. coli* VIP3A and ~93% of the maize VIP3A. Neither the MS analysis nor the DIG Glycan analysis showed any evidence for the existence of post-translational glycosylation of either protein.

In Figure 1, the smaller protein bands that the study author attributed to VIP3A degradation products were not visible in determining the MW via SDS-PAGE. The identity of the prominent band in the *E. coli*- produced VIP3A sample, with the protein concentration of 1.0 µg (lane 7) was also not addressed. There was a similar data discrepancy in Figure 2, where smaller bands were referred to in the text, but not present in the gel. Therefore, better reproductions of the gels should be provided by the registrant.

In the insect bioassay, the maize-derived protein appeared to be slightly less active than the *E. coli*-derived VIP3A. There were a few anomalous aspects of this experiment, which includes 1) why the % mortality was not evaluated for all of the test substances and 2) whether the experimental results were corrected for control mortality. These data discrepancies should be addressed by the registrant.

VI. CLASSIFICATION:

This data packet is classified as **SUPPLEMENTAL, but UPGRADEABLE**- pending submission of better reproductions of the SDS-PAGE and Western Blots of the *E. coli*- and maize-derived VIP3A test proteins; as well as, clarifications to data discrepancies noted in the insect bioassay.

VII. REFERENCES:

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immuoassays. *In practice and theory of enzyme immunoassays.* (Laboratory techniques in Biochemistry and Molecular Biology, V. 15) Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

Reference SOP:
SOP 2.1 BCA™ Protein Determination
SOP 2.3 Western Blot analysis
SOP 2.4 SDS-Polyacrylamide Gel Electrophoresis
SOP 2.23 Immunoaffinity Chromatography
SOP 2.33 Diet Surface Bioassay of Lepidopteran Insects
SOP 2.34 Amino Acid Sequence Analysis
SOP 2.38 Quantitative Analysis for VIP3A by ELISA
SOP 2.46 VIP3A Enrichment from VIP3 Transgenic maize leaves

Table 1. Bioactivity of VIP3A from recombinant *E. coli* (VIP3A-0199) and Pacha Maize (LPPACHA-0199) on sensitive first-instar Lepidoptera

Insect	VIP3A-0199[a]		LPPACHA-0199[b]		LPPACHA-0199C[c]
	96-h LC_{50}	96-h LC_{90}	96-h LC_{50}	96-h LC_{90}	% Mortality (96 h)
	ng VIP3A/cm^2 (95% confidence interval)				
Fall Armyworm	126.9 (93.4 – 155.0)	341.1 (264.4 – 555.8)	148.2 (100.1 – 188.1)	469.2 (336.8 – 1007.9)	3.3
Black Cutworm	70.4 (36.2 – 94.1)	180.3 (142.8 – 265.6)	88.5 (33.2 – 126.9)	318.4 (229.8 – 709.6)	10.0
Corn Earworm	110.2 (25.0 – 162.1)	872 (426.1 – 52417.1)	194.9 (19.8 – 466.0)	1817 (615.6 – NQ[d])	13.3

[a] Test substance concentration ranged from 85.3 to 341.3 ng VIP3A/cm^2

[b] Test substance concentration ranged from 91.5 to 366.1 ng VIP3A/cm^2

[c] The control test substance was applied at a total protein concentration corresponding to that of the highest test substance concentration applied, 1817 ng VIP3A/cm^2

[d] NQ = not quantifiable; the calculated value approached infinity

Table 2. Bioactivity of VIP3A from recombinant *E. coli* (VIP3A-0199) and Pacha Maize (LPPACHA-0199) on relatively insensitive first-instar Lepidoptera

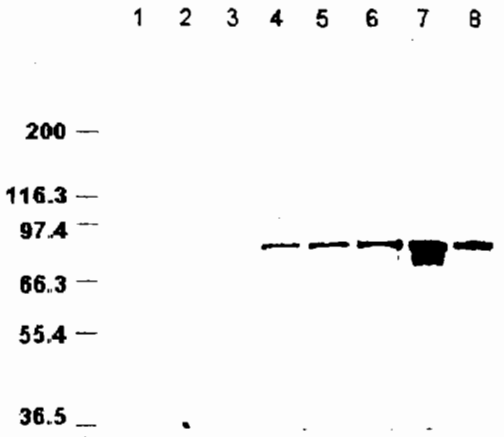
Insect	Test Concentration ng VIP3A/cm^2	% Mortality		
		VIP3A-0199	LPPACHA-0199	LPPACHA-0199C[c]
European Corn Borer	0	--	--	0
	872[a]	0.0	6.7	--
	1817[b]	3.3	6.7	--
Diamondback Moth	0	--	--	0
	872[a]	3.3	3.3	--
	1817[b]	3.3	6.7	--

[a] Corresponds to corn earworm estimated LC_{90} using VIP3A-0199

[b] Corresponds to corn earworm estimated LC_{90} using LPPACHA-0199

[c] The control test substance was applied at a total protein concentration corresponding to that of the highest test substance concentration applied, 1817 ng VIP3A/cm^2

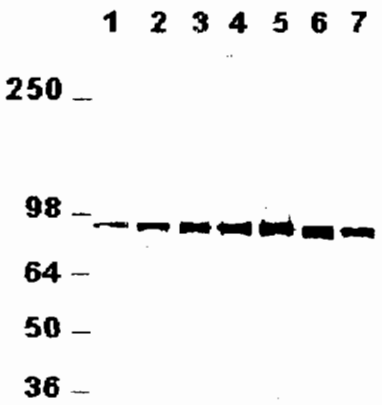
Figure 1. Coomassie blue stained poly-acrylamide gel comparing VIP3A protein from Pacha-derived maize and recombinant *E. coli*



Molecular weight of VIP3A protein from Pacha-derived maize (sample IAPPACHA-0199) and recombinant *E. coli* (sample VIP3A-0199) were evaluated by Coomassie blue staining of a SDS 8% polyacrylmide gel on which various protein concentrations had been subjected to electrophoresis. Lane 1, molecular weight (x 10^{-3}) markers; lanes 2 – 6: 0.1, 0.25, 0.5, 0.75 and 1.0 µg VIP3A, respectively, from Pacha-derived maize; lanes 7 and 8: 1.0 and 0.5 µg VIP3A, respectively, from recombinant *E. coli*. The molecular weight of VIP3A corresponds to *ca.* 89,000.

(from p. 20 of MRID 46864806).

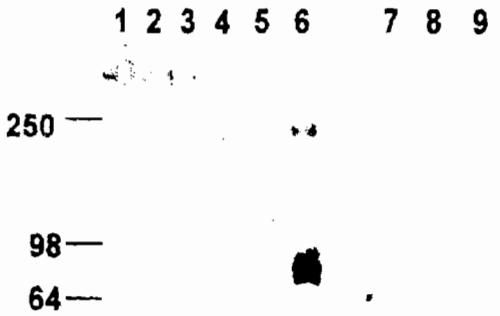
Figure 2. Western blot analysis of VIP3A protein from Pacha-derived maize and recombinant *E. coli*



Immunoreactivity of VIP3A protein from Pacha-derived maize (sample IAPPACHA-0199) and recombinant *E. coli* (sample VIP3A-0199) was compared by western blot analysis following electrophoresis on a SDS 8% polyacrylamide gel and electroblotting. Lanes 1 – 5: 5, 10, 15, 20 and 25 ng VIP3A, respectively, from Pacha-derived maize; lanes 6 and 7: 25 and 12.5 ng VIP3A, respectively, from recombinant *E. coli*. Molecular weight (x 10^{-3}) markers are indicated. The molecular weight of VIP3A corresponds to *ca.* 89,000.

