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

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

OCT 31 2008

MEMORANDUM

SUBJECT: Review of Product Characterization, Expression Analysis, and Human Health Data for Plant-Incorporated Protectant plants as expressed in the stacked products: Bt11 x MIR162 maize hybrid [EPA Reg. No. 67979-RE] and Bt11 x MIR162 x MIR604 maize hybrid [EPA Reg. No. 67979-RG] in support for Sec. 3 Registration, submitted by Syngenta Seeds, Inc. – Field Crops- NAFTA

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

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

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

ACTION REQUESTED: To review product characterization, protein expression analyses, and toxicological and allergenicity data, submitted by Syngenta Seeds, Inc.-Field Crops - NAFTA in support for Section 3 registrations for the PIP stacked products- Bt11 x MIR162 maize hybrid and Bt11 x MIR162 x MIR604 maize hybrid, crossed via traditional breeding methods with the *Bacillus thuringiensis* derived PIP Corn Events: Cry1Ab protein as expressed in Event Bt11, Vip3Aa20 protein as expressed in Event MIR162, and/or modified Cry3A protein as expressed in Event MIR604.

CONCLUSION: The data submitted for the transgenic protein expression levels and the Southern Analyses in Events Bt11, MIR162, MIR604 and the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 hybrids, crossed via traditional plant breeding, were found to be similar to the Cry1Ab protein expressed in Event Bt11 corn, the Vip3Aa protein expressed in Event MIR162 corn, and mCry3A protein as expressed in Event MIR604 corn. These data are classified as **ACCEPTABLE.** However, the data to support the characterization of the Cry1Ab test material, TRYCRY1AB-0105, is classified as **SUPPLEMENTAL.** The registrant should submit the mass spectra as visual

confirmation of the molecular weight for the TRYCRY1AB-0105 and conduct a direct and concurrent comparison of the biochemical and functional characteristics of a purified plant-expressed protein extracted from Bt11 maize leaves to the TRYCRY1AB-0105 test substance. These data can be used to address the equivalence between the plant- and microbial-produced Cry1Ab proteins in support of utilizing the TRYCRY1AB-0105, as a test material in the non-target organism studies for demonstration of lack of synergism in the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 PIP products.

DATA REVIEW RECORD:

Active Ingredients: 1) Plant-Incorporated Protectant (PIP) *Bacillus thuringiensis* Cry1Ab protein as expressed in Event Bt11 and the genetic material (*via* elements pZ01502) necessary for its production [OECD Unique ID. SYN-BTØ11-1],

2) PIP *Bt* Vip3Aa20 protein as expressed in Event MIR162 and the genetic material (*via* elements pZNOV1300) necessary for its production [OECD Unique ID. SYN-IR162-4], and/or

3) PIP *Bt* Modified Cry3A protein as expressed in Event MIR604 and the genetic material (*via* elements pZM26) necessary for its production [OECD Unique ID. SYN-IR6Ø4-8]

Product Names: *Agrisure* ™ 2100, Bt11 x MIR162 hybrid [EPA Reg. No. 67979-RE] and *Agrisure* ™ 3100, Bt11 x MIR162 x MIR604 hybrid [EPA Reg. No. 67979-RG]

Company Name: Syngenta Seeds, Inc. – Field Corps- NAFTA

ID No: 67979

Chemical Number: 006461, 006599, and/or 006509

Decision Number: 379488 and 379490

DP Barcode: 345909 and 345912

MRID No:

471374-01	**Comparative Southern Analysis of Bt11 x MIR162 Maize Hybrid with the Individual Events Bt11 Maize and MIR162 Maize.**
471374-02	**Comparison of Transgenic Protein Expression in Event Bt11, Event MIR162 and Stacked Bt11 x MIR162 Maize (corn) Hybrids**
471372-01	**Comparative Southern Analysis of Bt11 x MIR162 x MIR604 Maize Hybrid with the Individual Events Bt11 Maize, MIR162 Maize, and MIR604 Maize.**
471372-02	**Comparison of Transgenic Protein Expression in Event Bt11, Event MIR162, and MIR604 and Stacked Bt11 x MIR162 x MIR604 Maize (corn) Hybrids**
471372-11	**Characterization of Trypsinized Cry1Ab Test Substance TRYCRY1AB-0105**

BACKGROUND:

Syngenta Seeds, Inc. – Field Corps- NAFTA submitted an application and is seeking Sec. 3 Registration for two PIP stacked products- Bt11 x MIR162 maize hybrid and Bt11 x MIR162 x MIR604 maize hybrid, crossed via traditional breeding methods, with the *Bacillus thuringiensis* derived PIP Corn Events: Bt11, MIR162, and MIR604.

In 1996, EPA granted a registration for the *Bt* subsp. *kurstaki* strain HD-1 Cry1Ab protein and the genetic material necessary for its production in Event MON 810 corn (EPA Reg. No. 524-489). The Agency concluded that there were no adverse effects on human health from the use of the Cry1Ab protein expressed in corn. Therefore, an exemption from the requirement for a food tolerance was established when Cry1Ab protein is used as a plant-incorporated protectant [40CFR §174.511]. Syngenta bridged data from MON810 and provided additional product characterization data to register Cry1Ab expressed in Event Bt11 corn. The PAT protein is also co-expressed with Cry1Ab in Bt11 corn, as a PIP inert ingredient, and granted an exemption from the requirement of a tolerance in all plant raw agricultural commodities [40 CFR 174.522].

The product characterization, toxicological, and allergenicity data supporting the registration of *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production in corn (plasmid vector pZO1502), which includes the submitted study titles, conclusions, and their MRID numbers are found in the 2001 *Bt* Crops Reassessment. EPA determined that the human health data previously submitted for Cry1Ab produced in MON810 is applicable to the Cry1Ab produced in Event Bt11 (US EPA, 2001).

Syngenta is currently seeking a Sec. 3 registration for MIR162 maize, produced by *Agrobacterium*-mediated transformation using elements of a vector (pNOV1300) containing a variant of the *vip3Aa1* gene, which was isolated from *Bt* strain AB88. This gene encodes a vegetatative insecticidal protein Vip3Aa20 that is highly toxic to several Lepidopteran pests of maize. This protein is 99.8% identical to another Vip3Aa variant, Vip3Aa19 protein expressed in COT102 cotton, another PIP product that was registered by the EPA. The product characterization, toxicological, and allergenicity data supporting the registration of *Bt* Vip3Aa19 protein expressed in COT102 and the genetic material necessary for its production in cotton, which includes the submitted study titles, conclusions, and their MRID numbers are found in the VipCot BRAD (US EPA, 2008). The Agency established a permanent exemption from the requirement of a tolerance for Vip3Aa proteins and the genetic material necessary for its production in cotton and corn [40 CFR 174.505], as part of the Sec. 3 registration of Event COT102.

Event COT102 cotton and Event MIR162 maize also contain the *manA* gene from *Escherichia coli*, which encodes the selectable marker, phosphomannose isomerase (PMI). An existing permanent exemption from the requirement of a tolerance was established for PMI in all crops when used as a PIP inert ingredient [40 CFR 174.527]. The product characterization, toxicological, and allergenicity data to support the registration of *Bt* Vip3Aa20 protein and the genetic material necessary for its production in MIR162 corn were reviewed by the Agency and are pending registration [see EPA memorandum: from I. Barsoum, Ph.D., through J. Kough, Ph,D., to J. Kausch, dated September 11, 2008].

The other protein that is expressed in the combined trait PIP product- Bt11 x MIR162 x MIR604 is modified Cry3A as expressed in Event MIR604. Syngenta's Event MIR604 corn plants were a result of a corn plant transformation with the synthetic modified *cry3A* gene, which provides resistance to western corn rootworm and northern corn rootworm. The Agency established a permanent exemption from the requirement of a tolerance for Modified Cry3A protein and the genetic material necessary for their production in corn [40 CFR 174.505]. In addition, EPA issued a Sec. 3 Registration for *Bt* mCry3A protein and the genetic material necessary for their production (via plasmid pZM26) in Event MIR604 corn. The product characterization, toxicological, and allergenicity data supporting the registration of *Bacillus thuringiensis* mCry3A protein and the genetic material necessary for its production in corn (plasmid vector pZM26), which includes the submitted study titles, conclusions, and their MRID numbers are found in the mCry3A BRAD (US EPA, 2007).

In this report, Syngenta Seeds, Inc. has submitted data in support for two FIFRA Section 3 registrations- Bt11 x MIR162 hybrid, expressing containing *Bt* Cry1Ab and Vip3Aa in conjunction with the Bt11 x MIR162 x MIR604 hybrid, containing *Bt* Cry1Ab, Vip3Aa, and mCry3A proteins and the genetic material necessary for its production, respectively. The confirmation of molecular identity and protein expression levels for Bt11 x MIR162 corn hybrid and Bt11 x MIR162 x MIR604 corn hybrid are presented in this report.

The Bt11 x MIR162 hybrid cross combines Events Bt11 and MIR162 by conventional breeding and are expected to produce all four transgenic proteins coded by the individual events. The Bt11 x MIR162 x MIR604 hybrid cross combines Events Bt11, MIR162, and MIR604 by conventional breeding and are expected to produce all six transgenic proteins coded by the individual events. These proteins are known to act individually to affect a typical midgut pathology in susceptible insects like previously studied *Bt* delta-endotoxins (Lee, *et al.* 2003). Thus, no synergistic action or interaction of these proteins is known or expected to occur. Therefore, the existing exemptions from the requirement of a food tolerance for Cry1Ab, Vip3Aa20, mCry3A insecticidal proteins, as well as PAT and PMI inert proteins, can be amended to support the addition of the Bt11 x MIR162 hybrid and the Bt11 x MIR162 x MIR604 corn hybrid, when used as plant-incorporated protectants, respectively.

RECOMMENDATION: The data submitted for the comparison of transgenic protein expression levels and the Southern Analyses in Events Bt11, MIR162, MIR604 and the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 hybrids are classified as **ACCEPTABLE.** However, the data to support the characterization of the Cry1Ab test material, TRYCRY1AB-0105, is classified as **SUPPLEMENTAL**. The registrant should submit the mass spectra as visual confirmation of the molecular weight for the TRYCRY1AB-0105 and conduct a direct and concurrent comparison of the biochemical and functional characteristics of a purified plant-expressed protein extracted from Bt11 maize leaves to the TRYCRY1AB-0105 test substance. These data can be used to determine the equivalence between the plant- and microbial-produced Cry1Ab proteins in support for utilizing the TRYCRY1AB-0105, as a test material in the non-target organism studies for demonstration of lack of synergism in the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 PIP products.

SUMMARY OF DATA SUBMITTED:

Summaries of each review supporting the product characterization of these products are provided below.

MRID No. 471374-01 **Comparative Southern Analysis of Bt11 x MIR162 hybrid with the Individual Events Bt11 Maize and MIR162 Maize**

The purpose of this study was to use Southern blot analysis to confirm the presence of the *cry1Ab* and *pat* genes from the parental Event Bt11 and *vip3Aa20* and *pmi* genes from parental Event MIR162 in the hybrid Bt11 x MIR162 in a predictable manner. For the *cry1Ab*-specific probe, the *Nde*I, *Sph*I, and *Bgl*II + *Eco*RI digests of Bt11 DNA resulted in a single hybridization band of >4.4 kb, >4.5 kb, and ~4.7 kb, respectively, in both Event Bt11 and Bt11 x MIR16, indicating the presence of the *cry1Ab* gene. Likewise, for the *pat*-specific probe, the *Nde*I, *Sph*I, and *Bgl*II + *Eco*RI digests of Bt11 DNA resulted in a single hybridization band of >1.7 kb, >4.5 kb, and ~4.7 kb, respectively, in both Event Bt11 and Bt11 x MIR162 hybrid, indicating the presence of the *pat* gene. The MIR162 DNA digested with each restriction enzyme was negative because this event does not contain the *cry1Ab* or *pat* genes.

For the *vip3Aa19*-specific probe, the *Kpn*I, *Eco*RV, and *Nco*I digests of MIR162 DNA resulted in a single hybridization band of >4.7 kb, >6.9 kb, and ~4.6 kb, respectively, in both Event MIR162 and the Bt11 x MIR162 hybrid, indicating the presence of the *vip3Aa20* gene. Likewise, for the *pmi*-specific probe, the *Kpn*I, *Bam*HI, and *Hin*dIII + *Xma*I digests of MIR162 DNA resulted in a single hybridization band of >3.6 kb, >1.6 kb, and ~8.1 kb, respectively, in both Event MIR162 and the Bt11 x MIR162 hybrid, indicating the presence of the *pmi* gene. The Bt11 DNA digested with each restriction enzyme was negative because this event does not contain the *vip3Aa20* and *pmi* genes.

Therefore, the predicted DNA hybridization patterns were retained and stability of the transgenic locus from parent to progeny was demonstrated.

CLASSIFICATION: ACCEPTABLE

MRID No. 471374-02 **Comparison of Transgenic Protein Expression in Event Bt11, Event MIR162 and Stacked Bt11 x MIR162 Maize (corn) Hybrids**

The purpose of this study was to compare expression of the four transgenic proteins (Cry1Ab, Vip3Aa20, PAT, and PMI) in a Bt11 x MIR162 maize (field corn) hybrid with expression in corresponding near-isogenic hybrids derived from the individual transformation events. Four plants per each parental event (Bt11 and MIR162) and the stacked hybrid (Bt11 X MIR162) were collected at different developmental stages from each of five replicate planted blocks of maize. Plant tissue extracts from leaves, roots, pollen, and whole-plant samples at the anthesis stage and kernel samples at the physiological maturity stage were analyzed for each of the transgenic proteins from the appropriate hybrids via enzyme-linked immunosorbent assay (ELISA).

The average protein concentrations of Cry1Ab protein in Bt11 corn were measured in comparison to the stacked Bt11 x MIR162 hybrid of each plant tissue. Tissues analyzed for Cry1Ab protein included: leaves (141.7 to 154.2 µg/g dw), roots (12.8 to 11.9 µg/g dw), pollen (0.636 to 0.858 µg/g dw), kernels (6.94 to 6.79 µg/g dw) and the whole plant (19.6 to 17.8 µg/g dw). The average protein concentrations of PAT protein in Bt11 corn were measure in comparison to the stacked Bt11 x MIR162 hybrid of each plant tissue. Tissues analyzed for PAT protein included: leaves (0.657 to 0.629 µg/g dw), roots (0.580 to 0.403 µg/g dw), pollen (below LOD), kernels (below LOD) and the whole plant (0.872 to 0.751 µg/g dw).

The average protein concentrations of Vip3Aa20 protein in MIR162 corn were measured in comparison to the stacked Bt11 x MIR162 hybrid of each plant tissue. Tissues analyzed for Vip3Aa20 protein included: leaves (185.0 to 191.6 µg/g dw), roots (32.0 to 28.4 µg/g dw), pollen (107.6 to 157.0 µg/g dw), kernels (83.8 µg/g dw for both) and the whole plant (80.4 to 79.0 µg/g dw). The average protein concentrations of PMI protein in MIR162 corn were measured in comparison to the stacked Bt11 x MIR162 hybrid of each plant tissue. Tissues analyzed for PMI protein included: leaves (6.74 to 7.44 µg/g dw), roots (2.03to 2.15 µg/g dw), pollen (4.62 to 4.79 µg/g dw), kernels (1.84 to 1.77 µg/g dw) and the whole plant (3.94 µg/g dw for both).

Overall, concentrations of Cry1Ab, Vip3Aa20, PAT and PMI protein levels were found comparable and all control tissues were negative for the expression of Cry1Ab, Vip3Aa20, PAT and PMI proteins. Therefore, transgenic protein expression in the Bt11 x MIR162 hybrid are not substantially different from that of the hybrids derived from the individual Bt11 and MIR162 transformation events.

CLASSIFICATION: ACCEPTABLE

MRID No. 471372-01 **Comparative Southern Analysis of Bt11 x MIR162 x MIR604 Maize Hybrid with the Individual Events Bt11 Maize, MIR162 Maize, and MIR604 Maize.**

The purpose of this study was to use Southern blot analysis to confirm the presence of the *cry1Ab* and *pat* genes from the parental Event Bt11, *vip3Aa20* and *pmi* genes from parental Event MIR162, and *mcry3A* and *pmi* genes from parental Event MIR604 in the hybrid Bt11 x MIR162 x MIR604 in a predictable manner.

For the *cry1Ab*-specific probe, the *Nde*I, *Sph*I, and *Bgl*II + *Eco*RI digests of Bt11 DNA resulted in a single hybridization band of >4.4 kb, >4.5 kb, and ~4.7 kb, respectively, in Event Bt11 and the Bt11 x MIR162 x MIR604 hybrid, thus, indicating the presence of the *cry1Ab* gene. Likewise, for the *pat*-specific probe, the *Nde*I, *Sph*I, and *Bgl*II + *Eco*RI digests of Bt11 DNA resulted in a single hybridization band of >1.7 kb, >4.5 kb, and ~4.7 kb, respectively, in Event Bt11 and the Bt11 x MIR162 x MIR604 hybrid, thus, indicating the presence of the *pat* gene. The MIR162 DNA and MIR604 DNA digested with each restriction enzyme were negative because these events do not contain the *cry1Ab* or *pat* genes.

For the *vip3Aa19*-specific probe[1], the *Kpn*I, *Eco*RV, and *Bam*HI digests of MIR162 DNA resulted in a single hybridization band of >4.7 kb, >6.9 kb, and ~4.6 kb, respectively, in Event MIR162 and the Bt11 x MIR162 x MIR604 hybrid, thus, indicating the presence of the *vip3Aa20* gene. No banding was present in the either the Bt11 DNA or MIR 604 DNA, because neither event contains the *vip3Aa20* gene.

For the *pmi*-specific probe, the *Kpn*I, *Bam*HI, and *Hin*dIII + *Xma*I digests of MIR162 DNA resulted in a single hybridization band of >3.6 kb, >1.6 kb, and ~8.1 kb, respectively, in Event MIR162, Event MIR604, and the Bt11 x MIR162 x MIR604 hybrid, thus, indicating the presence of the *pmi* gene. The Bt11 DNA digested with each restriction enzyme was negative because this event does not contain the *pmi* gene.

For the *mcry3A*- specific probe, the *Kpn*I, *Eco*RV, and *Asc*I + *Xma*I digests of MIR604 DNA resulted in a single hybridization band of >4.8 kb, >7.0 kb, and ~8.2 kb, respectively, in both Event MIR604 and the stacked Bt11 x MIR162 x MIR604 hybrid, indicating the presence of the *mcry3A* gene. The Bt11 DNA digested with each restriction enzyme was negative because this event does not contain the *mcry3A* gene.

Therefore, the predicted DNA hybridization patterns were retained and stability of the transgenic locus from parent to progeny was demonstrated.

CLASSIFICATION: ACCEPTABLE

MRID No. 471372-02 **Comparison of Transgenic Protein Expression in Event Bt11, Event MIR162, Event MIR604 and Stacked Bt11 x MIR162 x MIR604 Maize (corn) Hybrids**

The purpose of this study was to compare expression of the each transgenic proteins (Cry1Ab, PAT, Vip3Aa20, PMI, and mCry3A) in a Bt11 x MIR162 x MIR604 maize (field corn) hybrid with expression in corresponding near-isogenic hybrids derived from the individual transformation events. Four plants per maize event (Bt11, MIR162, and MIR604) and hybrid (Bt11 x MIR162 x MIR604) were collected at different developmental stages from each of five replicate planted blocks of maize. Plant tissue extracts from leaves, roots, pollen, and whole-plant samples at the anthesis stage and kernel samples at the physiological maturity stage were analyzed for each of the transgenic proteins from the appropriate hybrids via ELISA.

