FINAL REPORT

Study Title

Characterization of proteins as expressed in *B.t.* Cry1F maize tissues

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Signatures of Approval:

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1.0 Summary

Maize lines have been modified to express the Cry1F protein from *Bacillus thuringiensis* subsp. *aizawai* and PAT protein. The Cry1F protein confers resistance to the European corn borer (*Ostrinia nubilalis*) insect pest while the PAT protein provides tolerance to herbicide glufosinate. Western analysis techniques were utilized to determine if the Cry1F and PAT proteins expressed in the plant were of the correct molecular weight and immunoreactivity. This study was also designed such that any Cry1F or PAT protein or peptide that is smaller (a partial protein) or larger (a fusion protein) in size than the expected full length protein would be detected by molecular weight. First, polyclonal antibodies were used for detection of the Cry1F or PAT proteins. Polyclonal antibodies recognize multiple antigenic epitopes on the protein thereby increasing the sensitivity of the assay. Second, experiments were conducted with Cry1F and PAT proteins that were denatured, which exposes linear epitopes on the protein to detection by the polyclonal antibody.

The results of western analysis of Cry1F protein expression in plant tissues from *B.t.* Cry1F maize line 1507 demonstrated that under denaturing conditions the Cry1F protein was detected as two bands or doublet of approximately 65 to 68 kD in leaf, pollen, whole plant, and grain tissue. No other bands indicative of a partial Cry1F protein or a fusion protein of greater molecular weight were observed in the maize line 1507 tissues. It appears that the doublet resulted from limited N-terminal processing by a plant protease with trypsin-like specificity.

PAT protein was detected by western analysis in leaf tissue of maize line 1507 as a protein of an approximate molecular weight of 22 kD, but no PAT protein was detect in pollen, whole plant and grain tissues. PAT protein has been shown to be expressed at detectable levels only in leaf tissue of maize line 1507. Based on the western analysis data, it can be concluded that a PAT protein of the expected molecular weight and immunoreactivity is expressed in *B.t.* Cry1F maize line 1507.

2.0 Introduction

The purpose of this study was to examine molecular weight and immunoreactivity of the Cry1F and PAT proteins expressed *in planta* in *B.t.* Cry1F maize line 1507. Leaf, pollen, grain and whole plant tissues of this maize line and a non-transgenic control were sampled for protein extraction. The protein extracts, along with negative control tissues from plants that do not express either protein, were then electrophoresed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS will denature the protein resulting in linearization of the protein molecule. The positive controls in the SDS-PAGE were either microbially-derived Cry1F or PAT protein. The protein extracts were also subjected to polyacrylamide gel electrophoresis under non-denaturing conditions that allow the protein to remain in its normal conformation. Western analysis techniques were utilized to determine if the Cry1F and PAT proteins expressed in the plant were of the correct molecular weight and immunoreactivity. Polyclonal antibodies were used that recognize multiple antigenic epitopes on the protein. The use of polyclonal antibodies increases the likelihood that a protein that is smaller in size (a partial protein) or larger in size (a fusion protein) than expected would be detected by its molecular weight.

3.0 Methods

Polyacrylamide gel electrophoresis (PAGE) of proteins was conducted using pre-cast gels from the Novex® Gel System according to manufacturer's instructions. Tris-Glycine polyacrylamide gels (4%-20%) were utilized in all experiments. Native or denaturing conditions were created by using a Tris-Glycine native sample and running buffer or a Tris-Glycine SDS sample and running buffer respectively. For reducing conditions beta-mercaptoethanol was added to the sample buffer.

Proteins were then transferred electrophoretically from the SDS-PAGE gel to a PVDF membrane for western blotting using tris-glycine transfer buffer. The Novex XCell II Blot Module and Mini-Cell apparatus were used for the transfer process.

Western blots were performed using the Tropix® Western-Light chemiluminescent detection system according to manufacturer's instructions. The binding of the antibodies to the protein bands is detected by a chemiluminescent reaction.

