

BIOPESTICIDES REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for
Their Production (Plasmid Insert PHP 17662) in Event DAS-59122-7 Corn

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

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Bacillus thuringiensis Cry34Ab1 and Cry35Ab1 Proteins and the Genetic Material Necessary for Their Production (Plasmid Insert PHP 17662) in Event DAS-59122-7 Corn Regulatory Action Team

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I. Overview

A. Executive Summary

EPA has conditionally registered Mycogen Seeds c/o Dow AgroSciences LLC and Pioneer Hi-Bred International's new active ingredient, *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn. The Agency has determined that the use of this pesticide is in the public interest and that it will not cause any unreasonable adverse effects on the environment during the time of conditional registration.

The new products are the second PIP to offer protection against corn rootworm (CRW), and they are expected to result in a further reduction of chemical insecticide use by growers. The reduced chemical pesticide use will benefit the environment directly and can mean less exposure to people who apply chemical pesticides to corn. The availability of multiple CRW-protected corn products will also increase grower choice and price competition, resulting in lower seed prices for growers and higher adoption rates.

The new corn plant-incorporated protectant, Event DAS-59122-7 Corn, produces its own insecticide within the corn plant derived from *Bacillus thuringiensis* (Bt), a naturally occurring soil bacterium. The Bt proteins used in this product, called Cry34Ab1 and Cry35Ab1 (Cry 34/35), control corn rootworm, a highly destructive pest responsible for the single largest use of conventional insecticides in the United States.

In order to reduce the possibility of corn rootworm developing resistance to Bt, EPA is requiring Mycogen and Pioneer to ensure that 20 percent of the planted acreage of this product be set aside where non-CRW-protected Bt corn will be grown to serve as a "refuge." These refuge areas will support populations of corn rootworm not exposed to the Bt corn. The insect populations in the refuges will help prevent resistance development when they cross-breed with insects in the Bt fields. This resistance management strategy was developed as a condition of the registration, and EPA will require routine monitoring and documentation that these measures are followed. The submitted insect resistance management data support a 5-year registration until 2010.

A tolerance exemption under 40 CFR Part 174.457 has also been approved for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn.

Product Characterization

Cry34Ab1 and Cry35Ab1 proteins are from *Bacillus thuringiensis* PS149B1 and have activity against certain beetles.

B.t. Cry34/35Ab1 corn was produced by *Agrobacterium tumefaciens*-mediated transformation of public corn line (Hi-II) with the T-DNA from plasmid PHP17662, which contains *cry34Ab1*, *cry35Ab1*, *pat*, and regulatory sequences necessary for the expression of the genes. The *cry34Ab1* and *cry35Ab1* transgenes were optimized for expression in maize, but the amino acid sequence of the expressed proteins is identical to the native proteins from *B.t.* Characterization of the DNA isolated from *B.t.* Cry34/35Ab1 corn using restriction enzyme digests and Southern blot analysis indicated that the T-DNA from plasmid PHP17662 inserted as a single, intact copy into the corn genome. In addition, DNA analysis indicated stability and inheritance of the inserted DNA within and across several generations.

Protein characterization data demonstrate that the plant-produced proteins have characteristics and activities that are equivalent to those of the proteins produced in *Pseudomonas fluorescens* transformed to produce Cry34Ab1 and Cry35Ab1.

Studies on the mode of action of Cry34Ab1 and Cry35Ab1 indicate that similar to other *B.t.* delta-endotoxins, Cry34Ab1 and Cry35Ab1 appear to target midgut epithelial cells in susceptible larvae. Cry34Ab1 appears to cause pore formation in phospholipid membranes, and addition of Cry35Ab1 resulted in pores remaining open longer and improved membrane permeability (Masson *et al.* 2004 Biochem. 43. 12349-12357). Ribosomal inhibition activity was also investigated. The results demonstrated that the insecticidal activity of Cry34Ab1 and Cry35Ab1 is not associated with the inhibition of protein synthesis.

Human Health Assessment

Based upon the human health data provided, the risk of toxic and/or allergenic effects to humans or animals due to exposure to the Cry34Ab1 and Cry35Ab1 proteins is minimal and there is a reasonable certainty of no harm to humans and animals posed by the aggregate exposure to residues of these proteins.

