SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Biochemical Characterization of Recombinant Cry1F Protein Expressed in Maize Line 1507 and *Pseudomonas fluorescens*

DATA REQUIREMENTS

None

AUTHOR(S)

Russell Essner, MS

STUDY COMPLETED ON

September 10, 2002

PERFORMING LABORATORY

Pioneer Hi-Bred International, Inc.
Analytical Biochemistry Protein Core Facility
7300 NW 62nd Ave
Johnston, IA 50131

HHMI / Keck Biotechnology Resource Laboratory
301 BCMM, 295 Congress Ave
New Haven, CT 06519-1418

LABORATORY STUDY ID

PHI-2002-032

Biochemical Characterization of Recombinant Cry1F Protein Expressed in Maize Line 1507 and *Pseudomonas fluorescens*

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Maize line 1507 was transformed with the *cry1*F gene from *Bacillus thuringiensis* var. *aizawai*; *in planta* expression of this protein confers resistance to certain lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*).

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The Cry1F protein derived from *P. fluorescens* and transgenic maize event TC1507 kernel and leaf was shown to be comparable with respect to size, immuno-recognition, and lack of glycosylation. The peptide mass finger print data obtained by MALDI-TOF MS analysis for the kernel and bacterial expressed Cry1F proteins confirms earlier MALDI-TOF MS analysis from leaf and bacterial expressed Cry1F (Schafer, 2001), therefore lending further support for the use of the *P. fluorescens* derived Cry1F protein for regulatory studies.

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Johnston, IA 50131

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New Haven, CT 06519-1418

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F δ-endotoxin

Title: Biochemical Characterization of Recombinant Cry1F Protein Expressed in Maize Line 1507 and *Pseudomonas fluorescens*

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

Company: Pioneer Hi-Bred International, Inc.

Company Agent: Angel Fuentes, Ph.D.

Title: Regulatory Scientist

Signature: [signature]

Date: 10th September 2002

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE
WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Biochemical Characterization of Recombinant Cry1F Protein Expressed in Maize Line 1507 and *Pseudomonas fluorescens*

Study Initiation Date:	April 2, 2002	Study Completion Date:	September 10, 2002
Experimental Start Date:	April 2, 2002	Experimental Termination Date:	June 25, 2002

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ISBN 92-64-12367-9, Paris 1982

At the time this study was conducted, it was not subject to the Good Laboratory Practice Standards and was, therefore, not monitored by the quality assurance unit.

Angel M. Fuentes — 10th Sept 2002
Angel Fuentes, Ph.D., Submitter — Date
Pioneer Hi-Bred International, Inc.

Lawrence Zeph — 9/16/2002
Lawrence Zeph, Ph.D., Sponsor — Date
Pioneer Hi-Bred International, Inc.

Tom — 9/16/2002
Thomas R. Davis, ~~Ph.D.~~, Study Director — Date
Pioneer Hi-Bred International, Inc.

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NON-GLP STUDY

SIGNATURE PAGE

Title: Biochemical Characterization of Recombinant Cry1F Protein Expressed in Maize Line 1507 and *Pseudomonas fluorescens*

Testing Facilities:

Pioneer Hi-Bred International, Inc.
7300 NW 62nd Ave.
Analytical Biochemistry Protein Core Facility
Johnston, Iowa 50131-1004

HHMI / Keck Biotechnology Resource Laboratory
301 BCMM, 295 Congress Ave
New Haven, CT 06519-1418

Contributors: Xiu-Qing Zhang, Ph.D., Pioneer Hi-Bred International, Inc.

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Tom — 9/16/2002
Thomas R. Davis, Ph.D., Study Director — Date
Pioneer Hi-Bred International, Inc.

Lawrence Zeph — 9/16/2002
Lawrence Zeph, Ph.D., Sponsor — Date
Pioneer Hi-Bred International Inc.

Russell Essner — 9/16/2002
Russell Essner, MS, Author — Date
Pioneer Hi-Bred International, Inc.

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ABBREVIATIONS

CBB	Coomassie brilliant blue
EDTA	ethylenediamine tetraacetate
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MW	molecular weight
µg	microgram
µl	microliter
MAb	Monoclonal antibody
ng	nanogram
PBST	phosphate buffered saline (10mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl) with 0.05% Tween 20, pH = 7.4
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis

Biochemical Characterization of Recombinant Cry1F Protein Expressed in Maize Line 1507 and *Pseudomonas fluorescens*

ABSTRACT

Maize line 1507 was transformed with the *cry1*F gene from *Bacillus thuringiensis* var. *aizawai*; *in planta* expression of this protein confers resistance to certain lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*).

The purpose of this study was to biochemically characterize Cry1F protein derived from *Pseudomonas fluorescens* and maize line 1507 leaf and kernel tissues. Cry1F protein from maize leaf and kernel tissue from three (3) US locations and one (1) European location and *P. fluorescens* was analyzed by sodium dodecyl sulfate –polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and glycoprotein detection methods. Monoclonal antibodies directed against Cry1F expressed in *Pseudomonas fluorescens* were selected and used to purify Cry1F protein from kernel tissue by immunoaffinity chromatography. The immunopurified Cry1F protein from kernel along with the bacterial expressed protein from *P. fluorescens* was further characterized by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The Cry1F protein derived from *P. fluorescens* and transgenic maize event TC1507 kernel and leaf was shown to be comparable with respect to size, immuno-recognition, and lack of glycosylation. The peptide mass finger print data obtained by MALDI-TOF MS analysis for the kernel and bacterial expressed Cry1F proteins confirms earlier MALDI-TOF MS analysis from leaf and bacterial expressed Cry1F (Schafer, 2001), therefore lending further support for the use of the *P. fluorescens* derived Cry1F protein for regulatory studies.