(from p. 21 of MRID 46864806).

Figure 3. Glycosylation analysis of VIP3A protein expressed in Pacha-derived maize and recombinant *E. coli*



VIP3A from Pacha-derived maize (sample IAPPACHA-0199) and VIP3A from recombinant *E. coli* (sample VIP3A-0199) were analyzed for the presence of glycosyl residues using the DIG Method. Lanes 1 - 6, with oxidation; lanes 7 - 9, without oxidation. Lanes 1 and 7: 2000 ng VIP3A from recombinant *E. coli*; lane 2: 896 ng VIP3A from recombinant *E. coli*; lanes 3 and 8: 896 ng VIP3A from Pacha-derived maize; lanes 4 – 6: 25, 50, and 500 ng transferrin, respectively; lane 9: 500 ng transferrin. Molecular weight markers (x 10^{-3}) are indicated. The molecular weight of VIP3A (not visible) corresponds to *ca.* 89,000. The molecular weight of transferrin corresponds to *ca.* 80,000.

(from p. 22 of MRID 46864806).

Figure 4. Amino acid sequence of VIP3A protein from *E. coli* and maize as confirmed by mass spectral analysis

1
MNKNNTKLSTRALPSFIDYFNGIYGFATGIKDIMNMIFKTDTGGDLTLDEILKNQQLLND

61
ISGKLDGVNGSLNDLIAQGNLNTELSKEILKIANEQNQVLNDVNNKLDAINTMLRVYLPK

121
ITSMLSDVMKQNYALSLQIEYLSKQLQEISDKLDIINVNVLINSTLTEITPAYQRIKYVN

181
EKFEELTFATETSSKVKKDGSPADILDELTELTELAKSVTKNDVDGFEFYLNTFHDVMVG

241 ↓
NNLFGRSALKTASELITKENVKTSGSEVGNVYNFLIVLTALQAQAFLTLTTCRKLLGLAD

301
IDYTSIMNEHLNKEKEEFRVNILPTLSNTFSNPNYAKVKGSDEDAKMIVEAKPGHALIGF

361
EISNDSITVLKVYEAKLKQNYQVDKDSLSEVIYGDMDKLLCPDQSEQIYYTNNIVFPNEY

421
VITKIDFTKKMKTLRYEVTANFYDSSTGEIDLNKKKVESSEAEYRTLSANDDGVYMPLGV

481
ISETFLTPINGFGLQADENSRLITLTCKSYLRELLLATDLSNKETKLIVPPSGFISNIVE

541
NGSIEEDNLEPWKANNKNAYVDHTGGVNGTKALYVHKDGGISQFIGDKLKPKTEYVIQYT

601
VKGKPSIHLKDENTGYIHYEDTNNNLEDYQTINKRFTTGTDLKGVYLILKSQNGDEAWGD

661
NFIILEISPSEKLLSPELINTNNWTSTGSTNISGNTLTLYQGGRGILKQNLQLDSFSTYR

721
VYFSVSGDANVRIRNSREVLFEKRYMSGAKDVSEMFTTKFEKDNFYIELSQGNNLYGGPI

781
VHFYDVSIK

The underlined amino acids were confirmed by analysis of VIP3A produced in recombinant *E. coli* (750/789 amino acids confirmed = 95.1%). The amino acids shown in bold were confirmed by analysis of VIP3A produced in transformed maize (731/789 amino acids confirmed = 92.6%). Amino acid 284, indicated by the arrow, is predicted to be "K" (lysine) instead of "Q" (glutamine) in test substance VIP3A-0199, which was produced using the native *vip3A(a)* gene from *B. thuringiensis*. Maize-derived VIP3A (test substance IAPACHA-0199 and/or IAPACHA-0100) and *E. coli*-produced test substance VIP3A-0100 were confirmed to have the predicted glutamine at this position, as encoded by the synthetic *vip3A(a)* gene.

(from p. 27 of MRID 46864806)

DATA EVALUATION RECORD

VIP3A Protein Expressed in *E. coli* (Vip3Aa1) and Pacha-derived Corn (Vip3Aa19)

STUDY TYPE: Product Characterization

MRID 468648-06

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T. Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S. Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader Signature: _____
Date: _____

Quality Assurance:
Angela M. Edmonds, B.S. Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

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	DATA EVALUATION RECORD
Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Residue Analytical Method (OPPTS 860.1340)
MRID NO:	468648-07
TEST MATERIAL:	*Bacillus thuringiensis* (*Bt*) Vip3Aa20 protein expressed in Event MIR162 Maize
STUDY NO:	SSB-126-06
SPONSOR:	Syngenta Seeds, Inc. Product Registration Group P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Analytical method for the detection of Vip3Aa20 protein in maize tissues from event MIR162
AUTHOR:	Kim Hill
STUDY COMPLETED:	June 6, 2006
STUDY SUMMARY:	An ELISA procedure was used to determine Vip3Aa20 levels in tissues of Event MIP162 maize and the Vip3Aa20 protein tissue extraction efficiency. The ELISA method used 96-well plates, coated with goat anti-VIP3A antibody, purified rabbit anti-Vip3A polyclonal primary antibody, donkey anti-rabbit alkaline phosphatase conjugated secondary antibody, and phosphatase substrate. Each plate included the standard test substance (MIR162-VIP3A-0106 or VIP3A-0104) that was used to generate a standard curve, but this data was not shown. The LOQ and LOD for Vip3Aa20 ranged from, respectively, 0.04-0.25 and 0.003-0.032 µg Vip3Aa20/gram fresh weight, and 0.21-0.35 µg and 0.029-0.045 Vip3Aa20/gram dry weight. The average extraction efficiency of Vip3Aa20 was 82.7% in leaves, 81.0% in roots, 79.5% in pith, 88.3% in silk, 79.7% in kernels, >98% in pollen, and 78.9% in whole plants at maturity.
CLASSIFICATION:	**UNACCEPTABLE-** The validation of analytical methods for Vip3A via ELISA should be repeated. The method should also be validated by an independent third party laboratory according to OPPTS 860.1340(c)(6) and PR Notice 96-1 with GLP compliance. A full report determining the protein concentrations of Vip3Aa20 and PMI at different stages of plant development should be submitted and reported on a dry weight basis.
GLP COMPLIANCE:	A signed GLP statement was provided stating that this report does not contain study results and GLP standards were not applicable.

THIS REPORT <u>DOES NOT</u> CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

The purpose of this study was to determine the Vip3Aa20 extraction efficiency from maize plant tissues in MIP162 maize, using an ELISA assay (Tijssen, 1985) to measure Vip3Aa20 levels.