Three acute oral toxicity studies on Cry34Ab1 and Cry35Ab1 in mice were submitted, which indicated that these proteins are non-toxic to humans. In addition, a study was submitted where the amino acid sequences of the Cry34Ab1 and Cry35Ab1 proteins were compared with protein sequences in publicly available sequence databases to identify any potential similarities with known toxins. No similarities were identified that would raise a safety concern. Toxic proteins typically act as acute toxins with low dose levels. Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry34Ab1 and Cry35Ab1 proteins are not considered toxic.

Regarding allergenicity potential, 1) Cry34Ab1 and Cry35Ab1 originate from a non-allergenic source; 2) Cry34Ab1 and Cry35Ab1 have no overall sequence similarities or homology at the level of 8 contiguous amino acid residues with known allergens; 3) Cry34Ab1 and Cry35Ab1 will only be present at low levels in food; 4) Cry35Ab1 is rapidly digested in simulated gastric fluid, and

Cry34Ab1 is digested at a moderate rate in simulated gastric fluid; and 5) Cry34Ab1 and Cry35Ab1 are not glycosylated when expressed in maize. EPA has concluded that the potential for the Cry34Ab1 and Cry35Ab1 proteins to be food allergens is minimal.

A tolerance exemption exists under 40 CFR Part 174.457 has also been approved for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production in corn that includes Event DAS-59122-7 corn.

Environmental Assessment

The Agency is using a Maximum Hazard Dose Tiered system for biopesticide non-target wildlife hazard assessment. When no adverse effects at the maximum hazard dose are observed, the Agency concludes that there are no unreasonable adverse effects from the use of the pesticide. From all of the required and voluntarily developed indicator and host range species test data on Cry34/35Ab1 corn, the Agency concludes that the levels of Cry34/35Ab1 protein in Event DAS-59122-7 corn will not pose unreasonable adverse effects to corn field flora and fauna. Available data also indicate that there should be minimal short-term accumulation of Cry34/35Ab1 protein in agricultural soil. In addition, no adverse effect on endangered and threatened species listed by the US Fish and Wildlife Service is expected from the Event DAS-59122-7 corn registration.

At present, the Agency is aware of no identified significant adverse effects of Cry34/35Ab1 proteins on the abundance of non-target beneficial organisms in any population in the field, whether they are pest parasites, pest predators, or pollinators. Field testing and field census data submitted to the Agency show minimal to undetectable changes in the beneficial insect abundance or diversity. To date, available field test data show that compared to crops treated with conventional chemical pesticides, the transgenic crops have no detrimental effect on the abundance of non-target insect populations.

The Agency believes that cultivation of Event DAS-59122-7 corn may result in fewer adverse impacts to non-target organisms than result from the use of chemical pesticides. Under normal circumstances, Event DAS-59122-7 corn requires substantially fewer applications of chemical pesticides. This should result in fewer adverse impacts to non-target organisms because application of nonspecific conventional chemical pesticides is known to have an adverse effect on non-target beneficial organisms found living in the complex environment of an agricultural field. Many of these beneficial organisms are important integrated pest management controls (IPM) for secondary pests such as aphids and leafhoppers. The overall result of cultivation of corn expressing Cry34/35Ab1 proteins is that the number of chemical insecticide applications for non-target pest control is reduced for management of multiple pest problems.

The movement of transgenes from Cry34/35Ab1 host plants into weeds and other crops has also been considered. The Agency has determined that there is no significant risk of gene capture and expression of Cry34/35Ab1 protein by wild or weedy relatives of corn in the U.S., its possessions, or

territories. The fate of Cry34/35Ab1 protein in soils and indirect effects on soil biota have also been evaluated. Test data show that most of the Cry protein deposited into soil is quickly degraded, although a residual amount may persist in biologically active form for a much longer period of time. It is also reported that the same degree of Bt Cry protein persistence takes place in soils that have been exposed to repeat Bt spray applications when compared to soil exposed to growing Bt crop. Limited data do not indicate that Cry proteins have any measurable effect on microbial populations in the soil. Horizontal transfer of genes from transgenic plants to soil bacteria has not been demonstrated. Published studies of Bt Cry protein in soil show no effect on bacteria, actinomyces, fungi, protozoa, algae, nematodes, springtails or earthworms. In addition, new plants planted in Bt Cry protein containing soil do not take up the Bt protein.

