16) Silvanovich, A., K. Karunanandaa, R. S. Thoma, J. Blasberg and J. D. Astwood (2001). The absence of detectable ble translation products in corn grain containing event MON 863. MSL-17449, an unpublished study conducted by Monsanto Company.

NOTE TO FILE
STUDY: 01-01-39-42

Explanation for different titles used in study protocol and the final report:

Protocol title reflects the question we intend to address and the report title reflects the conclusion of the study.

OBSERVED BY: [signature] Date: Dec 7, 2001

Study Title

The Absence of Detectable *ble* Translation Products in Corn Grain Containing Event MON 863

Authors

Andre Silvanovich, Ph.D., Kanthasamy Karunanandaa, Ph.D., Richard S. Thoma, M.Sc., Jennifer Blasberg, B.Sc. and James D. Astwood, Ph.D.

Study Completed On

December 10, 2001

Performing Laboratory

Product Safety Center
Biotechnology Regulatory Sciences
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St. Louis, MO 63167, U.S.A.

Laboratory Project ID

MSL-17449
Study 01-01-39-42

Statement of No Data Confidentiality Claims

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10(d)(1)(A), (B), or (C).

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by company notwithstanding this submission to the EPA."

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Title: _____

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Statement of Compliance

This study meets the requirements under GLP as specified in 40 CFR Part 160 (EPA) except:

The microplate reader equipment records were not GLP compliant, in that no equipment SOP was available. Nevertheless, this instrument was calibrated at the time of use and records for calibration and maintenance have been retained. Collected data were well documented according to the Monsanto Company Guidelines for Keeping Research Records (GRR 10/1799).

The raw data for Figure 2, a Coomassie stained SDS-PAGE gel, was not retained.

Submitter: _____ Date: _____

Sponsor Representative: Ravinder S Sidhu Date: Dec 10, 2001
Ravinder S. Sidhu, Ph.D.

Study Director: _____ Date: Dec 10, 2001
Andre Silvanovich, Ph.D.

Quality Assurance Statement

Study Title: The Absence of Detectable *ble* Translation Products in Corn Grain Containing Event MON 863

Study Number: 01-01-39-42

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study report herein.

Date of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
09/10/2001	Protein extraction	10/01/2001	10/01/2001
11/28/2001	Raw data audit	12/10/2001	12/10/2001
11/28/2001	Draft report review	12/10/2001	12/10/2001

Barbara A Foy

Barbara A. Foy
Quality Assurance Unit
Monsanto Regulatory
Monsanto Company

10 Dec. 2001
Date

Study Number: 00-01-39-42

Title: The Absence of Detectable *ble* Translation Products in Corn Grain Containing Event MON 863

Testing Facilities: Monsanto Company
Product Safety Center
Biotechnology Regulatory Sciences
800 N. Lindbergh Blvd.,
St. Louis, MO 63167, USA

Sponsor Monsanto Company

Study Director Andre Silvanovich

Study Initiation Date: September 10, 2001

Study Completion Date: December 10, 2001

Records Retention: The protocol, all study specific raw data, final reports and facility records will be retained at Monsanto, St. Louis.

Signatures of Approval:

Study Director, Product Safety Center: Andre Silvanovich — Date: Dec 10, 2001

Principal Investigator, Product Safety Center: Kanthasamy Karunanandaa — Date: Dec 10, 2001

Director, Product Safety Center: James D. Astwood — Date: Dec 8, 2001

Sponsor Representative: Ravinder S. Sidhu — Date: Dec 10, 2001

Testing Facility Manangement: Patrick T. Weston — Date: Dec 10, 2001

Table of Contents

Abbreviations

≈	Approximately
BLE	Bleomycin binding protein from transposon Tn5
ble	The gene encoding the Tn5 Bleomycin binding protein
BLE 10.25	A putative 10.25 kDa protein containing amino acids 1-51 of Tn5 bleomycin binding protein
cDNA	Complementary DNA
COA	Certificate of Analysis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
HIS6 BLE	Tn5 bleomycin binding protein tagged near the amino terminal with a linker and 6 histidine residues
IPTG	Isopropylthio-β-D-galactoside
IRES	Internal ribosome entry site
LOD	Limit of detection
MW	Molecular Weight
mRNA	messenger ribonucleic acid
NFDM	Nonfat dried milk
NFDM-PBS	Nonfat dried milk- phosphate-buffered saline
NOS 3'	The transcriptional terminator of nopaline synthase
NPTII	Neomycin phosphotransferase II
*npt*II	The gene encoding neomycin phosphotransferase II
ORF	Open reading frame
pET23b-ble 10.25	A plasmid that encodes a putative 10.25 kDa protein containing aminoacids 1-51 of Tn5 bleomycin binding protein
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween-20
ppm	Parts per million (µg/g)
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TE	10 mM Tris-HCl-1mM EDTA buffer
Tris-HCl	Tris(hydroxymethyl)aminomethane as the hydrochloride salt
Tween-20	Polyoxyethylenesorbitan monolaurate
UTB	urea-tris-sodium tetraborate

1.0 Summary

Monsanto has genetically improved corn (*Zea mays* L.) to produce a variant of the wild type Cry3Bb1 protein, isolated from *Bacillus thuringiensis*, that has insecticidal activity against corn rootworm (CRW, *Diabrotica*), a major North American insect pest. The vector that was used to introduce the *cry3Bb1* gene into the corn genome (event MON 863) contains two expression cassettes: one encodes the Cry3Bb1 protein and the other encodes the dominant selectable plant transformation marker, neomycin phosphotransferase II (NPTII). Sequence analysis confirmed that the NPTII expression cassette has the potential to produce an mRNA that contains two open reading frames (ORFs). One ORF encodes the NPTII protein, while the other ORF, whose start codon is located 20 nucleotides downstream of the NPTII stop codon, encodes a segment of the bleomycin binding protein (BLE). Specifically, if translated, this second ORF would yield a 10.25 kDa protein containing amino acids 1-51 of the BLE protein linked to four amino acid residues encoded by a DNA cloning linker, followed by 34 amino acid residues encoded by the NOS 3' transcriptional terminator. Hereafter, this putative 10.25 kDa protein will be referred to as BLE 10.25.

Although the *nptII* transcriptional unit could potentially yield an mRNA that contains both *nptII* and *ble 10.25* coding sequences, it is predicted that only the *nptII* coding sequence will be translated *in planta*. This prediction is based on a substantial body of published data which demonstrates that eukaryotic translational initiation occurs via a ribosomal mRNA scanning mechanism. In eukaryotes, ribosomal subunits identify a unique structure at the 5' end of the mRNA called a "cap". Once bound to the 5' end of the mRNA, a ribosome scans the mRNA until the first contextually correct AUG start codon is identified and translation is initiated. Translation then continues until a stop codon is encountered. Three mechanisms that facilitate the translation of more than one ORF in eukaryotic polycistronic mRNA (mRNAs that contain two or more non-overlapping ORFs) have been described. Two translational mechanisms are entirely dependent upon the size and positioning of the ORFs within the mRNA. The first translational mechanism involves ribosome frame shifting and/or the bypass of the stop codon in the 5' ORF such that translation yields a polyprotein composed of both ORFs. The second translational mechanism involves translational reinitiation at a second ORF whose start codon is located downstream of the 5' ORF stop codon. The final mechanism involves the presence of an internal ribosome entry site (IRES) in the mRNA that permits bypass of the 5' "cap". Examination of the *nptII* transcriptional unit mRNA sequence shows that neither the size of the *ble 10.25* coding sequence nor positioning relative to the NPT II ORF would facilitate its translation. Moreover, the distance separating the NPTII stop codon and the BLE 10.25 start codon is insufficient to contain an IRES. Given these constraints, it is predicted that only NPTII will be translated from the mRNA expressed by the *nptII* transcriptional unit.