INTRODUCTION

Hybrid maize line 1507 was transformed with a gene that encodes a truncated version of the insecticidal protein Cry1F isolated from *Bacillus thuringiensis* var. *aizawai.* This protein (approximately 66 kDa) when expressed in maize confers resistance to certain lepidopteran pests including the European corn borer (*Ostrinia nubilalis*).

The purpose of this study was to biochemically characterize Cry1F protein derived from the transgenic maize line 1507 and the expressed Cry1F protein purified from *Pseudomonas fluorescens*. The maize samples were collected from three (3) US locations and one (1) European location. The results obtained for Cry1F from maize leaf and kernel tissue were compared to Cry1F protein from *P. fluorescens.*

The protein methods used to characterize Cry1F from maize leaf, kernel and *P. fluorescens* included sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, glycoprotein detection, and peptide mass fingerprinting by MALDI-TOF MS. To separate and resolve the expressed Cry1F protein from a heterogeneous protein extract, SDS-PAGE was used. This technique separates proteins based on size allowing for the mass of an unknown protein of interest to be determined (Scopes, 1982). The presence of Cry1F protein was verified by a technique known as Western blotting. With this method, protein can be further characterized by specific antibody/antigen interactions. Cry1F positive maize leaf and kernel samples along with negative control samples from each location and the bacterial expressed Cry1F were run on SDS-PAGE, transferred to PVDF membranes and probed with a monoclonal antibody specific for Cry1F. To check for evidence of post-translational glycosylation, which is a major contributor to protein heterogeneity, Cry1F purified from kernel from all 4 geographic locations was assayed. Glycosylation is easily detected by protein electrophoresis followed by periodate oxidation and staining of the attached oligosaccharides (Roe, 2001). Also, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) was conducted by the Keck Facility (301 BCMM, 295 Congress Ave, New Haven, CT 06519-1418) on immunopurified Cry1F protein from kernel from all locations, along with the bacterially expressed Cry1F protein. The instrument used was a reflectron time-of-flight analyzer with a resolving power in the range of 1000-300,000 Daltons (Siuzdak, 1996; Keck Facility).

The results of this study support earlier Cry1F protein characterization studies, demonstrating that Cry1F expressed in maize and bacteria are comparable (Shafer and Schwedler, 2001).

MATERIALS AND METHODS

Test and Control Materials

- *Pseudomonas fluorescens* derived Cry1F protein: Lot# 112800-1
- Plant derived CryF1: See Table 1

Test of Monoclonal Antibodies

Four monoclonal antibodies raised against the microbial expressed Cry1F protein (lot # 112800) were tested by Western blot for specificity, relative affinity, and ability to recognize native and denatured Cry1F protein. Of the four monoclonal antibodies tested, Clone 205A158 was able to recognize native Cry1F protein, and Clone 205A62 recognized the denatured Cry1F protein (data not shown). Therefore, the former was used for affinity purification of Cry1F from maize kernel and the latter was used for Western blotting analysis.

Antibody Preparation and Column Coupling

Anti-Cry1F monoclonal antibody Clone 205A158 was coupled to immunoaffinity support Affi-Gel 10 (Bio-Rad 153-6046) according to the manufacturer's instructions. Briefly, the antibody preparation containing 50% glycerol was diluted with coupling buffer (0.1 M MOPS, pH 7.4, 0.1M NaCl). The diluted antibody was precipitated with ammonium sulfate (70% saturation), re-dissolved, and desalted with a HiPrep 26/10 column (Amersham Biosciences catalog #17-5087-01). The antibody was then coupled to the Affi-Gel 10 support resin.

Cry1F Purification from Maize Kernel

Transgenic maize kernels from the IA1, IA2, IL2, and EU locations were ground and defatted by hexane extraction (Zhang, 2001). The defatted kernel meal was homogenized with a mortar and pestle in buffer A (50 mM phosphate, pH 7.8, 5% glycerol) containing 1 mM EDTA, 0.2% Triton X-100 and a cocktail of protease inhibitors (Complete, E64, Leupeptin, Pepstatin, and Pefabloc SC) (Boehringer Mannheim). The homogenate was filtered through four layers of cheesecloth and the filtrate was centrifuged for 20 min at 38000 g. The supernatant was filtered through a 0.2 micron membrane. The clarified extract was loaded onto a column packed with the immunoaffinity resin. The column was sequentially washed with buffer B (25 mM phosphate, pH 7.2, 0.05% Triton X-100, 1 M NaCl) and buffer C (buffer A containing 0.2 M sodium thiocyanate, 0.5% Triton X-100 and 0.5% sodium deoxycholate). Finally, Cry1F protein was eluted with the Actisep Elution Medium (Sterogene #9701). The eluted protein was desalted with Econo-Pac 10DG (Bio-Rad #732-2010) columns and concentrated with CentriPlus concentrators (Millipore).

Gel Electrophoresis and Western Blotting Analysis

Tris-HCl Ready Gels (10-20% gradient, Bio-Rad 161-1160) were used for SDS-PAGE. Samples were dissolved in Laemmli buffer and heated for 5 min at 95°C. The denatured proteins were then separated using the Bio-Rad Ready Cell system, with approximately equivalent amounts of protein loaded per lane (Bio-Rad#165-3125). Following electrophoresis of immunopurified Cry1F, the gel was either stained for total proteins with the GelCode Blue Stain Reagent (Pierce #24592) or processed for Western blotting.

