Application for an Extension of the Determination of Nonregulated Status for *B.t.* Cry1F Insect-Resistant, Glufosinate-Tolerant Maize (00-136-01p): Maize Line 6275

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The undersigned submits this petition under 7 CFR 340.6 (e) to request that the Adminstrator, Animal and Plant Health Inspection Service, make a determination that the article should not be regulated under 7 CFR 340.

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CLAIM OF DATA CONFIDENTIALITY

The Freedom of Information Act (FOIA) specifically exempts federal agencies from releasing information that are "trade secrets and commercial or financial information obtained from a person and privileged or confidential" 5 U.S.C. 552(b)(4). This exemption from FOIA's disclosure requirements for federal agencies applies where the disclosure of information would likely cause harm to the competitive position of the person from whom the information was obtained, or where, in the case of voluntarily submitted information, the submitter would be less likely in the future to voluntarily share data with the agency.

Appendix 2 of this Application for an Extension of the Determination of Nonregulated Status for B.t. Cry1F Insect-Resistant, Glufosinate-Tolerant Maize (00-136-01p):Maize Line 6275 is claimed by Dow AgroSciences LLC (DAS) as proprietary, trade secret and confidential information exempt from disclosure under FOIA. Appendix 2 contains proprietary information and research data that must be maintained as confidential as disclosure of this information would likely result in substantial competitive harm to DAS and provide a significant unfair competitive advantage to DAS' competitors. Disclosure of this information would allow DAS' competitors access to extensive proprietary research data which could enable such competitors to duplicate DAS' products or to develop competing products without incurring the millions of dollars and years of research and development expended by DAS. Disclosure of this information would also provide DAS' competitors with commercially valuable non-public information concerning DAS' product research, development and commercialization strategies, techniques, methods, operations, development timelines, etc. Keeping this information confidential is necessary for DAS to maintain its competitive position in a highly competitive high tech industry. DAS further desires to keep this information confidential to the extent patent applications have not been filed or patents are pending and have not been published. For these reasons, DAS claims the information contained in Appendix 2 is exempt from disclosure under FOIA.

Summary

Mycogen Seeds c/o Dow AgroSciences LLC (Dow AgroSciences) is submitting an Extension Petition for Determination of Nonregulated Status to the Animal and Plant Health and Inspection Service (APHIS) for insect-resistant, glufosinate-tolerant *B.t.* Cry1F maize line 6275 (Event DAS-06275-8). To provide farmers with a simple, inexpensive, highly effective and environmentally benign means of controlling the European corn borer (ECB; *Ostrinia nubilalis* [Hubner]) and certain other Lepidopteran pests of corn, Dow AgroSciences has developed *B.t.* Cry1F maize line 6275, containing the transformation event DAS-06275-8, which expresses the Cry1F protein from the bacterium *Bacillus thuringiensis* var. *aizawai*. A prior *B.t.* Cry1F maize line, known as 1507 (derived from Event DAS-01507-1; petition number 00-136-01p), received a determination of nonregulated status on June 14, 2001 from APHIS.

The DAS-06275-8 event contains a synthetic truncated *cry*1F gene optimized for maize expression (mo*cry*1F) which was transformed into maize plants using *Agrobacterium* transformation. The mo*cry*1F gene encodes a truncated, core insecticidal crystal protein (ICP) that is identical in amino acid sequence to the native Cry1F protein and to the Cry1F protein expressed in Event 1507. Cry1F expression levels are efficacious for control of the lepidopteran pest European corn borer and other pests such as black cutworm (*Agrostis ipsilon*), fall armyworm (*Spodoptera frugiperda.*) and southwestern corn borer (*Diatraea grandiosella*). The DAS-06275-8 event also contains the phosphinothricin acetyltransferase *bar* gene isolated from the bacterium *Streptomyces hygroscopicus* which is used as a selectable marker and to confer tolerance to glufosinate-ammonium. The BAR protein, expressed from the *bar* gene, acetylates phosphinothricin conferring tolerance to a chemically synthesized phosphinothricin such as the herbicide glufosinate-ammonium. Glufosinate-ammonium is a broad spectrum, non-systemic, non-selective herbicide.