The Agency finds no hazard to the environment at the present time from cultivation of Event DAS-59122-7 corn for a time-limited registration.

Insect Resistance Management

Dow and Pioneer proposed a Cry34/35 Event 59122-7 corn durability plan has the following elements: 1) structured refuge, 2) resistance monitoring, 3) remedial action plan, and 4) compliance and education. Simulation models were used to assist in evaluating and comparing structured refuge options. In order to reduce the possibility of corn rootworm developing resistance to Bt, EPA is requiring Mycogen and Pioneer to ensure that 20 percent of the planted acreage of this product be set aside where non-CRW-protected Bt corn will be grown to serve as a “refuge.” These refuge areas will support populations of corn rootworm not exposed to the Bt proteins. The insect populations in the refuges will help prevent resistance development when they mate with any potentially resistant adult rootworm beetles emerging from the Bt fields. This resistance management strategy was developed as a condition of the registration, and EPA will require routine monitoring and documentation that these measures are followed. The submitted insect resistance management data support a 5-year registration until 2010.

Benefits

In assessing the potential benefits from Event DAS-59122-7 corn, EPA compared the efficacy of Event DAS-59122-7 corn to other chemical controls for CRW, evaluated the human health and environmental benefits compared to registered alternatives, estimated the grower benefits, and estimated the chemical pesticide use reduction from adoption of Event DAS-59122-7 corn. EPA made a determination that the registration of Event DAS-59122-7 corn was in the public interest and that the benefits outweigh the risks.

Cry34/35Ab1-protected corn provides effective control of key rootworm pests of field corn and may prove more efficacious than chemical insecticides presently registered for this purpose.

Economic models suggest that, under conditions of high rootworm pressure, use of Cry34/35Ab1-protected corn will provide greater net returns to farmers. Cost benefits include reduced expenditures on insecticides, application equipment, and personnel, complemented by greater potential corn yields. Under high rootworm pressure, these benefits are expected to outweigh the higher cost of seed.

Registration of Cry34/35Ab1-protected corn is expected to result in further reduction of chemical insecticide use by growers. This is of special importance since many pesticides registered for CRW control are highly toxic to humans and the environment, while Cry34/35Ab1 expressing corn poses no foreseeable human health or environmental risks.

Cry34/35Ab1 corn is the second CRW-protected corn PIP to be registered (the first was Cry3Bb1). The availability of multiple CRW-protected corn products will increase grower choice and price competition, likely resulting in lower seed prices for growers and higher adoption rates.

The Cry34/35Ab1 CRW-protected corn will provide a different mode of action and extend the durability of other CRW control measures, including other Bt CRW-protected corn hybrids.

B. Use Profile

- ! **Pesticide Name:** *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 proteins and the genetic material necessary for their production (plasmid insert PHP 17662) in Event DAS-59122-7 corn

- ! **Trade and Other Names:** Event DAS-59122-7 corn, Herculex Rootworm, Herculex RW, Herculex RW Rootworm Protection

- ! **OPP Chemical Code:** 006490

- ! **Basic Manufacturers:** Mycogen Seeds c/o Dow AgroSciences LLC
330 Zionsville Road, Indianapolis, IN 46268

Pioneer Hi-Bred International, A Dupont Company
7250 N.W. 62nd Ave., P.O. Box 552, Johnston, IA

- ! **Type of Pesticide:** Plant-Incorporated Protectant

- ! **Uses:** Field Corn

- ! **Target Pest(s):** Corn Rootworm.