This study was conducted to confirm that BLE 10.25 is not produced in the grain of corn event MON 863. Rabbit antiserum that is capable of detecting the BLE protein was used in this study to demonstrate the absence of detectable plant-produced BLE 10.25, while *E. coli*-produced BLE 10.25, identical to the plant putative BLE 10.25, was used as the positive control. Through spiking experiments, it was also shown that corn grain extracts do not mask the immunoreactivity of *E. coli*-produced BLE 10.25. Using western blot analysis, it was determined that BLE 10.25 is not detectable in grain from corn event MON 863 above the limit of detection of 1.7 ppm. These data corroborate the prediction that BLE 10.25 protein is not produced in grain from corn event MON 863.

2.0 Introduction

2.1 Background

The full-length *ble* gene encodes a protein named bleomycin binding protein (BLE, alternatively known as BLMT) that confers resistance to bleomycin. Bleomycin belongs to a class of glycopeptide antibiotics produced by *Streptomyces verticillus* (Genilloud, 1984) which function by generating free radicals (Harrison 1991) that catalyze double strand DNA breakage (Bansal, 1997; Vanderwall, 1997; and Wu, 1998). Despite being a potent antibacterial agent, bleomycin has found only limited use in therapeutic and experimental settings. In a clinical setting, bleomycin is one of a large group of drugs known as "antineoplastics" or cancer drugs. In the laboratory, bleomycin and the related homologue phleomycin have been used as selective agents for transformation events that are scored using the dominant selectable marker *ble.* However, unlike the antibiotics ampicillin and kanamycin, that are used almost universally for transformant selection, currently less than 20 publications describe the use of bleomycin or phleomycin for this purpose.

The dominant selectable marker (*nptII)* that was used for the identification of corn rootworm-protected transformants including event MON 863 was originally cloned from the transposon Tn5 into a backbone derived from pBR322, yielding the plasmid pKC7. This *E. coli*-derived Tn5 transposon sequence included an additional sequence downstream of the *nptII* ORF. Through subsequent stages of transformation plasmid construction, a linker and the NOS 3' transcriptional terminator were inserted into a *Sma* I restriction site downstream of the *nptII* coding sequence. Due to this selection of the *Sma* I restriction site and the subsequent cloning steps required to construct the MON 863 transformation vector, the *nptII* transcriptional unit contained in corn event MON 863 yields an mRNA that contains two open reading frames (ORFs) when transcribed. The first is a 792 nucleotide ORF located at the 5' end of the mRNA encodes the NPTII protein. A second ORF, whose start codon is located 20

nucleotides downstream of the *nptII* stop codon, encodes a segment of the bleomycin binding protein joined through the linker to the NOS 3' transcriptional terminator. This *ble*-linker-NOS 3' ORF encodes an 89 amino acid polypeptide named BLE 10.25 (Figure 1). Presently, there exists a large body of published scientific data related to the mechanism of mRNA translation in eukaryotes (Hinnebusch, 1997; and Kozak, 1987). These data demonstrated that, with a small number of well-characterized exceptions, only one ORF per mRNA is translated to yield a protein in eukaryotes. Examination of the *nptII* transcriptional unit sequence shows that the size and spatial arrangement of the NPTII and BLE 10.25 ORFs are inconsistent with any known eukaryotic mRNA that, when translated, would yield two proteins. In the unlikely event that the *ble 10.25* coding sequence were translated, it would be contained in a NPTII-BLE 10.25 polyprotein. However, previous western blot studies have shown no evidence of such a polyprotein (Holleschak *et al.*, 2001 and references therein). Therefore, it is predicted that the *ble 10.25* ORF will not be translated, and consequently, will have no impact on the physiology or safety of crops containing this DNA.

2.2 Purpose

The purpose of this study was to determine whether the BLE 10.25 protein is produced and, if so, at what level in the grain of corn event MON 863.

3.0 Materials

3.1 Test Substance

The test substance was the grain of corn hybrid CRW0586, containing event MON 863 (MON 863 grain; LIMS #: 00ZMGRO02934, Production plan #: 00-01-39-18, archival sample lot #: 1746LDRP), that was stored at room temperature prior to protein extraction. Identity of the test material was confirmed by event specific PCR and the certificate of analysis (COA) records archived with this study.

3.2 Control Substance

The control substance used in this study was the grain of non-transgenic corn hybrid RX670 (LIMS#: 00ZMGRO02937, Production plan #: 00-01-39-18, archival sample lot #: SCP71A99IT) which has a genetic background similar to that of the test substance. Identity of the test material was confirmed by event specific PCR and the certificate of analysis (COA) records archived with this study.

3.3 Reference Substance

The reference substance was the *E. coli*-produced BLE 10.25 protein (Lot #: 6949365-A), which is identical in its amino acid sequence to the putative BLE 10.25 protein in corn event MON 863. The DNA that encodes BLE 10.25 was amplified by PCR from MON 863 genomic DNA using a nested primer methodology. The initial round of PCR employed a primer pair that amplified a 1230 bp segment of DNA that contained the BLE 10.25 coding sequence. This 1230 bp PCR product was subsequently amplified using a pair of primers that specifically amplified the BLE 10.25 ORF and incorporated *Nde* I and *Hind* III restriction sites into the 5' and 3' flanks of the PCR product, respectively. The PCR product was subjected to restriction enzyme digestion with *Nde* I and *Hind* III and cloned into the corresponding restriction sites contained in pET23b. The expression vector carrying the *ble 10.25* coding sequence was transformed into an *E. coli* host strain, BL21 pLysS, for gene expression.

Overexpression of the *ble10.25* coding sequence to produce BLE 10.25 protein was achieved by inducing the *E. coli* cells with isopropyl-β-D-thiogalactopyranoside (IPTG) adjusted to a final concentration of 1 mM. Following induction, bacterial cells were harvested and inclusion bodies were prepared by lysing the cells in CelLytic™ B Bacterial Cell Extraction Reagent (Sigma, St. Louis, MO, product #: B-3553). Inclusion bodies were resuspended in Tris-EDTA (TE) buffer and serial dilutions of this preparation were analyzed by gel electrophoresis using a 16% tricine gel along with the known HIS6-BLE (Lot #: 6965119-T, see section 3.4.1) standards to estimate purity and concentration of the expressed BLE 10.25 protein. The HIS6-BLE was histidine-tagged full length bleomycin binding protein expressed in *E. coli* and subsequently affinity purified to near homogeneity. The concentration and purity of the BLE 10.25 protein was estimated to be 3.3 mg/ml and 25%, respectively. Under non-reducing conditions (result not shown), or in the absence of complete reduction, BLE 10.25 forms a dimer and migrates at ≈ 20 kDa molecular size on a 16% tricine gel. Prior to the study, dimerization was confirmed by N-terminal sequencing of both protein bands, 10 kDa (monomer) and 20 kDa (dimer), that were separated on a 16% tricine gel and transblotted onto a PVDFM membrane. The stability of the BLE 10.25 (reference substance) was evaluated by performing western blot analysis at the beginning and end of the experimental phase of the study. As expected, the detection limit was within the acceptable range of between 4 ng to 10 ng and thus the stability of the reference substance did not change during the study period. Therefore, data pertaining to stability-experiment was not reported, but the results were archived with this study.