II. MATERIALS AND METHODS:

A. Extraction and extraction efficiency of Vip3Aa20 from MIP162 maize tissues: The plant material was powdered and lyophilized (procedure not stated) and ~0.1 g was suspended in 3 mL extraction buffer (500 mM Tris, 20 mM EDTA, 1 M NaCl, 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonylfluoride HCl], 2 mM DTT (dithiothreitol), 1 µM leupeptin, pH 9.5) and homogenized in a 15-mL polypropylene tube. An additional 3 mL buffer was added, the samples vortexed and incubated on ice 30 minutes, centrifuged 15 minutes at 10,000 x g at 4°C, and the supernatants subjected to ELISA. Pollen samples were prepared similarly except the homogenization step was replaced by passage of the pollen + buffer suspension three times through a French pressure cell.

Extraction efficiency was determined using three or more replicate samples per tissue type, except duplicate samples for silk. Following extraction and centrifugation, the first supernatant was collected for ELISA, and remaining insoluble material was extracted two more times and the extracts subjected to ELISA.

The percent extraction efficiency for Vip3Aa20 was calculated as the ng Vip3Aa20/mL 1st extraction ÷ [ng Vip3Aa20/mL 1st + 2nd + 3rd extraction] x 100. If the Vip3Aa20 content was below the LOQ or LOD, the respective LOQ and LOD values were used in the calculation.

B. ELISA (enzyme linked immunosorbent assay) procedure: ELISA was conducted using Nunc Maxisorp 96-well plates (Fisher Scientific, Agawam, MA) coated with goat anti-Vip3A polyclonal antibodies [2µg/mL in Borate Buffered Saline (BBS- 100 mM boric acid, 25 mM NaCl, 75 mM sodium borate, pH 8.5)]. The plates were incubated overnight at 2-8°C, washed 5 times with wash buffer (10 mM Tris-HCl, 0.05% Tween-20, 0.02% sodium azide, pH 8.0), and blocked ≥45 minutes with diluent (10 mM sodium phosphate, 140 mM NaCl, 1% bovine serum albumin, 0.02% sodium azide, and 0.05% Tween-20, pH 7.4). After five washes with wash buffer, triplicate samples (100µL) of each tissue or standard were placed per well, incubated 1.5 hr at 2-8°C and then 0.5 hr at room temperature, and the plates were again washed five times. Protein A-purified rabbit anti-Vip3A polyclonal antibody (100 µL of 0.25 µg/mL in diluent) was added to each well and incubated ~1 hr at 35-39°C, washed five times, and then incubated ~1 hr at 35-39°C with donkey anti-rabbit alkaline phosphatase conjugated antibody (100 µL of 1µg/mL in diluent; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The plates were washed five times, incubated with phosphatase substrate (Sigma Fast™ p-nitrophenyl phosphate) for 30 minutes at room temperature, and the reaction stopped with 3N NaOH. Sample absorbance was measured at 405-495 nm with a Tecan Sunrise® multi-well plate reader (Tecan Corp., Research Triangle Park, NC). Each plate included a series of dilutions of the standard test substance (MIR162-VIP3A-0106; VIP3A-0104 for extraction efficiency experiments) that was used to generate a standard curve with the DeltaSoft Curve fitting program (BioMetallics, Inc., Princeton, NJ).

Tissue sample data were considered acceptable if their mean delta OD lay within the linear range of the standard curve, and the coefficient of variation was <10%.

EPA Reviewer's Comment: *There were no standard curve data provided to demonstrate that the tissue sample data were considered valid (based on their mean delta OD laying within the linear range).*

The limit of quantitation (LOQ) (µg Vip3Aa20/gram dry weight) was calculated as the lowest concentration on the linear portion of the standard curve (ng Vip3Aa20/mL) X the dilution factor where the negative control was below the LOQ, X the mL buffer used in extraction ÷ the grams tissue extracted, ÷ 1000.

The limit of detection (LOD) (µg Vip3Aa20/gram dry weight) was calculated as the optical density plus two standard deviations of the lowest concentration used in the standard curve (ng Vip3Aa20/mL) X dilution factor where the negative control was below the LOD, X mL buffer used in extraction, ÷ the grams tissue extracted, ÷ 1000.

EPA Reviewer's Comment: *The procedure for calculating the dry weight conversion factor from the fresh weight tissue samples was not provided.*

III. RESULTS:

The LOQ and LOD for Vip3Aa20 in various tissues of Event MIR162 maize ranged from 0.04-0.25 and 0.003-0.032 µg Vip3Aa20/gram fresh weight, and 0.21-0.35 and 0.029-0.45 µg Vip3Aa20/gram dry weight, respectively. The LOQ and LOD for maize tissues at various developmental stages are shown in Table 1.

Using ELISA to determine Vip3Aa20 protein concentration, the average extraction efficiency of Vip3Aa20 was determined to be 82.7% in leaves, 81.0% in roots, 79.5% in pith, 88.3% in silk, 79.7% in kernels, >98% in pollen, and 78.9% in whole plants at maturity. The extraction efficiency of the 1st, 2nd, and 3rd extractions for each tissue is shown in Table 2.

IV. CONCLUSION:

The LOQ and LOD for Vip3Aa20 have been established in various tissues of Event MIR162 maize. Moreover, the extraction efficiency of Vip3Aa20 was >79% for each of the tested Event MIR162 maize tissues, using the developed ELISA method.

Regarding the ELISA method, the study report states that standards were run concurrently with the test samples on the 96-well plates and that standard curves were generated. However, none of this data was shown, but should have been to confirm the validity of the assay. Moreover, the calculation method for determining the dry weight conversion factor from the fresh weight tissue samples was not reported.

The reviewer also disagrees with the author's GLP statement that this report does not contain study results and therefore GLP standards were not applicable. An experiment was conducted and data were obtained and reported in MRID 46864807, and therefore the report should have stated whether the experiment was conducted per GLP. Moreover, there was no conclusion reported, no statement of retention of all raw data and records, and no verification (with approval) from the study author who conducted the analyses and report.

V. CLASSIFICATION:

In regards to validation of the residue analytical method, this data packet is classified as **UNACCEPTABLE.** A new study should be submitted (concurrently with the Sec. 3 registration of Event MIR162) and specifically conducted on the MIR162 transgenic grain (single seed and composite) in order for the ELISA to be verified as a suitable analytical method. This experiment should also be validated by an independent third party laboratory according to OPPTS 860.1340(c)(6) and PR Notice 96-1 with GLP compliance. The report should also include the following:

1) Qualitative data to represent positive vs. negative transgenic specific event results with percent accuracy;
2) Utilization of a negative control (non- transgenic convention corn line) and positive control (confirmed transgenic corn line);
3) Testing of cross-reactivity against other transgenic events and other proteins; and
4) The intra- and inter- assay coefficient of variation should be reported.

Once the recommended report has been submitted and found acceptable, EPA's Analytical Method Laboratory located in Fort Meade (Maryland) will have to independently validate Syngenta's ELISA protocol for accuracy, precision, and sensitivity.