II. Science Assessment

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

II A. PRODUCT CHARACTERIZATION

The Agency’s detailed assessment of the product characterization for *Bacillus thuringiensis* Cry34/35Ab1 is found in Edelstein (2004b). Portions of the product characterization data were also reviewed in Wozniak (2001), Wozniak (2002a), and Wozniak (2003a, c, d, and e). *B.t.* Cry34/35Ab1 corn was produced by *Agrobacterium tumefaciens*-mediated transformation of public corn line (Hi-II) with the T-DNA from plasmid PHP17662^a, which contains *cry34Ab1*, *cry35Ab1*, *pat*, and regulatory sequences necessary for the expression of the genes. The *cry34Ab1* and *cry35Ab1* transgenes were optimized for expression in maize, but the amino acid sequence of the expressed proteins is identical to the native proteins from *B.t.* Characterization of the DNA isolated from *B.t.* Cry34/35Ab1 corn using restriction enzyme digests and Southern blot analysis (MRIDs 461239-08 and 461239-09, reviewed in Edelstein, 2004b) indicated that the T-DNA from plasmid PHP17662 inserted as a single, intact copy into the corn genome. In addition, DNA analysis indicated stability and inheritance of the inserted DNA within and across several generations.

Protein characterization data demonstrate that the plant-produced proteins have characteristics and activities that are equivalent to those of the proteins produced in *Pseudomonas fluorescens* transformed to produce Cry34Ab1 and Cry35Ab1 (MRIDs 461239-05 and 461239-06, reviewed in Edelstein, 2004b). The following techniques were used to characterize and compare the plant-

^a Early in the development of this product, other plasmids were used and other events tested. Event DAS-59122-7 where plasmid PHP17662 was used has been chosen for commercialization.

produced proteins and the microbially-produced proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), N-terminal amino acid sequencing, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), glycosylation analysis, enzyme-linked immunosorbent assays (ELISAs), and western blot analysis. Glycosylation analysis indicated that the Cry34Ab1 and Cry35Ab1 proteins are not glycosylated. Biological activity of the plant-produced and *P. fluorescens*-produced proteins was also assessed and compared. Cry34Ab1 and Cry35Ab1 from both sources displayed similar insecticidal activity to western corn rootworm larvae. These analyses justified the use of microbially-produced proteins in toxicity studies.

Studies on the mode of action of Cry34Ab1 and Cry35Ab1 indicate that similar to other *B.t.* delta-endotoxins, Cry34Ab1 and Cry35Ab1 appear to target midgut epithelial cells in susceptible larvae (MRID 457906-01, reviewed in Wozniak, 2003c). Cry34Ab1 appears to cause pore formation in phospholipid membranes, and addition of Cry35Ab1 resulted in pores remaining open longer and improved membrane permeability (Masson *et al.* 2004 Biochem. 43. 12349-12357). Ribosomal inhibition activity was also investigated (461239-10, reviewed in Edelstein, 2004b). The results demonstrated that the insecticidal activity of Cry34Ab1 and Cry35Ab1 is not associated with the inhibition of protein synthesis.

Submitted studies on heat stability of the Cry34Ab1 and Cry35Ab1 proteins demonstrate that these proteins are inactivated at 90 °C and 60 °C, respectively (MRIDs 453584-01, 455845-01, 458086-01, and 458602-01, reviewed in Wozniak (2001, 2002a, and 2003a).

Data on expression levels of the proteins in transgenic corn tissues were also provided for both inbred and hybrid lines from several different events (MRID 461239-04, reviewed in Edelstein, 2004b). The protein expression levels are comparable between hybrid and inbred lines. Data on expression levels in a hybrid line are summarized in Table 1.

Table 1. Mean expression levels of Cry34Ab1 and Cry35Ab1 in plant tissues from a hybrid line containing event DAS-59122-7

Tissue	Cry34Ab1 (ng/mg tissue dry weight ± standard deviation)*	Cry35Ab1 (ng/mg tissue dry weight ± standard deviation)*
Leaf	50 ± 8 – 220 ± 38	41 ± 7 – 85 ± 19
Root	37 ± 9 – 50 ± 20	3 ± 2 – 8 ± 3
Whole Plant	32 ± 16 – 77 ± 10	7 ± 2 – 14 ± 2
Pollen	74 ± 7	0.02 ± 0.04
Stalk	33 ± 4	10 ± 2
Forage	53 ± 19	12 ± 3
Grain	50 ± 16	1 ± 0.3

* Ranges reflect the range of means at different growth stages

The product characterization studies that were submitted in support of this product registration are summarized below in Table 2. Some of the studies were conducted on events other than DAS-

59122-7, and some of the studies refer to the Cry34Ab1 and Cry35Ab1 proteins as PS149B1 13.6 or 14 kDa and 43.8 or 44 kDa insecticidal crystal protein. The term PS149B1 proteins was used prior to the proteins receiving their *B. thuringiensis* toxin nomenclature designations (Crickmore, et al., 2005). The PS149B1 13.6 kDa or 14 kDa and 43.8 or 44kDa proteins are the same as the Cry34Ab1 and Cry35Ab1 proteins, respectively.