The average protein concentrations of Cry1Ab protein in Bt11 corn were measured in comparison to the stacked Bt11 x MIR162 x MIR604 hybrid of each plant tissue. Tissues analyzed for Cry1Ab included: leaves (92.7 to 88.4 µg/g dw), roots (11.5 to 11.3 µg/g dw), pollen (0.0764 to 0.0801 µg/g dw), kernels (1.78 to 1.57 µg/g dw) and the whole plant (15.9 to 15.2 µg/g dw). The average protein concentrations of PAT protein in Bt11 corn were measured in comparison to the stacked Bt11 x MIR162 x MIR604 hybrid of each plant tissue. Tissues analyzed for PAT protein included:

[1] A *vip3Aa19*- probe was used for the *vip3Aa20* analysis. The nucleotide sequences of *vip3Aa19* and *vip3Aa20* differ by 2 amino acids and are 99.9% identical, which should not affect the ability of the *vip3Aa19*- specific probe to hybridize to the *vip3Aa20* sequence present in MIR162 corn or its associated stacks.

leaves (0.596 to 0.603 µg/g dw), roots (0.905 to 0.739 µg/g dw), pollen (below LOD), kernels (below LOD) and the whole plant (0.912 to 0.873 µg/g dw).

The average protein concentrations of Vip3Aa20 protein in MIR162 corn were measured in comparison to the stacked Bt11 x MIR162 x MIR604 hybrid of each plant tissue. Tissues analyzed for Vip3Aa20 included: leaves (165.6 to 159.7 µg/g dw), roots (52.1 to 53.1 µg/g dw), pollen (97.2 to 85.4 µg/g dw), kernels (123.8 to 140.1 µg/g dw) and the whole plant (73.0 to 72.6 µg/g dw). The average protein concentrations of PMI protein in MIR162 corn were measured in comparison to the stacked Bt11 x MIR162 x MIR604 hybrid of each plant tissue. Tissues analyzed for PMI protein included: leaves (7.72 to 16.3 µg/g dw), roots (2.58 to 5.37 µg/g dw), pollen (5.07 to 48.1 µg/g dw), kernels (2.48 to 5.18 µg/g dw) and the whole plant (3.87 to 8.54 µg/g dw).

The average protein concentrations of mCry3A protein in MIR604 corn were measured in comparison to the stacked Bt11 x MIR162 x MIR604 hybrid of each plant tissue. Tissues analyzed for mCry3A protein included: leaves (35.8 to 34.0 µg/g dw), roots (22.6 to 25.4 µg/g dw), pollen (below LOD), kernels (0.717 to 0.620 µg/g dw) and the whole plant (18.1 to 16.2 µg/g dw). The average protein concentrations of PMI protein in MIR604 corn were measured in comparison to the stacked Bt11 x MIR162 x MIR604 hybrid of each plant tissue. Tissues analyzed for PMI included: leaves (5.13 to 10.0 µg/g dw), roots (2.41 to 4.08 µg/g dw), pollen (43.3 to 50.4 µg/g dw), kernels (2.33 to 4.74 µg/g dw) and the whole plant (4.37 to 7.20 µg/g dw).

Overall, concentrations of Cry1Ab, Vip3Aa20, mCry3A, and PAT protein levels were found comparable and all control tissues were negative for the expression of Cry1Ab, Vip3Aa20, and PAT proteins. Mean total PMI (PMI + MIR604 PMI) concentrations were consistently higher, as expected, in the tissues of the Bt11 x MIR162 x MIR604 hybrid as compared to PMI concentrations in the MIR162 hybrid or MIR604 PMI concentrations in Event MIR604. This reflected the inheritance of both PMI and MIR604 PMI in the Bt11 x MIR162 x MIR604 hybrid. Generally, the mean total PMI concentrations in the stacked hybrid was approximately double that in the individual MIR162 and MIR604 parental events. This effect is most likely due to the two sources of the *pmi* gene from the MIR162 and MIR604 parental events. Therefore, transgenic protein expression in the Bt11 x MIR162 x MIR604 hybrid are not substantially different from that of the hybrids derived from the individual Bt11, MIR162, and MIR604 transformation events.

CLASSIFICATION: ACCEPTABLE

MRID No. 471372-11 **Characterization of Trypsinized Cry1Ab Test Substance TRYCRY1AB-0105**

The purpose of this study was to characterize test substance TRYCRY1AB-0105, containing the *ca.* 66 kDa truncated form of the full length (*ca.* 130 kDa) Cry1Ab insecticidal protein. The identity of test substance TRYCRY1AB-0105 was evaluated using SDS-PAGE, Western blot analysis, mass analysis, peptide mapping, N-terminal sequence analysis, an insecticidal bioassay, and analysis of its lipopolysaccharide (endotoxin) content.

The test substance purity was calculated as 127 µg Cry1Ab/mL TRYCRY1AB-0105 or 74.1% Cry1Ab protein/total protein. Protein levels were determined by measuring sample absorption at 280 nm (A_{280} method) in conjunction with densitometry data after electrophoretic separation.

Western blot analysis of the test substance showed a dominant immunoreactive band corresponding to the predicted molecular weight of Cry1Ab of *ca.* 66 kDa. Total mass analysis of the Cry1Ab in test substance TRYCRY1AB-0105 found two predominant CrylAb species, with molecular weights of 66.3 and 65.8 kDa. In addition, two putative CrylAb breakdown fragments corresponding to *ca.* 39 kDa and 27 kDa were present. The 65.8 and 66.3 kDa CrylAb protein species both contain the biologically active portion of the insecticidal CrylAb protein and, therefore, were considered throughout the study as active CrylAb protein and were included in the purity estimate of CrylAb protein in test substance TRYCRY1AB-0105.

N-terminal sequencing of Cry1Ab in test substance TRYCRY1AB-0105 confirmed that the first 10 amino acids of the protein corresponded to the predicted N-terminal sequence of Cry1Ab. Peptide mapping of the Cry1Ab in test substance TRYCRY1AB-0105, representing 26% coverage of the Cry1Ab protein, gave peptide spectra identical to that of the known sequence of trypsinized Cry1Ab. TRYCRY1AB-0105 was also evaluated for lipopolysaccharide concentration and found to contain 4.8 ng endotoxin/mL test substance TRYCRY1AB-0105. Lastly, the CrylAb bioactivity against the European Corn Borer was confirmed. The 96-hour LC_{50} was 6.2 ng Cry1Ab/cm^2 diet surface (95% confidence interval: 3.8 – 8.9 ng/cm^2) against first instar larvae of the European corn borer.

EPA Reviewer's Comments:

EPA previously notified Syngenta of data deficiencies in the product characterization study for the bacterial-expressed test substance, TRYCRY1AB-0105, in a letter dated October 30, 2007 [from Dr. S. Reilly, Branch Chief of BPPD in EPA's Office of Pesticide Programs, to Mr. E. Dunder, Regulatory Affairs Manager of Syngenta Seeds, Inc.]. In a letter dated January 8, 2008 [from Mr. S. Huber of Syngenta Biotechnology, Inc. to Dr. S. Reilly of EPA's Office of Pesticide Programs], Syngenta responded to EPA's questions (see the DER for MRID No. 471372-11 for details). Syngenta concluded that the purpose of submitting the characterization of the Cry1Ab test material was to confirm the identity of the trypsinized Cry1Ab of the microbial-expressed test substance, TRYCRY1AB-0105, and not to demonstrate equivalence (via SDS-PAGE, Western blot analysis, mass analysis, peptide mapping, N-terminal sequence analysis, and a susceptible insect bioassay). Syngenta indicated that their previous supply of bacterial-expressed test substance was exhausted, therefore, no subsequent analyses were performed to confirm the equivalence between the original and new bacterial-expressed test substance.

Syngenta also confirmed that the test substance TRYCRY1AB-0105 is utilized in only two ecotoxicity studies. All other toxicity data that used the Cry1Ab test substance derived from *Bt* subspecies *kurstaki* strain HD-1 are applicable to the stacked PIP products because the equivalence was previously determined for the test materials used in those tests.

However, for every new microbial-expressed test substance that is used as a surrogate test material for a plant-expressed protein, the equivalence between the surrogate form and that expressed in the plant must be demonstrated in order to justify its use in the toxicity studies. In this case, the TRYCRY1AB-0105 test material was used in two non-target organism studies that were submitted to support the lack of synergism when Cry1Ab, Vip3Aa20, and mCry3A are combined in the stacked PIP products- Bt11 x MIR162 and Bt11 x MIR162 x MIR604. While the identity and characterization of the test material is established for TRYCRY1AB-0105, there remains no direct

comparison of the identity of the microbial-expressed Cry1Ab test substance to the Cry1Ab protein expressed in Event Bt11 corn. Therefore, the registrant must address the following data discrepancies:

1) The total mass analysis of the test substance TRYCRY1AB-0105 showed two forms of Cry1Ab proteins, with masses of 65.8 and 66.3 kDa, however, the mass spectra demonstrating these peaks were not provided. While these results indicated that the test substance contains the truncated insecticidal portion of Cry1Ab protein (albeit two forms) with the predicted MW of 66 kDa, the registrant should submit the mass spectra as visual confirmation of the molecular weights for the two forms of Cry1Ab for the test substance TRYCRY1AB-0105.

2) In the Western blot results of the Bt11 maize leaf plant extract, the study authors concluded that the presence of intact Cry1Ab was shown as corresponding bands in the Bt11 maize plant extract. However, the biologically active portion of the insecticidal Cry1Ab does not appear to have a corresponding band at the predicted MW of 66 kDa. The closest in mobility was a faint *ca.* 54 kDa band and a *ca.* 39 kDa band with much stronger intensity. In addition, the extract of Bt11 maize leaf was not characterized for its biochemical and functional similarities to demonstrate its equivalence to this microbial-expressed substance. There were few details of the preparation of the plant extract, such as whether the plant extract was purified and lyophilized for purity. Therefore, the registrant should concurrently analyze a purified plant-expressed protein extracted from Bt11 maize for its biochemical and functional characteristics (such as molecular weight analysis and biological activity) as confirmation of its equivalence to the microbial-expressed TRYCRY1AB-0105 protein.

Despite the deficiencies noted, the test substance TRYCRY1AB-0105 is only utilized in two ecotoxicity studies to determine if there is any enhanced toxicity to lady beetles and rove beetles from exposure to the single parental events (Bt11, MIR162, and MIR604) in comparison to the combined PIP products: Bt11 x MIR162 and Bt11 x MIR162 x MIR604 corn hybrids. Another study using pollen from the stacked product Bt11 x MIR162 x MRI604 showed no effects to larval mortality and biomass of the monarch butterfly (*Danaus plexippus*) via dietary exposure in comparison to the parental events (MRID No. 471372-10). All other toxicity studies utilize Bt11 plant tissue as the test material or the data are bridged to the microbial-expressed test substance containing Cry1Ab protein as expressed in Event MON810 (MRID No. 435332-04 and US EPA, 2001). Those data support the food safety assessment for the lack of toxicity or potential for allergenicity in the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 products. Further, a three-year field study conducted on Bt11 x Event Pacha maize hybrid (expressing Cry1Ab and Vip3Aa19 proteins) showed no differences on densities of non-target arthropod communities when compared with an isogenic conventional corn control (Dively *et al.* 2005). The results also showed that biodiversity and community-level responses were not significantly affected by expression of the stacked Vip3Aa19 and Cry1Ab proteins.

In regards to bioactivity, the TRYCRY1AB-0105 was tested against a susceptible pest species (European corn borer) in diet-incorporation bioassays with an LC_{50} of 6.2 ng Cry1Ab /cm^2 (95 % confidence interval of 3.8 – 8.9 ng Cry1Ab /cm^2), with concentrations of $\geq$ 32.9 ng Cry1Ab /cm^2 causing 100 % lethality. In addition, a published study was conducted to establish a baseline of

susceptibility to CrylAb and LC_{50} values for ECB populations exposed to purified CrylAb protein ranged from 2.22 ng /cm^2 to 7.89 ng /cm^2 (Marcon *et al.* 1999). The bioactivity of the microbial produced test substance FLCRY1AB-0103 (containing the full length Cry1Ab insecticidal protein encoded by the synthetic *cry1Ab* gene in a recombinant *E. coli* over-expression system, used in toxicity testing of Event COT67B cotton) against the ECB showed a range of LC_{50} values from 3.7 to 16.5 ng Cry1Ab/ cm^2 (see MRID No 470176-04). Therefore, both of these studies demonstrate very similar ranges of toxicity against the same target pest, thus, equating their functional equivalence.

As previously noted, TRYCRY1AB-0105 was prepared from archived cell paste of *E. coli* over-expressing the full-length Cry1Ab protein (The test substance for FLCry1Ab, FLCRY1AB-0103, contains 1181 amino acids and was originally characterized in Graser, 2005, MRID No. 470176-04). EPA previously determined that Cry1Ab protein produced FLCRY1AB-0103 is a suitable surrogate for Cry1Ab expressed in COT67B cotton based on similar molecular weight, immunorecognition via Western blot, and strong insecticidal activity against ECB (see MRID No. 47017608 in EPA memorandum: from R. Edelstein Ph.D., through J. Kough, Ph.D., to A. Reynolds, M.S., dated Feb.7, 2008 and US EPA, 2008). Since the TRYCRY1AB-0105 test substance is a trypsinized form of the FLCry1Ab and demonstrated identical insecticidal activity with the FLCRY1AB-0103 test substance, the weight-of-evidence supports the use of TRYCRY1AB-0105 as a test material based on similar biological activity, i.e. functional equivalence. Therefore, the effects of the TRYCRY1AB-0105 in other toxicity studies to non-target organisms would be equivalent to exposure to the FLCRY1AB-0103 test substance. In addition, there were two studies on protein interaction between Vip3Aa19 and FLCry1Ab by comparing the larval mortality of observed for the mixed proteins with the predicted responses based on the bioassay of each protein individually. In those reports, EPA determined there was no evidence of either a synergistic or antagonistic interaction between Vip3Aa and FLCry1Ab in cotton bollworm or tobacco budworm. The results demonstrated that the effect of a mixture of Vip3Aa and FLCry1Ab on non-target Lepidoptera can be predicted from the effects of the individual proteins alone (see MRID No. 470176-21 and 470176-22 in EPA memorandum: from A. Waggoner, through Z. Vaituzis, Ph.D., to A. Reynolds, dated May 15, 2008; and US EPA, 2008). Thus, the weight-of-evidence demonstrates that the effects of the TRYCRY1AB-0105 test substance in other toxicity studies to both target and non-target organisms would be at least functionally equivalent to exposure with FLCRY1AB-0103 test substance.

In summary, the total mass analysis showed that the TRYCRY1AB-0105 test substance contained the truncated Cry1Ab protein, with the characteristic 66.3 kDa molecular weight. The TRYCRY1AB-0105 test substance also confirmed the insecticidal activity against European corn borer and the range of mortality and LC_{50} values were similar to previously conducted susceptible insect bioassays. However, there was no direct and concurrent testing in between the purified Cry1Ab protein from Event Bt11 plant tissue and the TRYCRY1AB-0105 microbial-expressed protein. The biochemical equivalence of TRYCRY1AB-0105 and the plant-expressed Cry1Ab protein in Event Bt11 has not been fully demonstrated and should be addressed by the registrant.

CLASSIFICATION: The data in this study is classified as **SUPPLEMENTAL.** The registrant should submit the mass spectra as visual confirmation of the molecular weights for the two forms of Cry1Ab for the test substance TRYCRY1AB-0105 and conduct a direct and concurrent analysis of the biochemical and functional characteristics of a purified plant-expressed protein extracted from

Bt11 maize leaves to the TRYCRY1AB-0105 test substance. These data can be used to determine the equivalence between the plant- and microbial-produced Cry1Ab proteins in support of utilizing the TRYCRY1AB-0105, as a test material in the non-target organism studies for demonstration of lack of synergism in the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 PIP products.

REFERENCES:

Dively, G.P. (2005). Impact of transgenic VIP3A x Cry1Ab lepidopteran-resistant field corn on the nontarget arthropod community. *Environmental Entomology* 34, 1267-1291. MRID 46784601

Lee, M.K., F.S. Walters, 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. *App. Environ. Micro.* 69(8): 4648-4657.

Marcon, P.C.R.G., L. J. Young, K. L. Steffey, and B. D. Siegfried. (1999) Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. J. *Econ. Entomol.* 92: 279 - 285.

US EPA, (2001) "Biopesticides Registration Action Document for the *Bacillus thuringiensis* (*Bt*) Plant-Incorporated Protectants," dated October 15, 2001.

US EPA, (2007) "Biopesticides Registration Action Document for the *Bacillus thuringiensis* (*Bt*) Plant-Incorporated Protectant Modified Cry3A Protein and the Genetic Material Necessary for its Production (via Elements of pZM26) in Event MIR604 Corn SYN-IR6 Ø4-8," dated March, 2007.

US EPA, (2008) Biopesticides Registration Action Document for *Bacillus thuringiensis* modified Cry1Ab (SYN-IR67B-1) and Vip3Aa19 (SYN-IR102-7) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton, Dated June 26, 2008.

DATA EVALUATION RECORD

Maize Events Bt11, MIR162, MIR604, and GA21 and the Stacked Hybrid Bt11 x MIR162 x MIR604 x GA21

STUDY TYPE: Product Identity (OPPTS 885.1100)
MRID 471372-01

Prepared for
Biopesticides and Pollution Prevention Division
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Disclaimer

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Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract number DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer: Gary Sega, Ph.D., Oak Ridge National Laboratory
Secondary Reviewer: Annabel Waggoner, Environmental Protection Specialist, BPPD (AJW)

STUDY TYPE:	Product Identity (OPPTS Guideline No. 885.1100)
MRID NO:	471372-01
DECISION NO:	379490
DP BARCODE:	345912
TEST MATERIAL:	Transgenic maize lines included: Event Bt11, Event MIR162, Event GA21, and Event MIR604; and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21. Leaf tissues from ten plants of each genotype were used for the analyses.
PROJECT STUDY NO:	SSB-112-07
SPONSOR:	Syngenta Seeds, Inc. - Field Crops - NAFTA
TESTING FACILITY:	Syngenta Biotechnology, Inc., Regulatory Science, P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709-2257
TITLE OF REPORT:	Comparative Southern Analyses of Bt11 x MIR162 x MIR604 x GA21 Maize with the Individual Events Bt11 Maize, MIR162 Maize, MIR604 Maize, and GA21 Maize.
AUTHOR:	Mulcahy, K.
STUDY COMPLETED:	May 3, 2007
CONFIDENTIALITY CLAIMS:	None
GOOD LABORATORY PRACTICE:	GLP was not followed, but the study was conducted according to accepted scientific methods and the raw data and study records have been retained.
CONCLUSION:	Molecular analyses (restriction enzyme digests and Southern blots) were performed to compare the integrity of the transgenic inserts in the individual maize lines Event Bt11, Event MIR162, Event MIR604, and GA21 maize with the transgenic inserts in stacked Bt11 x MIR162 x MIR604 x GA21 maize, which was produced by conventional plant breeding techniques. Data from the Southern blot analyses of the individual events Bt11, MIR162, MIR604, GA21 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize

confirmed the integrity of the inserts from the individual events to the stacked Bt11 x MIR162 x MIR604 x GA21 hybrid during conventional breeding. The predicted DNA hybridization patterns from the individual events were confirmed in the stacked hybrid, demonstrating preservation of the integrity of the transgenic insert from each individual event to the stacked hybrid.

CLASSIFICATION: ACCEPTABLE

I. STUDY DESIGN:

Molecular analyses (via restriction enzyme digests and Southern blots) were performed to compare the integrity of the transgenic inserts in various parental lines with the transgenic inserts in the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 maize. The individual parental lines were: Event Bt11, Event MIR162, Event MIR604, and Event GA21 maize.