3.4 HIS6-BLE Antigen and Rabbit Anti-HIS6-BLE Serum Production

3.4.1 HIS6-BLE Antigen Production

HIS6-BLE protein encoded by plasmid pET14b-ble and produced in *E. coli* was used as an antigen for antibody production and as a standard for BLE 10.25 concentration estimation. DNA encoding the full length BLE protein was cloned from plasmid pKC7 using PCR and a primer pair that introduced *Nde* I and *Eco* RI restriction sites into the 5' and 3' flanks of the PCR product, respectively. Following PCR, the product was restricted and cloned into an *Nde* I and *Eco* RI restricted pET14b plasmid. HIS6-BLE was purified from a soluble protein extract of pET14b-ble transformed strain BL21 DE3 *E. coli* using an immobilized nickel ion matrix.

3.4.2 Rabbit Anti-HIS6-BLE Serum Production

Rabbit anti-HIS6-BLE serum was produced by Harlan Bioproducts for Science Inc. (Madison, WI) using HIS6-BLE as the antigen. Two rabbits were immunized with HIS6-BLE antigen and a 112-day protocol was followed in the production of polyclonal antibody. Serum from Rabbit # 371 (lot #: R-371) was used in this study as the primary antibody for the detection of the BLE 10.25 protein.

4.0 Methods

4.1 Grain Extraction

Ground and defatted grain tissue, stored in a –80°C freezer, was used as the starting material for the extraction of protein. The chemical composition of the extraction buffer was as follows: urea-tris-borate (UTB) containing buffer [Urea 1 M; Tris 50 mM; Sodium Tetraborate 50 mM; and dithiothreitol (DTT) 50 mM]. Ground tissue was weighed in a Nalgene screw cap tube and extracted at a 1:5 tissue weight to buffer ratio, at room temperature with rocking on a nutator (Clay Adams Brand, Serial #: 110011227) for an hour. Extracted protein was separated from solid material by centrifugation at approximately 25, 000 x g for 15 minutes. The supernatant was filtered through a 0.45 µm filter (Pall Corporation, Ann Arbor MI) to remove any floating particulate material. Prior to storage in a –80 °C freezer, aliquots of samples were diluted with an equal volume of 2x tricine sample buffer that contained sodium dodecylsulfate (SDS) and DTT for analysis by western blot. Another set of aliquots was stored without the addition of sample buffer for the estimation of total protein content. Total protein content of the supernatant was determined using the colorimetric dye binding assay of BioRad Protein Assay according to SOP #: GEN-

PRO-015-00, with bovine serum albumin prepared in UTB as the standard. The total protein content in the grain extract of corn event MON 863 was estimated to be 4.0 mg/ml.

The protein extraction and storage procedures for the non-transgenic control grain RX670 were identical to that described above. The total protein content of the supernatant of control grain extract, as determined by BioRad Protein Assay (SOP #: GEN-PRO-015-00), was 3.8 mg/ml.

4.2 SDS-PAGE Analysis

Protein extracts of control grain and grain from corn event MON 863 were loaded on a 10-well 16% tricine gel (Invitrogen; product # EC 6695) and electrophoretically separated according to SOP #: PB-EQP-005-01. The amount loaded per lane was 23 µg of total protein, equivalent to the extraction of 1200 µg of grain tissue. Seven different dilutions, including 25, 10, 5, 4, 3, 2, and 1 nanograms per lane of *E. coli*-produced BLE 10.25 protein were spiked into the MON 863 protein extracts for comparison with unspiked MON 863 and control corn grain protein extracts. Protein separation was performed at a constant voltage of 125 V, until the dye front reached the bottom of the gel. A pre-stained molecular weight marker (BioRad, Hercules, CA, product # 1610-318) was loaded to identify the migration of the BLE 10.25 protein. Two identical SDS-PAGE analyses were performed: one for staining with Coomassie blue solution (BioRad, Hercules, CA, product #: 161-0436, Figure 2) and the other for transblotting onto a PVDF membrane for western blot analysis (Figure 3).

4.3 Western Blotting

Following the electrophoretic separation, proteins were electro-transferred onto a 0.2 µm polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA; product #: LC 2002) at ≈ 301 milliamps for 45 minutes in Tris-Glycine transfer buffer (Invitrogen, Carlsbad, CA product #: LC 3675). Non-specific binding sites on the PVDF membrane were blocked overnight at 4°C in 5% (w/v) non-fat dry milk (NFDM) dissolved in PBS (Roche, Indianapolis, IN., product #: 1666789) containing 0.05% (v/v) Tween 20 (Sigma, St. Louis, MO, product #: P1379). After blocking, the PVDF membrane was hybridized for an hour with the primary antibody, rabbit anti-HIS6-BLE IgG (Lot #: R371), at a dilution of 1:25000 in 2% NFDM containing PBST. The membrane was then washed three times, each for 15 minutes, in PBST and then hybridized for an hour with secondary antibody, goat anti-rabbit IgG, conjugated with horse radish peroxidase (Sigma, St. Louis, MO, product #: A 9169) at a dilution of 1:50,000 in 2% NFDM in PBST. After three washes in PBST, each

for 15 minutes, the bound secondary antibody was detected by incubating the membrane in Supersignal® West Dura Extended Duration Substrate (Pierce, Rockford, IL, product #: 34075) for 5 minutes and subsequently exposing the membrane to Hyperfilm™ ECL high performance chemiluminescence film (Amersham, Buckinghamshire, UK, product #: RPN 3114k).

4.4 Data Recording

The exposed film and the Coomassie stained gel were scanned into Hewlett Packard ScanJet 4c, Deskscan II version 2.4 software, (Hewlett Packard Co.) and the images were converted to PowerPoint figures for Windows, version 7b (Microsoft) to add annotation for molecular weight marker sizes and lane identification.

4.5 Limit of Detection (LOD)

The LOD of the western blot was established by spiking known quantities of the reference protein (*E. coli*-produced BLE 10.25) into the protein extracts of test substance. The lowest amount of BLE 10.25 spiked into corn grain protein extracts that could be detected by visual inspection of the X-ray film was 2 nanograms. Based on a per lane loading of 2 ng of BLE 10.25 spike plus 23 µg of corn grain protein extract (or an extraction equivalent of 1200 µg tissue), the limit of detection of BLE 10.25 was 1.7 µg of BLE 10.25 protein per gram fresh weight of corn grain tissue, or 1.7 parts per million (ppm). Therefore, the presence of a putative plant BLE 10.25 protein would have been readily visible at or above the LOD of 1.7 ppm. However, when unspiked MON 863 was immunoblotted, no visible band was detected at the molecular weight where *E. coli*-produced BLE 10.25 was readily detected (Figure 3).

5.0 Results

5.1 SDS-PAGE Analysis

SDS-PAGE analyses of protein extracts of test and control substances along with BLE 10.25 spikes are shown in Figure 2. Although the BLE 10.25 protein spiked at nanogram quantities were not readily visible in a Coomassie stained gel, the purpose of spiking was to compensate for any matrix effects that may have an impact on the migration and/or immunodetectability of BLE 10.25, and also to demonstrate that each lane contained approximately the same amount of total protein. Lower molecular weight proteins, including the BLE 10.25 protein, that ranged in molecular weight between between 7 kDa and 20 kDa, were resolved using the 16% tricine gel (Figure 2). An identical gel to that of Figure 2 was transblotted onto a PVDF membrane for western blot analysis (Figure 3).