Table 2. Submitted Product Characterization Data

Study Title	Summary	MRID #
Product characterization data for <i>Bacillus thuringiensis</i> PS149B1 13.6 kDa and 43.8 kDa insecticidal crystal protein expressed in transgenic maize plants	Maize immature embryo tissues were transformed using a linearized fragment from plasmid PHP12560 encoding two ICP genes, <i>8v6</i> (13.6 kDa) and <i>7v7</i> (43.8 kDa), each driven by the maize ubiquitin promoter, and the phosphinothricin acetyl transferase gene (<i>pat</i>), driven by the CaMV 35S promoter. The two ICP genes from <i>B. thuringiensis</i> PS149B1 are modified in coding sequence to optimize for plant expression. Amino acid sequences are, however, identical to the bacterial source proteins. Microprojectile bombardment of tissues followed by selection on glufosinate, produced plants expressing the two ICP of PS149B1 and PAT. Maize plants expressing these genes were also produced by <i>Agrobacterium</i> -mediated transformation of immature embryos. Western corn rootworm (WCR) bioassays were used to assess the bioactivity of the expressed ICP. Within 48 hours of feeding on transgenic maize plants, WCR larvae had midgut symptoms consistent with feeding on d-endotoxins (e.g., swelling, lysis, blebbing, vacuolization). WCR feeding on control plants did not show pathological changes in midgut epithelium. The two ICP were also tested for ADP ribosylating or glucosylating activities as well as other enzymic activities, but none were detected. Classification: Acceptable.	452422-01
Characterization of inserted DNA of event 5639 transgenic corn plants– interim report	Based upon the Southern blot data presented, the most likely conclusion is that there are three insertions of the <i>8v6</i> , <i>7v7</i> and <i>pat</i> genes into event 5639, although only one may be fully intact. The complexity of the hybridization patterns does not allow one to say with certainty that three insertions are present at this time. A more detailed analysis of gene insertion will be required as this product develops to fully ascertain the nature of the integration events. Data indicated that the event 5639 does not contain a <i>kan^r</i> sequence. Classification: Acceptable.	452422-02
Equivalency of microbial- and maize- expressed PS149B1 proteins	Biochemical, molecular and serological data presented indicate that the proteins produced in the <i>P. fluorescens</i> system are equivalent to the proteins produced in transgenic maize plants. Relative molecular mass and serological reactivity to polyclonal antibodies were the same for the 14 kDa proteins produced in either system. SDS-PAGE and Western blots indicated identity between the two production systems. This was also true for the 44 kDa protein and its proteolytic cleavage product, the 42 kDa protein. The 44 kDa protein predominated in the fresh plant material and in the <i>P. fluorescens</i> production system. Storage of leaf material led to the production of the 42 kDa protein as the predominant form due to proteolytic processing. No glycosylation was detected on either protein from the maize system. Tryptic digestion of PS149B1 proteins and subsequent analysis by MALDI-TOF MS, N-terminal sequencing and ESI MS/MS spectra all indicated that PS149B1 proteins produced by <i>P. fluorescens</i> and the transgenic maize are equivalent for toxicological testing to be performed with proteins from either source. In insect bioassays, proteins produced in the transgenic maize system and the microbial system both showed activity against the Southern corn rootworm (SCR) and WCR larvae. While the transgenic maize assay indicated no reduction in damage from	452422-03