In regards to establishing field protein expression levels in MIR162 corn tissues and plants, the study is supplemental. It does provide useful information for tissue expression levels to determine exposure for non-target organisms, for IRM dose levels, and dietary exposure estimates. However, it does not include quantification of Vip3Aa20 protein levels expressed in various plant tissues and the whole corn plant. A full report determining the protein concentrations of Vip3Aa20 and PMI at different stages of plant development should be submitted (including: the mean, range, and standard deviations) and reported on a dry weight basis (µg protein/g tissue) with GLP compliance. This data requirement can be addressed in the Sec. 3 Registration of Vip3Aa20. The study should also include the following:

1) Standard curve data for the ELISA;
2) The calculation method for determining the dry weight conversion factor from the fresh weight tissue samples; and
3) Identification of the specific seed line and lot utilized as the test material with number of field sites and replicates.

VI. REFERENCES:

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immuoassays. *In practice and theory of enzyme immunoassays.* (Laboratory techniques in Biochemistry and Molecular Biology, V. 15) Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

TABLE 1. Approximate Limits of Quantitation and Detection for Vip3Aa20 in Various Tissue Types of Event MIR162 Plants				
Tissue	**Developmental Stage**			
	V9-V12	**Anthesis**	**Seed Maturity**	**Senescence**
	Limits of Quantitation µg Vip3Aa20/gfw (µg Vip3Aa20/gdw)			
Leaves	0.05 (0.22)	0.06 (0.27)	0.13 (0.33)	0.15 (0.24)
Roots	0.04 (0.22)	0.04 (0.27)	0.06 (0.23)	0.05 (0.24)
Pith	N/A	0.03 (0.24)	0.04 (0.21)	N/A
Silk	N/A	0.03 (0.24)	N/A	N/A
Kernels	N/A	N/A	0.17 (0.24)	0.25 (0.30)
Pollen	N/A	0.32 (0.35)	N/A	N/A
Whole Plants	0.03 (0.22)	0.04 (0.24)	0.10 (0.24)	0.10 (0.21)
	Limits of Detection µg Vip3Aa20/gfw (µg Vip3Aa20/gdw)			
Leaves	0.007 (0.030)	0.007 (0.030)	0.012 (0.030)	0.025 (0.039)
Roots	0.005 (0.030)	0.005 (0.030)	0.007 (0.029)	0.009 (0.045)
Pith	N/A	0.003 (0.030)	0.006 (0.030)	N/A
Silk	N/A	0.004 (0.030)	N/A	N/A
Kernels	N/A	N/A	0.021 (0.030)	0.025 (0.030)
Pollen	N/A	0.032 (0.035)	N/A	N/A
Whole Plants	0.004 (0.030)	0.005 (0.030)	0.013 (0.030)	0.014 (0.030)

N/A = Not analyzed at this stage.

(Data from p. 10 of MRID 46864807)

TABLE 2. Extraction efficiency of Vip3Aa20 from event MIR162 plant tissues

Tissue (Stage Analyzed)	ng Vip3Aa20 /mL			% Extraction Efficiency	Average % Extraction Efficiency
	1st Extraction	2nd Extraction	3rd Extraction*		
Leaves (anthesis)	2721.20	433.05	131.56	82.8	82.7
	2761.40	452.34	107.04	83.2	
	2847.20	496.88	125.38	82.1	
Roots (anthesis)	764.10	138.58	31.82	81.8	81.0
	796.00	157.72	35.85	80.4	
	776.60	133.30	29.76	82.6	
	401.32	101.66	13.65	77.7	
	350.42	56.57	7.66	84.5	
	370.24	85.27	12.99	79.0	
Pith (anthesis)	594.95	130.41	19.11	79.9	79.5
	501.90	117.84	15.93	79.0	
	592.90	134.78	16.53	79.7	
Silk (anthesis)	3334.80	343.56	61.02	89.2	88.3
	2898.20	335.46	84.26	87.3	
Kernels seed maturity)	850.00	165.44	90.84	76.8	79.7
	1178.70	189.88	94.86	80.5	
	1251.80	273.14	89.78	77.5	
	696.00	127.90	23.02	82.8	
	566.23	120.34	22.11	79.9	
	599.15	119.56	21.35	81.0	
Pollen (anthesis)	1499.71	29.00	<1.00	>98.0	>98.0
	1749.76	25.86	<1.00	>98.5	
	1685.82	42.45	<1.00	>97.5	
Whole plants (seed maturity)	1344.68	245.77	61.15	81.4	78.9
	1325.48	304.10	66.88	78.1	
	1128.16	269.22	66.26	77.1	

*The Vip3Aa20 concentrations in the third extraction of pollen were <LOD. Therefore, the LOD value for pollen, expressed as ng Vip3Aa20/mL, was used to estimate the extraction efficiency.

(Data from p. 11 of MRID 46864807)

DATA EVALUATION RECORD

Vip3Aa20 Protein Expressed in Event MIR162 Maize

STUDY TYPE: Analytical Method to Detect Vip3Aa20 Protein in Maize Tissues

MRID 468648-07

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T.

Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S.

Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader

Signature: _____
Date: _____

Quality Assurance:
Eric B. Lewis, M.S

Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory managed and operated by UT-Battelle, LLC., for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer: Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer: Annabel Waggoner, Environmental Protection Specialist, BPPD

STUDY TYPE:	Amino acid homology to known toxins
MRID NO:	468648-08
TEST MATERIAL:	*Bacillus thuringiensis* (*Bt*) Vip3A protein expressed in Event MIR162 Maize
STUDY NO:	SSB-112-06
SPONSOR:	Syngenta Seeds, Inc. Product Registration Group P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known toxins.
AUTHORS:	Brian Harper
STUDY COMPLETED:	February 14, 2006
STUDY SUMMARY:	No relevant similarities between the Event MIR162 Vip3A query sequence (Vip3Aa20) and known protein toxins were found other than with other insect-specific vegetative insecticidal proteins of *B. thuringiensis*.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating that this was a non-GLP study conducted using accepted scientific methods, and the raw data and study records have been retained.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

The purpose of the study was to determine if Event MIR162 Vip3A protein had any significant amino acid sequence homology to known or putative protein toxins.

II. MATERIALS AND METHODS:

The 789 amino acid sequence of Event MIR162 Vip3A (Vip3Aa20- see Figure 1) was compared to known amino acid protein sequences contained in the latest posting of the National Center for Biotechnology Information (NCBI) Entrez Protein Database (NCBI, 2006) [accessed on 02/10/2006]. The Vip3A protein was originally identified in *B. thuringiensis* strain AB88. It is a naturally occurring insecticidal protein produced during vegetative growth. The MIR162 Vip3A query sequence was compared using version 2.2.6 of the BLASTP program[1] (Altschul et al., 1997). All database sequences with a conservative Expect (E) value of 10 or lower were identified. To assess which database "hits" likely represented proteins with insignificant, random amino acid similarity (background) rather than statistically significant sequence homology, control BLASTP searches were done using five different "shuffled" versions of the MIR162 Vip3A query sequence in place of the original MIR162 Vip3A sequence. The five shuffled versions of the sequence were created using the Shuffle Protein Program (IFOM-IEO, 2000) of the Sequence Manipulation Suite 2 program (Stothard, 2000). This program retained the overall amino acid composition but rearranged its sequence. The shuffled sequences were used to determine the background control range of E values of 6×10^{-6} to 8.9. Protein identity[2] and function, if known, were determined for all individual entries with an E value below 6×10^{-6}.