1. Test materials

Maize Lines:

1) Bt11 maize was generated by transformation of a non-transgenic maize line (NP2673/NP2171) using the transformation vector pZO1502 (Figure 1a) and its *Not*I restriction fragment. Bt11 maize contains the *cry1Ab* transgene that expresses the Cry1Ab protein that confers resistance to certain lepidopteran insect pests. The *cry1Ab* gene was originally cloned from *Bacillus thuringiensis* var. *kurstaki* HD-1. The native coding sequence was truncated at the 3'-end and modified to enhance expression in plants. The amino acid sequence of the truncated Cry1Ab protein is unaltered. Bt11 maize also contains the *pat* gene that encodes the selectable marker, phosphinothricin acetyltransferase (PAT) protein, which confers resistance to herbicides containing glufosinate.

2) MIR162 maize was generated by transformation of a non-transgenic maize line (NP2673/NP2171) using the transformation vector pNOV1300 (Figure 2a). An 8.4 kb T-DNA from pNOV1300 was used to create MIR162 maize. MIR162 maize contains the *vip3Aa20* transgene that differs from the *vip3Aa19* gene present in pNOV1300 shown in Figure 2a by two single nucleotide changes. One of these nucleotide changes resulted in an amino acid change in the encoded protein, while the other was a silent mutation. MIR162 maize also contains the *E. coli pmi* gene encoding the enzyme phosphomannose isomerase (PMI) which catalyzes the isomerization of mannose-6-phosphate to fructose-6-phosphate. The *pmi* gene present in MIR162 differs from the *pmi* gene present in MIR604 by two single nucleotides, which results in two amino acid differences: in MIR604 PMI valine at position 61 is replaced by alanine and glutamine at position 210 is replaced by histidine.

3) MIR604 maize was generated by transformation of a non-transgenic maize line (NP2673/NP2171) using the transformation vector pZM26 (Figure 3a). An 8.4 kb T-DNA from pZM26 was used to create MIR604 maize. MIR604 maize contains the modified *cry3A* gene expressing a modified Cry3A (mCry3A) protein and a MIR604 *pmi* gene that produces

an MIR604 PMI protein that differs by two amino acids from the PMI protein expressed in MIR162.

4) GA21[1] maize was generated by transformation of a non-transgenic maize line (NP2673/NP2171) using the transformation vector pDPG434 (Figure 4a). A 3492 bp fragment generated from a *NotI* restriction digest of pDPG434 containing the *mepsps* gene was used to create GA21 maize. The *mepsps* gene expresses a doubly mutated 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS) protein that confers tolerance to herbicides containing glyphosate.

5) The stacked maize line Bt11 x MIR162 x MIR604 x GA21 was produced by conventional breeding of the individual transgenic maize lines and contains all seven of the transgenic proteins Cry1Ab, PAT, Vip3Aa20, PMI, mCry3A, MIR604 PMI, and mEPSPS.

6) NP2673/NP2171 maize is a near-isogenic, nontransgenic control maize line.

2. Methods

A. Processing of Plant Material

Plants from Bt11, MIR162, MIR604, GA21, stacked Bt11 x MIR162 x MIR604 x GA21, and NP2673/NP2171 non-transgenic control maize were grown under standard greenhouse conditions. Leaves from 10 plants of each maize line were collected and pooled. Genomic DNA was extracted from 8 g of leaves from each line. Prior to pooling, all plants were individually analyzed using TaqMan® PCR to confirm the presence of the appropriate genes for the transgenic lines and the absence of these genes from the nontransgenic, negative control plants.

B. DNA Quantification

DNA concentrations of the samples were measured using the PicoGreen QuantiT (Molecular Probes) technology with a Turner Biosystems TBS-380 Fluorometer. A Lambda DNA standard was used to calibrate the instrument prior to quantitation.

C. Southern Analyses

For each Southern analysis performed, genomic DNA samples from the individual events (Bt11, MIR162, MIR604, and GA21 maize), the stacked hybrid, and the nontransgenic control were analyzed *via* three restriction enzyme digestion strategies. These digests give a characteristic hybridization pattern for each probe. The hybridization patterns of Bt11 maize and the stacked hybrid were compared using *cry1Ab*- and *pat*-specific probes. The hybridization patterns of MIR162 maize and the stacked hybrid were compared using *vip3Aa19*- and *pmi*-specific probes. The hybridization patterns of MIR604 maize and the stacked hybrid were compared using *mcry3A*- and *pmi*-specific probes. The hybridization

[1] Event GA21 expresses mEPSPS, an inert PIP protein used to confer resistance to glyphosate containing herbicides. Although this event is crossed with Bt11, MIR162, and MIR604 events to produce the combined trait stacked product- Bt11x MIR162 x MIR604 x GA21, the data for GA21 is reported in this DER, but not analyzed. This is because Event GA21 does not express an active PIP pesticidal substance, which deems Event GA21 out of EPA's jurisdiction under the U.S. Coordinated Framework for regulation of biotechnology products.

patterns of GA21 maize and the stacked hybrid were compared using a *mepsps*-specific probe. A negative control (nontransgenic maize) was included in each Southern analysis to identify any endogenous maize sequences that might cross-hybridize with the element-specific probes.

Southern analyses were carried out using standard molecular biology techniques. Genomic DNA (7.5 µg) was digested with the appropriate restriction enzymes. Digested DNA was electrophoretically separated on 1% agarose gels, depurinated with 0.25 M HCl for 10 minutes, and denatured with 0.5M NaOH + 1.5M NaCl for 30 minutes. The DNA was then transferred to a Zeta-Probe GT membrane (Bio-Rad) using alkaline transfer and crosslinked to the membrane using UV radiation. Gene-specific, full-length PCR-generated probes were labeled with [α-^{32}P] dCTP by random priming. Radiolabeled probe was incubated with the membranes for a minimum of 3 hours at 65°C, followed by extensive washing and then autoradiography.

Positive hybridization controls for each component were included on each Southern blot. For the Bt11 line, a quantity of plasmid pZO1502, representing one copy equivalent per maize genome, was included as a positive hybridization control for the *cry1Ab*- and *pat*-specific probes and to demonstrate the method sensitivity. (With a genomic sample size of 7.5 µg of DNA applied to the gels this required a spike of 10.2 pg of pZO1502 DNA.) For the MIR162 line, a quantity of plasmid pNOV1300 representing one copy equivalent per maize genome was included to serve as a positive control for the *vip3Aa19*- and *pmi*-specific probes. (With a genomic sample size of 7.5 µg of DNA applied to the gels this required a spike of 20.2 pg of pNOV1300 DNA.) For the MIR604 line, a quantity of plasmid pZM26 representing one copy equivalent per maize genome was included to serve as a positive control for the *mcry3A*- and *pmi*-specific probes. (With a genomic sample size of 7.5 µg of DNA applied to the gels this required a spike of 19.4 pg of pZM26 DNA.) For the GA21 line, a quantity of T-DNA representing one copy equivalent per maize genome was included to serve as a positive control for the *mepsps*-specific probe. (With a genomic sample size of 7.5 µg of DNA applied to the gels this required a spike of 2.84 pg of the GA21 insert.)

II. RESULTS:

A. *Southern analysis:*

cry1Ab-specific probe

Figure 5 shows the Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *cry1Ab*-specific probe. The MIR162, MIR604, and GA21 DNA digested with *Nde*I in lanes 4, 5, and 6, respectively, show no band, as expected, since these maize lines do not contain the *cry1Ab* gene. The Bt11 DNA, which contains the *cry1Ab* gene, when digested with *Nde*I (lane 3) shows a band >4.4 kb, as expected, based on the restriction site map for pZO1502 shown in Figure 1b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Nde*I in lane 7 also shows the same band (>4.4 kb), indicating that the stacked maize line also contains the *cry1Ab* gene.

The MIR162, MIR604, and GA21 DNA digested with *Sph*I in lanes 10, 11, and 12, respectively, show no band, as expected, since these maize lines do not contain the *cry1Ab* gene. The Bt11 DNA, which contains the *cry1Ab* gene, when digested with *Sph*I (lane 9) shows a band >4.5 kb, as expected, based on the restriction site map for pZO1502 shown in Figure 1b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Sph*I in lane 13 also shows the same band (>4.5 kb), indicating that the stacked maize line also contains the *cry1Ab* gene.

The MIR162, MIR604, and GA21 DNA digested with *Bgl*II + *Eco*RI in lanes 16, 17, and 18, respectively, show no band, as expected, since these maize lines do not contain the *cry1Ab* gene. The Bt11 DNA, which contains the *cry1Ab* gene, when digested with *Bgl*II + *Eco*RI (lane 15) shows a band ~4.7 kb, as expected, based on the restriction site map for pZO1502 shown in Figure 1b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Bgl*II + *Eco*RI in lane 19 also shows the same band (~4.7 kb), indicating that the stacked maize line also contains the *cry1Ab* gene.

Lane 21, containing non-transgenic DNA spiked with 10.2 pg of pZO1502 DNA (equivalent to a single copy of the plasmid in the maize genome) and digested with *Bgl*II + *Eco*RI, also demonstrates that the Southern blot is sensitive enough to detect a single copy of the plasmid. In addition, the intensity of this band is comparable to the bands seen in lanes 15 and 19, verifying that a single copy of the *cry1Ab* gene was present in both the Bt11 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize. The hybridization patterns for Bt11 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize in this Southern analysis were identical, demonstrating that the integrity of the *cry1Ab* cassette was maintained in going from the individual component line to the stack during conventional breeding.

***pat*-specific probe**

Figure 6 shows the Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *pat*-specific probe. The MIR162, MIR604, and GA21 DNA digested with *Nde*I in lanes 4, 5, and 6, respectively, show no band, as expected, since these maize lines do not contain the *pat* gene. The Bt11 DNA, which contains the *pat* gene, when digested with *Nde*I (lane 3) shows a band >1.7 kb, as expected, based on the restriction site map for pZO1502 shown in Figure 1c. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Nde*I in lane 7 also shows the same band (>1.7 kb), indicating that the stacked maize line also contains the *pat* gene.

The MIR162, MIR604, and GA21 DNA digested with *Sph*I in lanes 10, 11, and 12, respectively, show no band, as expected, since these maize lines do not contain the *pat* gene. The Bt11 DNA, which contains the *pat* gene, when digested with *Sph*I (lane 9) shows a band >4.5 kb, as expected, based on the restriction site map for pZO1502 shown in Figure 1c. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Sph*I in lane 13 also shows the same band (>4.5 kb), indicating that the stacked maize line also contains the *pat* gene.

The MIR162, MIR604, and GA21 DNA digested with *Bgl*II + *Eco*RI in lanes 16, 17, and 18, respectively, show no band, as expected, since these maize lines do not contain the *pat* gene. The Bt11 DNA, which contains the *pat* gene, when digested with *Bgl*II + *Eco*RI (lane 15) shows a band ~4.7 kb, as expected, based on the restriction site map for pZO1502 shown in Figure 1c.

The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Bgl*II + *Eco*RI in lane 19 also shows the same band (~4.7 kb), indicating that the stacked maize line also contains the *pat* gene.

Lane 21, containing non-transgenic DNA spiked with 10.2 pg of pZO1502 DNA (equivalent to a single copy of the plasmid in the maize genome), and digested with *Bgl*II + *Eco*RI, also demonstrates that the Southern blot is sensitive enough to detect a single copy of the plasmid. In addition, the intensity of this band is comparable to the bands seen in lanes 15 and 19, verifying that a single copy of the *pat* gene was present in both the Bt11 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize. The hybridization patterns for Bt11 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize in this Southern analysis were identical, demonstrating that the integrity of the *pat* cassette was maintained in going from the individual component line to the stack during conventional breeding.

***vip3Aa19*-specific probe**

Figure 7 shows the Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *vip3Aa19*-specific probe[2]. The Bt11, MIR604, and GA21 DNA digested with *Kpn*I in lanes 3, 5, and 6, respectively, show no band, as expected, since these maize lines do not contain the *vip3Aa20* gene. The MIR162 DNA, which contains the *vip3Aa20* gene, when digested with *Kpn*I (lane 4) shows a band >4.7 kb, as expected, based on the restriction site map for pNOV1300 shown in Figure 2b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Kpn*I in lane 7 also shows the same band (>4.7 kb), indicating that the stacked maize line also contains the *vip3Aa20* gene.

The Bt11, MIR604, and GA21 DNA digested with *Eco*RV in lanes 9, 11, and 12, respectively, show no band, as expected, since these maize lines do not contain the *vip3Aa20* gene. The MIR162 DNA, which contains the *vip3Aa20* gene, when digested with *Eco*RV (lane 10) shows a band >6.9 kb, as expected, based on the restriction site map for pNOV1300 shown in Figure 2b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Eco*RV in lane 13 also shows the same band (>6.9 kb), indicating that the stacked maize line also contains the *vip3Aa20* gene.

The Bt11, MIR604, and GA21 DNA digested with *Bam*HI in lanes 15, 17, and 18, respectively, show no band, as expected, since these maize lines do not contain the *vip3Aa20* gene. The MIR162 DNA, which contains the *vip3Aa20* gene, when digested with *Bam*HI (lane 16) shows a band ~4.6 kb, as expected, based on the restriction site map for pNOV1300 shown in Figure 2b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Bam*HI in lane 19 also shows the same band (~4.6 kb), indicating that the stacked maize line also contains the *vip3Aa20* gene.

Lane 21, containing non-transgenic DNA spiked with 20.2 pg of pNOV1300 DNA (equivalent to a single copy of the plasmid in the maize genome) and digested with *Bam*HI, also demonstrates

[2] A *vip3Aa19*- probe was used for the *vip3Aa20* analysis. The nucleotide sequences of *vip3Aa19* and *vip3Aa20* differ by 2 amino acids and are 99.9% identical, which should not affect the ability of the *vip3Aa19*- specific probe to hybridize to the *vip3Aa20* sequence present in MIR162 corn or its associated stacks.

that the Southern blot is sensitive enough to detect a single copy of *vip3Aa19* in the plasmid. In addition, the intensity of this band is comparable to the bands seen in lanes 16 and 19, verifying that a single copy of the *vip3Aa20* gene was present in both the MIR162 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize. The hybridization patterns for Bt11 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize in this Southern analysis were identical, demonstrating that the integrity of the *vip3Aa20* cassette was maintained in going from the individual component line to the stack during conventional breeding.

***mcry3A*-specific probe**

Figure 8 shows the Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *mcry3A*-specific probe. The Bt11, MIR162, and GA21 DNA digested with *Kpn*I in lanes 3, 4, and 6, respectively, show no band, as expected, since these maize lines do not contain the *mcry3A* gene. The MIR604 DNA, which contains the *mcry3A* gene, when digested with *Kpn*I (lane 5) shows a band >4.8 kb, as expected, based on the restriction site map for pZM26 shown in Figure 3b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Kpn*I in lane 7 also shows the same band (>4.8 kb), indicating that the stacked maize line also contains the *mcry3A* gene.

The Bt11, MIR162, and GA21 DNA digested with *Eco*RV in lanes 9, 10, and 12, respectively, show no band, as expected, since these maize lines do not contain the *mcry3A* gene. The MIR604 DNA, which contains the *mcry3A* gene, when digested with *Eco*RV (lane 11) shows a band >7.0 kb, as expected, based on the restriction site map for pZM26 shown in Figure 3b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Eco*RV in lane 13 also shows the same band (>7.0 kb), indicating that the stacked maize line also contains the *mcry3A* gene.

The Bt11, MIR162, and GA21 DNA digested with *Asc*I + *Xma*I in lanes 15, 16, and 18, respectively, show no band, as expected, since these maize lines do not contain the *mcry3A* gene. The MIR604 DNA, which contains the *mcry3A* gene, when digested with *Asc*I + *Xma*I (lane 17) shows a band ~8.2 kb, as expected, based on the restriction site map for pZM26 shown in Figure 3b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Asc*I + *Xma*I in lane 19 also shows the same band (~8.2 kb), indicating that the stacked maize line also contains the *mcry3A* gene.

Lane 21, containing non-transgenic DNA spiked with 19.4 pg of pZM26 DNA (equivalent to a single copy of the plasmid in the maize genome) and digested with *Asc*I + *Xma*I, also demonstrates that the Southern blot is sensitive enough to detect a single copy of the plasmid. In addition, the intensity of this band is comparable to the bands seen in lanes 17 and 19, verifying that a single copy of the *mcry3A* gene was present in both the MIR604 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize. The hybridization patterns for MIR604 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 maize in this Southern analysis were identical, demonstrating that the integrity of the *mcry3A* cassette was maintained in going from the individual component line to the stack during conventional breeding.

***pmi*-specific probes**

Figures 9a and 9b show a side by side comparison of the location of restriction sites and position of the 1176 bp *pmi*-specific probe for MIR162 and MIR604 maize, respectively. An 8.4 kb insert of pNOV1300 was used to create MIR162 maize and an 8.4 kb insert of pZM26 was used to create MIR604 maize. The vertical arrows indicate the sites of restriction digestion. Sizes of the predicted restriction fragments, calculated from the linear map, are indicated.

Figure 10 shows the Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *pmi*-specific probe. The Bt11 and GA21 DNA digested with *Kpn*I in lanes 3, and 6, respectively, show no band, as expected, since these maize lines do not contain the *pmi* gene. The MIR162 DNA, which contains the *pmi* gene, when digested with *Kpn*I (lane 4) shows a band >3.6 kb, as expected, based on the restriction site map of the 8.4 kb insert of pNOV1300 shown in Figure 9a. The MIR604 DNA, which contains the *pmi* gene, when digested with *Kpn*I (lane 5) shows a band >3.6 kb, as expected, based on the restriction site map of the 8.4 kb insert of pZM26 shown in Figure 9b. The bands in lanes 4 and 5 are different sizes because the 8.4 kb inserts from pNOV1300 and pZM26 plasmids used to generate MIR162 and MIR604, respectively, have been inserted at different locations in the maize genome. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Kpn*I in lane 7 shows both of the bands shown in Lanes 4 and 5, indicating that the stacked maize line contains the *pmi* gene from both the MIR162 and MIR604 lines.

The Bt11 and GA21 DNA digested with *BamH*I in lanes 9, and 12, respectively, show no band, as expected, since these maize lines do not contain the *pmi* gene. The MIR162 DNA, which contains the *pmi* gene, when digested with *BamH*I (lane 10) shows a band >1.6 kb, as expected, based on the restriction site map of the 8.4 kb insert of pNOV1300 shown in Figure 9a. The MIR604 DNA, which contains the *pmi* gene, when digested with *BamH*I (lane 11) shows a band >1.6 kb, as expected, based on the restriction site map of the 8.4 kb insert of pZM26 shown in Figure 9b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *BamH*I in lane 13 shows both of the bands shown in Lanes 10 and 11, indicating that the stacked maize line contains the *pmi* gene from both the MIR162 and MIR604 lines.

The Bt11 and GA21 DNA digested with *Hin*dIII + *Xma*I in lanes 15, and 18, respectively, show no band, as expected, since these maize lines do not contain the *pmi* gene. The MIR162 DNA, which contains the *pmi* gene, when digested with *Hin*dIII + *Xma*I (lane 16) shows a band ~8.1 kb, as expected, based on the restriction site map for pNOV1300 shown in Figure 9a. The MIR604 DNA, which contains the *pmi* gene, when digested with *Hin*dIII + *Xma*I (lane 17) shows a band ~8.2 kb, as expected, based on the restriction site map of the 8.4 kb insert of pZM26 shown in Figure 9b. The stacked Bt11 x MIR162 x MIR604 x GA21 maize DNA digested with *Hin*dIII + *Xma*I in lane 19 shows both of the bands shown in Lanes 16 and 17. In this case, the individual bands are not resolved in the stacked hybrid (lane 19), making the band about twice as intense as the separate bands in lane 16 and 17. The data again indicates that the stacked maize line contains the *pmi* gene from both the MIR162 and MIR604 lines.