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	<p>European corn borer (ECB) feeding, the diet based overlay with the microbially produced protein indicated some slight inhibition of growth for ECB larvae. Additionally, the 149B1 maize plants sustained greater leaf damage from ECB, based upon visual estimates, than the control plants. It is not clear if this difference was statistically significant since there was no analysis provided.</p> <p>Classification: Acceptable.</p>	
<p>Microbial PS149B1 binary delta-endotoxin: maize-insect pest susceptibility study</p>	<p>The relative sensitivity of the test insects in the feeding bioassays suggests that larvae of coleopteran species are far more sensitive to the 14 kDa and 44 kDa proteins of PS149B1 than the lepidopteran or homopteran larval insects evaluated. Adult WCR were not sensitive to the d-endotoxins, however. Rootworms (NCR, WCR and SCR) were the most susceptible to the PS149B1 proteins. ECB and Corn ear worm (CEW) demonstrated some inhibition of growth at higher concentrations of the test substance than the rootworm larvae. Black cut worm (BCW) was the least sensitive of the lepidopteran species tested. No activity was seen against the Corn leaf aphid (CLA), a homopteran. Relative susceptibilities of the insect species to PS149B1, based upon the more sensitive measure of GI₅₀, were as follows: (most susceptible) WCR, NCR, SCR > ECB, CEW >> WCR adult, CLA, BCW (least susceptible).</p> <p>Classification: Acceptable.</p>	452422-04
<p>Quantitative ELISA analysis of PS149B1 protein expression levels in hybrid and inbred lines of maize event TC5639</p>	<p>Leaf, pollen, root and senescent whole plant (WPS) tissue samples were taken from field grown plants cultured in the U.S. corn belt. Both test hybrid and inbred lines of event 5639 and control hybrid and inbred lines were compared for expression of the 14 kDa and 44 kDa PS149B1 proteins. Leaf, pollen, root and WPS tissues expressed the 14 kDa and 44 kDa proteins at measurable levels in the modified maize lines. Hybrid and inbred lines had comparable levels of expression of these proteins when analyzed on a dry weight basis. On a total extractable protein basis, though, root tissues had the highest expression and pollen the lowest. Control tissues were negative for the presence of the 14 kDa or the 44 kDa proteins. Coefficients of variation (CV) were lacking for data presented and need to be included in order to adequately assess the results of the ELISA assays. The use of samples spiked with known quantities of the two proteins separately is also needed to assess the possible influence of maize tissue extracts on the ELISA values observed. These data and an evaluation of possible cross-reactivity of the primary antibody with other Cry proteins will be needed for Section 3 registration.</p> <p>Classification: Acceptable.</p>	452422-13
<p>Lateral flow test kit for the detection of the PS149B1 protein in maize grain</p>	<p>The double antibody sandwich format of the lateral flow test strip from Strategic Diagnostics was capable of correctly detecting 48 positive aliquots of 24 extracts from 24 single kernel samples of PS149B1 maize. Conversely, 48 aliquots of 24 extracts from 24 kernels of a non-PS149B1 inbred line all tested negative, as expected. Ratios of positive (PS149B1) and negative (control) kernels were mixed and extracted. When 0.5 % or greater PS149B1 kernels were present in the mixture, they were correctly detected as containing Cry34Ab1 protein. No ratios of positive : negative below 0.5% were evaluated in this study.</p> <p>Classification: Acceptable.</p>	453834-01
<p>Characterization of Cry34Ab1 and Cry35Ab1 from recombinant <i>Pseudomonas fluorescens</i> and transgenic maize</p>	<p>Cry34Ab1 and Cry35Ab1 proteins were separated from maize leaf extracts and from microbial production sources on SDS-PAGE gels, then blotted to nitrocellulose for serological detection. Both proteins were detected on the Western blots by polyclonal antibody preparations and appeared at the appropriate M_r for both proteins on individual blots (<i>i.e.</i>, 14 kD - Cry34Ab1; 44 kD Cry35Ab1). A second protein band reacted with the Cry35Ab1 specific antibody in the microbially produced protein preparation at approximately 40 kD, which is presumably a degradation product from protease activity. This band or signal was noted in the Western blot of the 3 of 6 maize leaf extracts representing the six events, but at</p>	457904-01