Western blotting was performed following electrophoresis by incubating the gels with transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS and 20% methanol) for 10 min. Proteins were then transferred to PVDF membranes using the Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell system (Bio-Rad #170-3940). Membranes were then probed with a monoclonal antibody (Clone 205A62) for detection of Cry1F protein, followed with a goat anti-mouse IgG Alkaline

Phosphatase conjugate (Promega #S3721) and developed with Tropix CSPD reagent (Tropix #CD100RN). The membrane blots were exposed to Roche Lumi-Film.

Glycosylation Detection

Immunoaffinity purified Cry1F from kernel and Cry1F protein derived from *P. fluorescens*, along with the appropriate controls (Horseradish peroxidase and Soybean Trypsin Inhibitor) were resolved by SDS-PAGE. The gel was then stained for the presence of glycoproteins using the GelCode Glycoprotein Staining Kit (Pierce #24562) (Lot# CJ50744).

The detection procedure included SDS-PAGE, fixing the gel with 50% methanol for 30 min and then washing with 3% acetic acid. The gel was then incubated with Oxidizing Solution for 15 min and washed with 3% acetic acid. The gel was then stained with Staining Reagent to visualize the presence of glycoproteins. Following staining, the gel was treated with Reducing Reagent and extensively washed with 3% acetic acid and water. Following glycoprotein detection, the same gel was stained with the GelCode Blue Stain Reagent (Pierce #24592) for total protein detection.

MALDI-TOF MS Peptide Mass Fingerprinting of Kernel Derived Cry1F

To prepare samples for MALDI-TOF analysis the bacterial expressed Cry1F protein along with the immunoaffinity purified Cry1F proteins from kernel from all 4 geographic locations were run by SDS-PAGE. Respective bands at approximately 66 kDa were excised from the gel, placed in 1.5 ml Eppendorf tubes, and sent to the Keck Biotechnology Resource Laboratory (301 BCMM, 295 Congress Ave, New Haven, CT 06519-1418) for MALDI-TOF MS analysis.

The MALDI-TOF MS procedures used by the Keck Laboratories (Keck Laboratories, 2002) were as follows. The gel slice samples were prepared for analysis by the addition of 50% H_2O:50% acetonitrile to each tube. This was washed for 5 minutes on a tilt table, aspirated, and 50% H_2O:50% acetonitrile was again added to each tube. The tubes were placed on a tilt table for 30 minutes, aspirated, and followed by the addition of 50% CH_3CN:10 mM NH_4HCO_3. The gel slices were then washed for 30 minutes on a tilt table, aspirated and dried in a SPEEDVAC.

Once dry, trypsin enzyme stock at 0.1 mg/ml and diluted 1:14 10 mM NH_4HCO_3. Equal volumes of enzyme dilution were added to the gel slices and incubated for 5-10 minutes to allow the enzyme/buffer to absorb into the gel. A sufficient amount of 10 mM NH_4HCO_3 was added to cover the gel slices. This was incubated for 18 hours at 37°C. At this point MALDI-TOF-MS analysis was performed on 10-20% of the sample digest using a reflectron MALDI-MS instrument.

RESULTS AND DISCUSSION

Purification and Confirmation of Cry1F Protein from Transgenic Maize Line 1507

Analysis of leaf and kernel extracts by Western blot confirmed the presence of Cry1F protein in positive samples from all four locations with an expected band at approximately 66 kDa (Figures 1, 2). Furthermore, Cry1F protein was not detected in the negative control samples. In Figure 3, a side-by-side comparison was done with Cry1F protein from bacteria, maize leaf and kernel illustrating the size similarity between the proteins from various sources. Although Figure 1 showed moderate cross reactivity with non-specific bands appearing in both the Cry1F positive and negative samples this did not impact the interpretation of the immunoblots. The monoclonal antibody (205A62) clearly recognized both the plant and bacterial derived Cry1F protein. In Figures 2 and 3, doublet bands were detected for leaf and kernel derived Cry1F from several locations. Previous studies have shown that the Cry1F protein expressed in corn (approximately 66 kDa) is subject to truncation resulting in the loss of 27 amino acids from the amino terminus to yield the trypsin resistant Cry1F core toxin (approximately 65 kDa) (Schafer 2001, Evans 1998). The bacterial derived Cry1F protein was subjected to trypsin truncation during purification and therefore represents the core toxin. Plant-derived Cry1F preparations may consist of a mixture of the full length Cry1F and the truncated core toxin, depending upon exposure to plant proteases.

Using monoclonal antibody 205A158, Cry1F protein was purified from maize kernel tissue by immunoaffinity chromatography (IAC). Recovery of the purified Cry1F protein from kernel tissue from each location was confirmed SDS-PAGE (Figure 4), and by Western analysis using monoclonal antibody 205A62 (Figure 5). A major band at approximately 66 kDa was detected in the known positive samples and was apparent in both the Coomassie stained gel and the immunoblot. The bacterial expressed Cry1F was also recognized by the same antibody and identified in the same blot. The size of Cry1F from both kernel and *P. fluorescens* is comparable.

Detection of Glycosylation

Purified Cry1F protein extracts were tested for glycosylation (Figure 6) and were verified for the presence of Cry1F protein, (Figures 4 and 5) by Coomassie blue staining and Western blot (see previous section). Evidence of post-translational glycosylation was not detected for Cry1F protein expressed in maize kernel and *P. fluorescens*. The results of this experiment were consistent with previous findings showing no evidence of glycosylation of Cry1F derived from maize leaf tissue (Schafer, 2001). Results for the positive and negative controls were as expected, horseradish peroxidase was stained and clearly visible, while the negative control, soybean trypsin inhibitor, did not show any staining for glycosylation.