Lane 21, contains non-transgenic DNA spiked with 19.4 pg of pZM26 DNA (equivalent to a single copy of the plasmid in the maize genome) and digested with *Hin*dIII + *Xma*I; Lane 22,

contains non-transgenic DNA spiked with 20.2 pg of pNOV1300 DNA (equivalent to a single copy of the plasmid in the maize genome) and digested with *Hin*dIII + *Xma*I. Both lanes show a single band at ~8.1- 8.2 kb demonstrating that the Southern blot is sensitive enough to detect single copies of the *pmi*-containing cassettes from pZM26 and pNOV1300 plasmids. In addition, the intensity of these bands is comparable to the bands seen in lanes 16 and 17, verifying that a single copy of the *pmi* gene was present in both the MIR162 and MIR604 lines.

Figure 11 shows the Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *mepsps*-specific probe. All of the transgenic lines as well as the non-transgenic maize show common bands from unknown, endogenous DNA. These common bands appear at different molecular weights, depending on the particular restriction digest. DNA from GA21 and stacked maize, digested with *Hin*dIII (Lanes 6 and 7) produced three unique bands of ~ 3.5, 4.7, and 6.7 kb, corresponding to the multiple copies of the *mepsps* gene present in the GA21 maize. DNA from GA21 and stacked maize, digested with *Sac*I (Lanes 12 and 13) produced two unique bands of ~ 2.1 and 3.5 kb. (The 2.1 kb band could not be seen by the reviewer.) DNA from GA21 and stacked maize, digested with *Sph*I (Lanes 18 and 19) produced three unique bands of ~ 2.1, 3.5, and 30 kb. (The report author puts the last band at ~18.0 kb, but the reviewer could only see a very faint band at 30 kb.) In all digests, the hybridization pattern was identical between GA21 maize and the stacked Bt11 x MIR162 x MIR604 x GA21 hybrid, demonstrating that the integrity of the insert from GA21 containing the *mepsps* gene was maintained in going from the individual component line to the stack during conventional breeding.

III. CONCLUSION:

Data from the Southern analyses of the individual events Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 maize confirmed the integrity of the insert from the individual events to the stacked hybrid during conventional breeding. The predicted DNA hybridization patterns from the individual events were confirmed in the stacked Bt11 x MIR162 x MIR604 x GA21 hybrid, demonstrating preservation of the integrity of the transgenic insert from each individual event to the stacked hybrid.

IV. REVIEWER'S COMMENTS:

The data support the conclusions of the study and no deficiencies were noted.

V. CLASSIFICATION: ACCEPTABLE

Figure 1. Transformation Vector pZO1502 and Map of the T-DNA Region containing the *cry1Ab* and *pat* genes

Figure 1a shows the transformation vector pZO1502 used to generate the Bt11 transgenic maize line. Figures 1b and 1c show maps of the T-DNA region of the transformation vector, indicating the locations of the *Cry1A*- and *pat*-specific probes, respectively.

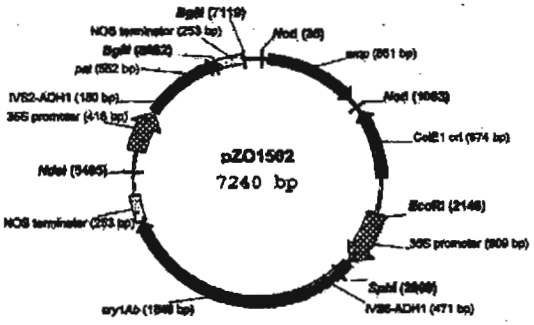


Figure 1a. Plasmid map of pZO1502 indicating the restriction sites used for Southern analysis.

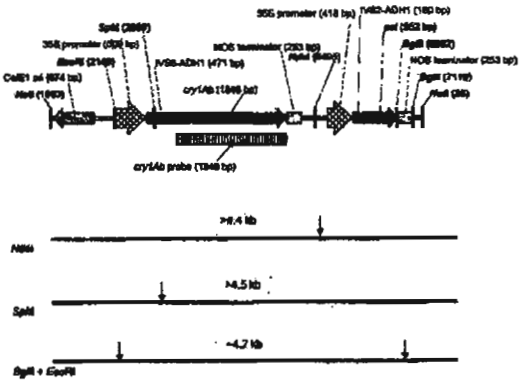
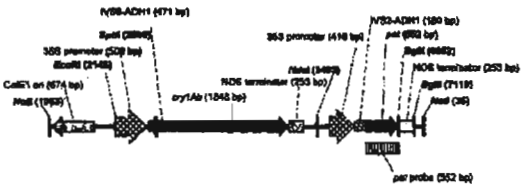


Figure 1b. Location of restriction sites and position of *Cry1Ab*-specific probe.



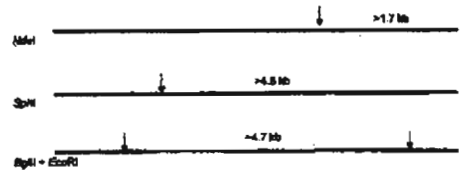


Figure 1c. Location of restriction sites and position of *pat*-specific probe.

from Figure 1, page 27, Figure 7, page 31, and Figure 9, page 34 of MRID 47137201.

Figure 2. Transformation Vector pNOV1300 and Map of the T-DNA Region containing the *vip3Aa19* and *pmi* Genes

Figure 2a shows the transformation vector pNOV1300 used to generate the MIR162 transgenic maize line. Figures 2b and 2c show maps of the T-DNA region of the transformation vector, indicating the locations of the *vip3Aa19*- and *pmi*-specific probes, respectively.

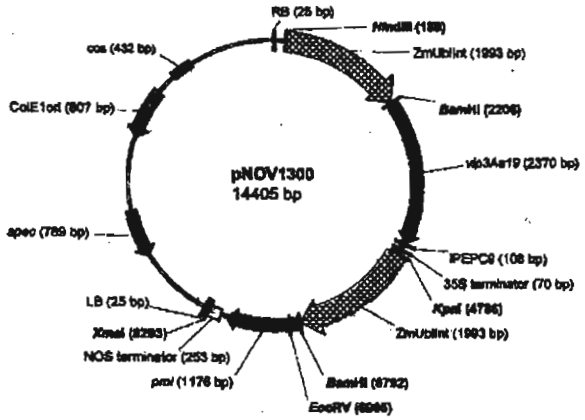


Figure 2a. Plasmid map of pNOV1300 indicating the restriction sites used for Southern analysis.

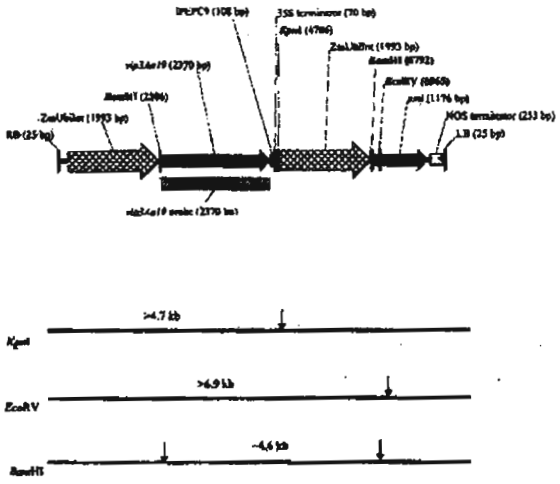


Figure 2b. Location of restriction sites and position of *vip3Aa19*-specific probe.

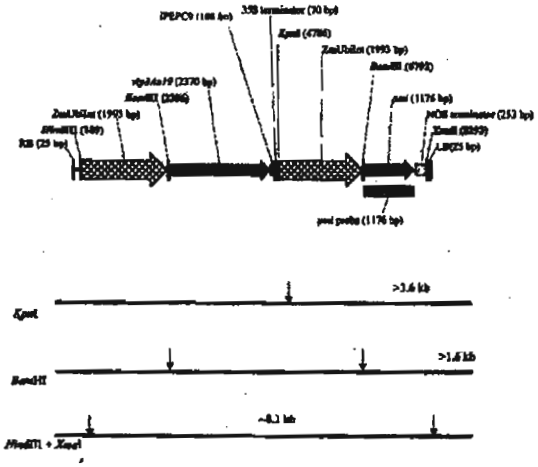


Figure 2c. Location of restriction sites and position of *pmi*-specific probe.

from Figure 2, page 28, Figure 11, page 37, and Figure 15, page 43 of MRID 47137201.

Figure 3. Transformation Vector pZM26 and Map of the T-DNA Region containing the *mcry3A* and *pmi* Genes

Figure 3a shows the transformation vector pZM26 used to generate the MIR604 transgenic maize line. Figures 3b and 3c show maps of the T-DNA region of the transformation vector, indicating the locations of the *mcry3A*- and *pmi*-specific probes, respectively. Note that the caption for figure 3c is incorrect, and should state "Location of restriction sites and position of *pmi*-specific probe."

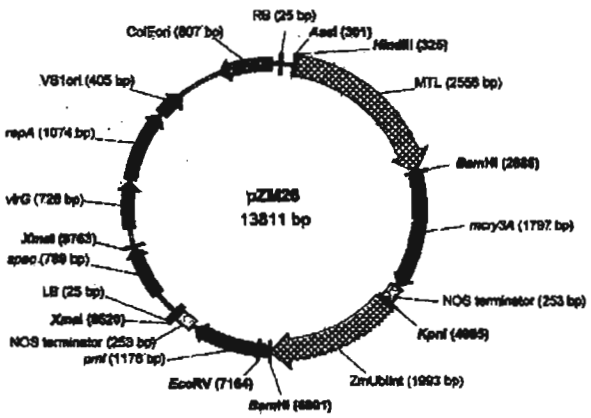


Figure 3a. Plasmid map of pZM26 indicating the restriction sites used for Southern analysis.

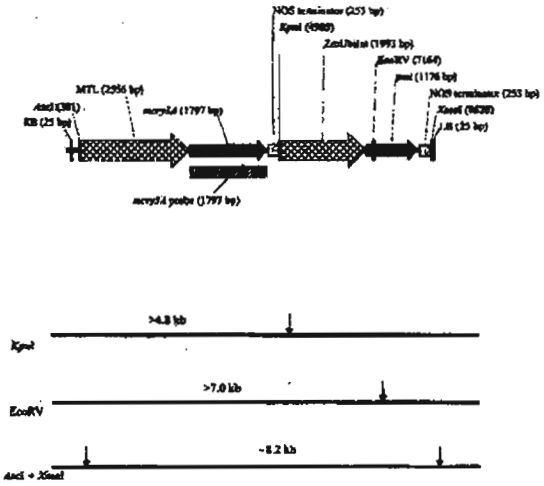


Figure 3b. Location of restriction sites and position of *mcry3A*-specific probe.

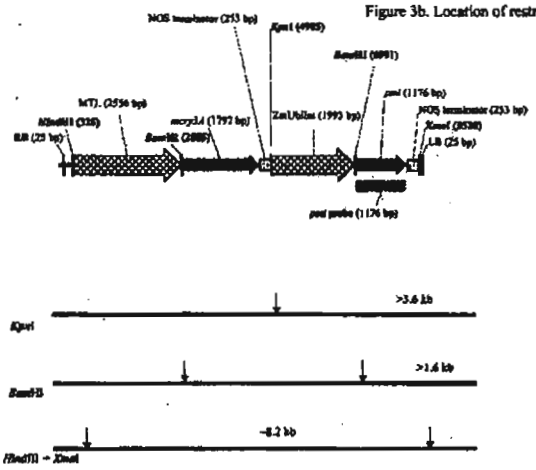


Figure 3c. Location of restriction sites and position of *pat*-specific probe.

from Figure 3, page 29, Figure 13, page 40 and Figure 16, page 44 of MRID 47137201.

Figure 4. *GA21 Maize Transformation Vector*

Figure 4a shows the *Not*I restriction fragment of pDPG434 used to generate the GA21 transgenic maize line. Figures 4b and 4c show the transgenic insert in the GA21 maize, and the 2022 bp DNA fragment containing the *mepsps*-specific probe, respectively.

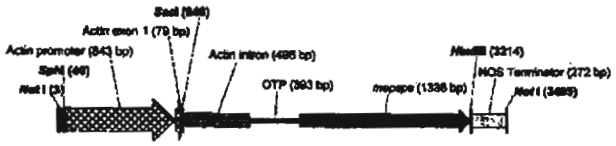


Figure 4a. Map of the original NotI fragment used to create the GA21 maize.

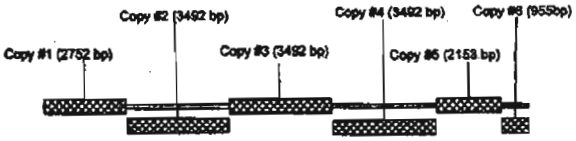


Figure 4b. Location of restriction sites and position of *Cry1Ab*-specific probe.

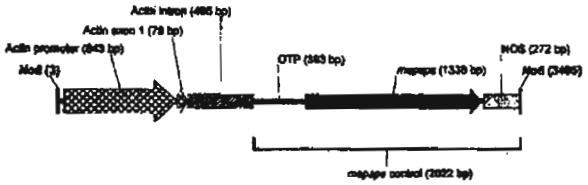
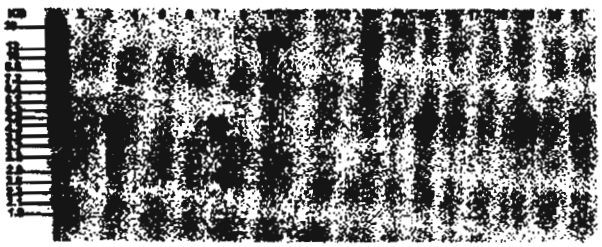


Figure 4c. DNA fragment used as a positve control for GA21 maize.

from Figures 4, 5, and 6, respectively, on page 30 of MRID 47137201.

Figure 5. Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *cry1Ab*-specific probe.

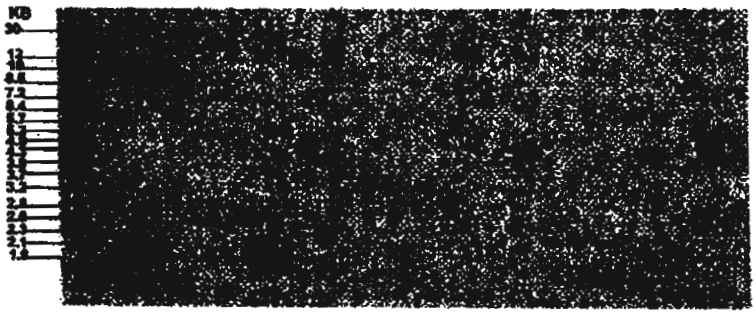


Lane 1: Molecular weight markers.
Lane 2: Blank.
Lane 3: Bt11 DNA digested with *NdeI*.
Lane 4: MIR162 DNA digested with *NdeI*.
Lane 5: MIR604 DNA digested with *NdeI*.
Lane 6: GA21 DNA digested with *NdeI*.
Lane 7: Stacked hybrid digested with *NdeI*.
Lane 8: Non-transgenic control DNA digested with *NdeI*.
Lane 9: Bt11 DNA digested with *SphI*.
Lane 10: MIR162 DNA digested with *SphI*.
Lane 11: MIR604 DNA digested with *SphI*.
Lane 12: GA21 DNA digested with *SphI*.
Lane 13: Stacked hybrid digested with *SphI*.
Lane 14: Non-transgenic control DNA digested with *SphI*.
Lane 15: Bt11 DNA digested with *BglII* + *EcoRI*.
Lane 16: MIR162 DNA digested with *BglII* + *EcoRI*.
Lane 17: MIR604 DNA digested with *BglII* + *EcoRI*.
Lane 18: GA21 DNA digested with *BglII* + *EcoRI*.
Lane 19: Stacked hybrid digested with *BglII* + *EcoRI*.
Lane 20: Non-transgenic control DNA digested with *BglII* + *EcoRI*.
Lane 21: Non-transgenic control DNA spiked with 10.2 pg of pZO1502 DNA, digested with *BglII* + *EcoRI*.

Figure 5. Southern analysis of transgenic Bt11, MIR162, MIR604, and GA21 maize and the stacked hybrid using the *cry1Ab*-specific probe.

from Figure 8, page 33 of MRID 47137201.

Figure 6. Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *pat*-specific probe.

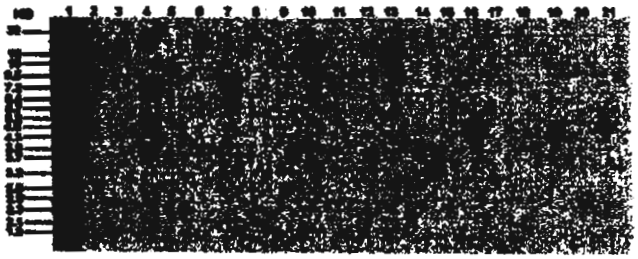


Lane 1: Molecular weight markers.
Lane 2: Blank.
Lane 3: Bt11 DNA digested with *NdeI*.
Lane 4: MIR162 DNA digested with *NdeI*.
Lane 5: MIR604 DNA digested with *NdeI*.
Lane 6: GA21 DNA digested with *NdeI*.
Lane 7: Stacked hybrid digested with *NdeI*.
Lane 8: Non-transgenic control DNA digested with *NdeI*.
Lane 9: Bt11 DNA digested with *SphI*.
Lane 10: MIR162 DNA digested with *SphI*.
Lane 11: MIR604 DNA digested with *SphI*.
Lane 12: GA21 DNA digested with *SphI*.
Lane 13: Stacked hybrid digested with *SphI*.
Lane 14: Non-transgenic control DNA digested with *SphI*.
Lane 15: Bt11 DNA digested with *BglII* + *EcoRI*.
Lane 16: MIR162 DNA digested with *BglII* + *EcoRI*.
Lane 17: MIR604 DNA digested with *BglII* + *EcoRI*.
Lane 18: GA21 DNAdigested with *BglII* + *EcoRI*.
Lane 19: Stacked hybrid digested with *BglII* + *EcoRI*.
Lane 20: Non-transgenic control DNA digested with *BglII* + *EcoRI*.
Lane 21: Non-transgenic control DNA spiked with 10.2 pg of pZO1502 DNA, digested with *BglII* + *EcoRI*.

Figure 6. Southern analysis of transgenic Bt11, MIR162, MIR604, and GA21 maize and the stacked hybrid using the *pat*-specific probe.

from Figure 10, page 36 of MRID 47137201.

Figure 7. Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *vip3Aa19*-specific probe

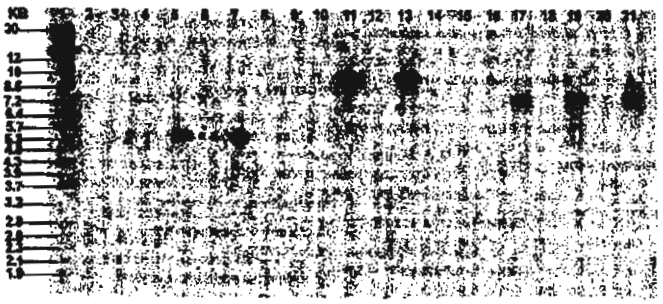


Lane 1: Molecular weight markers.
Lane 2: Blank.
Lane 3: Bt11 DNA digested with *KpnI*.
Lane 4: MIR162 DNA digested with *KpnI*.
Lane 5: MIR04 DNA digested with *KpnI*.
Lane 6: GA21 DNA digested with *KpnI*.
Lane 7: Stacked hybrid digested with *KpnI*.
Lane 8: Non-transgenic control DNA digested with *KpnI*.
Lane 9: Bt11 DNA digested with *EcoRV*.
Lane 10: MIR162 DNA digested with *EcoRV*.
Lane 11: MIR604 DNA digested with *EcoRV*.
Lane 12: GA21 DNA digested with *EcoRV*.
Lane 13: Stacked hybrid digested with *EcoRV*.
Lane 14: Non-transgenic control DNA digested with *EcoRV*.
Lane 15: Bt11 DNA digested with *BamHI*.
Lane 16: MIR162 DNA digested with *BamHI*.
Lane 17: MIR604 DNA digested with *BamHI*.
Lane 18: GA21 DNA digested with *BamHI*.
Lane 19: Stacked hybrid digested with *BamHI*.
Lane 20: Non-transgenic control DNA digested with *BamHI*.
Lane 21: Non-transgenic control DNA spiked with 20.2 pg of pNOV1300 DNA, digested with *BamHI*.