	<p>reduced amounts relative to the microbial preparation. Both Cry proteins from the maize extracts migrated very similarly to the Cry proteins produced in the microbial production system and by this criterion (<i>i.e.</i>, mobility in SDS-PAGE) are considered as indistinguishable. Classification: Acceptable.</p>	
<p>Characterization of DNA inserted into transgenic corn events E4497.42.1.34, E4497.45.2.16, E4497.59.1.10, E4497.66.1.27, E4497.71.1.29, and E4497.71.1.33</p>	<p>Southern blots were used to discern the number of insertions from each of the two plasmid constructs, PHP17662 and PHP17658, used in the transformation of maize. An analysis of integration sites in Events E4497.42.1.34, E4497.45.2.16, and E4497.59.1.10 indicated a single integration of the T-DNA sequence into the maize genome. Each reactive band present in the blot results from the <i>XhoI</i> cleavage of the incorporated T-DNA region (containing <i>cry34Ab1</i>, <i>cry35Ab1</i> and <i>pat</i> from PHP17622 in a single fragment) and a second <i>XhoI</i> restriction site in the adjacent maize genome. Hence, the bands represent individual, unique integrations of T-DNA in the maize genome. The size of the reactive bands cannot be determined <i>a priori</i> since the size of the flanking or border fragment is not known as it is determined by the distance from the single <i>XhoI</i> site within the T-DNA region. Events E4497.42.1.34 and E4497.45.2.16 each had single reactive hybridization events (bands) on the blot, indicating the presence of a single T-DNA insertion. The commonality of band sizes (<i>i.e.</i>, 9.4 or > 22 kb) suggests that the T-DNA insertion is the result of a single insertional event. A 9.4 kb signal in E4497.1.10 also indicates the integration of one full T-DNA containing <i>cry34Ab1</i>, <i>cry35Ab1</i> and <i>pat</i>. The presence of a second hybridizing signal (7.4 kb) in the blot of genomic DNA from E4497.1.10 probed with <i>cry35Ab1</i> suggests that a partial construct insertion occurred containing only the <i>cry35Ab1</i> portion and not <i>cry34Ab1</i> or <i>pat</i>. <i>StuI</i> cuts once in PHP17658 and was used to define integration sites in events E4497.66.1.27, E4497.71.1.29, and E4497.71.1.33. Events E4497.71.1.29 and E4497.71.1.33 appear to both contain a single T-DNA insertion based on the reactive band signals being present at 11 and 8.3 kb for all three probes. Additionally, there was a reactive band at 14 kb and 2.7 kb for the <i>pat</i> probed blots of E4497.71.1.29 and E4497.71.1.33, respectively. The second reactive band in the <i>pat</i> probed blots is a consequence of the position of the cleavage site for the <i>StuI</i> enzyme being within the <i>pat</i> ORF. Genomic DNA from control plants (non-transgenics) were spiked with restricted plasmid DNA of PHP17661 and PHP17657 at a concentration of one copy per genome equivalent to serve as positive controls. Classification: Acceptable.</p>	<p>457902-02</p>
<p>Product characterization data for <i>Bacillus thuringiensis</i> Cry34Ab1 and Cry35Ab1 proteins expressed in transgenic maize plants (PHP17662)</p>	<p>Genes encoding the PAT, Cry34Ab1 and Cry35Ab1 proteins were transferred into the T-DNA region of the disarmed <i>Agrobacterium tumefaciens</i> strain LBA4404. This Ti-plasmid was then referred to as PHP17662 and is approximately 50 kb in size. This strain carrying PHP17662 was used to transform immature maize embryo tissues by co-cultivation for 6 days. Coding sequences for the Cry34Ab1 (14 kD) and Cry35Ab1 (44 kD) proteins were synthesized from the native sequence to optimize expression in maize. The PAT protein produced from a synthetic gene is not altered in its amino acid sequence as compared to the native protein. The gene and protein are considered as inert ingredients in this plant-incorporated protectant. Gut cells of the control maize-fed insects and the starved insects appeared normal, although the latter group showed some collapse of the gut lumen. After 48h of feeding on the transgenic maize plants, WCRW larvae had evidence of cell lysis, blebbing and vacuolization, as would be expected from intoxication with δ-endotoxins in a susceptible insect. Subsequently the gut tissues showed further collapse and more fragmentation. Enzyme assays were evaluated to determine if either Cry34Ab1 or Cry35Ab1 proteins were capable of activity: glucosylating or ADP-ribosylating of midgut proteins, protease at acid and neutral pH, neuraminidase (sialidase), acid and alkaline phosphatase activity. Protein synthesis inhibition of an <i>in vitro</i> system was also tested. All assays were negative for their corresponding enzyme activities.</p>	<p>457906-01</p>