III. RESULTS:

The database identified 32 entries[3] with E values below 6×10^{-6}, of which 30 were vegetative insecticidal proteins of *B. thuringiensis* and had E values of 0.0 to 1×10^{-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, see Table 2) 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[4].

IV. CONCLUSION:

No relevant similarities between the Event MIR162 Vip3A query sequence and known protein toxins were found other than with other insect-specific vegetative insecticidal proteins of *B. thuringiensis*.

V. CLASSIFICATION: ACCEPTABLE

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[1] BLASTP Search Parameters: No complexity filter; *expect score = 10; word size= 3; gap costs: existence* = 11 and extension = 1. The similarity matrix was Blosum 62.

[2] Identities were calculated using a local alignment comparing only the regions of the two proteins that are most similar and not the entire protein sequence as in a global alignment.

[3] Refer to MRID No. 468648-08, Table 1 for a complete detailed list of all 32 hits that gave an E value less than 6×10^{-6}.

[4] Refer to MRID No. 468648-08, Figures 2 and 3 for the global alignments of MIR162 and the rhoptry proteins from *P. yoelii*

VI. REFERENCES:

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller W., Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.

Doolittle, R.F. (1990). Searching through Sequence Databases. *Methods in Enzymology*, 183: 99-110.

IFOM-IEO (2000). FIRC Institute of Molecular Oncology and the European Institute of Oncology. (http://bio.ifom-firc.it/TOOLS/sms/shuffle_protein.html). Accessed May 10, 2006.

Myers, E.W. and W. Miller (1988). Optimal alignments in linear space. Comput. Appl. Biosci. 4: 11-17.

NCBI (2006). National Center for Biotechnology Information GenBank Database (http://www.ncbi.nlm.nih.gov/BLAST/); non-redundant GenBank coding sequence translations (PDB+SwissProt+PIR+PRF) containing 3,284,813 entries on February 26, 2006.

Ogun, S.A. and A.A. Holder (1996). A high molecular mass *Plasmodium yoelii* rhoptry protein binds to erythrocytes. *Mol. Biochem. Parasitol.* 76: 321-324.

Stothard, P. (2000). The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques*, 28: 1102-1104.

Table 2. Percent sequence identity between Vip3A and *Plasmodium yoelii* rhoptry proteins[1]		
Protein 1	**Protein 2**	**% Sequence Identity between Proteins 1 and 2**
VIP3A	Rhoptry protein accession number AAB41263 (E value 2 X 10^{-6})	3.9 %
VIP3A	Rhoptry protein accession number XP_724976 (E value 2 X 10^{-6})	11.4%

[1] Note: identities are calculated using a global alignment comparing both proteins' amino acid sequence in entirety.

Figure 1. Amino acid sequence of Vip3A as expressed in Event MIR162.