Figure 7. Southern analysis of transgenic Bt11, MIR162, MIR604, and GA21 maize and the stacked hybrid using the *vip3Aa19*-specific probe.

from Figure 12, page 39 of MRID 47137201.

Figure 8. Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *mcry3A*-specific probe.



Lane 1: Molecular weight markers.
Lane 2: Blank.
Lane 3: Bt11 DNA digested with *Kpn*I.
Lane 4: MIR162 DNA digested with *Kpn*I.
Lane 5: MIR604 DNA digested with *Kpn*I.
Lane 6: GA21 DNA digested with *Kpn*I.
Lane 7: Stacked hybrid digested with *Kpn*I.
Lane 8: Non-transgenic control DNA digested with *Kpn*I.
Lane 9: Bt11 DNA digested with *EcoRV*.
Lane 10: MIR162 DNA digested with *EcoRV*.
Lane 11: MIR604 DNA digested with *EcoRV*.
Lane 12: GA21 DNA digested with *EcoRV*.
Lane 13: Stacked hybrid digested with *EcoRV*.
Lane 14: Non-transgenic control DNA digested with *EcoRV*.
Lane 15: Bt11 DNA digested with *Asc*I + *Xma*I.
Lane 16: MIR162 DNA digested with *Asc*I + *Xma*I.
Lane 17: MIR604 DNA digested with *Asc*I + *Xma*I.
Lane 18: GA21 DNA digested with *Asc*I + *Xma*I.
Lane 19: Stacked hybrid digested with *Asc*I + *Xma*I.
Lane 20: Non-transgenic control DNA digested with *Asc*I + *Xma*I.
Lane 21: Non-transgenic control DNA spiked with 19.4 pg of pZM26 DNA, digested with *Asc*I + *Xma*I.

Figure 8. Southern analysis of transgenic Bt11, MIR162, MIR604, and GA21 maize and the stacked hybrid using the m*cry3A*-specific probe.

from Figure 14, page 42 of MRID 47137201.

Figures 9a and 9b. Location of restriction sites and position of the 1176 bp *pmi*-specific probe for MIR162 and MIR604 maize, respectively.

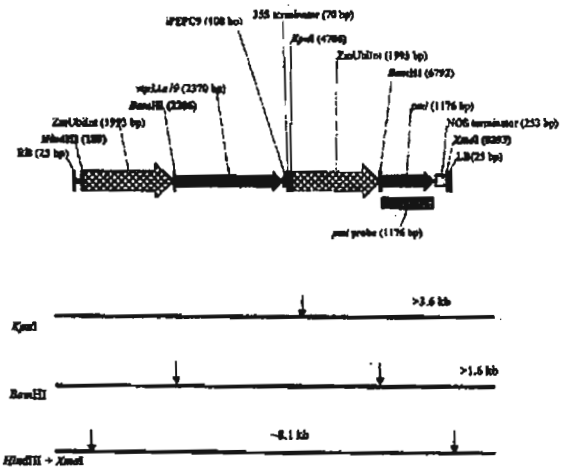


Figure 9a. Location of restriction sites and position of *pmi*-specific probe for MIR162 maize.

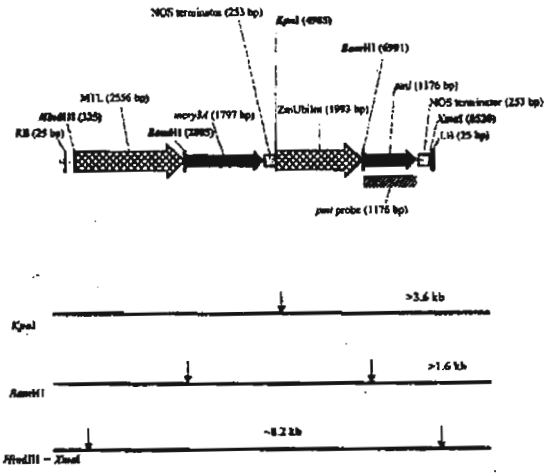
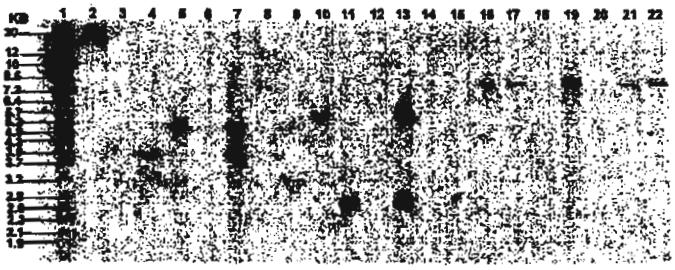


Figure 9b. Location of restriction sites and position of *pmi*-specific probe for MIR604 maize.

from Figure 15, page 43, and Figure 16, page 44, respectively, of MRID 47137201.

Figure 10. Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *pmi*-specific probe.



Lane 1: Molecular weight markers.
Lane 2: Blank.
Lane 3: Bt11 DNA digested with *Kpn*I.
Lane 4: MIR162 DNA digested with *Kpn*I.
Lane 5: MIR04 DNA digested with *Kpn*I.
Lane 6: GA21 DNA digested with *Kpn*I.
Lane 7: Stacked hybrid digested with *Kpn*I.
Lane 8: Non-transgenic control DNA digested with *Kpn*I.
Lane 9: Bt11 DNA digested with *BamH*I.
Lane 10: MIR162 DNA digested with *BamH*I.
Lane 11: MIR604 DNA digested with *BamH*I.
Lane 12: GA21 DNA digested with *BamH*I.
Lane 13: Stacked hybrid digested with *BamH*I.
Lane 14: Non-transgenic control DNA digested with *BamH*I.
Lane 15: Bt11 DNA digested with *Hind*III + *Xma*I.
Lane 16: MIR162 DNA digested with *Hind*III + *Xma*I.
Lane 17: MIR604 DNA digested with *Hind*III + *Xma*I.
Lane 18: GA21 DNA digested with *Hind*III + *Xma*I.
Lane 19: Stacked hybrid digested with *Hind*III + *Xma*I.
Lane 20: Non-transgenic control DNA digested with *Hind*III + *Xma*I.
Lane 21: Non-transgenic control DNA spiked with 19.4 pg of pZM26 DNA, digested with *Hind*III + *Xma*I.
Lane 22: Non-transgenic control DNA spiked with 20.2 pg of pNOV1300 DNA, digested with *Hind*III + *Xma*I.

Figure 10. Southern analysis of transgenic Bt11, MIR162, MIR604, and GA21 maize and the stacked hybrid using the *pmi*-specific probe.

from Figure 17, page 46 of MRID 47137201.

Figure 11. Southern analyses of the individual transgenic maize lines Bt11, MIR162, MIR604, GA21, and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21 using the *mepsps*-specific probe.

Lane 1: Molecular weight markers.
Lane 2: Blank.
Lane 3: Bt11 DNA digested with *Hind*III.
Lane 4: MIR162 DNA digested with *Hind*III.
Lane 5: MIR04 DNA digested with *Hind*III.
Lane 6: GA21 DNA digested with *Hind*III.
Lane 7: Stacked hybrid digested with *Hind*III.
Lane 8: Non-transgenic control DNA digested with *Hind*III.
Lane 9: Bt11 DNA digested with *Sac*I.
Lane 10: MIR162 DNA digested with *Sac*I.
Lane 11: MIR604 DNA digested with *Sac*I.
Lane 12: GA21 DNA digested with *Sac*I.
Lane 13: Stacked hybrid digested with *Sac*I.
Lane 14: Non-transgenic control DNA digested with *Sac*I.
Lane 15: Bt11 DNA digested with *Sph*I.
Lane 16: MIR162 DNA digested with *Sph*I.
Lane 17: MIR604 DNA digested with *Sph*I.
Lane 18: GA21 DNA digested with *Sph*I.
Lane 19: Stacked hybrid digested with *Sph*I.
Lane 20: Non-transgenic control DNA digested with *Sph*I.
Lane 21: Non-transgenic control DNA spiked with 2.84 pg of *mepsps* positive control DNA.

Figure 11. Southern analysis of transgenic Bt11, MIR162, MIR604, and GA21 maize and the stacked hybrid using the *mepsps*-specific probe.

from Figure 19, page 49 of MRID 47137201.

DATA EVALUATION RECORD

Maize Events Bt11, MIR162, MIR604, and GA21 and the Stacked Hybrid Bt11 x MIR162 x MIR604 x GA21

STUDY TYPE: Product Identity (OPPTS 885.1100)
MRID No. 471372-02

Prepared for
Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
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Primary Reviewer:
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Disclaimer

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

Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract number DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer: Gary Sega, Ph.D., Oak Ridge National Laboratory

Secondary Reviewer: Annabel Waggoner, Environmental Protection Specialist, BPPD

STUDY TYPE:	Product Identity (OPPTS Guideline No. 885.1100)
MRID NO:	471372-02
DECISION NO:	379490
DP BARCODE:	345912
TEST MATERIAL:	Transgenic maize lines: Event Bt11, Event MIR162, Event MIR604, and Event GA21; and the stacked hybrid Bt11 x MIR162 x MIR604 x GA21- leaves, roots, pollen, kernels, and whole plants were analyzed.
PROJECT STUDY NO:	Study No. BT162604GA-06-01; Report No. SSB-001-07
SPONSOR:	Syngenta Seeds, Inc. - Field Crops - NAFTA
TESTING FACILITY:	Syngenta Biotechnology, Inc. Regulatory Science, P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709-2257.
TITLE OF REPORT:	Comparison of Transgenic Protein Expression in Event Bt11, Event MIR162, Event MIR604, Event GA21, and Bt11 x MIR162 xMIR604 x GA21 Maize Hybrids
AUTHOR:	McDonald, J.
STUDY COMPLETED:	May 11, 2007
GOOD LABORATORY PRACTICE:	GLP was followed except for the field aspects of the study, the statistical analyses, and the determination of percent purity for the microbially produced PMI reference protein. Also, whorl stages were received, separated and weighed before the protocol was signed, but all the work was performed according to GLP.
CONFIDENTIALITY CLAIMS:	None
CONCLUSION:	The purpose of this study was to use an enzyme-linked immunosorbent assay (ELISA) to compare protein expression in a Bt11 x MIR162 x MIR604 x GA21 stacked maize hybrid (produced by conventional plant breeding), with expression in corresponding, near-isogenic hybrids derived from the individual transformation events: Bt11, MIR162, MIR604, and GA21. Four plants per maize event and hybrid were collected at different developmental stages from each of five replicate planted blocks of maize. Tissues analyzed included leaves, roots, kernels, whole plants, and pollen. While two statistically significant differences (out of 25 statistical analyses) were seen between the concentrations of the transgenic proteins expressed in the maize

plant tissues of the individual-event hybrids and the stacked hybrid, these differences were slight and were not considered to be biologically significant. Overall, the transgenic protein concentrations were generally similar between the Bt11 x MIR162 x MIR604 x GA21 stacked hybrid and the four individual component event hybrids.

CLASSIFICATION: ACCEPTABLE

I. STUDY DESIGN:

The purpose of this study was to use enzyme-linked immunosorbent assay ELISA to compare transgenic protein expression in several plant tissues from a Bt11 x MIR162 x MIR604 x GA21 maize hybrid with expression in corresponding, near-isogenic lines derived from the component individual transformation events: Bt11, MIR162, MIR604, and GA21. Bt11 expresses a truncated Cry1Ab protein and a phosphinothricin acetyltransferase (PAT) protein; MIR162 expresses a Vip3Aa20 protein and a phosphomannose isomerase (PMI) protein; MIR604 expresses a modified Cry3A (mCry3A) protein and a MIR604 PMI protein that differs by two amino acids from the PMI protein expressed in MIR162; GA21 expresses a doubly mutated 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS) protein. The Bt11 x MIR162 x MIR604 x GA21 combined-trait hybrid was obtained through conventional breeding. Depending on growth stage, leaves, roots, whole plants, and kernels were analyzed for the concentration of these transgenic proteins. In addition, whorl stage leaves were analyzed for Cry1Ab and Vip3Aa20 and whorl stage roots were analyzed for mCry3A to compare the concentrations of these insecticidal proteins at relevant feeding times of the target insects.

1. Test materials

Maize Lines:

Event Bt11 expressing a truncated Cry1Ab protein and a phosphinothricin acetyltransferase (PAT) protein.

Event MIR162 expressing a Vip3Aa20 protein and a phosphomannose isomerase (PMI) protein.

MIR604 expressing a modified Cry3A (mCry3A) protein and a PMI protein that differs by two amino acids from the PMI protein expressed in MIR162.

Event GA21[1] expressing a doubly mutated 5-enol pyruvylshikimate-3-phosphate synthase (mEPSPS) protein.

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[1] Event GA21 expresses mEPSPS, an inert PIP protein used to confer resistance to glyphosate containing herbicides. Although this event is crossed with Bt11, MIR162, and MIR604 events to produce the combined trait stacked product- Bt11x MIR162 x MIR604 x GA21, the data for GA21 is reported in this DER, but not analyzed. This is because Event GA21 does not express an active PIP pesticidal substance, which deems Event GA21 out of EPA's jurisdiction under the Coordinated Framework for regulation of biotechnology products.

Maize hybrid Bt11 x MIR162 x MIR604 x GA21, obtained by conventional breeding, and expressing all seven proteins: Cry1Ab, PAT, Vip3Aa20, mCry3A, mEPSPS, PMI (from MIR162), and PMI (from MIR604).

2. Methods

A. Plant Tissues Sampled

The hybrid maize plants used in this study were grown from seeds planted on June 4, 2006 at Syngenta's research station in Bloomington, IL. Five blocks of maize were planted, each containing a two-row plot of each hybrid. Four plants per hybrid were selected randomly from each block at various developmental stages shown in Table 1. Pollen was collected and pooled from less than ten plants of the same genotype at anthesis, creating one pooled sample per replicate block per hybrid. The pollen samples were passed through 80 μ sieves to remove non-pollen debris. The harvested, whole plants were shipped on ice packs and pollen was shipped on dry ice to Syngenta's Regulatory Science Lab. Two plants per hybrid from each replicate block were kept as whole plant samples, while the other two plants were separated into parts. Samples were weighed, and then stored at -80 °C until processing. Pollen was also stored in the same manner.

TABLE 1. Maize Plant Tissues Sampled at Different Developmental Stages.

Stage	Tissues Sampled
Anthesis	Leaves, Roots, and Whole Plants
Physiological Maturity	Kernels
Whorl	Leaves [a], Roots [b]

Data taken from text Table, pg. 10, MRID 47137402.

[a] Leaves were analyzed from Event Bt11, Event MIR162, and the stacked hybrid: Bt11 x MIR162 x MIR604 x GA21.

[b] Roots were analyzed from Event MIR604, and the stacked hybrid: Bt11 x MIR162 x MIR604 x GA21.

B. Plant Tissue Processing

All processing was done with frozen samples. Each tissue sample (except pollen) was reduced to a fine powder using a Retsch Cutting Mill for large samples and a GRINDOMIX Knife Mill (Retsch, Inc.) for smaller samples. Each sample was mixed to ensure homogeneity and a sub-sample from each was lyophilized for subsequent analysis. Processed samples were stored at -80 ± 10°C. Lyophilized samples were stored in the same way.

The percent dry-weight of each sample was determined by recording the weight of the sample before and after lyophilization (fresh weight (FW) and dry weight (DW), respectively). The percent dry weight was used to convert protein concentrations between gram dry weight and gram fresh weight.

For all plant tissues except pollen, 3 mL of CAPS buffer [50 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS), 0.1 M NaCl, 1.54 mM ethylenediaminetetraacetic acid (EDTA), pH 10, 2 mM dithiothreitol (DTT), 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride·HCl (AEBSF), 1 μM leupeptin] was combined with 100 mg of

lyophilized tissue and kept on ice for 30 min. The samples were then homogenized using an Autogizer (Tomtec; Hamden, CT) and centrifuged at *ca.* 10,000 xg at 2 – 8°C. The supernates were stored at 2 – 8°C until ELISA analysis.

Pollen extracts were prepared by suspending *ca.* 100 mg frozen, dry pollen in 3 mL of CAPS buffer and subjecting the suspension to *ca.* 15,000 psi in a French Pressure Cell pre-cooled at 2 – 8 °C. After two additional pressings, the extracts were centrifuged at *ca.* 10,000 x g and the supernatants were collected and stored at 2 – 8 °C until analysis by ELISA.

Plant tissue extracts from leaves, roots, pollen, whole-plant samples at the anthesis stage and kernel samples at the physiological maturity stage were analyzed for each of the transgenic proteins from the appropriate hybrids. In addition, extracts of whorl-stage leaves from the corresponding hybrids were analyzed for Vip3Aa20 and Cry1Ab proteins, and whorl-stage roots from the corresponding hybrids were analyzed for mCry3A protein. (Expression of insecticidal proteins in the whorl-stage was more relevant to times when insects might be feeding on the plants.)

Data points from the ELISA plates were considered acceptable if the mean optical density (OD) value, obtained from three aliquots of each dilution of sample extract lay within the linear range of the standard curve. Only analyses where the OD coefficient of variance was less than 10% were accepted. If one of the three OD values was an outlier, then the mean of the remaining two OD values was used. The mean absorbance per sample was read and plotted against the standard curve to give a value for the concentration of the protein of interest (POI).

Concentrations of all proteins analyzed using lyophilized tissue (Cry1Ab, PAT, Vip3Aa20, and PMI) were calculated as follows:

ng POI/mL = diluted ng POI/mL x dilution factor
µg POI/g DW = ng POI/mL x 3 mL (volume of extraction buffer) ÷ amount extracted (g) ÷ 1000

Concentrations of proteins analyzed using fresh, frozen tissue (mCry3A, MIR604 PMI, and mEPSPS) were calculated as follows:

ng POI/mL = diluted ng POI/mL x dilution factor
µg POI/g FW = ng POI/mL x 3 mL (volume of extraction buffer) ÷ amount extracted (g) ÷ 1000
µg POI/g DW = µg POI/g FW ÷ % dry weight

Each transgenic protein was extracted and then analyzed by ELISA using methods developed specifically for quantification of each protein, as described below.

C. Quantification of Transgenic Proteins in Plant Tissues

1. Cry1Ab Quantification

Extracts were quantitatively analyzed for Cry1Ab protein by ELISA. Nunc MaxiSorp plates were coated with mouse anti-HO4 antibody. (HO4 is a hybrid protein comprised of the 1st and 2nd domains of Cry1Ab and the 3rd domain of Cry1C). After blocking the plates with powdered milk and washing, dilutions of each tissue extract and appropriate serial dilutions of microbially produced Cry1Ab standard were applied to each plate (total volume was 100 µL per well). After incubation the plates were washed and 100 µl per well of rabbit anti-Cry1Ab antisera was added. Following incubation with the rabbit antibody, the plates were again washed and goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (100 µL per well) was added. After incubation for 1 hour and washing, TMB (3,3',5,5'-tetramethylbenzidine) substrate solution (100 µL per well) was added. Color was allowed to develop for 30 minutes at ambient temperature in the dark and the reaction was then stopped by the addition of 3M H_2SO_4 (50 µL per well). Absorbance at 450 nm was measured using a Tecan Sunrise multi-well plate reader (Tecan; Research Triangle Park, NC). The results were analyzed using DeltaSOFT PC Microplate Analysis software (BioMetallics, Inc.; Princeton, NJ).