MALDI-TOF MS Analysis

MALDI-TOF analysis was conducted on trypsin digested Cry1F protein purified from *P. fluorescens* and maize kernel from the IA1, IA2, IL2 and EU locations. The trypsin digest of *P. fluorescens*-derived Cry1F protein resulted in 16 identified peptides that matched the theoretical peptide masses of Cry1F (Table 2). The peptide fragments detected were between residues (32 and 494). The trypsin digest of the Cry1F protein derived from transgenic maize kernel resulted in 14 identified peptides from the EU location, 15 identified peptides from IL2, 16 identified peptides from IA1, and 16 identified peptides from IA2. Detected peptides were considered a match if the observed experimental mass was within a 0.1 error range to the theoretical peptide mass (Table 2). Overall, the peptides matched very well between the *P. fluorescens* and maize kernel Cry1F proteins from all geographic locations. In a few random instances, matching peptides were not detected. Possible causes for peptide non-match are that the missing peptide fragments are small and near the lower size limit for detection resulting in inconsistent peptide detection, low protein concentration, over digestion, and random breakage of the peptides during ionization.

CONCLUSIONS

- Cry1F protein expressed in *P. fluorescens*, maize kernel and maize leaf are of similar size as resolved by SDS-PAGE electrophoresis and are each recognized by a monoclonal antibody specific to Cry1F.
- Cry1F protein expressed in *P. fluorescens* and maize kernel shows no evidence of post-translational glycosylation.
- MALDI-TOF MS analysis indicated that the Cry1F protein expressed in *P. fluorescens* and maize kernel are comparable in terms of the peptide fragments identified when compared to each other and their theoretical masses.
- Using the analytical techniques of SDS-PAGE, Western blotting, glycoprotein staining, and MALDI-TOF MS, Cry1F proteins from maize line 1507 across four test locations in both leaf and kernel tissues, are comparable to bacterial derived *P. fluorescens* Cry1F protein.

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APPENDIX 1: TABLES

Table 1. List of Plant Materials Used for this Study

Location	Sample ID	Tissue	Maize plants
EU	EU-1507 Composite	Kernel	Transgenic
EU	EU-CHPH09B	Kernel	Control
IL2	IL2-1507	Kernel	Transgenic
IL2	IL2-CHPH09B	Kernel	Control
IA1	IA1-1507	Kernel	Transgenic
IA1	IA1-CHPH09B	Kernel	Control
IA2	IA2-1507	Kernel	Transgenic
IA2	IA2-CHPH09B	Kernel	Control
EU	FR3-1507-1-1-L9	Leaf	Transgenic
EU	FR3-CHPH09B-1-1-L9	Leaf	Control
IL2	IL2-1507-1-1-L9	Leaf	Transgenic
IL2	IL2-CHPH09B-1-1-L9	Leaf	Control
IA1	IA1-1507-1-1-L9	Leaf	Transgenic
IA1	IA1-CHPH09B-1-1-L9	Leaf	Control
IA2	IA2-1507S-1-1-L9	Leaf	Transgenic
IA2	IA2-CHPH09B-1-1-L9	Leaf	Control

Table 2. Tryptic Peptide Mass Data (m/z $[M+H]^+$) of Cry1F Proteins Obtained by MALDI-TOF MS

AA Residue #	Theoretical mass (*m/z*)	*P. Fluorescens* Cry1F [M+H]	EU Maize Cry1F [M+H]	IL Maize Cry1F [M+H]	IA1 Maize Cry1F [M+H]	IA2 Maize Cry1F [M+H]
32-42	1227.73	1227.78	1227.75	*ND*	1227.77	1227.78
100-113	1612.82	1612.86	1612.85	1612.85	1612.86	1612.86
114-125	1441.68	1441.72	1441.71	1441.72	1441.72	1441.72
172-193	2434.15	2434.15	*ND*	2434.13	2434.15	2434.15
204-217	1675.79	1675.82	1675.80	1675.81	1675.82	1675.81
223-232	1408.73	1408.66	1408.66	1408.66	1408.66	1408.67
252-263	1394.73	1394.77	1394.76	1394.76	1394.76	1394.76
264-286	2509.22	2509.22	2509.15	2509.21	2509.22	2509.22
312-324	1413.72	1413.76	1413.76	1413.76	1413.76	1413.77
367-379	1386.73	1386.77	1386.77	1386.77	1386.77	1386.78
380-392	1416.70	1416.74	1416.73	1416.74	1416.74	1416.74
431-442	1376.62	1376.67	1376.62	1376.67	1376.67	1376.67
443-451	1132.54	1132.61	1132.61	1132.61	1132.61	1132.62
452-463	1301.63	1301.67	1301.67	1301.67	1301.67	1301.67
472-483	1269.69	1269.72	*ND*	1269.73	1269.73	1269.73
484-494	1089.57	1089.64	1089.63	1089.64	1089.64	1089.64

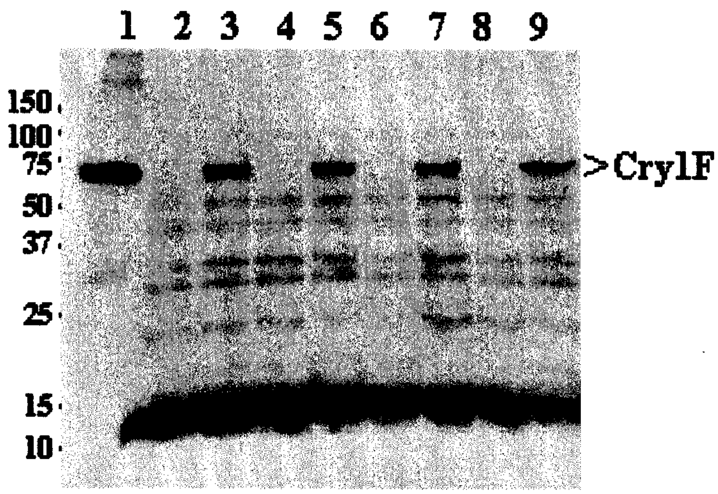
Note:

1. Two digit decimals were used for mass data in this table although raw data obtained from MALDI-MS were shown in 3 digit decimals. A peptide was considered a match if its *m/z* is within 0.1 error range of its theoretical *m/z*.