This protein differs from the intended *Bacillus thuringiensis*-derived Vip3A protein, Accession Number AAQ23140.2, by one amino acid. Methionine at position 129 has been substituted by isoleucine. The amino acid substitution is highlighted.

```
   1 MNKNNTKLST RALPSFIDYF NGIYGFAIGI KDIMNMIFKT DTGGDLTLDE ILKNQQLLND
  61 ISGKLDGVNG SLNDLIAQGN LNTELSKEIL KIANEQNQVL NDVNNKLDAI NTMLRVYLPK
 121 ITSMLSDVMK QNYALSLQIE YLSKQLQEIS DKLDIINVNV LINSTLTEIT PAYQRIKYVN
 181 EKFEELTFAT ETSSKVKKDG SPADILDELT ELTELAKSVT KNDVDGFEFY LNTFHDVMVG
 241 NNLFGRSALK TASELITKEN VKTSGSEVGN VYNFLIVLTA LQAQAFLTLT TCRKLLGLAD
 301 IDYTSIMNEH LNKEKEEFRV NILPTLSNTF SNPNYAKVKG SDEDAKMIVE AKPGHALIGF
 361 EISNDSITVL KVYEAKLKQN YQVDKDSLSE VIYGDMDKLL CPDQSEQIYY TNNIVFPNEY
 421 VITKIDFTKK MKTLRYEVTA NFYDSSTGEI DLNKKKVESS EAEYRTLSAN DDGVYMPLGV
 481 ISETFLTPIN GFGLQADENS RLITLTCKSY LRELLLAIDL SNKETKLIVP PSGFISNIVE
 541 NGSIEEDNLE PWKANNKNAY VDHTGGVNGT KALYVHKDGG ISQFIGDKLK PKTEYVIQYT
 601 VKGKPSIHLK DENTGYIHYE DTNNNLEDYQ TINKRFTTGT DLKGVYLILK SQNGDEAWGD
 661 NFIILEISPS EKLLSPELIN TNNWTSTGST NISGNTLTLY QGGRGILKQN LQLDSFSTYR
 721 VYFSVSGDAN VRIRNSREVL FEKRYMSGAK DVSEMFTTKF EKDNFYIELS QGNNLYGGPI
 781 VHFYDVSIK
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DATA EVALUATION RECORD

Bacillus thuringiensis Vip3A Protein (Vip3Aa20) Expressed in Event MIR162 Maize

STUDY TYPE: Amino Acid Homology to Known Toxins

MRID 468648-08

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T. Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S. Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader Signature: _____
Date: _____

Quality Assurance:
Eric B. Lewis, M.S. Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory managed and operated by UT-Battelle, LLC., for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Amino acid homology to known allergens
MRID NO:	468648-09
TEST MATERIAL:	*Bacillus thuringiensis* (*Bt*) Vip3Aa20 protein expressed in Event MIR162 Maize
STUDY NO:	SSB-115-06
SPONSOR:	Syngenta Seeds, Inc. Product Registration Group P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Vip3A as expressed in Event MIR162 maize: Assessment of amino acid sequence homology with known allergens.
AUTHORS:	Brian Harper
STUDY COMPLETED:	March 2, 2006
STUDY SUMMARY:	No similarities were found between the MIR162 Vip3Aa20 query sequence and any known or putative protein allergens.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided. This was a non-GLP study conducted using accepted scientific methods, and the data and study records have been retained.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

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.

II. MATERIALS AND METHODS:

The 789 amino acid sequence of Event MIR162 Vip3A protein was systematically compared to the latest version of the Syngenta Biotechnology, Inc. Allergen Database (SBI, version 4.0, last updated March 2005). The SBI Allergen Database[1] contains 1414 non-redundant amino acid sequences of known and putative protein allergens, including gliadins, and was initially compiled from entries from the following sources:

1. All entries identified as allergens or putative allergens in the publicly available GenPept, PIR, or SWISS-PROT protein databases. Allergen sequence entries from these public databases were identified using the program Lookup from the GCG Wisconsin Package version 10.1 as part of the SeqWeb Bioinformatics package (Accelrys, Inc., 2001; further citation was not reported);

2. Entries in the SWISS-PROT Allergen database (SWISS-PROT, 2001);

3. Entries in the List of Allergens database (International Union of Immunological Societies, 2001);

4. Entries in the FARRP Protein Allergen database (Food Allergy Research and Resource Program, 2001); and

5. Additional entries identified in the scientific literature as putative allergens, but which are not found in public databases.

Two different searches were done to compare the amino acid sequence of MIR162 Vip3A. The first search compared the sequence homology of the protein divided into 80 amino-acid blocks to allergen sequences using the FASTA search[2] algorithm (Pearson and Lipman, 1988). Each successive block was offset from the previous by one residue and the search repeated. Any 80 amino acid MIR162 Vip3A peptide with >35% homology to an allergen was by definition significantly similar (FAO/WHO, 2001) and was further examined.

In the second search, the MIR162 Vip3A amino acid sequence was screened for matches of eight or more contiguous amino acids (Hileman *et al.*, 2002) using a Syngenta program that compares every possible peptide of eight contiguous amino acids between the query sequence and the allergen sequences in the SBI Allergen Database. This was done to search for short, local regions of amino acids which could indicate the presence of common T-cell epitopes.

III. RESULTS:

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

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[1] Refer to MRID No. 468848-09, Table 1 for a detailed list of all sequences contained in the SBI Allergen Database, version 4.0.

[2] FASTA search parameters: the scoring matrix was blosum 62, the gap extension penalty was two and the gap creation penalty was 12.

contiguous identical amino acids were identified between MIR162 Vip3A and proteins in the SBI Allergen Database.

IV. CONCLUSION:

No significant amino acid sequence homology was found between the MIR162 Vip3A and any known or putative protein allergens.

V. CLASSIFICATION: ACCEPTABLE

VI. REFERENCES:

FAO/WHO (2001). Evaluation of allergenicity of genetically modified foods. Report of a joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology. 22-25 January, 2001. Rome, Italy.

Food Allergy Research and Resource Program (2001). FARRP Protein Allergen Database, version 4.0. http://www.allergenonline.com, last updated January, 2006.

Hileman, R. E., Silvanovich, A., Goodman, R. E., Rice, E. A., Holleschak, G., Astwood, J. D., and Hefle, S. L. (2002). Bioinformatic methods of allergenicity assessment using a comprehensive allergen database. *Int. Arch. Allergy Immunol.* 128: 280-291.

International Union of Immunological Societies, Allergen Nomenclature Sub-Committee (2001). http://www.allergen.org/List.htm. Accessed March 2001.

Pearson, W.R. and Lipman, D.J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci.* 85(8): 2444-2448.

SWISS-PROT Protein Knowledgebase (2001). Nomenclature and index of allergen sequences. http://us.expasy.org/cgi-bin/lists?allergen.txt. Swiss Institute of Bioinformatics, Geneva, Switzerland and European Bioinformatics Institute, Hinxton, UK.

DATA EVALUATION RECORD

Bacillus thuringiensis Vip3Aa20 Protein Expressed in Event MIR162 Maize

STUDY TYPE: Amino Acid Homology to Known Allergens

MRID 468648-09

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T. Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S. Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader Signature: _____
Date: _____

Quality Assurance:
Eric B. Lewis, M.S. Signature: _____
Date: _____

Disclaimer

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Oak Ridge National Laboratory managed and operated by UT-Battelle, LLC., for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Amino acid homology to known toxins
MRID NO:	468648-10
TEST MATERIAL:	Phosphomannose isomerase (PMI) protein derived from *Escherichia coli*
STUDY NO:	SSB-114-06
SPONSOR:	Syngenta Seeds, Inc. Product Registration Group P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Phosphomannose isomerase protein: Assessment of amino acid sequence homology with known toxins.
AUTHORS:	Brian Harper
STUDY COMPLETED:	February 22, 2006
STUDY SUMMARY:	No similarities were found between phosphomannose isomerase (PMI) protein (from *Escherichia coli)* query sequence and known protein toxins.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating that this was a non-GLP study conducted using accepted scientific methods, and the raw data and study records have been retained.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

The purpose of this study was to determine if phosphomannose isomerase (PMI) derived from *Escherichia coli* had significant amino acid sequence homology to known protein toxins.

II. MATERIALS AND METHODS:

The 391 amino acid sequence of *E. coli* phosphomannose isomerase (PMI) was compared to known amino acid protein sequences contained in the latest posting of the National Center for Biotechnology Information (NCBI) Entrez Protein Database (NCBI, 2006) [accessed on 02/10/2006]. The PMI query sequence was compared using version 2.2.6 of the BLASTP search program[1] (Altschul et al., 1997). All database sequences with a conservative Expect (E) value of 10 or lower were identified. To assess which database "hits" likely represented proteins with insignificant, random amino acid similarity (background) rather than statistically significant sequence homology, control BLASTP searches were done using five different "shuffled" versions of the *E. coli* PMI query sequence in place of the original *E. coli* PMI sequence. The shuffled versions of the sequence were created using the Shuffle Protein Program (IFOM-IEO, 2000) of the Sequence Manipulation Suite 2 program (Stothard, 2000). This program retained the overall amino acid composition but rearranged its sequence. The shuffeled sequences were used to determine the background control range of E values of 0.087 to 8.1. Protein identity[2] and function, if known, were determined for all individual entries with an E value below 0.087.

III. RESULTS:

The database identified 186 entries[3] with E values below 0.087. All of these entries were known or putative PMI enzymes (including mannose-6-phosphate isomerase (MPI) and ManA) with no known toxic activity from 126 species[4], with E values of 0.0 – 0.067.

IV. CONCLUSION:

No relevant similarities were found between the *E. coli* PMI query sequence and known protein toxins.

V. CLASSIFICATION: ACCEPTABLE

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[1] BLASTP Search Parameters: No complexity filter; expect score = 10; word size= 3; gap costs: existence = 11 and extension = 1. The similarity matrix was Blosum 62.

[2] Identities were calculated using a local alignment comparing only the regions of the two proteins that are most similar and not the entire protein sequence as in a global alignment.

[3] Refer to MRID No. 468648-10, Table 1 for a complete detailed list of all 186 hits that gave an E value below 0.087 (the lowest E value for randomly shuffled PMI proteins).