2. PAT Quantification

Extracts were quantitatively analyzed for PAT protein by ELISA. Nunc MaxiSorp plates were coated with rabbit anti-PAT antibody. After blocking the plates with bovine serum albumin (BSA) and washing, dilutions of each tissue extract and appropriate serial dilutions of microbially produced PAT reference protein were applied to each plate (total volume was 100 µL per well). After incubation, the plates were washed and 100 µl per well of goat anti-PAT antibody was added. Following incubation with the goat antibody, the plates were again washed and donkey anti-goat IgG conjugated with horseradish peroxidase (100 µL per well) was added. After incubation and washing, TMB substrate solution (100 µL per well) was added. Color was allowed to develop for 30 minutes at ambient temperature in the dark and the reaction was stopped by the addition of 3M H_2SO_4 (50 µL per well). Absorbance at 450 nm was measured using a Tecan Sunrise multi-well plate reader. The results were analyzed using DeltaSOFT PC Microplate Analysis software.

3. Vip3Aa20 Quantification

Extracts were quantitatively analyzed for Vip3Aa20 protein by ELISA. Nunc MaxiSorp plates were coated with goat anti-Vip3Aa19 antibody (Vip3Aa19 differs from Vip3Aa20 by one amino acid). After washing and blocking the plates with bovine serum albumin (BSA), dilutions of each tissue extract and appropriate serial dilutions of microbially produced Vip3Aa20 standard were applied to each plate (total volume was 100 µL per well). After incubation the plates were washed and 100 µl per well of rabbit anti-Vip3Aa19 antibody was added. Following incubation with the rabbit antibody, the plates were again washed and donkey anti-rabbit IgG conjugated with alkaline phosphatase (100 µL per well) was added. After incubation and washing, phosphatase substrate solution (100 µL per well) was added. Color was allowed to develop for 30 minutes at ambient temperature and the reaction was then stopped by the addition of 3N sodium hydroxide (100 µL per well). Absorbance at 405

and 492 nm was measured using a Tecan Sunrise multi-well plate reader. The results were analyzed using DeltaSOFT PC Microplate Analysis software.

4. PMI Quantification

Extracts were quantitatively analyzed for PMI protein by ELISA. Nunc MaxiSorp plates were coated with rabbit anti-MIR604 PMI antibody [MIR604 PMI differs from PMI by two amino acids (Rabe, 2003)]. After blocking the plates with powdered milk and washing, dilutions of each tissue extract and appropriate serial dilutions of microbially produced PMI reference protein were applied to each plate (total volume was 100 µL per well). After incubation, the plates were washed and 100 µl per well of mouse anti-MIR604 PMI antibody was added. Following incubation with the mouse antibody, the plates were again washed and rabbit anti-mouse IgG conjugated with horseradish peroxidase (100 µL per well) was added. After incubation and washing, TMB substrate solution (100 µL per well) was added. Color was allowed to develop for 30 minutes at ambient temperature in the dark and the reaction was then stopped by the addition of 3M H_2SO_4 (50 µL per well). Absorbance at 450 nm was measured using a Tecan Sunrise multi-well plate reader. The results were analyzed using DeltaSOFT PC Microplate Analysis software.

5. mCry3A Quantification

Extracts were quantitatively analyzed for mCry3A protein by ELISA. Nunc MaxiSorp plates were coated with mouse anti-mCry3A antibody. After blocking the plates with powdered milk and washing, dilutions of each tissue extract and appropriate serial dilutions of microbially produced mCry3A standard were applied to each plate (total volume was 100 µL per well). After incubation the plates were washed and 100 µl per well of mouse anti-mCry3A antibody conjugated with horseradish peroxidase was added. After incubation for 1 hour and washing, TMB (3,3',5,5'-tetramethylbenzidine) substrate solution (100 µL per well) was added. Color was allowed to develop for 30 min. at ambient temperature in the dark and the reaction was then stopped by the addition of 3M H_2SO_4 (50 µL per well). Absorbance at 450 nm was measured using a Tecan Sunrise multi-well plate reader (Tecan; Research Triangle Park, NC). The results were analyzed using DeltaSOFT PC Microplate Analysis software (BioMetallics, Inc.; Princeton, NJ).

6. MIR604 PMI Quantification

Extracts were quantitatively analyzed for MIR604 PMI protein by ELISA. Nunc MaxiSorp plates were coated with rabbit anti-MIR604 PMI antibody [MIR604 PMI differs from PMI by two amino acids (Rabe, 2003)]. After blocking the plates with powdered milk and washing, dilutions of each tissue extract and appropriate serial dilutions of microbially produced MIR604 PMI reference protein were applied to each plate (total volume was 100 µL per well). After incubation, the plates were washed and 100 µl per well of mouse anti-MIR604 PMI antibody was added. Following incubation with the mouse antibody, the plates were again washed and rabbit anti-mouse IgG conjugated with horseradish peroxidase (100 µL per well) was added. After incubation and washing, TMB substrate solution (100 µL per well) was added. Color was allowed to develop for 30 minutes at ambient temperature in the dark and the reaction was then stopped by the addition of 3M H_2SO_4 (50 µL per well). Absorbance at 450 nm was measured using a Tecan Sunrise multi-well plate reader. The results were analyzed using DeltaSOFT PC Microplate Analysis software.

D. Statistical Analysis

Transgenic protein concentrations, with the exceptions of PMI and MIR604 PMI, were subjected to analysis of variance. Each individual dataset (consisting of the data from beach Bt11, MIR162, or MIR604 corn event and the Bt11 x MIR162 x MIR604 x GA21 stacked hybrid) was subjected to a two-tailed analysis of variance, in which the effect of the genotype was assessed using an F-test. An F-test probability less than 5% indicated that the genotypes were significantly different at the usual 5% level. All tests of significance used plot-level variation, not plant-level variation, as the error term. Only the dry weight data were statistically analyzed since the dry weight controls for variability in water content. All mean protein concentrations were calculated as least squares means.

II. RESULTS:

The ranges of concentrations of the Cry1Ab, PAT, Vip3Aa20, mCry3A, and mEPSPS transgenic proteins found in the various tissues of the individual parental events and in the stacked hybrid are shown in Table 2. These protein concentrations are presented on a dry-weight basis, which is appropriate because dry-weight data eliminates variability due to water content.

TABLE 2. Ranges of Transgenic Protein Concentrations Measured in Tissues of Individual-Event and Stacked Maize Hybrids on a Dry-Weight Basis (µg/g DW).

Tissue Type (Stage)	Hybrid	Cry1Ab [a]	PAT [a]	Vip3Aa20 [b]	mCry3A [c]	mEPSPS [d]
Leaves (Whorl)	Event	78.2 – 112.9	N/A	144 – 251	N/A	N/A
	Stack [e]	71.8 – 101.4	N/A	143 – 184	N/A	N/A
Leaves (Anthesis)	Event	25.8 – 35.2	0.50 – 0.82	137 – 280	30.7 – 46.4	40.3 – 59.7
	Stack	22.0 – 38.6	0.46 – 0.76	133 – 259	24.4 – 47.4	26.5 – 57.7
Roots (Whorl)	Event	N/A	N/A	N/A	28.7 – 51.9	N/A
	Stack	N/A	N/A	N/A	28.4 – 40.1	N/A
Roots (Anthesis)	Event	9.6 – 13.3	0.50 – 1.1	36.0 – 65.9	16.4 – 25.4	10.3 – 23.5
	Stack	7.3 – 13.5	0.58 – 1.0	38.6 – 65.8	17.0 – 31.6	8.8 – 17.8
Pollen (Anthesis)	Event	0.07 – 0.09	<LOD [f]	82.2 - 118	<LOQ [g]	64.0 – 113.7
	Stack	0.06 – 0.12	<LOD	74.6 – 95.9	<LOQ	73.1 – 129.6
Kernels (Physiological Maturity)	Event	1.2 – 2.3	<LOQ [h]	54.2 – 165.9	0.19 – 1.1	3.6 – 7.6
	Stack	1.2 – 2.5	<LOQ	89.7 – 164.7	0.40 – 0.77	3.0 – 7.7
Whole Plant (Anthesis)	Event	13.6 – 18.4	0.71 – 1.3	56.7 – 87.6	14.5 – 21.5	19.6 – 25.66
	Stack	13.6 – 17.1	0.75 – 1.0	57.0 – 87.5	12.8 – 21.0	19.3 – 25.3

Data were taken from Table 1, page 19 of MRID 47137202.

[a] Cry1Ab and PAT proteins are expressed in Event Bt11.

[b] Vip3Aa20 and PMI proteins are expressed in Event MIR162. PMI concentrations are presented in Table 3.

[c] mCry3A and MIR604 PMI proteins are expressed in Event MIR604. MIR604 PMI concentrations are presented in Table 3.

[d] mEPSPS protein is expressed in Event GA21. These data are reported but not subject to analysis and review.

[e] Stack is the hybrid containing Bt11 x MIR162 x MIR604 x GA21, expressing all 7 transgenic proteins.

[f] The limit of detection (LOD) for PAT protein in pollen (anthesis) was 0.07 µg/g DW.

[g] The limit of quantitation (LOQ) for mCry3A protein in pollen (anthesis) was 0.34 µg/g DW.

[h] The limit of quantitation (LOQ) for PAT protein in kernels (physiological m turity)was 0.44 µg/g DW.

N/A = not analyzed.

The average concentrations and the range of concentrations of the PMI and MIR604 PMI transgenic proteins found in various tissues of the individual MIR162 and MIR604 corn events

and in the stacked hybrid (Bt11 x MIR162 x MIR604 x GA21) are shown in Table 3. The ELISA used for measuring PMI in Event MIR162 used purified PMI as a reference standard. The ELISA used for measuring MIR604 PMI in Event MIR604 used purified MIR604 PMI as a reference standard and was done on a separate day from the PMI analyses for Event MIR162. Data presented for the stacked hybrid are for total PMI (PMI + MIR604 PMI), which was measured on two separate days: (1) concurrently with the PMI analyses of Event MIR162 (using the PMI reference standard) and (2) concurrently with the MIR604 PMI analyses of Event MIR604 (using the MIR604 PMI reference standard). The two forms of PMI can't be distinguished in the stacked hybrid, because the ELISA antibodies recognize both forms. The data in Table 3 were not subjected to statistical analysis.

Although a statistical comparison of PMI and MIR604 PMI concentrations between the Bt11 x MIR162 x MIR604 x GA21 and corresponding individual-events was not conducted, mean total PMI (PMI + MIR604 PMI) concentrations were consistently higher, as expected, in the tissues of the Bt11 x MIR162 x MIR604 hybrid as compared to PMI concentrations in the MIR162 hybrid or MIR604 PMI concentrations in Event MIR604 . This reflected the presence of both PMI and MIR604 PMI in the Bt11 x MIR162 x MIR604 hybrid. Generally, the mean total PMI concentrations in the stacked hybrid was approximately double that in the individual MIR162 and MIR604 parental events. The only exception to this was in the pollen (anthesis stage) where the mean total PMI was more than 9 times the PMI concentration in the Event MIR162 and the mean total PMI in the stacked hybrid was just slightly greater than the MIR604 PMI found in the individual Event MIR604.

TABLE 3. Concentrations of PMI and/or MIR604 PMI Protein in Events MIR162, MIR604, and the Bt11 x MIR162 x MIR604 x GA21 Stacked Hybrid on a Dry-Weight Basis (µg/g DW).

Tissue Type (Stage)	Hybrid	Mean PMI and/or MIR604 PMI Concentration in (µg/g DW ± SD (Range)	
		Measured with the PMI Reference Protein	Measured with the MIR604 PMI Reference Protein
Leaves (Anthesis)	Event MIR162	7.72 ± 0.87 (6.5 – 9.2)	N/A [a]
	Event MIR604	N/A [a]	5.03 ± 0.99 (3.8 – 6.1)
	Stacked Hybrid	16.3 ± 2.9 (11.8 – 19.8)	10.0 ± 2.0 (7.5 – 14.0)
Roots (Anthesis)	Event MIR162	2.58 ± 0.43 (1.9 – 3.3)	N/A [a]
	Event MIR604	N/A [a]	2.41 ± 0.42 (1.7 – 3.1)
	Stacked Hybrid	5.37 ± 0.77 (4.3 – 6.8)	4.08 ± 0.84 (3.0 – 5.7)
Pollen (Anthesis)	Event MIR162	5.07 ± 0.21 (4.7 – 5.2)	N/A [a]
	Event MIR604	N/A [a]	43.3 ± 3.8 (37.4 – 47.8)
	Stacked Hybrid	48.1 ± 1.5 (46.1 – 49.8)	50.4 ± 7.2 (41.0 – 58.2)
Kernels (Physiological Maturity)	Event MIR162	2.48 ± 0.64 (1.1 – 3.2)	N/A [a]
	Event MIR604	N/A [a]	2.33 ± 0.53 (1.6 – 3.0)
	Stacked Hybrid	5.18 ± 1.27 (3.4 – 6.5)	4.74 ± 1.43 (1.2 – 5.9)
Whole Plant (Anthesis)	Event MIR162	3.87 ± 0.51 (3.0 – 4.6)	N/A [a]
	Event MIR604	N/A [a]	4.37 ± 0.68 (3.5 – 5.5)
	Stacked Hybrid	8.54 ± 0.69 (7.5 – 9.5)	7.20 ± 0.76 (6.2 – 8.8)

Data was taken from Table 3, page 21 of MRID 47137202.
[a] N/A= not applicable.

The average concentrations of Cry1Ab and PAT proteins in the Bt11 event and in the stacked hybrid (Bt11 x MIR162 x MIR604 x GA21) of the various plant tissues analyzed are shown and compared in Table 4. A statistically significant difference ($p<0.05$) between the concentrations of the Cry1Ab protein in the Bt11 event and in the stacked hybrid was only observed in leaves (whorl stage) and in whole plants (anthesis stage). Statistically significant differences ($p<0.05$) between the concentrations of the PAT protein in the Bt11 event and in the stacked hybrid were observed in kernels (physiological maturity). A single standard deviation was also reported for each set of Bt11 and stacked hybrids for each tissue (stage).

TABLE 4. Statistical Analysis of Cry1Ab and PAT Protein Concentrations Measured in Event Bt11 and in the Stacked Bt11 x MIR162 x MIR604 x GA21 Maize Hybrid

Tissue (Stage)	Protein of Interest (POI)	Maize Hybrid Line	Mean µg POI/ gdw [1]	p-value (F-test)
Leaves (Whorl)	Cry1Ab	Bt11	92.7	0.303
		Bt11 x MIR162 x MIR604 x GA21	88.4	
Leaves (Anthesis)	Cry1Ab	Bt11	30.7	0.569
		Bt11 x MIR162 x MIR604 x GA21	29.7	
	PAT	Bt11	0.596	0.856
		Bt11 x MIR162 x MIR604 x GA21	0.603	
Roots (Anthesis)	Cry1Ab	Bt11	11.5	0.427
		Bt11 x MIR162 x MIR604 x GA21	11.3	
	PAT	Bt11	0.905	0.152
		Bt11 x MIR162 x MIR604 x GA21	0.739	
Pollen (Anthesis)	Cry1Ab	Bt11	0.0764	0.701
		Bt11 x MIR162 x MIR604 x GA21	0.0801	
	PAT	Bt11	N/A [2]	N/A [2]
		Bt11 x MIR162 x MIR604 x GA21	N/A [2]	
Kernels (Physiological Maturity)	Cry1Ab	Bt11	1.78*	0.029
		Bt11 x MIR162 x GA21	1.57*	
	PAT	Bt11	N/A [2]	N/A [2]
		Bt11 x MIR162 x MIR604 x GA21	N/A [2]	
Whole Plant (Anthesis)	Cry1Ab	Bt11	15.9	0.423
		Bt11 x MIR162 x MIR604 x GA21	15.2	
	PAT	Bt11	0.912	0.212
		Bt11 x MIR162 x MIR604 x GA21	0.873	

Data taken from Table 5, pg. 23, MRID 47137202.

[1] POI/gdw = Protein of interest/grams dry weight

[2] Protein expression was either below the limit of quantitation or the limit of detection and, therefore, no statistical analysis was possible.

* Mean µg POI/gdw of the Bt11 maize and the stacked hybrid maize lines are significantly different from each other: $p < 0.05$.

The average concentrations of Vip3Aa20 protein in the MIR162 event and in the stacked hybrid (Bt11 x MIR162 x MIR604 x GA21) of the various plant tissues analyzed are shown in Table 5. No statistically significant differences ($p<0.05$) were seen between the concentration of the Vip3Aa20 protein in the MIR162 parental event and in the stacked hybrid. A single standard deviation was also reported for each set of MIR162 and stacked hybrids for each tissue (stage).

TABLE 5. Statistical Analysis of Vip3Aa20 Protein Concentration Measured in Event MIR162 and in the Stacked Bt11 x MIR162 x MIR604 Maize Hybrid				
Tissue (Stage)	**Protein of Interest (POI)**	**Maize Hybrid Line**	**Mean µg POI/gdw [1]**	**p-value (F-test)**
Leaves (Whorl)	Vip3Aa20	MIR162	165.6	0.664
		Bt11 x MIR162 x MIR604 x GA21	159.7	
Leaves (Anthesis)	Vip3Aa20	MIR162	182.4	0.850
		Bt11 x MIR162 x MIR604 x GA21	187.2	
Roots (Anthesis)	Vip3Aa20	MIR162	52.1	0.734
		Bt11 x MIR162 x MIR604 x GA21	53.1	
Pollen (Anthesis)	Vip3Aa20	MIR162	97.2	0.067
		Bt11 x MIR162 x MIR604 x GA21	85.4	
Kernels (Physiological Maturity)	Vip3Aa20	MIR162	123.8	0.212
		Bt11 x MIR162 x MIR604 x GA21	140.1	
Whole Plant (Anthesis)	Vip3Aa20	MIR162	73.0	0.945
		Bt11 x MIR162 x MIR604 x GA21	72.6	

Data taken from Table 6, pg. 23, MRID 47137202.
[1]POI/gdw = Protein of interest/grams dry weight

The average concentrations of mCry3A protein in the MIR604 hybrid and in the stacked hybrid (Bt11 x MIR162 x MIR604) of the various plant tissues analyzed are shown in Table 6. A statistically significant difference ($p<0.05$) between the concentrations of the mCry3A protein in the MIR604 hybrid and in the stacked hybrid was only observed in the whole plants (anthesis stage). A single standard deviation was also reported for each set of MIR604 and stacked hybrids for each tissue (stage).

TABLE 6. Statistical Analysis of mCry3A Protein Concentration Measured in Event MIR604 and in the Stacked Bt11 x MIR162 x MIR604 x GA21 Maize Hybrid				
Tissue (Stage)	**Protein of Interest (POI)**	**Maize Hybrid Line**	**Mean µg POI/gdw [1]**	**p-value (F-test)**
Leaves (Whorl)	mCry3A	MIR604	35.8	0.477
		Bt11 x MIR162 x MIR604 x GA21	34.0	
Leaves (Anthesis)	mCry3A	MIR604	37.0	0.108
		Bt11 x MIR162 x MIR604 x GA21	31.0	
Roots (Anthesis)	mCry3A	MIR604	22.6	0.102
		Bt11 x MIR162 x MIR604 x GA21	25.4	
Pollen (Anthesis)	mCry3A	MIR604	N/A [2]	N/A [2]
		Bt11 x MIR162 x MIR604 x GA21	N/A [2]	
Kernels (Physiological Maturity)	mCry3A	MIR604	0.717	0.566
		Bt11 x MIR162 x MIR604 x GA21	0.620	
Whole Plant (Anthesis)	mCry3A	MIR604	18.1*	0.021
		Bt11 x MIR162 x MIR604 x GA21	16.2*	

Data taken from Table 7, pg. 24, MRID 47137202.

[1]POI/gdw = Protein of interest/grams dry weight

[2]Protein expression was either below the limit of quantitation or the limit of detection and, therefore, no statistical analysis was possible.

* Mean µg POI/gdw of the MIR604 maize and the stacked hybrid maize lines are significantly different from each other: $p < 0.05$.