4.0 Results

The results of western analysis of Cry1F protein expression in plant tissues from *B.t.* Cry1F maize line 1507 are shown in Figure 1. Under denaturing conditions the Cry1F protein was detected as two bands of approximately 65 to 68 kD in leaf, pollen, whole plant, and grain tissue. Expression of Cry1F protein was measurable in all four of these tissues as shown by Stauffer and Rivas, 1999. No other bands indicative of a partial Cry1F protein or a fusion protein of greater molecular weight were observed in the maize line 1507 tissues. Moreover, no immunoreactive proteins were detected in the negative control tissues, as expected, with the exception of a possible weakly reactive band in the negative control for grain tissue. Although this weakly reactive band is not readily visible in the figure, it has an apparent molecular weight that is slightly greater than the Cry1F protein. However, the western analysis conducted with Cry1F protein under non-denaturing conditions clearly shows that no immunoreactivity occurs with the

Cry1F antibody in any of the negative control tissues from *B.t.* Cry1F maize line 1507 (Figure 2). It can be concluded that the weakly reactive band observed under denaturing conditions for the grain negative control is due to possible binding of the Cry1F antibody to an epitope present on an endogenous maize protein.

The Cry1F protein detected in western analyses of maize line 1507 plant tissues was present as two bands of nearly identical molecular weight, commonly referred to as a "doublet." Protein doublets typically occur during gel electrophoresis if terminal amino acid residues have been removed from the protein as a result of the activity of proteases released during processing of the plant tissue for analysis. In a separate study, N-terminal amino acid sequence analysis of Cry1F protein derived from plant tissue showed that a five amino acid sequence corresponding to the expected N-terminus of proteolytically cleaved Cry1F was obtained (Evans, 1998). The observed sequence was ^{28}STGRL (the superscript denotes the amino acid residue in the protein). This N-terminal sequence would be expected if cleavage of the Cry1F protein occurred during its purification from plant tissue due to the presence of trypsin-like enzyme activity (trypsin cleaves after arginine residues). The N-terminal sequence of the full-length 68 kD Cry1F protein expressed *in planta* was blocked and therefore could not be sequenced. Therefore, it appears that the doublet resulted from limited N-terminal processing by a plant protease with trypsin-like specificity.

The PAT protein is known to be a homodimer of approximately 43 kD in its native form, comprised of two components of approximately 22 to 23 kD (Wehrmann et al., 1996). The results of western analysis of PAT protein expression in plant tissues from *B.t.* Cry1F maize line 1507 are shown in Figure 3. PAT protein was detected by western analysis in leaf tissue of maize line 1507 as a band of approximately 22 kD under denaturing conditions. No PAT protein was observed in pollen, whole plant, or grain of maize line 1507. These results are consistent with the levels of PAT protein expressed in maize line 1507 tissues (see Stauffer and Rivas, 1999) and the estimated detection limit of < 0.32 ng for the western analysis procedure used in this study. One additional band was observed to react with the PAT antibody in leaf tissue samples, but not in the negative control. This band may represent the 43 kD form of the protein that did not denature during gel electrophoresis. All other bands observed in pollen and grain tissue samples had corresponding bands of the same relative mobility in the negative control; indicating that the polyclonal antibody used to detect the PAT protein also recognized an epitope on a limited number of endogenous maize proteins. The PAT protein standard used in this study also contained bands corresponding to both the 22 kD PAT protein and a 43 kD form that did not denature during gel electrophoresis. From this data, it can be concluded that a PAT protein of the expected molecular weight and immunoreactivity is expressed in *B.t.* Cry1F maize line 1507.

5.0 Conclusions

- Cry1F protein of the correct molecular weight and immunoreactivity is expressed in *B.t.* Cry1F maize line 1507.

- The Cry1F protein appears as a doublet in western blots. Most likely the doublet consists of the expected 68 kD protein and a second 65 kD protein that resulted from limited N-terminal processing by a plant protease with trypsin-like specificity.

- No other bands indicative of a partial Cry1F protein or a fusion protein of greater molecular weight were observed in the maize line 1507 tissues.

- PAT protein of the expected molecular weight and immunoreactivity is expressed in *B.t.* Cry1F maize line 1507.

6.0 References

Evans, S.L. 1998. Equivalency of Microbial and Maize Expressed Cry1F Protein; Characterization of Test Substances for Biochemical and Toxicological Studies. Report number MYCO98-001, an unpublished technical report by Mycogen Seeds c/o Dow AgroSciences.

Stauffer, C., and J. Rivas. 1999. Quantitative ELISA Analysis of Cry1F and PAT Expression Levels in, and Compositional Analysis of, Maize Inbred and Hybrid Lines 1362 and 1507. Report number 98-09-RA-NGLP-012, an unpublished technical report by Pioneer Hi-Bred International Inc.