ND: not detected.

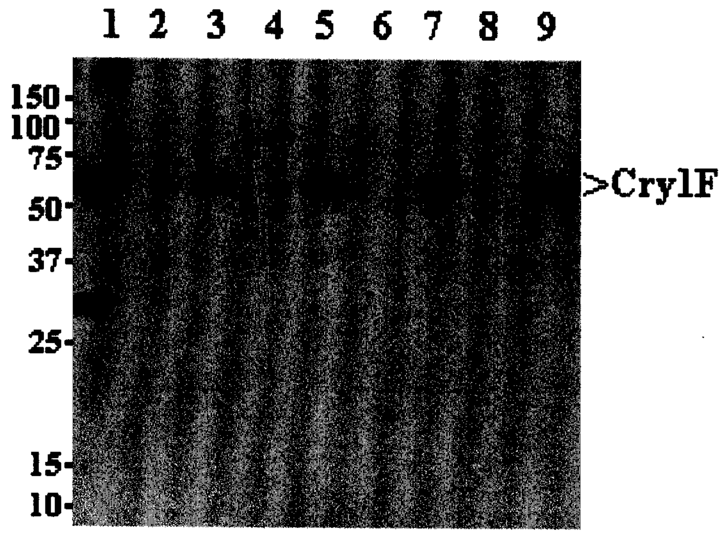
APPENDIX 2: FIGURES

Figure 1. Western Blot Analysis of Cry1F in Maize Line 1507 Kernel Extracts



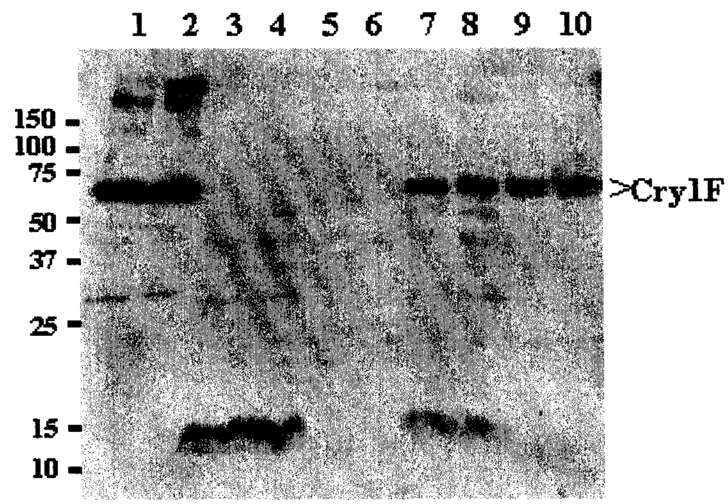
Lanes	Sample
1.	Purified *P. fluorescens*-expressed Cry1F, 5ng
2.	EU, CHPH09B kernel tissue (Cry1F negative)
3.	EU, 1507 Composite kernel tissue (Cry1F positive)
4.	IL2, CHPH09B kernel tissue (Cry1F negative)
5.	IL2, 1507 kernel tissue (Cry1F positive)
6.	IA1, CHPH09B kernel tissue (Cry1F negative)
7.	IA1, 1507 kernel tissue (Cry1F positive)
8.	IA2, CHPH09B kernel tissue (Cry1F negative)
9.	IA2, 1507 kernel tissue (Cry1F positive)

Figure 2. Western Blot Analysis of Cry1F in Maize Line 1507 Leaf Extracts



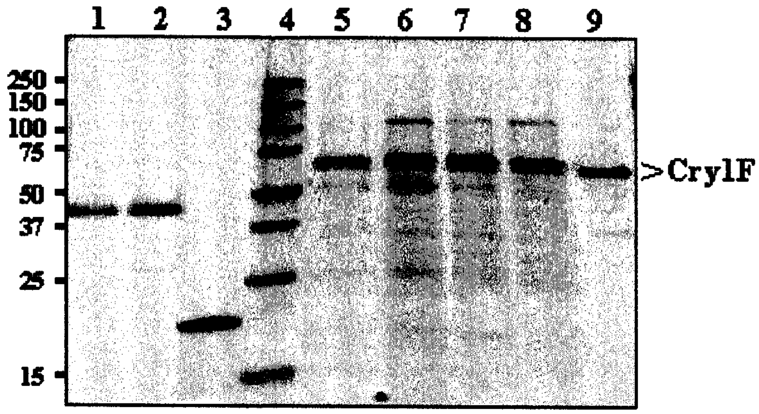
Lanes	Sample
1.	Purified *P. fluorescens*-expressed Cry1F, 5ng
2.	EU, FR3-CHPH09B 1-1-L9 Leaf tissue (Cry1F negative)
3.	EU, FR3-1507 1-1-L9 Leaf tissue (Cry1F positive)
4.	IL2, CHPH09B-1-1-L9 Leaf tissue (Cry1F negative)
5.	IL2, 1507-1-1-L9 Leaf tissue (Cry1F positive)
6.	IA1, CHPH09B-1-1L9 Leaf tissue (Cry1F negative)
7.	IA1, 1507-1-1-L9 Leaf tissue (Cry1F positive)
8.	IA2, CHPH09B-1-1-L9 Leaf tissue (Cry1F negative)
9.	IA2, 1507S-1-1-L9 Leaf tissue (Cry1F positive)

Figure 3. Comparison of Cry1F from *P. fluorescens* and Maize Line 1507 Kernel and Leaf Extracts