Maize line 6275 has been field tested by Dow AgroSciences since 1999 in the primary corn growing regions of the midwestern US as well as in Hawaii and Puerto Rico. These tests have occurred under field notifications granted by USDA APHIS. Information collected during these trials indicates that *B.t.* Cry1F maize line 6275 exhibits no plant pathogenic properties and is unlikely to harm other insects that are beneficial to agriculture. *B.t.* Cry1F maize line 6275 is no more likely to be a weed than non-transgenic maize and expression of the Cry1F protein is unlikely to increase the weediness potential of any other cultivated plant or wild species. In summary, *B.t.* Cry1F maize line 6275 is not likely to:

- become a weed of agriculture or be invasive of natural habitats,
- cross with wild relatives and create hybrid offspring which may become weedy or invasive,
- become a plant pest,
- have an impact on non-target species, including humans,
- have an impact on biodiversity.

Dow AgroSciences requests a determination from APHIS that *B.t.* Cry1F maize line 6275 and any progeny derived from crosses of this line with traditional maize lines, and any progeny derived from crosses of this line with transgenic maize lines that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR 340.

Certification

The undersigned certifies, that to the best knowledge and belief of the undersigned, this petition includes all information and views on which to base a determination, and that it includes relevant data and information known to the petitioner which are unfavorable to the petition.

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ACRONYMS AND SCIENTIFIC TERMS

6275	transgenic maize line containing the Cry1F protein
bar	gene isolated from *Streptomyces hygroscopicus* which encodes the enzyme phosphinothricin acetyltransferase (PAT).
BAR	phosphinothricin acetyltransferase protein (PAT) encoded by the *bar* gene.
BCW	*Agrotis ispilon;* black cutworm
CEW	*Heliothis zea;* corn earworm
*cry*1F	synthesized, truncated gene originally isolated from *Bacillus thuringiensis* var. *aizawai* which encodes for the insecticidal crystal protein Cry1F
Cry1F	Insecticidal crystal protein encoded from the *cry*1F gene
DIG	digoxigenin; used to label radionuclides for Southern hybridizations
DNA	deoxyribonucleic acid
ECB	*Ostrinia nubilalis;* European corn borer
ELISA	Enzyme Linked Immunosorbent Assay
FAW	*Spodoptera frugiperda;* fall armyworm
Hi-II	publicly available corn line
ICP	insecticidal crystal protein; δ-endotoxin
Mfe I	restriction enzyme used to release the gene transcription units for mo*cry*1F and *bar* genes
mo	maize optimized gene
MW	molecular weight
PAT	phosphinothricin acetyltransferase protein encoded by the *bar* and *pat* genes
PCR	polymerase chain reaction
Pf	*Pseudomonas fluorescens;* bacterial expression system used to produce Cry1F protein
PHP12537	plasmid used in *Agrobacterium*-mediated transformation to obtain event DAS-06275-8
PHP8999	plasmid used in biolistic bombardment transformation to obtain event DAS-01507-1
po	plant optimized gene
RCB	randomized complete block statistical design
Sac I	restriction enzyme that digests once in the T-DNA insert; used to determine number of insertions
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
spc	spectinomycin; antibiotic resistance gene contained in the vector backbone of PHP12537
SWCB	*Diatraea gradiosella;* southwestern corn borer
TC1507	transformation event DAS-01507-1
TC6275	transformation event DAS-06275-8
T-DNA	DNA region of the Ti plasmid from *Agrobacterium tumefaciens*
tet	tetracycline; antibiotic resistance gene contained in the vector backbone of PHP12537

I. RATIONALE FOR SUBMISSION OF REQUEST FOR EXTENSION

There are no changes in the rationale from the previously approved petition number 00-136-01p. The specific differences between *B.t.* Cry1F maize line 6275 (event DAS-06275-8) and the *B.t.* Cry1F maize line of the previous petition, 1507 (event DAS-01507-1), are discussed in the appropriate sections. Event DAS-06275-8 is a regulated article since it contains DNA sequences from the plant pathogens *Agrobacterium tumefaciens* and cauliflower mosaic virus (CaMV).

II. THE CORN FAMILY

There are no changes from the previously approved petition submission (00-136-01p).