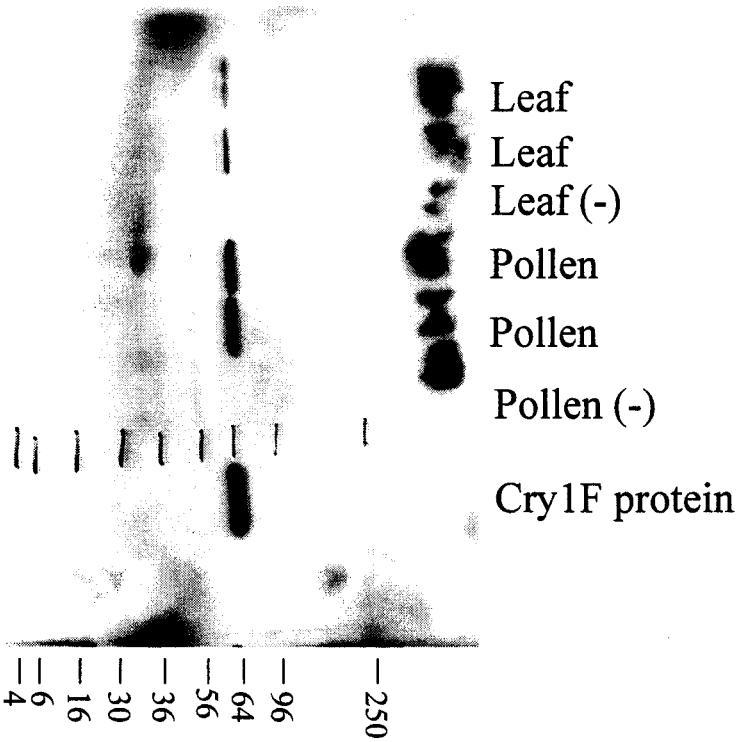
Wehrmann, A., A. Van Vliet, C. Opsomer, J. Botterman, A. Schulz. 1996. The similarities of *bar* and *pat* genes products make them equally applicable for plant engineers. Nature Biotechnology 14:1274-1278.

Figure 1. Immunoreactivity of the Cry1F Protein Expressed in Tissues of *B.t.* Cry1F Maize Line 1507. Electrophoresis was conducted under denaturing conditions.

Molecular weight standards from 4 kilodaltons (kD) to 250 kD are indicated.

Lanes are labeled as follows:

Leaf:	**Maize line 1507 leaf tissue**
Leaf (-):	**Leaf tissue from negative control maize line**
Pollen:	**Maize line 1507 pollen tissue**
Pollen (-):	**Pollen tissue from negative control maize line**
Whole plant:	**Maize line 1507 whole plant tissue**
Whole plant (-):	**Whole plant tissue from negative control maize line**
Grain:	**Maize line 1507 grain tissue**
Grain (-):	**Grain tissue from negative control maize line**
Cry1F protein:	**Purified Cry1F protein**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Leaf
Leaf (-)
Pollen
Pollen
Pollen (-)
Cry1F protein
4
6
16
30
36
56
64
96
250