5.2 Western Blot Analysis of Corn Grain Extracts

The reference substance, *E. coli*-produced BLE 10.25, was clearly detectable in all spiked samples except at the 1 ng level in lane 9 of Figure 3. The migration of BLE 10.25 corresponds to its estimated molecular weight of ≈ 10.25 kDa and it appears that the protein matrix has no effect on the migration or on the immunoreactvity of BLE 10.25. There is evidence of nonspecific binding of the primary antibody to plant proteins as revealed by detection of protein at ≈ 80 kDa for both the control and test substances. Nonspecific detection is not unexpected for a polyclonal antibody raised in rabbit, as corn grain is a likely component of a laboratory rabbit diet. There was an additional band (MW ≈ 20 kDa) detected in lanes 2 and 3 which contained corn grain tissue extracts spiked with BLE 10.25 protein at 25 and 10 nanograms per lane, respectively. This band is likely due to the BLE 10.25 protein dimer. However, this BLE-dimer band was not visible in lanes containing less than 10 nanograms of BLE 10.25 protein. Furthermore, it was demonstrated (data not shown here but archived with this study) that when the BLE 10.25 protein was reduced by the addition of DTT, only the band corresponding to the monomer was observed. In the absence of DTT reduction, bands due to the dimer and monomer migrating at 20 kDa and 10 kDa, respectively, were observed.

6.0 Discussion

Due to the selection of a *Sma* I restriction site for the insertion of a linker and NOS 3' transcriptional terminator downstream of *nptII*, the dominant selectable marker used for the identification of corn event MON 863 transformants is encoded by an mRNA that contains two ORFs. The 792 nucleotide ORF located near the 5' end of the mRNA encodes NPTII protein. A second ORF whose start codon is located 20 nucleotides downstream of the *nptII* stop codon has the potential to encode an 89 amino acid, 10.25 kDa protein, BLE 10.25. The *ble 10.25* ORF is composed of a small segment of the Tn5 *ble* gene joined through a linker to the NOS 3' transcriptional terminator which contains a stop codon. If translated, BLE 10.25 would contain amino acids 1-51 of the BLE protein linked to four amino acid residues encoded by a DNA cloning linker and 34 amino acid residues encoded by the NOS 3' transcriptional terminator (Figure 1).

The purpose of this study was to confirm the prediction that the *BLE 10.25* ORF is not translated in corn event MON 863. To assay BLE 10.25 levels, corn grain extracts were probed with a rabbit anti-HIS6-BLE polyclonal antiserum. To control for the potential masking of the immunoreactivity of BLE 10.25 by the corn seed protein matrix, extract samples were spiked with series of known quantities of *E. coli*-produced BLE 10.25 protein. As shown in Figure 3, the protein extract of MON 863 in lane 5 did not contain any detectable plant-produced BLE 10.25 protein, as expected. However, the *E. coli*-

produced BLE 10.25 protein that was spiked into the corn protein extracts was detectable as low as 2 ng per lane. It was also evident that in lanes 2 and 3, spiked with BLE 10.25 protein, there was an additional band detected at ≈ 20 kDa molecular weight. This band is due to the dimerization of BLE 10.25, as confirmed by N-terminal sequence. However, only a fraction of the BLE 10.25 was detected as a dimer. Moreover, it was not detectable in lanes where the spiked protein was less than 10 ng. A series of spiked samples was used to estimate the lower LOD for BLE 10.25 in corn grain extracts. Based upon the LOD, corn grain event MON 863 does not contain BLE 10.25 protein above a level of 1.7 ppm. This absence of detectable BLE 10.25 protein in corn grain was expected and likely indicative of the complete absence of BLE 10.25 given our current understanding of the mechanism of mRNA translation in eukaryotic organisms.

6.1 Constraints on Polycistron Translation in Eukaryotes

With the exception of internal ribosome entry sites (IRES), translation in eukaryotes involves a 5' cap-dependent scanning mechanism. Ribosomal components identify the "cap", a unique nucleotide located at the 5' end of a message, and scan the message usually to the first AUG codon where translation begins. Translation then continues through the ORF that is defined by this first AUG codon until a stop codon is encountered and the ribosome detaches from the mRNA (Kozak, 1999). Since the BLE 10.25 ORF is located downstream of the NPTII stop codon, translation of BLE 10.25 in corn would require that the *nptII* coding sequence and/or the 20 intercistronic ribonucleotides function as an IRES, or that BLE 10.25 translation be linked to the translation of NPTII through one of two additional mechanisms.

Presently, the number of mRNAs known to contain IRES elements is ≈ 30 (Gingras *et al.*, 1999). Although no consensus IRES element sequence exists, all IRES elements share at least two characteristics. First, IRES elements are contained in non-coding portions of mRNA such as 5' untranslated or intercistronic regions. The second characteristic is that the minimum size for an IRES element appears to be 34 nucleotides (Chappell *et al.*, 2000). In this instance, the 34 nucleotides can be subdivided into 9 nucleotides that function as the ribosome entry site and 25 nucleotides that separate the entry site from the translation start codon. Since the intercistronic spacing between the *nptII* and *ble 10.25* coding sequences is 20 nucleotides, it is physically impossible for this region to function as an IRES given our current understanding of such elements.

The second mechanism would require that a ribosome that is translating the NPTII coding sequence shift its reading frame near the NPTII stop codon, thereby bypassing the NPTII stop codon and translating through the BLE 10.25 ORF (see Garcia-Rios, 1997). The protein product derived from this event would be an NPTII-BLE 10.25

polyprotein. Such a frame-shift would require that a ribosome translate a nucleotide doublet rather than a codon which is a nucleotide triplet. Therefore, using trinucleotide codons we have the following situation where the C-terminal of NPT II and the N-terminal of BLE 10.25 are underlined:

CTT CTT GAC GAG TTC TTC TGA gcgggactctggggttcgaa ATG ACC
NPT II BLE 10.25

If a frame-shift were caused by the translation of a dinucleotide as shown in boldface, the stop codon will be bypassed and a NPTII-BLE 10.25 polyprotein would be produced:

CTT CTT **GA** CGA GTT CTT CTG Agc ggg act ctg ggg ttc gaa ATG ACC
NPT II-BLE 10.25 polyprotein

However, if a dinucleotide translation event were to occur, it would reside in the 12 nucleotides that lie upstream of the NPTII stop codon. If a nucleotide doublet translation were to occur further than 12 nucleotides upstream of the NPTII stop codon, a stop codon on the same reading frame as the BLE 10.25 start codon would be encountered. As shown below, NPT II would be truncated by four amino acids and no NPT II-BLE 10.25 polyprotein would be produced:

CTT **CT** TGA CGAGTTCTTCTGAgcgggactctggggttcgaa ATG ACC
NPT II BLE 10.25

Based on the second mechanism, if a NPTII-BLE 10.25 polyprotein was produced, it would contain at least 260 of the 264 amino acids that comprise the full length NPTII and have a MW of ≈ 39.7 kDa. This is significant because polyclonal antisera that were used for NPT II bridging studies would identify the NPT II-BLE 10.25 fusion if it were produced. No protein displaying a MW greater than 29 kDa was detected in protein gel blots of MON 863 corn grain extracts probed with anti-NPTII antibody (Holleschak *et al.*, 2001). Likewise, the anti-HIS6-BLE serum used in this study would also detect such a polyprotein if present in corn grain extracts. Although, a band of ≈ 80 kDa was detected using anti-HIS6-BLE serum, this band could not be the result of an NPTII-BLE 10.25 fusion as it is also present in the lane loaded with non-transgenic grain extract. No band of ≈ 39.7 kDa was detected using the anti-HIS6-BLE serum.