III. DESCRIPTION OF THE TRANSFORMATION SYSTEM

Event DAS-06275-8 was transformed, with plasmid PHP12537 (Figure 1), using *Agrobacterium*-mediated transformation (Zupan and Zambryski, 1995; 1997), unlike the previous deregulated event DAS-01507-1 which was transformed using plasmid PHP8999 and microprojectile bombardment. The maize recipient line used in both transformations was the public line designated Hi-II. Plasmid PHP12537 contains the *cry*1F and *bar* gene coding sequences and the regulatory components necessary for their expression in the corn genome. *Agrobacterium tumefaciens* strain LBA4404 was disarmed by the removal of its native T-DNA. Instead, a T-DNA region was introduced on a binary plasmid that contained a selectable marker gene (the *bar* gene) and the *cry*1F gene (Figure 2). The *bar* gene confers tolerance to the herbicide glufosinate-ammonium.

Immature embryos of various maize genotypes were aseptically removed from the developing caryopsis and treated with *A. tumefaciens* strain LBA4404 containing plasmid PHP12537 (Figure 1). After a period of embryo and *Agrobacterium* co-cultivation on solid culture medium, the embryos were transferred to fresh culture medium that contained antibiotics and the herbicide glufosinate-ammonium. The antibiotics killed any remaining *Agrobacterium*. The culture medium was stimulatory to the maize somatic embryogenesis and was selective for those cells that contain an integrated *bar* gene. Therefore, callus that survived the herbicide, proliferated and produced embryogenic tissue which was presumably genetically transformed. The embryonic tissue was then manipulated to regenerate whole transgenic plants, which were transferred to the greenhouse. Leaf samples were taken for molecular analysis to verify the presence of the transgenes by PCR and to confirm the expression of the foreign protein by ELISA. Plants were then subjected to a whole plant bioassay using European corn borers (ECB). Positive plants were crossed with inbred lines to obtain seed from the initially transformed plants. A number of lines were evaluated in the field which resulted in the selection of line 6275, based on its good agronomic characteristics and excellent resistance to European corn borer.

Figure 1. Plasmid map of PHP12537 indicating restriction enzyme sites for *Mfe* I and *Sac* I and coding regions for (mo)*cry*1F, *bar*, spectinomycin (*spc*), and tetracycline (*tet*).