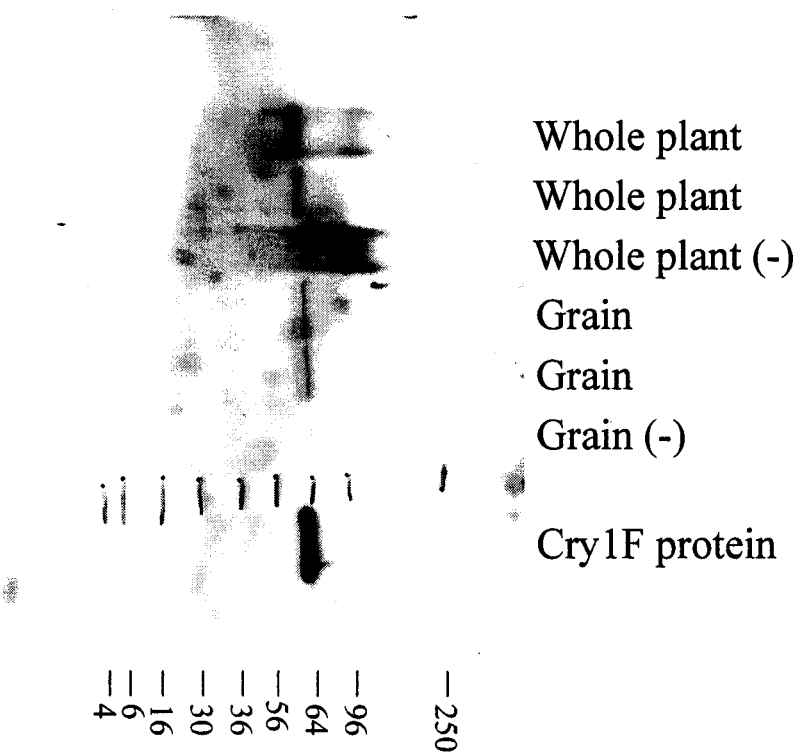
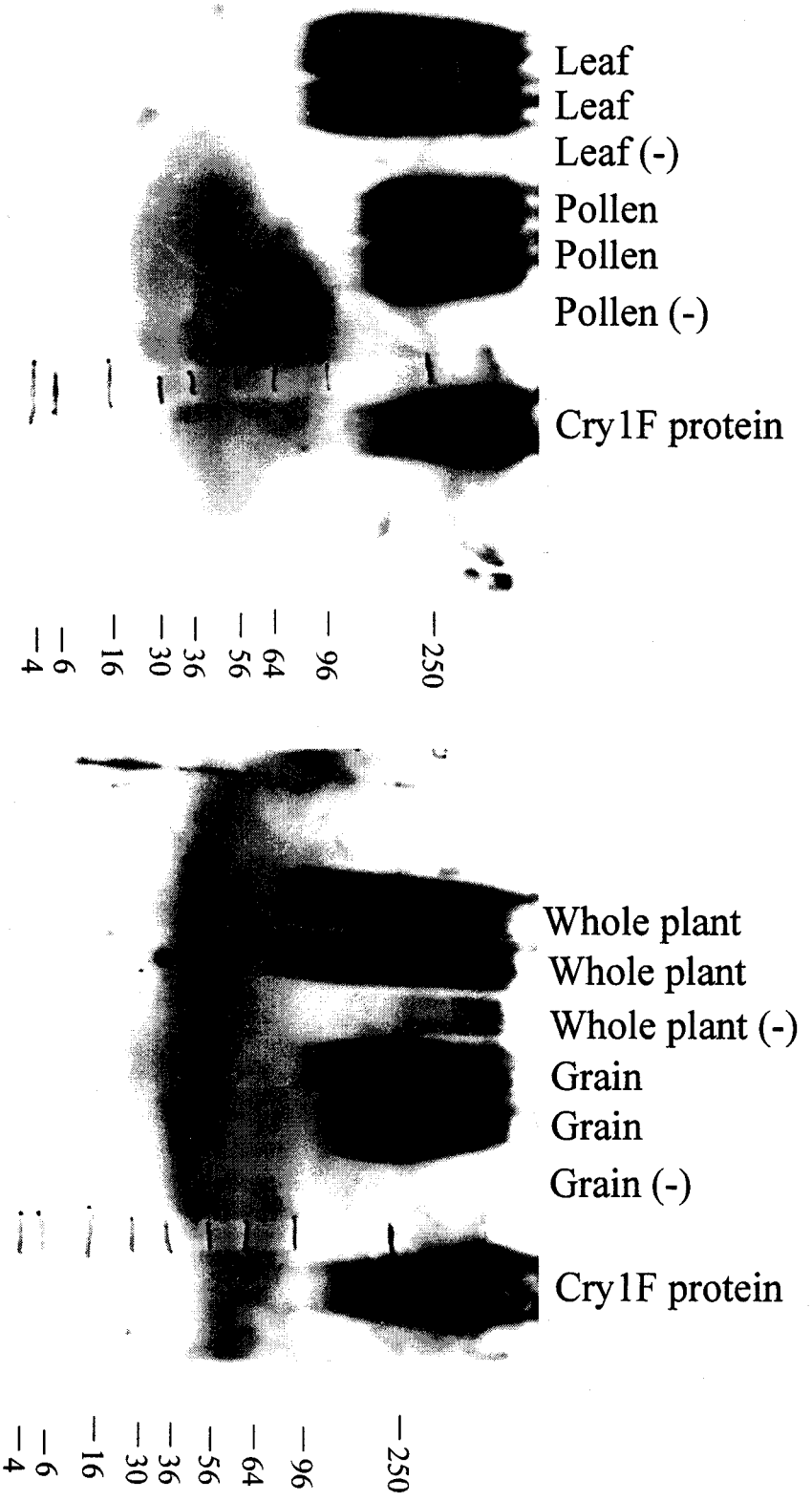

Whole plant
Whole plant
Whole plant (-)
Grain
Grain
Grain (-)
Cry1F protein
4
6
16
30
36
56
64
96
250

Figure 2. Immunoreactivity of the Cry1F Protein Expressed in Tissues of *B.t.* Cry1F Maize Line 1507. Electrophoresis was conducted under non-denaturing conditions.