[4] Refer to MRID No. 468648-10, Table 2 for a complete detailed list of all source organisms with potentially significant sequence homology to PMI.

VI. REFERENCES:

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller W., Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.

IFOM-IEO (2000). FIRC Institute of Molecular Oncology and the European Institute of Oncology. (http://bio.iform-firc.it/TOOLS/sms/shuffle_protein.html). Accessed May 10, 2006.

NCBI (2006). National Center for Biotechnology Information GenBank Database (http://www.ncbi.nlm.nih.gov/BLAST/); non-redundant GenBank coding sequence translations (PDB+SwissProt+PIR=PRF) containing 3,284,813 entries on February 26, 2006.

Stothard, P. (2000). The Sequence Manipulation Suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques*, 28: 1102-1104.

DATA EVALUATION RECORD

Phosphomannose Isomerase (PMI) Protein derived from *Escherichia coli*

STUDY TYPE: Amino Acid Homology to Known Toxins

MRID 468648-10

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
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One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T. — Signature: _____ Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S. — Signature: _____ Date: _____

Robert H. Ross, M.S., Group Leader — Signature: _____ Date: _____

Quality Assurance:
Angela M. Edmonds, B.S. — Signature: _____ Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

Oak Ridge National Laboratory managed and operated by UT-Battelle, LLC., for the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Amino acid homology to known allergens
MRID NO:	468648-11
TEST MATERIAL:	*Escherichia coli* phosphomannose isomerase protein
STUDY NO:	SSB-140-05
SPONSOR:	Syngenta Seeds, Inc. Product Registration Group P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science & Product Support P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709
TITLE OF REPORT:	Phosphomannose isomerase: Assessment of amino acid sequence homology with known allergens.
AUTHORS:	Hope Hart
STUDY COMPLETED:	July 14, 2005
STUDY SUMMARY:	No significant sequence homology was found between any sequential PMI 80-amino acid peptide of *E. coli* phosphomannose isomerase (PMI) and any entry in the SBI Allergen Database. Screening for matches of eight or more contiguous amino acids revealed an alignment with the allergen α-parvalbumin from *Rana species* CH2001. The common amino acid sequence of DLSDKETT occurred at positions 327-334 in PMI, and at positions 77-84 in α-parvalbumin. Serum from the one person with IgE-mediated allergy to α-parvalbumin was not cross-reactive with *E. coli* PMI, and this 8-amino acid match is considered biologically irrelevant. It is therefore concluded that *E. coli*-derived PMI has no significant amino acid sequence homology to any known or putative allergenic proteins.
CLASSIFICATION:	**ACCEPTABLE**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating that this was a non-GLP study conducted using accepted scientific methods, and the raw data and study records have been retained.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY DESIGN:

The purpose of this study was to determine if phosphomannose isomerase (PMI) protein derived from *E. coli* had any significant amino acid sequence homology to known or putative protein allergens.

II. MATERIALS AND METHODS:

The 391 amino acid sequence of the *E. coli* PMI protein was systematically compared to the Syngenta Biotechnology, Inc. Allergen Database (SBI, version 4.0, last updated March 2005). This analysis supersedes a previous allergen homology analysis for PMI conducted in 2004 (Rabe, 2004) using an older version of the SBI allergen database. The current (March 2005) SBI allergen database[1] contains 1414 non-redundant amino acid sequences of known and putative protein allergens, and was initially compiled from entries from the following sources:

1. All entries identified as allergens or putative allergens in the publicly available GenPept, PIR, or SWISS-PROT protein databases. Allergen sequence entries from these public databases were identified using the program Lookup from the GCG Wisconsin Package version 10.1 as part of the SeqWeb Bioinformatics package (Accelrys, Inc., 2001; further citation was not reported);

2. Entries in the SWISS-PROT Allergen database (SWISS-PROT, 2001);

3. Entries in the List of Allergens database (International Union of Immunological Societies, 2001);

4. Entries in the FARRP Protein Allergen database (Food Allergy Research and Resource Program, 2001); and

5. Additional entries identified in the scientific literature as putative allergens, but which are not found in public databases.

Two different searches were done to compare the amino acid sequence of PMI. The first search compared the sequence homology of the protein divided into 80 amino-acid blocks to allergen sequences using the FASTA search algorithm[2] (Pearson and Lipman, 1988). Each successive block was offset from the previous by one residue and the search repeated. Any 80 amino acid PMI peptide with >35% homology to an allergen was by definition significantly similar (FAO/WHO, 2001) and was further examined.

In the second search, the PMI amino acid sequence was screened for matches of eight or more contiguous amino acids (Hileman, *et al.*, 2002) using a Syngenta program that compares every possible peptide of eight contiguous amino acids between the query sequence and the allergen sequences in the SBI Allergen Database. This was done to search for short, local regions of amino acids which could indicate the presence of common IgE-binding epitopes.

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[1] Refer to MRID No. 468848-11, Table 1 for a detailed list of all sequences contained in the SBI Allergen Database, version 4.0.

[2] FASTA search parameters: the scoring matrix was blosum 62, the gap extension penalty was two and the gap creation penalty was 12.

III. RESULTS:

No significant sequence homology was found between any sequential PMI 80-amino acid peptides and any entry in the SBI Allergen Database. One alignment of eight contiguous identical amino acids was identified, between PMI and a known allergen, the 110 amino acid sequence of α-parvalbumin from *Rana species* CH2001 (Hilger et al. 2002). The common amino acid sequence of DLSDKETT occurred at positions 327-334 in PMI (see Figure 1), and at positions 77-84 in α-parvalbumin (see Figure 2).

Hilger et al. (2002) identified α-parvalbumin as an allergen in an individual who had severe anaphylaxis after eating frog legs of Indonesian origin. This patient's serum was not cross-reactive to related parvalbumins from the common edible frog (*Rana esculenta*). In order to determine if the IgE antibodies present in this patient's serum recognized PMI, Syngenta sent a sample of PMI overexpressed in *E. coli* (PMI-098; containing 61% w/w PMI protein and having PMI enzymatic activity, as described in Novartis Seeds, 1999) to the Hilger group, along with a placebo sample of bovine serum albumin (BSA). The Hilger group found no cross-reactivity between this patient's serum and PMI-098 (report provided in Appendix B of MRID 46864811), but did find some cross-reactivity with the BSA. The latter was irrelevant to the PMI results. Neither the PMI nor BSA cross-reacted with the negative control serum. EPA previously reviewed this study and concurred with the study author's conclusion (see MRID No. 464252-01 and EPA 2005 a, b, and c).

IV. CONCLUSION:

No significant sequence homology was found between any sequential PMI 80-amino acid peptides and any entry in the SBI Allergen Database. Screening of PMI amino acid sequence for matches of eight or more contiguous amino acids with the allergen database revealed one alignment, that with the allergen α-parvalbumin from *Rana species* CH2001. The common amino acid sequence of DLSDKETT occurred at positions 327-334 in PMI, and at positions 77-84 in α-parvalbumin. Serum obtained from the one person with IgE-mediated allergy to α-parvalbumin was not cross-reactive with PMI overexpressed in *E. coli* (PMI-098; containing 61% w/w PMI protein and having PMI enzymatic activity). Therefore it is concluded that this 8-amino acid sequence identity with α-parvalbumin was not biologically relevant, and that there is no evidence that *E. coli*-derived PMI has significant amino acid sequence homology to any known or putative allergenic proteins.

V. CLASSIFICATION: ACCEPTABLE

VI. REFERENCES:

EPA, 2005a. "Review of Product Characterization and Expression Analysis for Modified Cry3A (mCry3A) *Bacillus thuringiensis* insect control protein and maize (corn) plants Derived from Event MIR604 in support for a temporary exemption from tolerances and an Experimental Use Permit (EUP) Application." Memorandum- from A. Fellman, through J. Kough. Ph.D., to M. Mendelsohn, dated February 11, 2005.

EPA, 2005b. "Addendum to Product Characterization and Expression Analysis for Modified Cry3A (mCry3A) *B.t.* insect control protein and maize (corn) plants Derived from Event MIR604." Memorandum- from A. Fellman, through J. Kough, Ph.D., to M. Mendelsohn, dated February 11, 2005.

EPA, 2005c. "Evaluation of Phosphomannose isomerase protein (PMI) as expressed in Transgenic maize Event MIR604 with *E. coli*-derived PMI protein in support for a temporary tolerance exemption for Modified Cry3A *B.t.* insecticidal protein and the genetic material necessary for its production in corn plants derived from Event MIR604." Memorandum-from A. Fellman, through J. Kough, Ph.D., to M. Mendelsohn, dated March 3, 2005.

FAO/WHO (2001). Evaluation of allergenicity of genetically modified foods. Report of a joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology. 22-25 January, 2001. Rome, Italy.

Food Allergy Research and Resource Program (2001). FARRP Protein Allergen Database, version 4.0. http://www.allergenonline.com, last updated October 1, 2004.

Hileman, R. E., A. Silvanovich, R. E. Goodman, E. A. Rice, G. Holleschak, J. D. Astwood and S. L. Hefle (2002). Bioinformatic methods of allergenicity assessment using a comprehensive allergen database. *Int. Arch. Allergy Immunol.* 128: 280-291.

Hilger, C., Grigioni, F., Thill, L., Mertens, L. and Hentges, F. (2002). Severe IgE-mediated anaphylaxis following consumption of fried frog legs: Definition of alpha-parvalbumin as the allergen in cause. *Allergen* 57 (11): 1053-1058.

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Figure 1. Amino acid sequence of phosphomannose isomerase protein.
Location of 8 amino-acid homology with α-parvalbumin from *Rana species* CH2001 is underlined.