The final mechanism of *ble 10.25* translation would involve the ribosome not detaching from the mRNA once the NPTII stop codon is reached, but rather resuming translation at the BLE 10.25 AUG codon. In the absence of an IRES element, some eukaryotic mRNAs have been shown to produce a small peptide and a protein. In such instances, the peptide ORF is never longer than 90 nucleotides and it is always

found upstream of the protein coding sequence. Furthermore, the intercistronic distance between the peptide ORF stop codon and the protein start codon has been found to dramatically affect translation reinitiation frequency (Kozak, 1987 and Luukkonen *et al.*, 1995). It has been proposed that by increasing the intercistronic distance, ribosomes have sufficient time to bind the Met-$tRNA_i$·eIF-2 (a methionine charged tRNA-elongation initiation factor-2) complex and reinitiate translation of the downstream ORF (Kozak, 1999 and Hinnebusch, 1997). Studies performed using tissue culture cells transfected with plasmid constructs have shown that with intercistronic distances of 45 nucleotides, reinitiation frequencies are ≈ 20 % while with intercistronic distances of 11 nucleotides reinitiation frequencies are ≈ 10 % (Kozak, 1987). Collectively, these literature data support the conclusion that in the event the *ble* fusion sequence were transcribed, it is extremely unlikely to be translated into a protein.

6.2 BLE 10.25 is Unlikely to Bind Bleomycin

Bleomycin is a glycopeptide antibiotic which forms a complex with Fe(II). In the presence of oxygen, the bleomycin-Fe(II) complex causes nucleotide-sequence specific DNA cleavage. It is the ability to cleave DNA that is the basis of bleomycin antibiotic activity and the reason that bleomycin has only found limited use as a therapeutic agent. Recently, the X-ray crystal structures of BLE and the BLE-bleomycin complex have been determined to the respective resolutions of 1.7 and 2.5 Å (Maruyama *et al.*, 2001). Based upon the X-ray crystal structure and other physicochemical data (Kumagai *et al.*, 1999), it has been determined that native BLE is homodimeric and that it binds two bleomycin molecules. When exposed to heat, or treated with proteases, bleomycin-Fe(II) released from the BLE-bleomycin-Fe(II) complex displays antibacterial activity. Therefore, BLE does not display an enzymatic activity. Rather, BLE confers bleomycin resistance by binding bleomycin-Fe(II) and inhibiting the production of hydroxyl radicals which cause DNA scission.

X-ray crystal analysis has shown that BLE monomer is composed of two α-helices and two β-sheets (β2-β5 and β6-β9) that are arranged into two highly similar domains that have a βαββββ topology (Figure 4). Several features distinguish the two domains. The domain nearer to the amino terminal is composed of amino acids 1-49 while the domain nearer the carboxyl terminal is composed of amino acids 61-121. The difference in domain size, 49 versus 60 amino acids, is attributable to the length of the loops that join the α-helices and β-strands within the two domains. A second feature that distinguishes the domains is related to β-strand organization. A proline residue at position 7 produces a twist in the polypeptide backbone of the β1-β2 strand. In turn, this twist in the backbone results in a so-called chain exchange that facilitates the dimerization of BLE monomers via reciprocal interactions between the

β1 strand of one monomer and the β6 strand of the second monomer (Figure 5a). The overall result of dimerization is that two identical clefts that bind bleomycin are formed at the interface between the BLE monomers (Figure 5A).

In this study, dimerization of BLE 10.25 was observed under both reducing conditions (in the presence of DTT see Figure 3, lanes 2 and 3) and to a far greater extent under non-reducing conditions (in the absence of DTT, result not shown). Visual inspection of the BLE 10.25 amino acid sequence revealed that positions 74, 75 and 78 contain cysteine residues that are likely to account for the DTT-sensitive dimerization. Given the position of these cysteine residues, several closely related dimeric structures may be formed. Regardless of the exact dimeric conformation of these disulfide stabilized dimers, none would be expected to bind bleomycin because they would not be of the correct three-dimensional structure (see Figure 5 A and B).

If BLE 10.25 were to be translated *in planta*, it would be composed of amino acids 1-51 of BLE and 38 amino acids encoded by the linker and NOS 3' transcriptional terminator. In terms of BLE topology, the break point between the BLE and linker-NOS derived sequences is located in a loop that joins the two domains that comprise the BLE monomer (Figure 4). When aligned without the insertion of gaps, the aforementioned 38 amino acids display four identities with amino acids 52-89 of BLE. When allowing for the insertion of gaps using the default parameters of the Pearson-Lipman protein alignment tool contained in the MegAlign module (Version 4.03) of the DNASTAR package, only the first 51 amino acid residues are aligned. For the remaining 38 amino acids, the extent of identity/similarity is insufficient to support an alignment. Given that the amino acid sequence encoded by the linker and NOS3' untranslated region shares insufficient identity with BLE to support an alignment and is of insufficient length to form a domain having an βαβββ topology, it is unlikely if not impossible for BLE 10.25 to yield the structure necessary to bind bleomycin (Figure 5B).

7.0 Conclusions

Although the DNA sequence that encodes the dominant selectable marker for the MON 863 corn transformation event contains ORFs that encode NPTII and BLE 10.25, the intercistronic distance and size of these ORFs is such that it is highly unlikely that the BLE 10.25 ORF would be translated as a unique protein or as a fusion with the NPTII protein. Data presented in this report and in a previous report (Holleschak *et al.*, 2001) demonstrate the absence of a detectable NPTII-BLE 10.25 polyprotein. Moreover, this report also demonstrates that BLE 10.25 is not produced (1.7 ppm LOD). The lack of detectable BLE 10.25 in MON 863 corn grain extracts is likely reflective of the complete absence of BLE 10.25 translation products and is consistent with our current understanding

of the behavior of cistronic messages in eukaryotes. In the event that the BLE ORF is translated either as a NPTII-BLE 10.25 polyprotein or as BLE 10.25 at some level below the LOD, neither protein is likely to bind bleomycin.

8.0 References

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Wu, W.. Vanderwall D. E., Teramoto S., Lui S. M., Hoehn S. T., Tang X.-J., Turner C. J., Boger D. L., Kozarich J. W., and Stubbe J., 1998. NMR Studies of Co·Deglycobleomycin A2 Green and Its Complex with d(CCAGGCCTGG). *J. Am. Chem. Soc.*, **120**, 2239-2250.

Figure 1. Schematic Diagram of BLE 10.25 Protein. Schematic diagram of the coding regions of BLE 10.25 protein and NPTII protein. The *ble 10.25* ORF is composed of a small segment of the Tn5 *ble* gene joined through a linker to the NOS 3' transcriptional terminator which contains a stop codon. If translated, BLE 10.25 would contain amino acids (AA) 1-51 of the BLE protein linked to four amino acid residues encoded by a DNA cloning linker and 34 amino acid residues encoded by the NOS 3' transcriptional terminator.

