5) Hileman, R. E. and J. D. Astwood (2001). Additional characterization of the Cry3Bb1 protein produced in corn event MON 863. MSL-17137, an unpublished study conducted by Monsanto Company.

Study Title

Additional Characterization of the Cry3Bb1 Protein Produced in Corn Event MON 863

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Summary of Quality Control Review

This report was reviewed to ensure that it accurately reflects the supporting documentation. The supporting documentation was audited for compliance to the Monsanto Company Guidelines for Keeping Research Records (GRR 10/1/99), and where applicable, to Monsanto SOP's.

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Abbreviations and Definitions

aa	amino acid
B.t.	*Bacillus thuringiensis*
Cry3Bb1	*Bacillus thuringiensis* derived insecticidal protein
cry3Bb1	DNA that encodes the Cry3Bb1 protein
IUPAC-IUB	International Union of Pure and Applied Chemistry - International Union of Biochemistry
kb	kilobase
MALDI-TOF	Matrix assisted laser desorption ionization time of flight
npt II	DNA that encodes for the neomycin phosphostransferase II (NPT II) protein

1.0 Summary

Corn plants protected from corn rootworm feeding damage were designed by insertion of a gene encoding a modified *Bacillus thuringiensis* Cry3Bb1 protein into the corn genome. Corn event MON 863, which produces the Cry3Bb1 protein referred to as Cry3Bb1.11098, is the focus of this report. Other corn events producing the Cry3Bb1.11098 protein as well as a separately modified Cry3Bb1 protein have been previously reported and are also described for completeness.

Initial modifications of the wild type *Bacillus thuringiensis* (*B.t.*) *cry3Bb1* gene (Donovan *et al.*, 1992) were performed using a *B.t.* cloning vector and expression system (English *et al.*, 2000). Bioactivity analyses were used to select variant *cry3Bb1* coding sequences that produced Cry3Bb1 proteins in *B.t.* with increased activity toward the larvae of a target insect, Southern corn rootworm. The modified *cry3Bb1* coding sequence contained in *B.t.* strain EG11098 (producing the Cry3Bb1.11098 protein) was selected for corn transformation as part of this extensive process.

The Cry3Bb1.11098 protein produced in *B.t.* differs from the wild type Cry3Bb1 protein by 5 amino acids. When this *B.t.* gene was modified for expression in corn, the resulting gene was predicted to encode an additional two amino acid changes relative to the *B.t.* produced Cry3Bb1.11098 protein sequence. Thus the Cry3Bb1.11098 protein produced in corn event MON 863 differs from the *B.t.* produced Cry3Bb1.11098 protein by 2 amino acids, and 7 amino acids relative to the wild type Cry3Bb1 protein.

Additional characterization of the DNA sequence of the *cry3Bb1* coding region in corn event MON 863 and the Cry3Bb1 protein produced in corn event MON 863 confirmed these described amino acid differences relative to the wild type Cry3Bb1 protein sequence deduced from the wild type *cry3Bb1* gene. The exact DNA and encoded protein sequence of this specific Cry3Bb1 allele is described in this report. Specific amino acid substitutions are shown in Table 1.

Table 1. Amino Acid Diversity of Cry3Bb1 Variants.

Cry3Bb1 Allele	Amino acid substitutions relative to wild type Cry3Bb1 protein[a]						
B.t. strain EG11098 Cry3Bb1.11098 protein		D165G	H231R	S311L	N313T	E317K	
B.t. strain EG11231Cry3Bb1.11231 protein			H231R	S311L	N313T	E317K	
Corn event MON 863 Cry3Bb1.11098 protein[b]	A2	D166G	H232R	S312L	N314T	E318K	Q349R
Corn event MON 853 Cry3Bb1.11231 protein[b]	A2		H232R	S312L	N314T	E318K	

[a] wild type Cry3Bb1 protein corresponds to GenBank Accession No. M89794.
[b] Amino acid positions are shifted upwards by one amino acid relative to the *B.t.* proteins due to the introduction of an alanine at position 2.