Lanes	Sample
1.	Purified *P. fluorescens*-expressed Cry1F, 5ng
2.	Purified *P. fluorescens*-expressed Cry1F, 5ng
3.	EU, CHPH09B kernel tissue (Cry1F negative)
4.	IA1, CHPH09B kernel tissue (Cry1F negative)
5.	EU, FR3-CHPH09B 1-1-L9 Leaf tissue (Cry1F negative)
6.	IA1, CHPH09B-1-1L9 Leaf tissue (Cry1F negative)
7.	EU, 1507 Composite kernel tissue (Cry1F positive)
8.	IA1, 1507 kernel tissue (Cry1F positive)
9.	EU, FR3-1507 1-1-L9 Leaf tissue (Cry1F positive)
10.	IA1, 1507-1-1-L9 Leaf tissue (Cry1F positive)

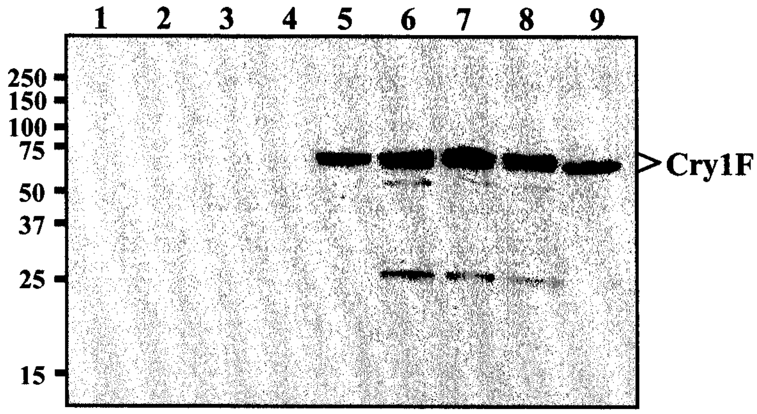
Figure 4. Gel of Immunopurified Cry1F from Kernel Stained for Total Protein



Note: Same gel was stained for glycoprotein detection (see Figure 6)

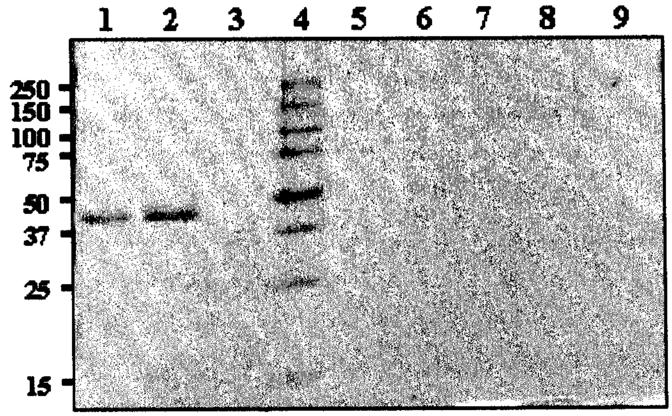
Lanes	Sample
1.	Horseradish Peroxidase, 0.8 μg
2.	Horseradish Peroxidase, 1.5 μg
3.	Soybean Trypsin Inhibitor, 1.5 μg
4.	Pre-stained Protein Markers, 10 μl
5.	Purified, EU, 1507 Composite kernel tissue (Cry1F positive)
6.	Purified, IL2, 1507 kernel tissue (Cry1F positive)
7.	Purified, IA1, 1507 kernel tissue (Cry1F positive)
8.	Purified IA2, 1507 kernel tissue (Cry1F positive)
9.	*P. fluorescens*-expressed Cry1F, 0.8 μg

Figure 5. Western Blot of Immunopurified Cry1F from Kernel



Lanes	Sample
1.	Horseradish Peroxidase, 8 ng
2.	Horseradish Peroxidase, 15 ng
3.	Soybean Trypsin Inhibitor, 15 ng
4.	Pre-stained Protein Markers, 10 µl
5.	Purified, EU,1507 Composite kernel tissue (Cry1F positive)
6.	Purified, IL2, 1507 kernel tissue (Cry1F positive)
7.	Purified, IA1, 1507 kernel tissue (Cry1F positive)
8.	Purified IA2, 1507 kernel tissue (Cry1F positive)
9.	*P. fluorescens*-expressed Cry1F, 5 ng

Figure 6. Gel Stained for Glycoprotein Detection of Immunoaffinity Purified Cry1F from Kernel



Note: Following glycoprotein stain, gel was stained for total protein

Lanes	Sample
1.	Horseradish Peroxidase, 0.8 μg
2.	Horseradish Peroxidase, 1.5 μg
3.	Soybean Trypsin Inhibitor, 1.5 μg
4.	Pre-stained Protein Markers, 10 μl
5.	Purified, EU, 1507 Composite kernel tissue (Cry1F positive)
6.	Purified, IL2, 1507 kernel tissue (Cry1F positive)
7.	Purified, IA1, 1507 kernel tissue (Cry1F positive)
8.	Purified IA2, 1507 kernel tissue (Cry1F positive)
9.	*P. fluorescens*-expressed Cry1F, 0.8 μg

MALDI-TOF MS Spectra of Cry1F Protein

Figure 7. MALDI Mass Spectra of Tryptic Peptides of Cry1F Derived from *P. fluorescens* (top) and EU Maize Kernel Tissue (bottom). Note: Not every peak was labeled.