III. CONCLUSION:

The measured concentrations of CrylAb, PAT, Vip3Aa20, and mCry3A were generally similar between the tissues of the corresponding individual events- Bt11, MIR162, and MIR604 maize and the Bt11 x MIR162 x MIR604 x GA21 hybrid. The two statistical differences observed between the concentrations of the transgenic proteins expressed in the plant tissues of the individual-events and the Bt11 x MIR162 x MIR604 x GA21 stacked hybrid were minimal and were the only differences observed out of 25 statistical comparisons. A valid statistical analysis of the PMI and/or MIR604 PMI data was not possible because the same antibody reacted with both proteins. However, the concentrations of total PMI (PMI + MIR604 PMI) in the tissues of the Bt11 x MIR162 x MIR604 hybrid were, as expected, consistently higher (typically 2-fold) than either the corresponding PMI concentration in the Event MIR162 or Event MIR604 PMI concentration in the MIR604 hybrid, reflecting the additive presence of both PMI and MIR604 PMI in the Bt11 x MIR162 x MIR604 x GA21 hybrid.

IV. REVIEWER'S COMMENTS:

There were two statistically significant differences between the concentrations of the Cry1Ab in kernels of Bt11 vs. the stacked hybrid and mCry3A in whole plants of MIR604 vs. the stacked hybrid), in which the p values were just slightly below the 0.05 level. However, the ranges of protein concentrations overlapped. Therefore, the statistically significant differences are not biologically relevant.

No statistical comparison of PMI and MIR604 PMI concentrations between the stacked hybrid and the corresponding individual event was conducted because the same antibody reacted with both proteins. The mean total PMI (PMI + MIR604 PMI) concentrations were consistently higher, as expected, in the tissues of the stacked hybrid as compared to PMI concentrations in Event MIR162 or MIR604 PMI concentrations in Event MIR604. Generally, the mean total PMI concentrations in the stacked hybrid was approximately double that in the individual MIR162 and MIR604 corn events. The only exception to this was in the pollen where the mean total PMI was more than 9 times the PMI concentration in the Event MIR162 and the mean total PMI in the stacked hybrid was just slightly greater than the MIR604 PMI found in the individual Event MIR604. Overall, transgenic protein concentrations were generally similar between the Bt11 x MIR162 x MIR604 x GA21 stacked hybrid and the four individual component event hybrids.

V. CLASSIFICATION: ACCEPTABLE

VI. REFERENCES:

Rabe, S. (2003) Molecular characterization of Event MIR604 maize (corn) expressing a modified Cry3A *Bacillus thuringiensis* protein. Syngenta Seeds Biotechnology Report No. SSB-033-03. US EPA MRID No. 46155602.

DATA EVALUATION RECORD

Cry1Ab
(Test Substance TRYCRY1AB-0105)

STUDY TYPE: Protein Characterization, Product Identity (885.1100)
MRID 471372-11

Prepared for

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

This record may have been altered subsequent to the contractor=s signatures above.

Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract number DE-AC05-00OR22725.

DATA EVALUATION RECORD

Primary Reviewer: Gary Sega, Ph.D., Oak Ridge National Laboratory
Secondary Reviewer: Annabel Waggoner, Environmental Protection Specialist, BPPD AFN

STUDY TYPE:	Product Identity (OPPTS Guideline No. 885.1100)
MRID NO:	471372-11
DECISION NO:	379490
DP BARCODE:	345912
TEST MATERIAL:	Test Substance TRYCRY1AB-0105, containing the truncated form of the full-length Cry1Ab protein
PROJECT STUDY NO:	BT11-05-01; Report No. SSB-010-06
SPONSOR:	Syngenta Seeds, Inc. - Field Crops - NAFTA
TESTING FACILITY:	Syngenta Biotechnology, Inc., Regulatory Science 3054 East Cornwallis Road, P.O. Box 12257 Research Triangle Park, NC 27709-2257.
TITLE OF REPORT:	Characterization of Trypsinized Cry1Ab Test Substance TRYCRY1AB-0105
AUTHOR:	Kramer, C. and Graser, G.
STUDY COMPLETED:	April 17, 2006
CONFIDENTIALITY CLAIMS:	None
GOOD LABORATORY PRACTICE:	GLP was followed except for the N-terminal sequence analysis conducted by Proseq, Inc.; peptide mass analysis done by the University of North Carolina; total mass analysis provided by Syngenta Protein Analysis Team.
CONCLUSION:	The purpose of this study was to characterize test substance TRYCRY1AB-0105, containing the *ca.* 66 kDa truncated form of the full length (*ca.* 130 kDa) Cry1Ab insecticidal protein. The test substance purity was calculated as 127 µg Cry1Ab/mL TRYCRY1AB-0105 or 74.1% Cry1Ab protein/total protein. Protein levels were determined by measuring sample absorption at 280 nm (A_{280} method) in conjunction with densitometry data after electrophoretic separation.

Western blot analysis of the test substance showed a dominant immunoreactive band corresponding to the predicted molecular weight of Cry1Ab of *ca.* 66 kDa. Total mass analysis of the Cry1Ab in test substance TRYCRY1AB found two predominant CrylAb species, with molecular weights of 66.3 and 65.8 kDa. In addition, two putative CrylAb breakdown fragments were found, corresponding to *ca.* 39 kDa and 27 kDa. The 65.8 and 66.3 kDa CrylAb protein species both contain the biologically active portion of the insecticidal CrylAb protein and, therefore, were considered throughout the study as active CrylAb protein and were included in the purity estimate of CrylAb protein in test substance TRYCRY1AB-0105.

N-terminal sequencing of Cry1Ab in test substance TRYCRY1AB-0105 confirmed that the first 10 amino acids of the protein corresponded to the predicted N-terminal sequence of Cry1Ab. Peptide mapping of the Cry1Ab in test substance TRYCRY1AB-0105, representing 26% coverage of the Cry1Ab protein, gave peptide spectra identical to that of the known sequence of trypsinized Cry1Ab.

CrylAb bioactivity against the European Corn Borer was confirmed. The 96-hour LC_{50} was 6.2 ng Cry1Ab/cm^2 diet surface (95% confidence interval: 3.8 – 8.9 ng/cm^2) against first instar larvae of the European corn borer.

TRYCRY1AB-0105 was also evaluated for lipopolysaccharide concentration and found to contain 4.8 ng endotoxin/mL test substance TRYCRY1AB-0105.

CLASSIFICATION: **SUPPLEMENTAL**- The registrant should submit the mass spectra as visual confirmation of the molecular weights for the two forms of Cry1Ab for the test substance TRYCRY1AB-0105 and conduct a direct and concurrent analysis of a purified plant-expressed protein extracted from Bt11 maize leaves to the TRYCRY1AB-0105 test substance. These data can be used to determine the equivalence between the plant- and microbial-produced Cry1Ab proteins in support of utilizing the TRYCRY1AB-0105, as a test material in the non-target organism studies for demonstration of lack of synergism in the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 PIP products.

I. STUDY DESIGN

The purpose of this study was to characterize test substance TRYCRY1AB-0105, containing the *ca.* 66 kDa truncated form of the full length (*ca.* 130 kDa) Cry1Ab insecticidal protein. The full-length protein is a protoxin that, when ingested by certain insects, is solubilized, cleaved and activated in the insect gut to its toxic form. The truncated form of Cry1Ab includes the

insecticidally-active 66 kDa N-terminal portion of the protein. The full-length Cry1Ab protein (FLCry1Ab) was encoded by the synthetic *cry1Ab* gene in a recombinant *E. coli* over-expression system. The FLCry1Ab protein was trypsinized to yield a truncated, trypsin-resistant core of *ca* 66 kDa, which is similar to the truncated Cry1Ab expressed in maize Event Bt11 (except that it lacks the first 28 amino acids, which are not required for insecticidal activity. The identity of test substance TRYCRY1AB-0105 was verified by N-terminal amino acid sequence analysis and peptide mass analysis, and its concentration and integrity were determined. TRYCRY1AB-0105 was also evaluated for bioactivity and lipopolysaccharide concentration.

Test materials:

Test substance TRYCRY1AB-0105 was prepared from archived cell paste of *E. coli* over-expressing the full-length Cry1Ab protein (The test substance for FLCry1Ab, FLCRY1AB-0103, contains 1181 amino acids and was originally characterized in Graser, 2005, MRID No. 470176-04). Bovine trypsin was used to partially hydrolyze the FLCry1Ab protein at two trypsin recognition sites: arginine-28 and arginine-619. The resulting, truncated protein was purified, pooled, and concentrated in 50mM NH_4HCO_3 (pH 9.25) and designated as trypsinized Cry1Ab or test substance TRYCRY1AB-0105.

Methods:

A. Protein Quantitation

Total protein in test substance TRYCRY1AB-0105 was quantified spectrophotometrically by measuring its absorption at 280 nm (A_{280} method) using a Genesys 6 spectrophotometer (Thermo Electron Corp., Madison, WI). Vector NTI software (Invitrogen, San Diego, CA) was used to calculate the Cry1Ab extinction coefficient at 280 nm. Based on these data, the approximate total protein concentration of the test substance was determined.

B. Densitometry and Purity Analysis

Aliquots of TRYCRY1AB-0105 (1.3 to 6.6 µg of Cry1Ab test substance per lane) were subjected to eletrophoretic separation via SDS-PAGE, protein bands were stained with Coomassie Blue, and the distribution of visible protein bands was estimated by densitometric analysis. The purity of test substance TRYCRY1AB-0105 was calculated from the total sample weight and the total protein as determined by the A_{280} method in conjunction with the densitometry data.

C. Immunoreactivity and Molecular Weight Determination

The integrity of the Cry1Ab protein in test substance TRYCRY1AB-0105 was determined using Western blot analysis. Aliquots of test substance TRYCRY1AB-0105 containing *ca.* 5 to 38 ng of Cry1Ab protein were subjected to SDS-PAGE along with SeeBlue® Plus2 protein standard for determining the approximate molecular weights. An extract from Event Bt11 maize leaf was used to compare the size of the trypsinized Cry1Ab in the test substance with the truncated Cry1Ab expressed in maize Event Bt11. After electroblotting, the membranes were incubated with sheep anti-Cry1Ab protein G-purified polyclonal antibodies raised against the FLCry1Ab protein. Alkaline phosphatase-conjugated donkey anti-sheep IgG was used to bind the sheep antibodies. Immunoreactive bands were visualized by development with alkaline phosphatase substrate solution.

D. N-Terminal Amino Acid Sequence

A sample of TRYCRY1AB-0105 was subjected to SDS-PAGE followed by electroblotting to a PVDF membrane. Predominant bands were cut from the blot and sent to ProSeq, Inc. (Boxford, MA) for N-terminal sequencing using Edman-based chemistry (Brauer, 1984). The *ca.* 66 and 39 kDa bands were excised from the blot as individual samples. The co-migrating, *ca.* 27 kDa bands were too close together to cut out separately, so they were combined in one sample. The N-terminal amino sequence analysis was done using non-GLP proprietary methods.

E. Total Mass

Protein mass measurements of TRYCRY1AB-0105 were done using a Waters Micromass Quadrupole Time-of-Flight (Q-TOF2) spectrometer connected to a capillary HPLC which separated the components of the test material prior to the mass spectrometry. The total mass analysis was performed by the Syngenta Protein Analysis Team and was not performed under GLP. The use of Waters Micromass Q-TOF2 also deviated from the original protocol to use MALDI-TOFF MS at the Michael Hooker Proteomics Core Facility.

F. Peptide Mass Analysis

Peptide mass analysis (peptide mapping) compares the masses of individual peptides resulting from proteolytic digestion of a test sample to the masses of known peptides in a database. This comparison is used to predict the amino acid sequences of unknown peptides. An aliquot of TRYCRY1AB-0105 was sent to the Michael Hooker Proteomics Core Facility (University of North Carolina, Chapel Hill, NC), where the test material was fully digested at additional trypsin recognition sites, with two overnight incubations in trypsin. The resulting peptides were separated by liquid chromatography and then analyzed by MS/MS (tandem mass spectrometry) with a Q-TOF mass spectrometer. The peptide spectra were compared to the predicted Cry1Ab peptide sequences using a Mascot database search engine (Matrix Science). This analysis was not done using GLP standards.

G. Lipopolysaccharide Measurement

Lipopolysaccharide (endotoxin) is a common contaminant in protein preparations from gram-negative bacteria such as *E. coli*. The PYROGENT Plus Single Test Limulus Amebocyte (LAL) kit (Cambrex Bio Science, Inc., Walkersville, MD) was used to estimate LPS present in TRYCRY1AB-0105 in accordance with the manufacturer's instructions.

H. Insecticidal Activity Assays

The bioactivity of TRYCRY1AB-0105 against first instar European corn borer (ECB) larvae was assessed as follows: Test substance TRYCRY1AB-0105 was diluted in MilliQ water to Cry1Ab concentrations ranging from 0 to 65.8 ng Cry1Ab/cm^2 diet surface. Aliquots of 100 µL of the test material were applied to the surface of artificial insect diet in 47 mm diameter Petri dishes containing 5 mL of insect diet. Untreated insect diets, and diets treated with buffer alone, were used as negative controls. Each treatment consisted of 3 replicate dishes of 10 larvae/dish. The culture dishes were maintained at room temperature and mortality was assessed after 96 hours.

II. RESULTS

A. Quantitation and Purity Analysis of Cry1Ab protein

Table 1 shows the total protein content and purity level for Cry1Ab protein, as measured by absorption at 280 nm, in test substance TRYCRY1AB-0105. Densitometric analysis indicated that the *ca.* 66 kDa Cry1Ab protein represented approximately 74% of the Cry1Ab protein present in TRYCRY1AB-0105, thereby, representing an overall concentration of *ca.* 127 µg/mL.

TABLE 1. Total Protein and Purity Analysis of Cry1Ab Protein in Test Substance TRYCRY1AB-0105.

Total Protein (µg protein/mL TRYCRY1AB-0105)	Densitometric Analysis (%Cry1Ab protein/total protein)	Purity (µg Cry1Ab / mL TRYCRY1AB-0105)
171	74.1	127

Data taken from Table 1, pg. 19 of MRID 47137211.

B. Immunoreactivity and Molecular Weight Determination

SDS-PAGE analysis of TRYCRY1AB-0105 (see Figure 1) showed a predominant Cry1Ab band at *ca.* 66 kDa and less abundant protein bands at *ca.* 39 kDa, and two co-migrating bands, representing proteins of *ca.* 27 kDa. The study author pointed out that while the 66 and 39 kDa bands showed slightly higher mobility (therefore, lower molecular weight) in comparison to the molecular weight standards, the difference can be explained by the limits of SDS-PAGE for the accurate determination of molecular weights. The reliability of SDS-PAGE for molecular weight determinations have been reviewed (Dube and Flynn, 1988) and it was concluded that the typical molecular weight of a protein is within about 10% of its true molecular weight using this method. The same argument can be made for the double protein band seen at >28 kDa (the author gives these proteins a molecular weight of *ca.* 27 kDa).

Total mass analysis by HPLC/Q-TOF2 found that TRYCRY1AB-0105 contained two major Cry1Ab protein species, both eluting at the same retention time of 13.6 min. The masses of these two species were found to be 65.8 and 66.3 kDa, and with an abundance ratio of *ca.* 2:1, respectively. The 66.3 kDa protein corresponded to the predicted molecular weight of trypsinized Cry1Ab protein, while the 65.8 kDa protein is consistent with a slightly shorter version of the Cry1Ab protein, missing the last four amino acids (DLER) at the C-terminal end. Since these two proteins could not be separated by SDS-PAGE or Western blot analysis, they are referred to collectively as the *ca.* 66 kDa band throughout the study. The second major peak in test substance TRYCRY1AB-0105 eluted at 14.3 min. and was identified as a protein with a mass of *ca.* 39.1 kDa.

Western blot analysis of the test substance revealed two immunoreactive bands (Figure 2) that cross-reacted to polyclonal antibodies to FLCry1Ab. These bands represented the ca. 66 kDa trypsinized Cry1Ab protein and the *ca.* 39 kDa Cry1Ab fragment derived from it. The 27 kDa Cry1Ab fragment identified in test substance TRYCRY1AB-0105 was not recognized by the antibodies used in this Western (compare Figures 1 and 2). Corresponding bands in the Bt11 maize leaf extract indicated the presence of intact Cry1Ab which was slightly larger than the Cry1Ab protein from test substance TRYCRY1AB-0105. This was consistent with the known 28 amino acid sequence added to the N-terminal end of the Cry1Ab protein in

Event Bt11 maize (see Figure 3). The plant extract also contained additional breakdown products not observed in test substance TRYCRY1AB-0105 (Figure 4).

C. N-Terminal Amino Acid Sequence Analysis

The N-terminal sequence analysis of the *ca.* 66 kDa band (containing both the 65.8 and 66.3 kDa Cry1Ab protein species) from test substance TRYCRY1AB-0105 showed a single sequence of IETGYTPIDI, and indicated that these two species have the same N-terminal sequence (see Figure 4). This sequence was identical to the predicted N-terminal amino acid sequence of the trypsinized Cry1Ab protein. The *ca.* 39 and 27 kDa bands from the SDS-PAGE gel were both identified as Cry1Ab-derived protein fragments. The 39 kDa protein had the same N-terminal sequence (IETGYTPI) as the *ca.* 66 kDa Cry1Ab protein, and likely consisted of the first 343 amino acids of the N-terminal portion. The N-terminal sequence of the *ca.* 27 kDa fragment (NIGINNQ) was consistent with the remaining C-terminal portion of the trypsinized Cry1Ab protein.

D. Peptide Analysis

After complete tryptic digestion of TRYCRY1AB-0105, LC/MS/MS on a Q-TOF mass spectrometer was used to map and sequence peptides derived from Cry1Ab. These peptide spectra were matched to the predicted sequence of trypsinized Cry1Ab, and represented 26% coverage of the Cry1Ab amino acid sequence (see Figure 5).

E. Lipopolysaccharide Measurement

The concentration of lipopolysaccharide in test substance TRYCRY1AB-0105 was determined to be 48 endotoxin units/mL test substance, corresponding to 4.8 ng endotoxin/mL test substance TRYCRY1AB-0105.

F. Insecticidal Activity

The result of the bioassay is summarized in Table 2. Test substance TRYCRY1AB-0105 showed a clear dose-response in insecticidal activity with an LC_{50} of 6.2 ng Cry1Ab /cm^2 (95 % confidence interval of 3.8 – 8.9 ng Cry1Ab /cm^2), with concentrations of ≥ 32.9 ng Cry1Ab /cm^2 causing 100 % lethality.

TABLE 2. Insecticidal Activity of Cry1Ab in Test Substance TRYCRY1AB-0105 Against First Instar European Corn Borer.

Treatment (concentration of Cry1Ab ng/cm^2)	% Mortality
0.13	7
0.26	7
0.51	10
1.03	27
2.06	30
8.23	50
16.45	70
32.90	100
65.80	100
Untreated Control Diet	7
Buffer Control Diet	10
LC_{50} (95% Confidence Interval)	**6.2 (3.8 – 8.9)**

Data taken from Table 2, page 19 of MRID 47137211.

III. CONCLUSION:

The identity of the trypsinized CrylAb protein in test substance TRYCRYIAB-0105 was confirmed in this study by SDS-PAGE, Western blot analysis, mass analysis, peptide mapping, and N-terminal sequence analysis. Also, CrylAb bioactivity against a known CrylAb-sensitive lepidopteran pest was confirmed. Two predominant CrylAb species, with molecular weights of 66.3 and 65.8 kDa, were identified in test substance TRYCRYIAB-0105, together with two putative CrylAb breakdown fragments, corresponding to *ca.* 39 kDa and 27 kDa. The 65.8 and 66.3 kDa CrylAb protein species both contain the biologically active portion of the insecticidal CrylAb protein (Bietlot *et al.*, 1989), and therefore were considered throughout the study as active CrylAb protein and were included in the purity estimate of CrylAb protein in test substance TRYCRYIAB-0105.