2.0 Background

A modified gene encoding a variant of the *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 protein was used to design corn event MON 863. A separate but similar *cry3Bb1* gene variant was used to design corn event MON 853 (Cavato *et al.*, 1999). Although the Cry3Bb1 protein produced in corn event MON 853 is not the focus of this report, DNA and protein sequences are provided for completeness because initial food, feed and environmental evaluations were performed using this protein variant. Plants producing these Cry3Bb1 protein variants are resistant to feeding damage from the coleopteran insect, corn rootworm.

The wild type *cry3Bb1* gene (GenBank Accession No. M89794) was modified initially utilizing a *B.t.* plasmid and expression system (English *et al.*, 2000). Proteins encoded by two modified *cry3Bb1* genes were observed to have increased bioactivity towards corn rootworm when expressed in *B.t.* These were the *cry3Bb1.11098* and *cry3Bb1.11231* genes, contained in *B.t.* strains EG11098 and EG11231 respectively, which produced the corresponding Cry3Bb1.11098 and Cry3Bb1.11231 proteins. The specific modifications that yielded these Cry3Bb1 protein variants are described in Table 1. The Cry3Bb1.11098 protein produced in *B.t.* strain EG11098 differs from the wild type Cry3Bb1 protein by 5 amino acids. The Cry3Bb1.11231 protein produced in *B.t.* strain EG11231 differs from the wild type Cry3Bb1 protein by 4 amino acids.

DNA encoding these Cry3Bb1 protein variants were then modified to design genes compatible for cloning and expression in plants prior to transforming corn. Corn events that have been previously described are summarized in Figure 1. These modifications resulted in an additional change to the predicted amino acid sequences of these variant proteins. That is, three nucleotides (GCC for the *cry3Bb1.11098* allele or GCA for the *cry3Bb1.11231* allele, at positions 4, 5 and 6) were inserted at the 5' end of the coding sequence to create an *Nco* I restriction endonuclease site and facilitate cloning of the *cry3Bb1* gene into a plant transformation vector. This manipulation resulted in the introduction of an alanine (A) residue at position 2 of the predicted plant protein sequences.

In addition to the *cry3Bb1* gene, other genetic elements were included for gene expression and selection, but are not the described in this report. For example, the neomycin phosphostransferase II (*nptII*) gene was used as the selectable marker (Cavato *et al.*, 2001), which enabled selection of cells in tissue culture containing the *cry3Bb1* gene.

Recent DNA sequencing of the *cry3Bb1* coding region and protein analyses of the Cry3Bb1 protein produced in corn event MON 863 have shown that the plant expressed 11098 gene encodes a Q349R substitution in the expected 11098 protein. The Cry3Bb1.11098 protein produced in *B.t.* strain EG11098 differs from the wild type Cry3Bb1 protein by 5 amino acids (Tables 2 and 3). When the coding region of this *B.t.* gene was modified for expression in corn, the resulting gene is predicted to encode an additional two changes relative to the *B.t.* strain EG11098 Cry3Bb1.11098 protein sequence. Thus, corn event MON 863 Cry3Bb1.11098 protein (as well as all other corn events producing the Cry3Bb1.11098 protein described in Figure 1) differs from the *B.t.* strain EG11098 Cry3Bb1.11098 protein by 2 amino acids, or 7 amino acids relative to the wild type Cry3Bb1 protein. The Q349R substitution is absent in corn events producing the Cry3Bb1.11231 protein.

3.0 Molecular Characterization of the *cry3Bb1* Insert in Corn Event MON 863

Additional molecular analyses have been recently performed on corn event MON 863 (Cavato and Lirette, 2001). Analysis of the insert confirmed the organization of the elements within the insert, as well as the complete DNA sequence of the insert in corn rootworm event MON 863 using PCR and DNA sequence analyses. The sequence of the 1.96 kb *cry3Bb1* DNA sequence and deduced 653 amino acid protein sequence is shown in Figure 2.