Molecular weight standards from 4 kilodaltons (kD) to 250 kD are indicated.

Lanes are labeled as follows:

Leaf:	**Maize line 1507 leaf tissue**
Leaf (-):	**Leaf tissue from negative control maize line**
Pollen:	**Maize line 1507 pollen tissue**
Pollen (-):	**Pollen tissue from negative control maize line**
Whole plant:	**Maize line 1507 whole plant tissue**
Whole plant (-):	**Whole plant tissue from negative control maize line**
Grain:	**Maize line 1507 grain tissue**
Grain (-):	**Grain tissue from negative control maize line**
Cry1F protein:	**Purified Cry1F protein**


Leaf
Leaf
Leaf (-)
Pollen
Pollen
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Cry1F protein
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30
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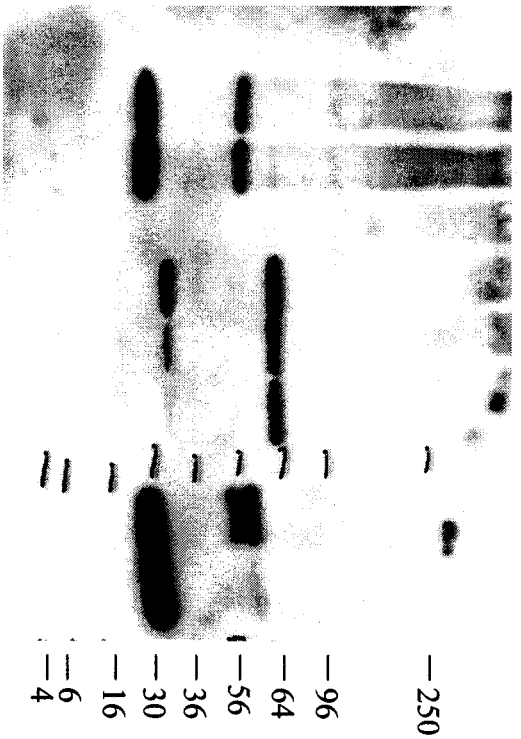
6
4
Whole plant
Whole plant
Whole plant (-)
Grain
Grain
Grain (-)
Cry1F protein
250
96
64
56
36
30
16
6
4

Figure 3. Immunoreactivity of the PAT Protein Expressed in Tissues of *B.t.* Cry1F Maize Line 1507. Electrophoresis was conducted under denaturing conditions.

Molecular weight standards from 4 kilodaltons (kD) to 250 kD are indicated.

Lanes are labeled as follows:

Leaf:	**Maize line 1507 leaf tissue**
Leaf (-):	**Leaf tissue from negative control maize line**
Pollen:	**Maize line 1507 pollen tissue**
Pollen (-):	**Pollen tissue from negative control maize line**
Whole plant:	**Maize line 1507 whole plant tissue**
Whole plant (-):	**Whole plant tissue from negative control maize line**
Grain:	**Maize line 1507 grain tissue**
Grain (-):	**Grain tissue from negative control maize line**
PAT protein:	**Purified PAT protein**
Data not used:	**Data generated for purposes other than this study.**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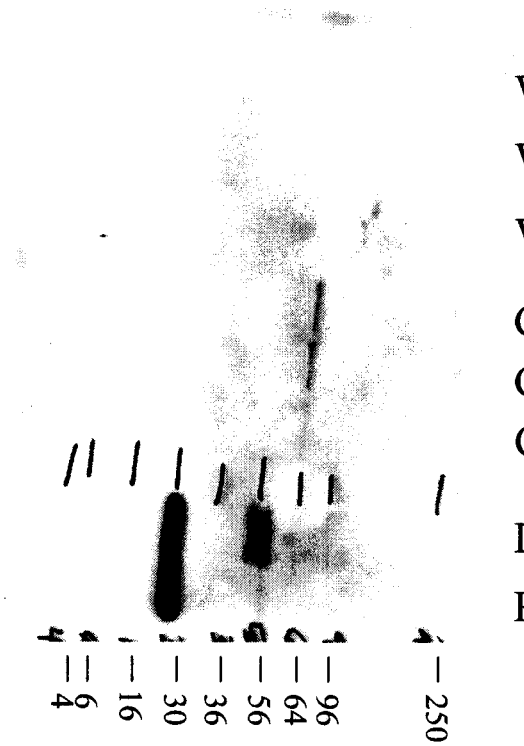
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