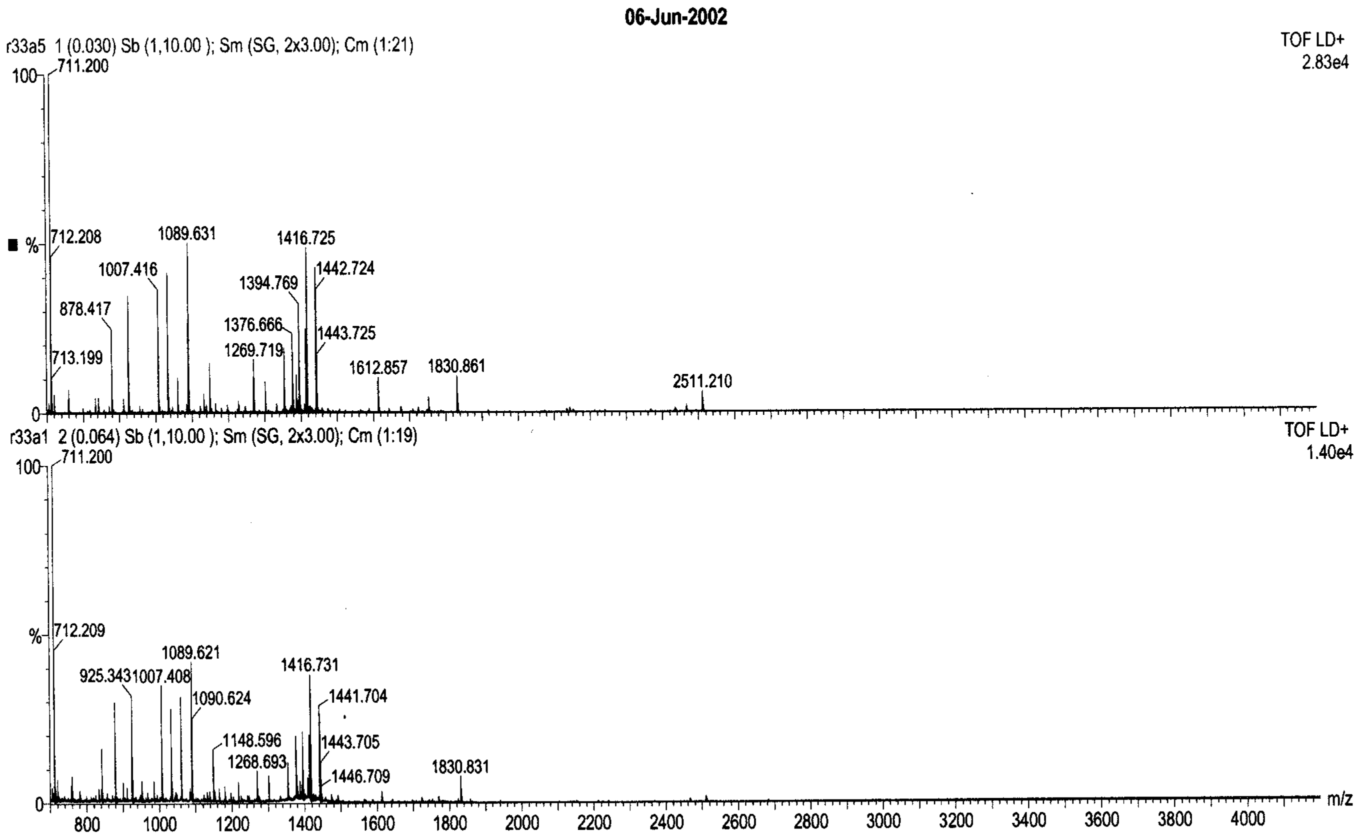


Figure 8. MALDI Mass Spectra of Tryptic Peptides of Cry1F Derived from *P. fluorescens* (top) and IL Maize Kernel Tissue (bottom). Note: Not every peak was labeled.