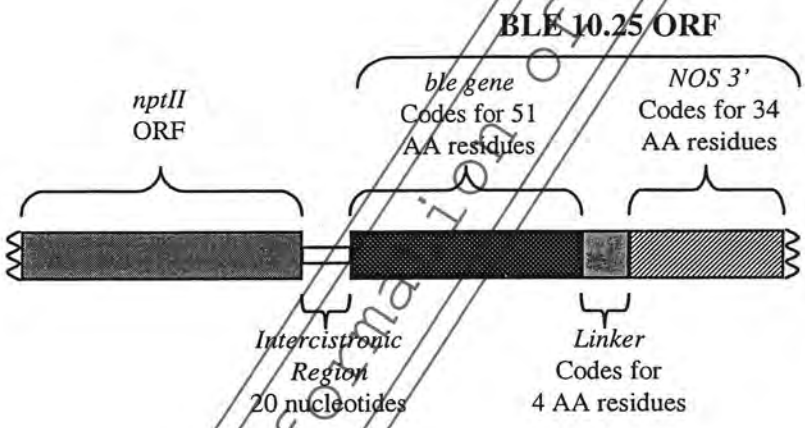
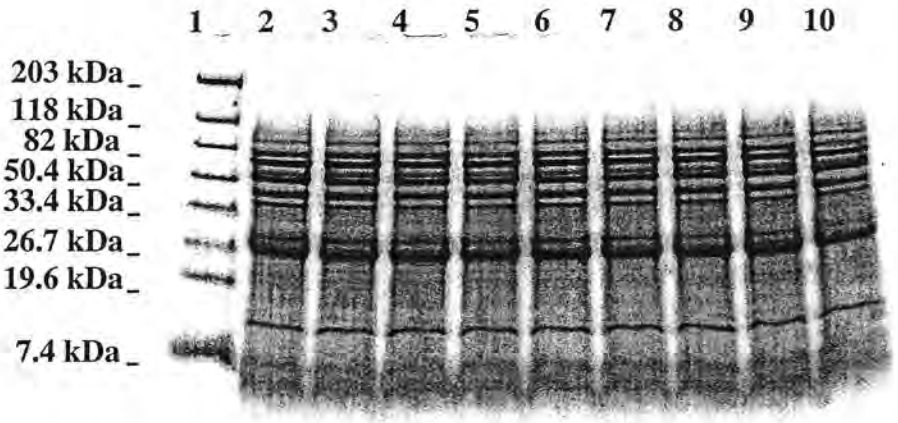


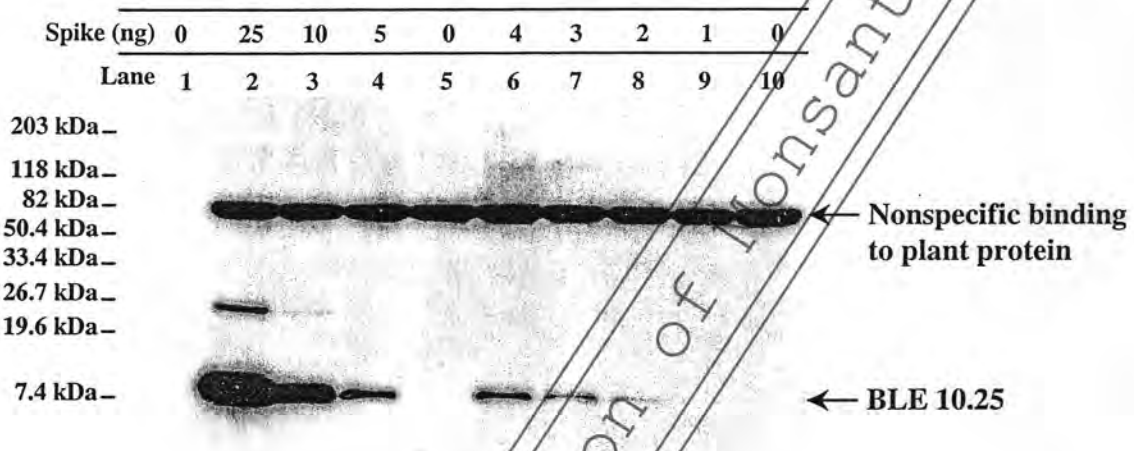
Figure 2. SDS-PAGE Analysis of Protein Extracts from Corn Grain. Equal volumes of extracts containing the equivalent of 1200 µg of extracted grain tissue per lane, were loaded on a 16% tricine gel and stained with Coomassie blue solution.



Lane #	Sample ID	Tissue Weight Equivalent* (µg/lane)	Reference Standard Protein Spike (ng/lane)
1	BioRad M.W.	0	0
2	Corn event MON 863 plus Reference Std	1200	25
3	Corn Rootworm Event 863 plus Reference Std	1200	10
4	Corn Rootworm Event 863 plus Reference Std	1200	5
5	Corn Rootworm Event 863 plus Reference Std	1200	0
6	Corn Rootworm Event 863 plus Reference Std	1200	4
7	Corn Rootworm Event 863 plus Reference Std	1200	3
8	Corn Rootworm Event 863 plus Reference Std	1200	2
9	Corn Rootworm Event 863 plus Reference Std	1200	1
10	Corn nontransgenic Control (CRW589)	1200	0

*Tissue weight of 1200 µg/lane is equivalent to an approximate loading of 23 – 24 µg total protein

Figure 3. Western Blot Analysis of Protein Extracts from Corn Grain. Equal volumes of extracts containing the equivalent of 1200 µg of grain tissue per lane were loaded on a 16% tricine gel, transblotted onto a PVDF membrane, and detected with rabbit polyclonal anti-HIS6-BLE serum



Lane #	Sample ID	Tissue Weight Equivalent* (µg/lane)	Reference Standard Protein Spike (ng/lane)
1	BioRad M.W.	0	0
2	Corn Rootworm Event 863 plus Reference Std	1200	25
3	Corn Rootworm Event 863 plus Reference Std	1200	10
4	Corn Rootworm Event 863 plus Reference Std	1200	5
5	Corn Rootworm Event 863 plus Reference Std	1200	0
6	Corn Rootworm Event 863 plus Reference Std	1200	4
7	Corn Rootworm Event 863 plus Reference Std	1200	3
8	Corn Rootworm Event 863 plus Reference Std	1200	2
9	Corn Rootworm Event 863 plus Reference Std	1200	1
10	Corn nontransgenic Control (CRW589)	1200	0

*Tissue weight of 1200 µg/lane is equivalent to an approximate loading of 23 – 24 µg total protein

Figure 4. The Topology of the Native BLE. The topology of native BLE, as determined by Maruyama *et al.*, 2001, shows the orientation and lengths of β-strand(▭), α-helices (▭) and random coils (⌣). The numerical values refer to the amino acid number at the junctions between α-helices, β-strands and random coils. The proline at position 7 places a twist between β-strands 1 and 2. The BLE monomer is composed of two domains that share a βαβββ topology and are connected by a loop between amino acids 49 and 61.