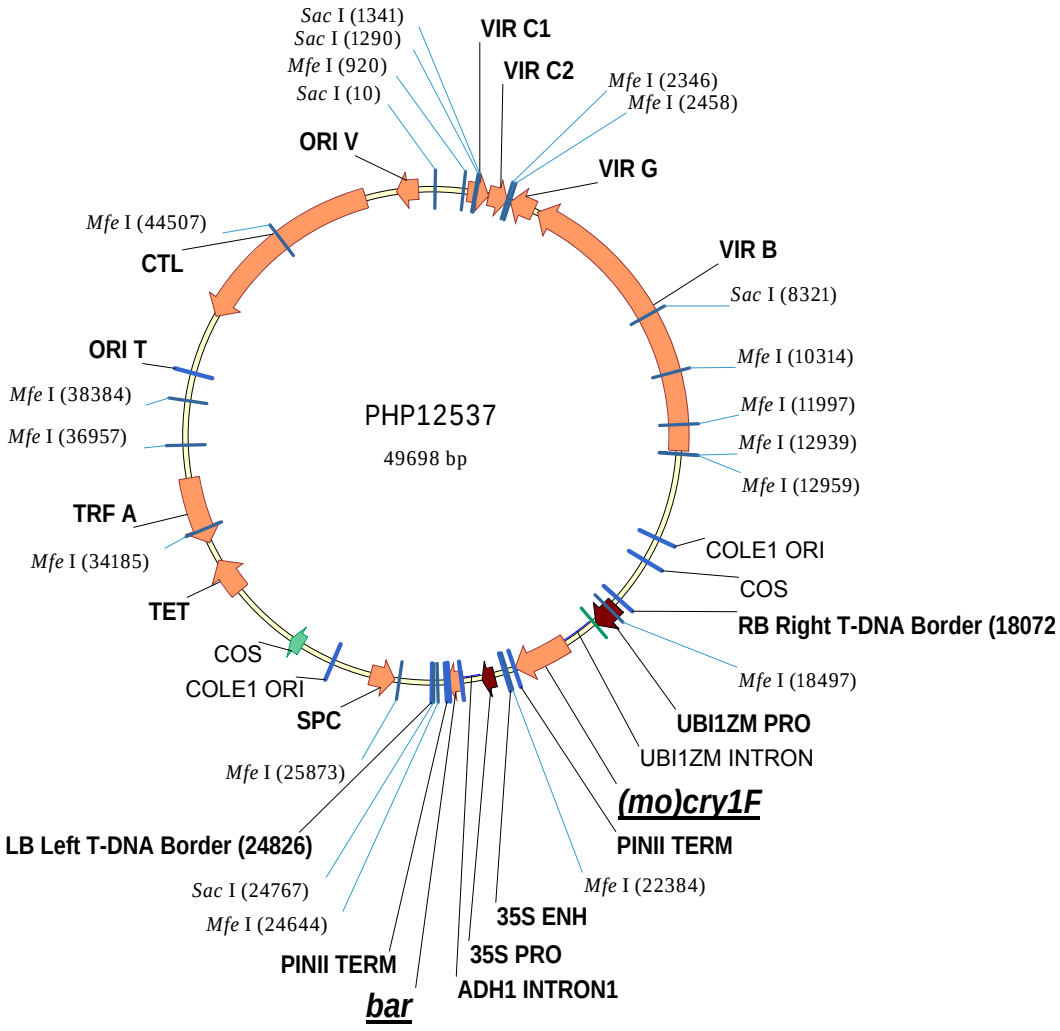
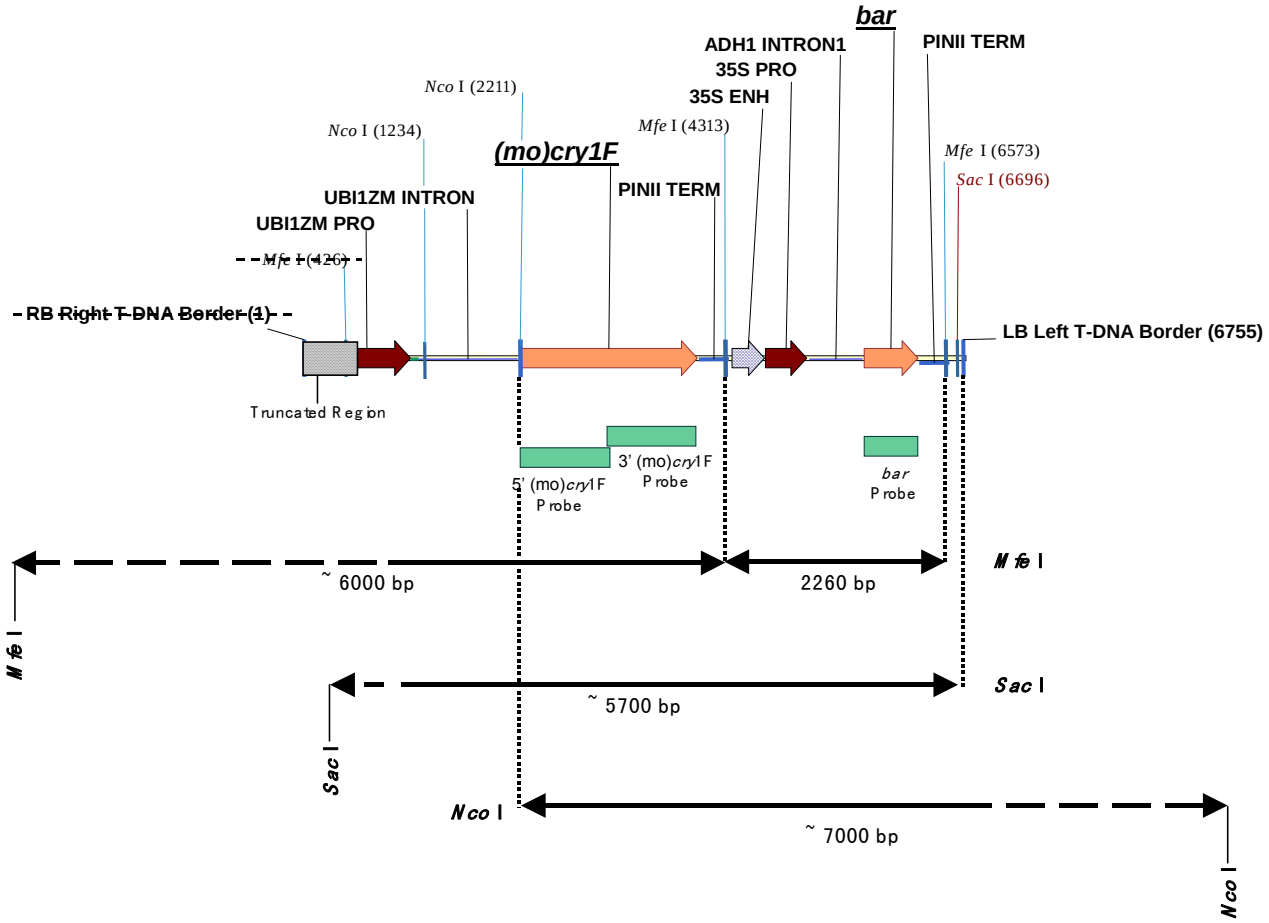