IV. REVIEWER'S COMMENTS:

EPA previously expressed concerns with the product characterization study for the bacterial-expressed test substance, TRYCRY1AB-0105, in a letter dated October 30, 2007 [from Dr. S. Reilly, Branch Chief of BPPD in EPA's Office of Pesticide Programs, to Mr. E. Dunder, Regulatory Affairs Manager of Syngenta Seeds, Inc.]. An excerpt of EPA's question to Syngenta is presented below:

EPA question number 2:

The intentions for submitting the study characterizing the bacterial-produced test substance TRYCRY1AB-0105 for various biochemical and functional parameters was not provided, see MRID No. 471372-11. This study characterized the bacterial-produced test substance, TRYCRY1AB-0105, for various biochemical and functional parameters (which included: purity, identity, molecular weight via SDS-PAGE and western blot, protein intactness and insecticidal activity, N-terminal amino acid sequence analysis, peptide analysis, and LPS determination). If Syngenta intends to use this study to demonstrate protein equivalence with the plant-produced test substance for Cry1Ab, then equivalence of the previously submitted bacterial-produced test substance for Cry1Ab protein and the new bacterial-produced test substance should be demonstrated for all previous human health studies (such as acute oral toxicity and in vitro digestibility studies) and environmental effect studies. Justification is needed in order to bridge to previously submitted studies. Several of the toxicity studies utilized the bacterial-produced test protein for Cry1Ab as a surrogate for the plant-produced test protein so that a sufficient amount of protein can be obtained to test at higher dose levels or the maximum hazard dose. This is especially important if the new Cry1Ab bacterial-produced test substance was used in the toxicity studies in support for registering MIR162 x Bt11 and MIR162 x Bt11 x MIR604 stacked products. Therefore, you must submit clarification of your intent for submitting this study and address the aforementioned data bridging concerns. As part of this clarification, submit a cross-walk describing your support of Cry1Ab data requirements for the MIR162 x Bt11 and MIR162 x Bt11 x MIR604 products and how this differs form your support of Cry1Ab data requirements for the MIR604 x Bt11 product.

In a letter dated January 8, 2008 [from Mr. S. Huber of Syngenta Biotechnology, Inc. to Dr. S. Reilly of EPA's Office of Pesticide Programs], Syngenta responded to EPA's questions and the excerpt of that response is presented herewith to:

Syngenta's response to question number 2:

Syngenta regrets any confusion caused by the submission of the test substance characterization report (MRID 471372-11) for the bacterially-produced Cry1Ab protein test substance TRYCRY1AB-0105. With regard to Syngenta's current applications for Section 3 registrations, this test substance was used as a source of Cry1Ab for the following two studies **only**:

- MRID 47137208; Patnaude, M. (2007). Laboratory study to determine the combined effects of Cry1Ab, Vip3Aa20 and mCry3A on the predatory beetle *Coleomegilla maculata.* Study No. 1781.6671.
- MRID 47153005; Stacey, D. and R. Blake (2007). A laboratory study to determine effects of Vip3Aa20 + Cry1Ab + mCry3A proteins on the rove beetle *Aleochara bilineata* (Coleoptera: Staphylinidae). Study No. T002321-06-REG.

These two nontarget organism studies were conducted in support of the Section 3 applications for Syngenta's Bt11xMIR162 and Bt11xMIR162xMIR604 products **only**, as these products contain multiple insecticidal proteins (Cry1Ab, Vip3Aa20, and mCry3A). These studies were conducted to determine whether the combination of the Vip3Aa20, Cry1Ab, and mCry3A proteins had any potential synergistic effect on the nontarget organisms *Coleomegilla maculate* and *Aleochara bilineata.* The purpose in submitting the TRYCRY1AB-0105 test substance characterization report was to demonstrate that the Cry1Ab test substance used in the above studies had been adequately characterized, i.e, that it contained the intended Cry1Ab protein, that its purity had been determined, and that the protein was bioactive against target pests. Our supply of the previous bacterially-produced Cry1Ab test substance (used in safety studies for Bt11 corn) had been exhausted, and a new test substance was produced in 2005 by a comparable process, i.e., microbial fermentation, purification, and trypsin cleavage of the protoxin. The test substance characterization reports for the Vip3Aa20 and mCry3A protein test substances used in the above-listed nontarget organism studies have been previously submitted to the Agency (MRID 47137801 and 46155603, respectively).

This characterization study was performed to confirm the identity and purity of the Cry1Ab test substance TRYCRY1AB-0105. Various biochemical and functional parameters were used to establish the identity and purity of this test substance (including molecular weight via SDS-PAGE, western blot analysis, protein intactness, insecticidal activity, N-terminal amino acid sequence analysis, peptide analysis, and lipopolysaccharide determination). This test substance characterization study was not conducted to demonstrate equivalency between plant and bacterial-produced Cry1Ab proteins; it was in essence a certificate of analysis. Equivalence between plant and bacterial-produced Cry1Ab proteins was previously demonstrated in the Lee et al.[1] Cry1Ab equivalence report (MRID 43533204). The Lee et al equivalence report is referenced in the data matrices of the Bt11 x MIR162 x MIR604, Bt11 x MIR162, and Bt11 x MIR604 applications for registration.

BPPD Reviewer Comments to Syngenta's response:

Syngenta concluded that the purpose of submitting the characterization of the Cry1Ab test material was to confirm the identity of the trypsinized Cry1Ab of the microbial-expressed test substance TRYCRY1AB-0105 and not to demonstrate equivalence (via SDS-PAGE, Western blot analysis, mass analysis, peptide mapping, N-terminal sequence analysis, and a susceptible insect bioassay). Syngenta also indicated that their previous supply of bacterial-expressed test substance was exhausted, therefore, no subsequent analysis was performed to confirm the equivalence between the original and new test substance. To satisfy the Agency's concerns,

Syngenta referenced a previously submitted Cry1Ab equivalence report[1] as justification for equivalence. However, for every new microbial-expressed test substance that is used as a surrogate test material for a plant-expressed protein, the equivalency between both forms of the protein must be demonstrated in order to justify its use in the toxicity studies. In this case, the TRYCRY1AB-0105 test material was used in two non-target organism studies that were submitted to support the lack of synergism when Cry1Ab, Vip3Aa20, and mCry3A are combined in the stacked PIP products- Bt11 x MIR162 and Bt11 x MIR162 x MIR604. While the identity and characterization of the test material is established, there remains no direct comparison of the identity of the microbial-expressed Cry1Ab test substance to the Cry1Ab protein expressed in Event Bt11 corn. In addition, the following data discrepancies were also identified in this study:

1) In the molecular weight determination, the study author attributed the difference in the predicted molecular weight (*ca.* 66 kD) to the observed typsinized Cry1Ab protein (*ca.* 60 kDa) [see Figures 1 and 2] due to limitations of SDS-PAGE in the accurate determination of the molecular weight2. Syngenta also referenced a study that reviewed the reliability of SDS-PAGE, which concluded that the apparent molecular weight of a protein is typically within 10% of its true molecular weight (Dube and Flynn, 1988). The study author also noted another literature reference that pointed out the similarity of the MW is dependent between the hydrodynamic properties of the protein of interest and the proteins in the standard set (Sadeghi, *et al.*, 2003).

The study by Sadeghi, *et al.* also indicated that the SDS-PAGE shortcomings are usually associated with proteins with molecular weights below 50 kDa or above 250 kDa, or post-translational modifications, differences in molecular charges among types of proteins (histones), and glycosylated proteins. None of these characteristics are applicable to the Cry1Ab protein. Sadeghi, *et al.* also constructed a calibration curve between known protein standards (both unstained and stained) after standardization of the MW by MALDI mass spectrometry in comparison to the estimation of masses of the 'apparent MW" in a variety of gels. The authors showed that the use of standard methods, such as calibration curves, can provide good estimates of MW and reduce some of the limitations of SDS-PAGE. This publication also recommended utilizing the pre-made spreadsheets that are publically accessible, which automatically perform the necessary calculations for MW determination, based on preselected non-linear equations for a variety of vendor, marker, and gel types. There was no mention of utilizing any of these techniques to reduce the limitations of SDS-PAGE.

2) The total mass analysis of the test substance TRYCRY1AB-0105 showed two forms of Cry1Ab proteins, with masses of 65.8 and 66.3 kDa, however, the mass spectra demonstrating these peaks were not provided. **While these results indicated that the**

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1 Lee, *et al.* 1995 Assessment of the equivalence of the *Bacillus thuringiensis* subsp. *kurstaki* HD-1 protein produced in *Escherichia coli* and European corn borer resistant corn. MSL-13879.

2 This trend was seen in the previous data submissions for establishing the molecular weight of MCRY3A-0102, the microbial-expressed test substance for use in toxicity studies for mCry3A protein as expressed in Event MIR604 corn. In that report, the lower MW observed for the test substance was also attributed to SDS-PAGE limitations (MRID No. 461556-03, -05, and -06; reviewed in EPA memoranda: from A. Fellman, through J. Kough, to M. Mendelsohn, dated 02/11/2005 and 02/23/2005). Therefore, this is a repeated concern among data submissions and it is highly recommended that a standardization of the materials and methods be utilized and/or the continued use of MALDI-TOF spectrometry with submission of the mass spectra for visual confirmation be provided in the future.

test substance contains the truncated insecticidal portion of Cry1Ab protein (albeit two forms) with the predicted MW of 66 kDa, the registrant should submit the mass spectra as visual confirmation of the molecular weights for the two forms of Cry1Ab for the test substance TRYCRY1AB-0105.

3) In the Western blot results of the Bt11 maize leaf plant extract, the study authors concluded that the presence of intact Cry1Ab was shown in the Bt11 maize plant extract as corresponding bands (see Figure 2, lane 2). However, the biologically active portion of the insecticidal Cry1Ab does not appear to have a corresponding band. The closest in mobility was a faint *ca.* 54 kDa band and an immunoreactive *ca.* 39 kDa band with stronger intensity. In addition, the extract of Bt11 maize leaf was not characterized for its biochemical and functional similarities to demonstrate its equivalence to the microbial-expressed substance. There were few details of the preparation of the plant extract, such as whether the plant extract was purified and lyophilized. **Therefore, the registrant should concurrently analyze a purified plant-expressed protein extracted from Bt11 maize for its biochemical and functional characteristics (such as molecular weight analysis and biological activity) as confirmation of its equivalence to the microbial-expressed TRYCRY1AB-0105 protein.**

V. CLASSIFICATION: SUPPLEMENTAL- The registrant should submit the mass spectra as visual confirmation of the molecular weights for the two forms of Cry1Ab for the test substance TRYCRY1AB-0105 and conduct a direct and concurrent analysis of a purified plant-expressed protein extracted from Bt11 maize leaves to the TRYCRY1AB-0105 test substance. These data can be used to determine the equivalence between the plant- and microbial-produced Cry1Ab proteins in support of utilizing the TRYCRY1AB-0105, as a test material in the non-target organism studies for demonstration of lack of synergism in the stacked Bt11 x MIR162 and Bt11 x MIR162 x MIR604 PIP products.

VI. REFERENCES:

Bietlot, H., P.R. Carey, C. Choma, H. Kaplan, T. Lessard, and M. Pozsgay (1989) Facile preparation and characterization of the toxin from *Bacillus thuringiensis* var. kurstaki. *Biochem. J.* 260: 87-91.

Brauer, A.W., Oman, C., Margolis, M.N. (1984) Use of o-phthalaldehyde to reduce background during automated Edman degradation. *Analytical Biochemistry* 137: 134-142.

Dively, G.P. (2005) Impact of transgenic VIP3A x Cry1Ab lepidopteran-resistant field corn on the nontarget arthropod community. *Environmental Entomology* 34, 1267-1291. MRID 46784601.

Dube, S. and E. Flynn (1988) Estimating protein molecular weights using SDS-PAGE. *Focus* 20: 24-25.

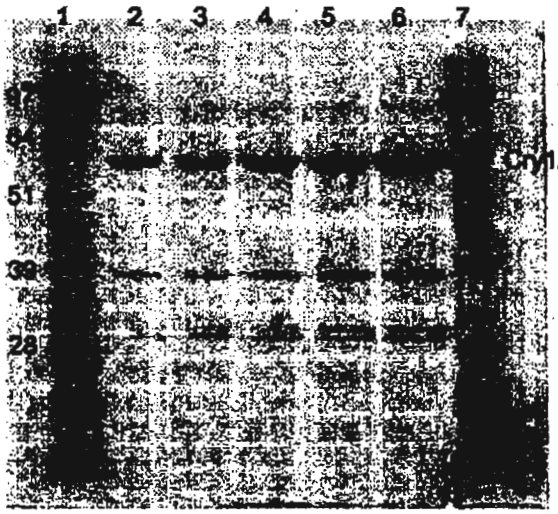
Marcon, P.C.R.G., L. J. Young, K. L. Steffey, and B. D. Siegfried. (1999) Baseline susceptibility of European corn borer (Lepidoptera: Crambidae) to *Bacillus thuringiensis* toxins. *J. Econ. Entomol.* 92: 279 - 285.

US EPA, (2001) Biopesticides Registration Action Document for the *Bacillus thuringiensis* (*Bt*) Plant-Incorporated Protectants, Dated October 15, 2001.

US EPA, (2008) Biopesticides Registration Action Document for *Bacillus thuringiensis* modified Cry1Ab (SYN-IR67B-1) and Vip3Aa19 (SYN-IR102-7) insecticidal proteins and the genetic material necessary for their production in COT102 X COT67B cotton, Dated June 26, 2008.

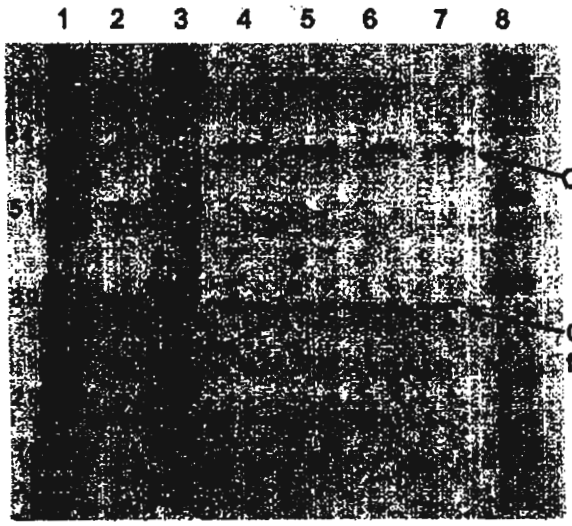
Graser, G. (2005) Characterization of Cry1Ab Test Substance FLCRY1AB-0103 and Certificate of Analysis. Syngenta Biotechnology, Inc., Report No. SSB-001-05 [see MRID No. 47017604].

Figure 1. SDS-PAGE gel of test substance TRYCRY1AB-0105 used for densitometric analysis.



Lane 1: Molecular weight markers.
Lane 2: 1.3 µg TRYCRY1AB-0105.
Lane 3: 2.6 µg TRYCRY1AB-0105.
Lane 4: 3.9 µg TRYCRY1AB-0105.
Lane 5: 5.3 µg TRYCRY1AB-0105.
Lane 6: 6.6 µg TRYCRY1AB-0105.
Lane 7: Molecular weight markers.

Figure 2. Immunoreactivity (Western blot) of the test substance TRYCRY1AB-0105.



Lane 1: Molecular weight markers.
Lane 2: Event Bt11 maize leaf extract (20 ng).
Lane 3: Molecular weight markers.
Lane 4: ~38 ng Cry1Ab protein.
Lane 5: ~29 ng Cry1Ab protein.
Lane 6: ~19 ng Cry1Ab protein.
Lane 7: ~5 ng Cry1Ab protein.
Lane 8: Molecular weight markers.

Figure 3. FLCry1Ab used to prepare the trypsinized Cry1Ab protein.

A partial tryptic digest of the FLCry1Ab protein (1181 amino acids) at the trypsin recognition sites of arginine-28 and arginine-619 was used to produce the truncated (trypsinized) Cry1Ab protein of 591 amino acids (shown in bold) in test substance TRYCRY1AB-0105. An additional Cry1Ab species (587 amino acids) apparently lacking the last four amino acids (DLER) was also identified in the test substance. The amino acid sequence corresponding to the truncated Cry1Ab protein in Bt11 (615 amino acids) is underlined. The insecticidal portion of the protein begins at the sequence IETG (in bold) after arginine-28 and is *ca.* 66 kDa in size (Bietlot *et al.*, 1989).

MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLG
LVDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWE
ADPTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSV
FGQRWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRREL
TLTVLDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPH
LMDILNSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQL
GQGVYRTLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEI
PPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQIT
QIPLTKSTNLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYAS
TTNLQFHTSIDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHV
FNSGNEVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVS
NLVECLSDEFCLDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITI
QGGDDVFKENYVTLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIR
YNAKHETVNVPGTGSLWPLSAPSPIGKCGEPNRCAPHLEWNPDLDCSCRDGEKCAHHSH
HFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFLEEKPLVGEALARVKRAEKK
WRDKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNIAMIHAADKRVHSIREAYL
PELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNGLSCWNVKGHVDVEEQNN
HRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVTIHEIENNTDELKFSNC
VEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSVPADYASAYEEKAYTD
GRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETEGTFIVDSVELLLME
E

Figure 4. Predicted and Actual N-terminal Amino Acid Sequences of Cry1Ab and Cry1Ab Fragments in Test Substance TRYCRY1AB-0105.

Underlined sequences confirmed the predicted amino acids by N-terminal sequencing. Theoretical cleavage site of the *ca.* 66 kDa protein into the *ca.* 39 and 27 kDa peptides indicated by symbol (*). Presence of four amino acids at the C-terminal (DLER) is consistent with a molecular weight of 66.3 kDa.

66 kDa Cry1Ab (587 and 591 amino acids)

IETGYTPIDISLSLTQFLLSEFVPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLINQRI
EEFARNQAISRLEGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLF
AVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLIGNYTDHAV
RWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVSLFPNYDSRTYPIRTVSQLTREIY
TNPVLENFDGSFRGSAQGIEGSIRSPHLMDILNSITIYTDAHRGEYYWSGHQIMASPVGF
SGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYRTLSSTLYRRPF*NIGINNQQLSVLDGTE
FAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSVSI
IRAPMFSWIHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKGPGFTGGDILRRTSPGQ
ISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNFSATMSSGSNLQSGSFR
TVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDRIEFVPAEVTFEAEY(DLER)

39 kDa Cry1Ab fragment (343 amino acids)

IETGYTPIDISLSLTQFLLSEFVPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLINQRI
EEFARNQAISRLEGLSNLYQIYAESFREWEADPTNPALREEMRIQFNDMNSALTTAIPLF
AVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLIGNYTDHAV
RWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVSLFPNYDSRTYPIRTVSQLTREIY
TNPVLENFDGSFRGSAQGIEGSIRSPHLMDILNSITIYTDAHRGEYYWSGHQIMASPVGF
SGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYRTLSSTLYRRPF

27 kDa Cry1Ab fragment (244 or 248 amino acids)

NIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNVPPRQGFSHRLS
HVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKSTNLGSGTSVVKG
PGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTSIDGRPINQGNF
SATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDRIEFVPAEVTF
EAEY(DLER)

Figure 5. Peptide Mapping of Tryptic Digest of TRYCRY1AB-0105.

TRYCRY1AB-0105 was completely digested[a] with trypsin. which cleaves at the carboxyl side of the basic amino acids lysine and arginine. The resulting peptides were analyzed by LC/MS/MS on a Q-TOF spectrometer and their spectra were matched against the predicted Cry1Ab sequence resulting in a total sequence coverage of 26% (underlined). Absence of the last four amino acids at the C-terminal (DLER) is consistent with the mass analysis results for the major protein species in TRYCRY1AB-0105.

[a] **TRYCRY1AB-0105 was completely digested with two overnight incubations in trypsin.**