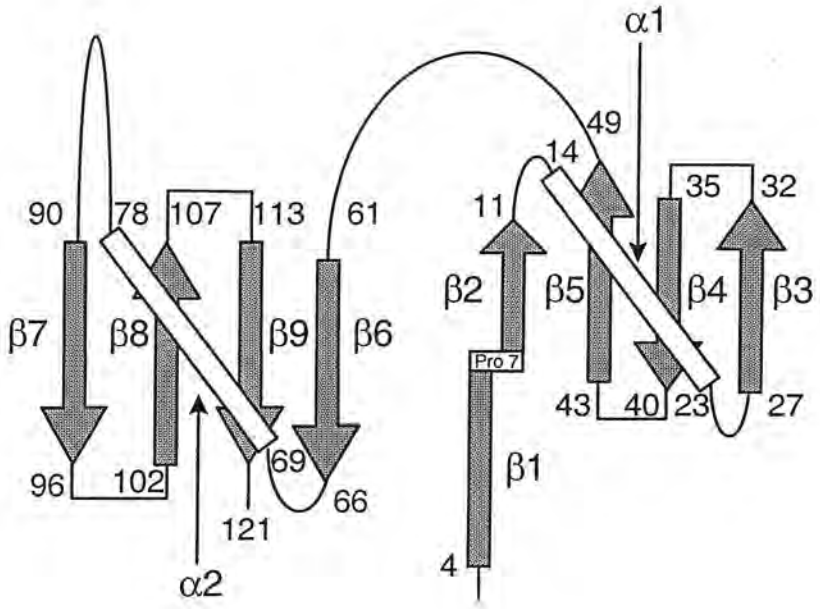
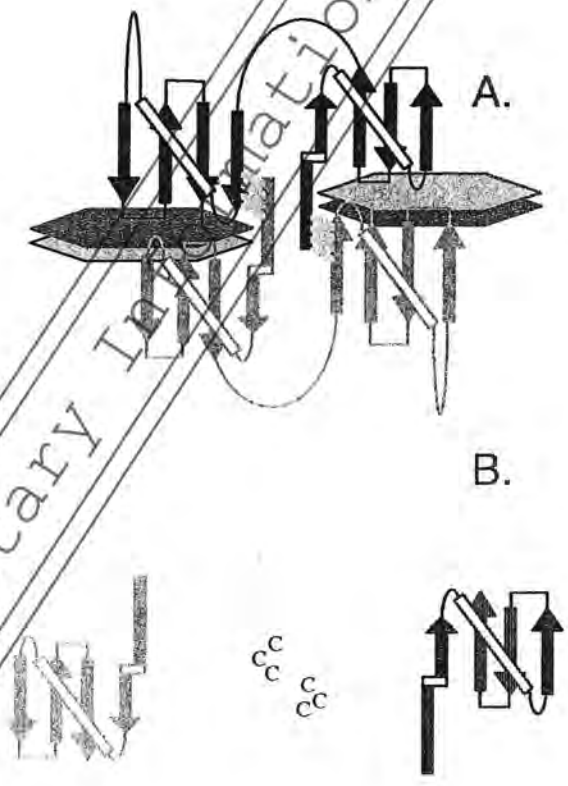


Figure 5. The Structural Relationship Between the Topology of Native BLE and the Amino Acid Sequence Encoding BLE 10.25. Using the topology map of native BLE of Maruyama *et al.*, (2001) as the point of reference, a schematic representation of native BLE-bleomycin binding is shown in panel A. Dimerization of BLE monomers, one shown in red and the other in black, is promoted by reciprocal chain exchange between the β1 and β6 strands, as indicated by the green lines. Bleomycin molecules are shown as stacked blue and yellow hexagons. These are bound in clefts formed by the amino terminal domain of the "red" monomer and the carboxyl terminal domain of the "black" monomer and the reciprocal cleft formed by the amino terminal domain of the "black" monomer and the carboxyl terminal domain of the "red" monomer. In stark contrast, the BLE 10.25 sequence depicted in panel B contains only amino acids 1-51 of BLE and 38 amino acids encoded by the linker and NOS 3' terminator. The aforementioned 38 amino acids shown in green would be in an unknown topology and would not be able to fold into a domain having the βαβββ topology found in BLE, since the assembly of such a domain requires ≈ 50-60 amino acids. As such, BLE 10.25 is unlikely to dimerize in a manner that would support bleomycin binding because it can not undergo chain exchange. In the event that BLE 10.25 were to dimerize through inter chain disulfide bonds (one possibility is depicted in panel B where "C" refers to cysteine residues), the cleft necessary for bleomycin binding would not be formed.



Appendix 1

Protocol for study 01-01-39-42 and Amendments #1 and #2

The following 9 pages are the study protocol

Monsanto Study #: **01-01-39-42**

Study Title: Evaluation of Corn Grain Containing Event MON 863 for the Presence of *ble* Translation Products

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Date

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Ravinder S. Sidhu
Sponsor Representative
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September 7, 2001
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James D. Astwood
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Sept. 7, 2001

Andre Silvanovich
Study Director
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Sept 7, 2001
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Reviewed By:

Quality Assurance Specialist
Monsanto Company

September 6, 2001
Date

Kanthasamy Karunanandaa
Principal Investigator
Monsanto Company

Sept. 7, 2001
Date

1.0 Regulatory Compliance

1.1 GLP Compliance

This is a product characterization study as defined by section §160.135(b) of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards (40 CFR Part 160) intended to characterize the physical and/or chemical properties of a potential commercial product. This study will be conducted in compliance with all requirements of section §160.135(b). Monsanto QAU will provide oversight and distribute QA reports according to the Monsanto Regulatory QAU SOPs.

2.0 Purpose

Monsanto has genetically improved corn (*Zea mays* L.) to produce a variant of the wild type Cry3Bb1 protein isolated from *Bacillus thuringiensis*, that has insecticidal activity against corn rootworm (CRW, *Diabrotica*), a major North American insect pest. The binary vector that was used to introduce the *Cry3Bb1* gene into the corn genome (event MON 863) contains two expression cassettes: one encodes the Cry3Bb1 protein and the other encodes the dominant selectable plant transformation marker neomycin phosphotransferase II (NPTII). Recent sequence analysis confirms that NPTII expression cassette has the potential to produce an mRNA that contains two open reading frames (ORFs). One ORF encodes the NPTII protein while the other ORF whose start codon is located 20 nucleotides downstream of the NPTII stop codon encodes a segment of the bleomycin binding protein (BLE). Specifically, if translated, this second ORF would yield a 10.25 kDa protein that would contain amino acids 1-51 of the BLE protein linked to four amino acid residues encoded by a DNA cloning linker and 34 amino acid residues encoded by the NOS 3' transcriptional terminator. Hereafter, this putative 10.25 kDa protein will be referred to as BLE 10.25. The purpose of this study is to determine if the BLE 10.25 protein is detectable in corn grain containing event MON 863

3.0 Timelines

3.1	Proposed Experimental Start Date:	September 7, 2001
3.2	Proposed Experimental Termination Date:	September 28, 2001

4.0 Test, Control and Reference Substances

4.1 Test Substance

The test substance is the grain of corn hybrid CRW0586, containing event MON 863 (MON 863 grain; LIMS #: 00ZMGRO02934), that will be stored at room temperature until protein extraction. Prior to extraction, the corn grain will be ground and defatted. The procedure for grinding and defattening will be archived with this study. The ground and defatted grain tissue will be extracted with a urea-tris-borate containing buffer (UTB; Urea 1 M; Tris 50 mM; Sodium Tetraborate 50 mM; and DTT 50

mM) using a tissue to buffer ratio of 1:5 (w:v). Once combined, the buffer and tissue will be thoroughly mixed to assure proper wetting and the extraction will be performed by rocking at room temperature for one hour in a nutator (Clay Adams Brand, Serial# 110011227). After the extraction, sample will be centrifuged for 15 minutes to separate the solid from the liquid phase and the supernatant will be filtered (0.45 µm filter). Prior to storage in a -80 °C freezer, one aliquot of the filtered supernatant will be diluted with an equal volume of 2x tricine protein loading sample buffer that contains sodium dodecylsulfate (SDS) and dithiothreitol (DTT) for analysis by western blot. The second aliquot will be retained for the estimation of total protein using SOP: GEN-PRO-015-00.

4.2 Control Substance(s)

The control substance is the grain of nontransgenic corn hybrid RX670 (LIMS#: 00ZMGRO02937) with a genetic background similar to that of the test substance. The protein extraction and storage procedure for the nontransgenic control grain RX670 will be identical to that described in section 4.1.

4.3 Reference Substance(s)

BLE 10.25, (*E. coli* produced lot #: 6949365-A) will be used as the reference substance in this study to establish limit of detection (LOD) by western blotting. All protein standards that are made from lot #: 6949365-A will be stored in -80 °C freezer.

4.4 Characterization of Test, Control and Reference Substances

The Study Director will determine the identity of the test and control substances by verifying the chain of custody documentation supplied with the samples. The identity of the test and control substances have been confirmed by analysis of the DNA and archived with the study files of production plan 00-01-39-18 by the Sponsor. Characterization data for the analytical reference standards are archived by the Sponsor. A certificate of analysis and the chain of custody documentation will be archived with this study.