Figure 2. T-DNA region from plasmid PHP12537 indicating restriction enzyme sites for *Mfe* I, *Sac* I and the coding regions and probe locations for mo*cry*1F and *bar*. The double arrow lines indicate the expected hybridizing fragment sizes for the listed enzyme digests. The dotted line indicates DNA outside of the T-DNA into the corn genome.



IV. DONOR GENES AND REGULATORY SEQUENCES

Event TC6275 was transformed with plasmid PHP12537 which differs from the Event TC1507 plasmid in several respects. PHP12537 contains the synthetic, truncated, maize-optimized *cry*1F gene and the *bar* gene as a selectable marker and to confer tolerance to glufosinate-ammonium herbicides between the left and right borders of the *Agrobacterium* T-DNA whereas PHP8999 (TC1507 plasmid) contained the synthetic, truncated, plant-optimized *cry*1F gene, the *pat* gene and the *npt*II gene. Event TC1507 does not contain the *npt*II gene and PHP12537 T-DNA does not contain an antibiotic-resistance marker gene. No element contained in PHP12537 is an allergen, toxicant, pathogenicity facor or irritant or causes injury or disease to plants, and there is a history of "safe use" of *Bacillus thuringiensis*. A summary of the genetic elements of PHP12537 is given in Table 1.

The synthetic *cry*1F gene contained in *B.t.* Cry1F maize line 6275 encodes for the same Cry1F protein that is expressed in the previously deregulated *B.t.* Cry1F maize line 1507 (00-136-01p). Codon changes were made to the gene to improve expression in moCry1F maize plants (See Section V.D.1 for specific codon changes), but these changes do not alter the amino acid sequence of the protein as compared to that expressed in poCry1F maize plants (Duck and Coats, 2000; Appendix 2). The promoter for the *cry*1F gene is the truncated intron of *ubi*ZM 1 promoter in maize line 6275 and the *ubi*ZM 1 promoter in maize line 1507.

The *bar* gene in PHP12537 is the native gene from *Streptomyces hygroscopicus.* The native gene uses a GTG codon to initiate translation. This was replaced with an ATG codon appropriate for translation initiation in plants (DeBlock, 1987). The promoter for the *bar* gene is the CaMV promoter of the 35S transcript from cauliflower mosaic virus (Piertrzak *et al*., 1986). The *bar* gene encodes a protein of 183 amino acids, the sequence of which is identical to the PAT protein present in commercial corn hybrids with tolerance to glufosinate-ammonium (Wehrman *et al*., 1996).

B.t. moCry1F maize line 6275 occurred as a simple integration of a partial copy of the T-DNA region from plasmid PHP12537, according to Southern blot data. Digestion with *Sac* I, an enzyme that digests once in the T-DNA, produced one hybridizing band for both the mo*cry*1F and *bar* gene probes indicating one insertion in event TC6275 (Locke and Tyree, 2002; Appendix 2). In contrast, event TC1507 contained one copy and a partial copy of the *cry*1F (plant optimized) gene and an intact copy and two partial copies of the *pat* gene.

Table 1. Genetic elements of the plasmid PHP12537

Name	Size (bp)	Location in PHP12537 (bp)	Description
RB	25	18072-18096	right border
UBIZM1(2)	1983	18271-20253	ubiquitin promoter (plus intron and 5' untranslated sequence) (Christensen *et al*., 1992) from *Zea mays*
mo*cry*1F (trunc)	1818	20283-22100	maize-optimized version of truncated Cry1F from *Bacillus thuringiensis* var. *aizawai*
PINII	309	22116-22424	terminator sequence from *Solanum tuberosum* proteinase inhibitor II (An *et al.*, 1989)
CAMV35S-1841 enhancer	330	22459-22788	upstream enhancer from Cauliflower Mosaic Virus strain 1841 (Pietrzak *et al*., 1986)
CaMV35S-1841 promoter	422	22801-23222	35S promoter from Cauliflower Mosaic Virus, strain 1841 (Pietrzak *et al*., 1986)
ADH1	538	23254-23791	alcohol dehydrogenase intron 1 from *Zea mays*
bar	552	23810-24361	phosphinothricin acetyltransferase gene isolated from *Streptomyces hygroscopicus* (Thompson *et al*., 1987)
PINII	309	24376-24684	terminator sequence from *Solanum tuberosum* proteinase inhibitor II (An *et al*., 1989)
LB	25	24826-24850	left border