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	<p>Amino acid sequences of the three proteins and micrographs of histopathological midgut responses were presented. Classification: Acceptable.</p>	
<p>Quantitative ELISA analysis of Cry34Ab1 and Cry35Ab1 proteins expressed in maize plants transformed with the vector PHP17662</p>	<p>ELISA was used to determine the quantity of the two ICPs present in leaf, pollen, root and seed of transgenic maize from three separate transformation events (E4497.42.1.34, E4497.45.2.16, E4497.59.1.10). Direct double antibody sandwich ELISA was used to quantify the Cry34Ab1 and Cry35Ab1 present in plant samples. The primary antibodies raised against Cry34Ab1 and Cry35Ab1 were used to capture the proteins (individually in separate reactions) in the microplate well and a second specific antibody was used to detect the protein that was bound. This second antibody was conjugated to biotin which was detected through the use of streptavidin linked to alkaline phosphatase (AP). The colorimetric reaction catalyzed by the AP provides for a means of measuring optical density or absorbance and correlating it to the amount of target protein present based upon comparison to a standard curve generated from reference standards. The values obtained from the ELISA readings are converted to amounts of Cry34Ab1 or Cry35Ab1 protein and expressed as ng Cry34Ab1 protein / mg total extractable protein (TEP). Since data for water content exists for the tissue types sampled, the conversion to ng Cry34Ab1 protein / mg dry tissue is possible with conversion factors. Reference standards used to generate the standard curve were run in triplicate within each 96-well microplate. For Cry34Ab1, the magnitude of expression for tissue types was as follows in descending order: leaf, pollen, kernel and root. All control tissues were negative for the expression of Cry34Ab1. Due to the Mendelian segregation of the transgenes, approximately 50 % of the seeds were found to be expressing Cry34Ab1. For Cry35Ab1, the magnitude of expression for tissue types was as follows in descending order: leaf, root and kernel. All control tissues were negative for the expression of Cry35Ab1. Cry35Ab1 protein was not detected in pollen. Due to the Mendelian segregation of the transgenes, approximately 50 % of the seeds were found to be expressing Cry35Ab1. Seeds that were observed to contain no Cry proteins by ELISA were not included in the summary statistics since they represent segregants from the heterozygous populations of the three events. Mean values are presented in tabular format for all three events for each tissue type. Classification: Acceptable.</p>	<p>458332-01</p>
<p>Probe MOA Studies to assess potential for protein synthesis inhibition by <i>B.t.</i> PS149B1 Cry34Ab1/Cry35Ab1 proteins in a rabbit reticulocyte assay: re-examination of lab notebook data</p>	<p>Two experiments were performed in order to assess the potential for ribosomal inhibition activity by either Cry34Ab1 or Cry35Ab1. The first experiment concluded, based upon a sensitive luciferase (chemiluminescence) assay, that the Cry 34Ab1 and Cry35Ab1 proteins did not show the properties of ribosomal inhibitory proteins (RIP). This was largely based upon comparison to a BSA control and PBS buffer control. A second experiment, with a different treatment regime and altered protocol concluded that there was some RIP activity associated with the PS149B1 proteins. Further, digesting the 14 kDa protein with trypsin may also reduce translation a little. The two experiments used different amounts of BSA (<i>i.e.</i>, 1x and 2x) and this added variable further confounds the interpretation of results. The lack of proper controls and different treatment regimes in the experiments makes any assessment of the potential RIP activity dubious. The experiments would also have benefitted by including a greater number of samples per treatment (at least triplicate) and appropriate controls. A positive control (<i>i.e.</i>, a protein with known RIP activity) is crucial to the interpretation of results. Note that the source (lot) of the proteins used for the two experiments was different as well. It is also probable that confounding stabilization reagents (<i>e.g.</i>, glycerol, azide) were present in the reaction during the second experiment, further complicating any analysis of results. It is not possible given the present data set and variance in methodologies to assess the results accurately. The RIP assay has not been considered as a typical part of the submission process for plant-incorporated protectants. There is no <i>a priori</i> reasoning</p>	<p>459428-01</p>