The deduced Cry3Bb1.11098 protein sequence in corn event MON 863 differs from the *B.t.* produced Cry3Bb1.11098 protein by 2 amino acids, or 7 amino acids relative to the wild type Cry3Bb1 protein. The deduced amino acid sequence of the Cry3Bb1.11098

protein in corn events MON 852, MON 858, MON 859 MON 862 and 96.040 is predicted to contain the same changes because the same *cry3Bb1.11098* gene was integrated into the transformation vectors used to create these corn events. Similarly, the deduced amino acid sequence of the Cry3Bb1.11231 protein in corn events MON 853, MON 854, MON 855 and MON 860 is predicted to contain the same changes because the same *cry3Bb1.11231* gene was integrated into the transformation vectors used to create these corn events. The deduced Cry3Bb1.11231 protein sequence in corn events producing the Cry3Bb1.11231 protein differs from the *B.t.* produced Cry3Bb1.11231 protein by 1 amino acid, or 5 amino acids relative to the wild type Cry3Bb1 protein. These amino acid changes are summarized in Table 3.

4.0 Characterization of the Cry3Bb1 Protein Produced in Corn Event MON 863

Additional analyses were also performed on the Cry3Bb1 protein produced in corn event MON 863. Cry3Bb1 protein was isolated from grain using immunoaffinity chromatography and analyzed by N-terminal sequencing. Peptide fragments produced by tryptic digestion were analyzed using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (Thoma *et al.*, 2001). Approximately 38% of the entire Cry3Bb1 protein sequence was identified by a combination of mass matching and N-terminal sequencing (Figure 2), confirming the amino acid sequence deduced from the DNA. Moreover, the mass spectral data were used to verify the presence of the alanine residue at position 2 and the Q349R substitution in corn event MON 863.

5.0 Conclusions

Previous reports did not indicate the presence of the intended Q349R substitution in the Cry3Bb1.11098 plant produced protein sequence. Recent additional analyses performed on both DNA and protein isolated from corn event MON 863 confirm that this substitution exists. The Q349R substitution is present in the predicted Cry3Bb1 protein sequence of all corn events producing the 11098 variant. This substitution was not intended for, and is therefore absent in corn events producing the Cry3Bb1.11231 protein variant.

Large quantities of Cry3Bb1.11098 and Cry3Bb1.11231 proteins were prepared from fermentation of *B.t.* (Hileman *et al.*, 2001) for mammalian and ecotoxicology evaluations as well as to serve as reference proteins in immunodiagnostic assays. These *B.t.* produced Cry3Bb1 protein variants were shown to be physicochemically and functionally equivalent to the respective corn produced proteins (Holleschak *et al.*, 2001a; Holleschak *et al.*, 2001b). Thus the Q349R substitution found in the plant produced Cry3Bb1.11098 protein is not expected to have changed the physicochemical and functional characteristics of this protein.

6.0 References

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Thoma, R. S., Holleschak, G., and Astwood, J. D. (2001). Primary structural protein characterization of Cry3Bb1.11098 protein from insect protected corn event MON 863 using MALDI time of flight mass spectrometric and N-terminal sequencing techniques. (Monsanto Company, St. Louis, MO) Technical Report MSL-17154.

Table 2. Specific nucleotide modifications and resulting amino acid changes used to produce the Cry3Bb1.11098 and Cry3Bb1.11231 proteins in *Bacillus thuringiensis*.

Cry3Bb1 Allele	*cry3Bb1* DNA modifications[a]	Resulting Cry3Bb1 aa modifications[a]
B.t. Strain EG11098	A494G, T687C, A692G, C932T, A938C, T942G, G949A, T954C	D165G H231R S311L N313T E317K
B.t. Strain EG11231	T687C, A692G, C932T, A938C, T942G, G949A, T954C	H281R S311L N313T E217K

[a] Standard biochemical nomenclature is shown using IUPAC-IUB single letter code. The first letter corresponds to the wild type base or amino acid, the following number corresponds to the position, and the second letter corresponds to the changed base or amino acid. The base and amino acid positions are shown relative to the wild type sequence.