V. GENETIC CHARACTERIZATION OF EVENT DAS-06275-8

Genetic analysis by Southern blots suggested that the insert in *B.t.* moCry1F maize line 6275 occurred as a simple integration of a partial copy of the T-DNA region from plasmid PHP12537 (Locke and Tyree, 2002; Appendix 2). Digestion with *Sac* I, an enzyme that digests once in the T-DNA, produced one hybridizing band for both the mo*cry*1F and *bar* gene probes indicating one insertion in DAS-06275-8. An additional digest with *Mfe* I, an enzyme that releases the gene transcription units for mo*cry*1F and *bar*, revealed that the *bar* transcription unit was inserted intact whereas the mo*cry*1F transcription unit was of a different size (larger) than the size predicted from the original transformation vector due to the loss of one of the *Mfe* I sites upon integration of the T-DNA. Results from a detailed molecular characterization study confirmed the presence of single, intact copies of mo*cry*1F, *bar*, the adh intron, the PinII terminator and the 35S promoter, and a single truncated copy of the intron of the maize ubiquitin promoter (Green *et al.*, 2003). The Southern blot data also suggested that the insert in *B.t.* moCry1F maize line 6275 occurred as a simple integration of a partial copy of the T-DNA region from plasmid PHP12537. The absence of

hybridization of the ubiquitin promoter and ubiquitin intron probes to fragment sizes predicted for an intact insert indicated truncation of the mo*cry*1F transcription unit occurred in that region. The remainder of the T-DNA insert appeared to be intact. Hybridization with four probes located outside of the T-DNA on plasmid PHP12537 did not detect any gene fragments indicating the absence of the tetracycline and spectinomycin resistance genes and the absence of regions immediately adjacent to the left and right T-DNA borders in *B.t.* moCry1F maize line 6275. The insert in event DAS-06275-8 was also shown to be stable across generations and within generations of corn plants.

V.A. SOUTHERN GEL ANALYSIS

Southern hybridization was used to determine the nature and number of mo*cry*1F and *bar* gene insertions which occur in transformation event DAS-06275-8 (Locke and Tyree, 2002; Appendix 2). The integration of genetically modified material into a plant genome can occur at virtually any site in the plant genome. In the *Agrobacterium* transformation method used to produce *B.t.* moCry1F maize line 6275, DNA located between the left and right border sequences of the vector is transferred and inserted into the plant cell genome. The transferred DNA is called T-DNA and once inserted into the plant genome the genes on this segment of DNA that contain plant expression signals are recognized by the plant and expressed.

Seeds were obtained from two generations of breeding of *B.t.* Cry1F maize line 6275, designated the T1S1 generation and the BC5 generation. The T1S1 generation seed consisted of the original Hi-II line containing event DAS-06275-8 (T0) crossed to elite inbred P38 to give an F1 hybrid (T1), and then selfed to give T1S1 seed. The BC5 generation consisted of the fifth backcross generation of the T1 of event DAS-06275-8 with recurrent parent PH09B Plants of both generations were grown in growth chambers and leaf samples were obtained for genomic DNA extraction and analysis. Leaf samples of the control plants (Hi-II, P38, PH09B inbred), which contain a genetic background representative of the transgenic plants, were also obtained for DNA extraction and analysis. Positive and negative plants were confirmed by testing with lateral flow test strips specific for Cry1F and leaf painting with the herbicide glufosinate ammonium.

Genomic DNA was isolated and analyzed from leaf samples from individual plants from each generation of *B.t.* moCry1F maize line 6275 (leaf samples for the T1S1 and BC5 generations were from 4 plants expressing Cry1F and BAR and from one null segregant) and from at least one leaf sample from individual plants for each of the three control substance lines. The isolated genomic DNA from the control and test substances and the reference substance (plasmid PHP12537) was digested with appropriate restriction enzymes to analyze the insertion number and arrangement of the T-DNA from PHP12537. Following digestion with the restriction enzymes, the fragments produced were electrophoretically separated by size through an agarose gel and transferred to nylon membranes. Southern hybridization was carried out using digoxigenin (DIG) labeled probes (Table 2 and Figure 2).