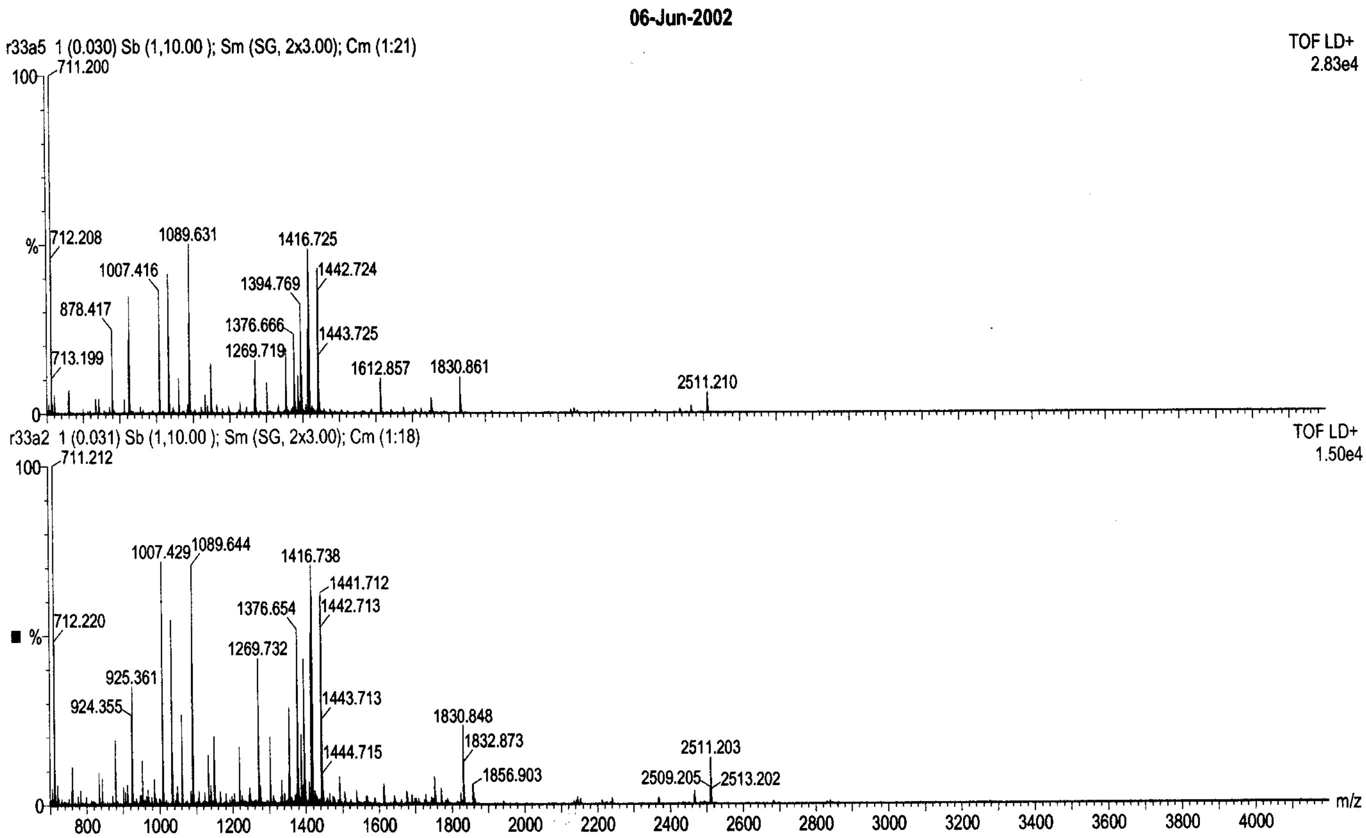


Figure 9. MALDI Mass Spectra of Tryptic Peptides of Cry1F Derived from *P. fluorescens* (top) and IA1 Maize Kernel Tissue (bottom). Note: Not every peak was labeled.

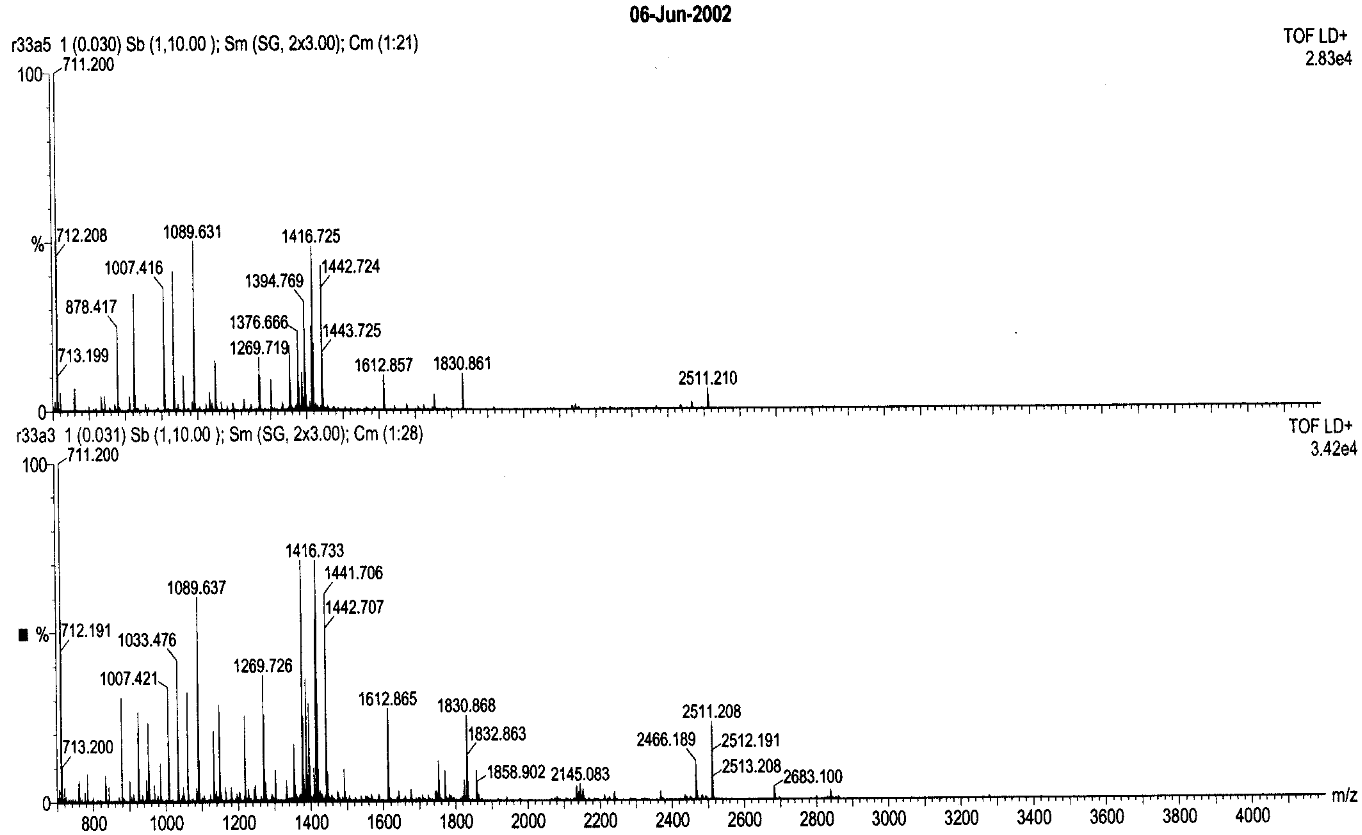


Figure 10. MALDI Mass Spectra of Tryptic Peptides of Cry1F Derived from *P. fluorescens* (top) and IA2 Maize Kernel Tissue (bottom). Note: Not every peak was labeled.