4.4.1 Test Substance: MON 863 Grain

The identity of the test substance will be verified as described in Section 4.4. Determining the stability of the test substance is unnecessary since the synthesis and use of test substance will be done immediately after the extraction of protein. Moreover the extracted protein will be diluted with protein loading buffer which contains denaturant and reducing agent. Protein stored with

reducing agent and denaturant has been shown to be effective in reducing protein degradation.

4.4.2 Control Substance: Corn Grain of hybrid RX670.
The identity of the control substance will be verified as described in Section 4.4.

4.4.3 Reference Substance: *E. coli* Produced BLE 10.25 kDa Protein (Lot# : 6949365-A).
The reference substance is a characterized *E. coli* produced BLE 10.25 protein identical to the hypothesized plant BLE 10.25 protein. The gene encoding the fusion protein, BLE 10.25 kDa, was cloned into a pET23b *E. coli* expression vector under the control of T7 promotor. The resulting vector was transformed into BL21 (DE3) pLysS cells for protein production. A majority of the expressed protein was found in the inclusion bodies. Procedures related to isolation of inclusion bodies and estimation of the BLE 10.25 protein will be archived with this study. The identity of BLE 10.25 was confirmed by sequencing the DNA of the expression plasmid, and by performing N-terminal sequencing on the translation product. Results showed that the sequence of the first 15 amino acids was identical to the deduced sequence of BLE 10.25. The N-terminal sequence and the DNA sequence results will be archived with this study. The stability of the BLE 10.25 will be evaluated by performing western blot analysis (limit of detection) on standards before and after the study. Detection between 4 ng and 10 ng of *E. coli* expressed BLE 10.25 will be acceptable to show the stability of the protein.

5.0 Description of Experimental Design

Evaluation of the sequence of the plasmid that was used for the transformation of corn resulting in event MON 863 indicates that the mRNA that encodes NPTII contains two ORFs. In corn event MON 863, the translation product of one ORF results in the production of functional NPTII protein while if translated, the second ORF would produce BLE 10.25 protein. This study employs immunoblot analysis to detect BLE 10.25 in MON 863 grain. The control substance RX670, will serve as the negative control for the detection of BLE 10.25. The *E. coli* produced BLE 10.25 will serve as the reference standard to establish the limit of detection when spiked into the protein extract of MON 863. Visual inspection of the autoradiograph of unspiked MON 863 will determine the presence or absence of the BLE 10.25 protein.

The limit of detection (LOD) will establish a minimum level of detectable protein (BLE 10.25) under the assay condition described in this study in the presence of corn grain matrix.

5.1 Analytical Methods

5.1.1 SDS-PAGE Analysis

Protein samples generated from *E. coli* expression and from plant tissues will be separated by SDS-PAGE in Novex brand pre-cast 16% Tricine gels under reducing conditions according to SOP#: PB-EQP-005-01. Two similar gels will be produced: one gel will be stained with coomassie blue stain and the other gel will be used in the transfer of proteins to a PVDF membrane.

5.1.2 Western blot analysis

Following separation of proteins by SDS-PAGE, the detection of the BLE 10.25 protein will be done according to SOP#: GEN-PRO-002-03. Immunoreactive band(s) will be visualized with enhanced chemiluminescence (ECL) detection system of Amersham Pharmacia Biotech or with Supersignal West Dura Extended of Pierce and will be exposed to Hyperfilm ECL high performance chemiluminescence film (Amersham Life Science).

5.1.3 Rabbit Antiserum against BLE 10.25 Protein

Antiserum (lot #: R371) will be used in this study to detect the BLE 10.25 protein. Characterization of this antibody has been reported previously (Silvanovich et al., MSL 16705).

5.1.4 Plant Protein Estimation.

In order to estimate plant tissue protein loading per well, immediately after the extraction of protein with UTB buffer, aliquots will be stored in -80°C freezer for later protein estimation by Bio-Rad Protein Assay (SOP #: GEN-PRO-015-00).

6.0 Proposed Statistical Methods

No statistical analysis is planned for this study.

7.0 Control of Bias

Standards of *E. coli* expressed BLE 10.25 will be spiked into plant tissue protein to confirm the migration pattern and detection that could be influenced by the plant protein matrix.

8.0 Records to be Maintained

Records will be maintained of all sample transfers, analyses, the protocol and all deviations and amendments thereto and copies of all letters memoranda and other correspondence related to this study. These documents may include: photocopies, computer generated hard copies or hand-written notes that describe the procedures used to generate data for this study. Upon completion of the study, all study records and final report will be archived at Monsanto Biotechnology Regulatory Sciences archives.

9.0 Changes to the Protocol

Planned changes to the protocol will be documented in the form of written protocol amendments and signed by the Study Director. Amendments become part of the protocol and will be archived with the protocol. All other changes will be in the form of written protocol deviations and will be filed with the raw data. All changes to the protocol will be addressed in the final report.

10.0 Reference:

Silvanovich, A., Turner, L.A., Blasberg, J., and Astwood, J.A. (2001) The absence of *ble* translation products in Bollgard, Roundup Ready and Bollgard-Roundup Ready Cotton Seed. Monsanto Technical Report: MSL-16705, St. Louis, MO.

Protocol Amendment Form **Amendment #: 1**

Monsanto Study #: **01-01-39-42**

Date changes implemented: September 13, 2001

Page number(s) and section(s): Page 6; Section 5.1.1

Protocol originally stated:

Two similar gels will be produced: one gel will be stained with coomassie blue stain and the other gel will be used in the transfer of proteins to a PVDF membrane.

Protocol amended as follows:

Three similar gels will be produced: one gel will be stained with coomassie blue stain and other two gels will be used in the transfer of proteins to two PVDF membranes. These two PVDF membranes will be hybridized with two different dilutions of primary antibody for the detection of BLE 10.25.

Reason for the amendment and what impact will result from this change:

Use of two different dilutions of primary antibody for hybridization will give the opportunity to select the optimum dilution of the primary antibody that is required for the detection of BLE 10.25.

Approved By:

Patrick T. Weston — Sep 17, 2001
Testing Facility Management Representative — Date

Andre Silvanovich — Sept 13, 2001
Study Director — Date

Ravinder S Sidhu — Sep 14, 2001
Sponsor Representative — Date

Protocol Amendment Form

Amendment #: 1

Reviewed By:

Barbara Foley

Quality Assurance Specialist

Sept. 13, 2001

Date

Protocol Amendment Form **Amendment #: 2**

Monsanto Study #:	**01-01-39-42**
Date changes implemented:	November 28, 2001
Page number(s) and section(s):	Page 6; Section 5.1.3 Page 7; Section 10
Protocol originally stated:	Characterization of this antibody has been reported previously (Silvanovich et al., MSL 16705).
Protocol amended as follows	The sentence that includes the citation was removed from the protocol, and the corresponding reference listed in Section 10 was also removed.

Reason for the amendment and what impact will result from this change:
The study was incomplete. There was no impact on the study, since the data related to the production of the antibody was archived with this study.

Approved By:

Patrick T. Weston — Dec 4, 2001
Testing Facility Management Representative — Date

Andre Silvanovich — Dec 5, 2001
Study Director — Date

Ravinder S Sidhu — Dec 3, 2001
Ravinder S. Sidhu
Sponsor Representative — Date

Reviewed By:

Barbara Foy — Dec. 4, 2001
Quality Assurance Specialist — Date

[redacted]