```
   1   MQKLINSVQN YAWGSKTALT ELYGMENPSS QPMAELWMGA HPKSSSRVQN
  51   AAGDIVSLRD VIESDKSTLL GEAVAKRFGE LPFLFKVLCA AQPLSIQVHP
 101   NKHNSEIGFA KENAAGIPMD AAERNYKDPN HKPELVFALT PFLAMNAFRE
 151   FSEIVSLLQP VAGAHPAIAH FLQQPDAERL SELFASLLNM QGEEKSRALA
 201   ILKSALDSQQ GEPWQTIRLI SEFYPEDSGL FSPLLLNVVK LNPGEAMFLF
 251   AETPHAYLQG VALEVMANSD NVLRAGLTPK YIDIPELVAN VKFEAKPANQ
 301   LLTQPVKQGA ELDFPIPVDD FAFSLHDLSD KETTISQQSA AILFCVEGDA
 351   TLWKGSQQLQ LKPGESAFIA ANESPVTVKG HGRLARVYNK L
```

Figure 2. Amino acid sequence of α-parvalbumin protein from Rana species CH2001.
Location of 8 amino-acid homology with PMI is underlined.

```
   1   MPMTDVLAAC DISKAMAAFP AAEPFNHKKF FELCGLKGKS QDDMKKVFHM
  51   LDKDQSGFIE KDELALILKG FTPEGRDLSD KETTALLAAG DKDGDGKIGV
 101   DEFVKLVSEC
```

DATA EVALUATION RECORD

Escherichia coli Phosphomannose Isomerase (PMI) Protein

STUDY TYPE: Amino Acid Homology to Known Allergens

MRID 468648-11

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
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Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

Primary Reviewer:
Sylvia Milanez, Ph.D., D.A.B.T. Signature: _____
Date: _____

Secondary Reviewers:
Anthony Q. Armstrong, M.S. Signature: _____
Date: _____

Robert H. Ross, M.S., Group Leader Signature: _____
Date: _____

Quality Assurance:
Angela M. Edmonds, B.S. Signature: _____
Date: _____

Disclaimer

This review may have been altered subsequent to the contractor's signatures above.

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DATA EVALUATION RECORD	
Primary Reviewer:	Sylvia Milanez, Ph.D., D.A.B.T.
EPA Secondary Reviewer:	Annabel Waggoner, Environmental Protection Specialist, BPPD
STUDY TYPE:	Product characterization
MRID NO:	468808-01
TEST MATERIAL:	*Bacillus thuringiensis* Cry1Ab and Vip3A proteins
STUDY NO:	162-EPA-2-VOL14
SPONSOR:	Published paper submitted by: Syngenta Seeds, Inc. P.O. Box 12257; 3054 East Cornwallis Road Research Triangle Park, NC 27709
TESTING FACILITY:	Not applicable -- published paper
TITLE OF REPORT:	The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-endotoxin.
AUTHOR / CITATION:	M.K. Lee, F.S. Walter, H. Hart, N. Palekar, and J.S. Chen (2003). The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-endotoxin. Applied and Environmental Microbiology 69 (8): 4648-4657.
STUDY COMPLETED:	Not applicable -- published paper
STUDY SUMMARY:	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**
GOOD LABORATORY PRACTICE:	A signed GLP statement was provided stating that, to the best of the registrant's knowledge, the study was not conducted in compliance with 40 CFR Part 160.

THIS REPORT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

I. STUDY PURPOSE:

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.

II. STUDY PUBLICATION SUMMARY:

The *Bacillus* Vip3A insecticidal protein exists as an 88-kDa full-length toxin (Vip3A-F) that can be proteolytically activated by trypsin (Vip3A-T) or extracts from lepidopteran gut (Vip3A-G) to form a 62-kDa core toxin. Biotinylated Vip3A-G was capable of competitive binding to Lepidopteran midgut brush border membrane vesicles (BBMV). Ligand blotting experiments showed that activated Cry1Ab and Vip3A-G bound to different receptor molecules in BBMV from Tobacco hornworm (*Manducta sexta*, Linnaeus). Additional blotting experiments showed that Vip3A-G did not bind isolated Cry1A receptors. Voltage clamping assays showed that Vip3A-G, but not Vip3A-F, formed distinct pores in dissected midgut from *M. sexta*, but Vip3A-G did not form pores with larvae from the monarch butterfly (*Danaus plexippus*, Linnaeus). The inherent ability of Vip3A-T and Vip3A-G to form pores was indicated by their ability to form stable ion channels in planar lipid bilayers containing no receptors. Cry1Ab and Vip3A channels were both voltage-independent and cation-selective, but their primary conductance state and cation specificity differed. Based on these results, the study authors concluded that Cry1Ab and Vip3A have different modes of action.

III. CONCLUSION:

The study concluded that Vip3A has a unique mode of action (which differs from Cry1Ab) in that in can form stable ion channels in the absence of any receptors in planar lipid bilayers. Specifically, the channels (which are structurally and functionally distinct from those of Cry1Ab) lead to pore formation and a loss of transmembrane potential, which causes histological changes in susceptible insect midgut tissue and ultimately, toxicity.

IV. CLASSIFICATION: ACCEPTABLE

V. REFERENCE:

Lee, M. K., F. S. Walter, H. Hart, N. Palekar, and J-S. Chen (2003). The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A from that of Cry1Ab delta-endotoxin. *Appl. Environ. Microbiol.* 69(8): 4648 – 4657.

DATA EVALUATION RECORD

Differences in the Mechanism of Insecticidal Activity of Cry1Ab and Vip3A Proteins

STUDY TYPE: Product Characterization

MRID 468808-01

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
One Potomac Yard
2777 South Crystal Drive
Arlington, VA 22202

Prepared by
Toxicology and Hazard Assessment Group
Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37830
Task Order No. 06-100

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Robert H. Ross, M.S., Group Leader

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Quality Assurance:
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Date: _____

Disclaimer

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