Table 3. Summary of the amino acid differences among the Cry3Bb1 protein variants produced in corn.

Cry3Bb1 Allele	Cry3Bb1 aa modifications[a]						
Corn event MON 863 Cry3Bb1.11098	A2	D166G	H232R	S312L	N314T	E318K	Q349R
Corn event MON 853 Cry3Bb1.11231	A2	–	H232R	S312L	N314T	E318K	–

[a] Standard biochemical nomenclature is shown using IUPAC-IUB single letter code as described in Table 2. Amino acid positions are shifted upwards by one amino acid relative to the *B.t.* proteins (Table 2) due to the introduction of an alanine at position 2.

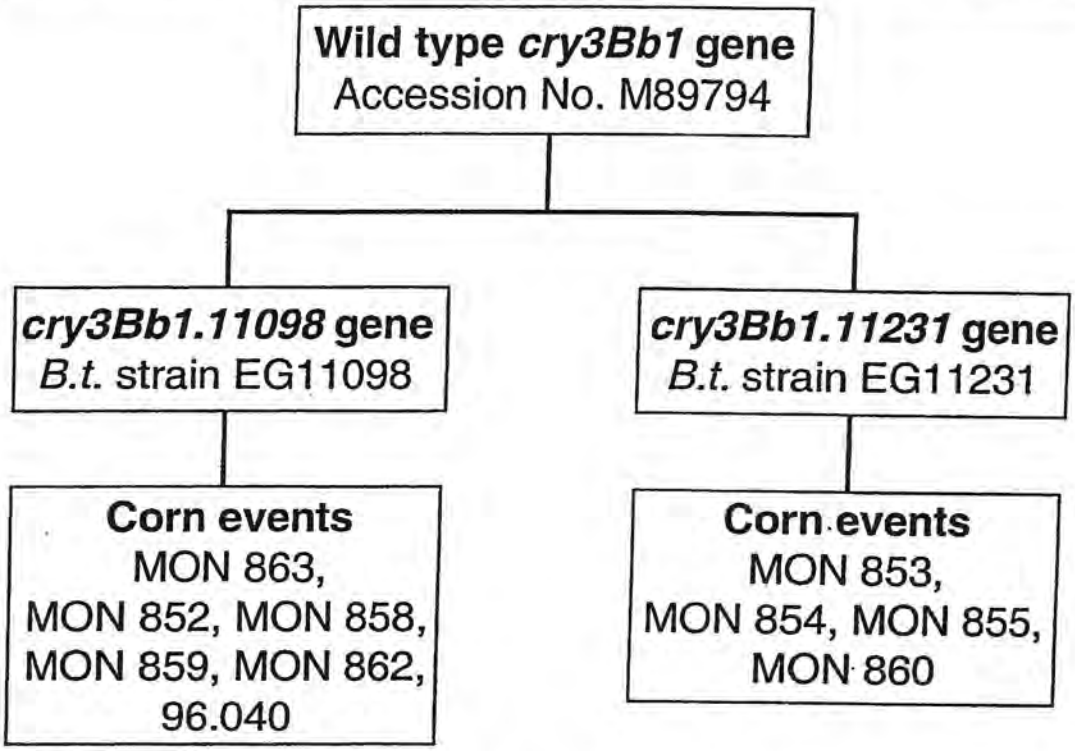

Figure 1. Summary of the origin of the Cry3Bb1 variant proteins present in corn events described in food, feed and environmental evaluations.

This place holder page in place of the following pages and can be found in **Cross Reference Number 1** of the **Confidential Attachment.**

Page	MSL-17137 Pages 17-22 of 22
Page Title	**Figure 2.** Sequences of the Cry3Bb1 protein variants
Reason for Deletion	Discloses commercial information
FIFRA Reference	10(d)(1)(A)

MSL-17137 Pages 17-22 of 22 are found in the **Confidential Attachment,** Pages 3-8 of 8.

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ATTACHMENT