Table 2: Description of DNA probes used in Southern hybridizations.

Probe	Genetic Element	Plasmid Source	Position (bp)	Length (bp)
5' and 3' (mo)*cry*1F	5' end of (mo)*cry*1F gene (encodes Cry1F protein)	PHP12537	20284 – 21202	919
	3' end of (mo)*cry*1F gene (encodes Cry1F protein)	PHP12537	21174 – 22084	911
bar	*bar* gene (encodes PAT protein)	PHP12537	23810 – 24359	550
spc	Spectinomycin resistance gene	PHP12537	26220-26629	410
tet	Tetracycline resistance gene	PHP12537	32408-32869	462
adh intron	Maize alcohol dehydrogenase intron	PHP12537	23254-23784	531
PinII terminator	Potato proteinase inhibitor II terminator	PHP12537	22115-22419 24375-24679	304
Ubi promoter	Maize ubiquitin promoter	PHP12537	18298-19155	858
Ubi intron and 5'	Maize ubiquitin intron	PHP12537	19167-20247	1081
35S promoter	Cauliflower mosaic virus, 35S promoter	PHP12537	22801-23225	424
Outside LB	Region immediately outside of the left T-DNA border	PHP12537	24875 – 25220	346
Outside RB	Region immediately outside of the right T-DNA border	PHP12537	17638 – 17902	265

Analysis of Integration Number for the mocry1F and bar Genes

DNA samples from *B.t.* moCry1F maize line DAS-06275-8, T1S1 and BC5 (back-cross 5) generations (4 DNA samples from plants expressing Cry1F and PAT and one null segregant per each generation), were cleaved with the restriction enzyme *Sac* I to determine the number of insertions of the mo*cry*1F and *bar* genes into the corn genome. The *Sac* I enzyme has one cleavage site located within the T-DNA region of plasmid PHP12537 (Figures 1 and 2) and any hybridizing DNA fragment on the Southern blot would be expected to result from digestion of the one site within the T-DNA and another site in the corn genome flanking the insert. Since the site in the corn genome flanking the insert is unique to the site of insertion, each independent insertion of the T-DNA will have a unique fragment, generally termed a border fragment. The number of bands produced from the enzyme digestion is directly related to the integration complexity, i.e., the greater the number of bands the more integrations that have occurred in the corn genome. In the case of T-DNA insertions, one hybridizing band produced from an enzyme that only cleaves once in the insert usually indicates the presence of one copy of the T-DNA inserted at a single locus in the genome. More than one copy of the T-DNA inserted at a single locus can result in one hybridizing band but this can usually be distinguished from one copy by additional digest and probe combinations or by the hybridizing fragment size which is usually a multiple of the T-DNA size (2x). Border fragments formed from the insertion of a full length T-DNA are typically larger than the predicted size of the T-DNA due to the inclusion of genomic DNA in the fragment. Hybridizing border fragments smaller than the predicted size based on the T-DNA sequence generally indicate a partial insertion of the T-DNA, i.e., a truncated T-DNA insert. The exact size of border fragments cannot be predicted in advance due to the unknown location of the cleavage site in the corn genome.

Southern blot hybridization results from a blot containing DNA digested with *Sac* I and hybridized with the 5' and 3' mo*cry*1F and *bar* probes are shown in Figure 3 and Figure 4, respectively. The hybridizing band sizes detected on the Southern blots are reported in Table 3 along with the expected hybridizing fragment size based on the original T-DNA sequence from plasmid PHP12537. Hybridization with either the 5' and 3' mo*cry*1F or the *bar* probe to DNA isolated from *B.t.* moCry1F maize line 6275 plants expressing Cry1F and BAR for both the T1S1 and the BC5 generations resulted in one hybridizing band with an approximate size of 5.7 kb. Null segregants of *B.t.* moCry1F maize line 6275 did not hybridize to either probe as expected. One hybridizing band with the *Sac* I digest and the two probes strongly suggested that a single T-DNA insert was present in *B.t.* moCry1F maize line 6275. The size of the hybridizing band, ~5.7 kb, was smaller than predicted from T-DNA sequence suggesting that the single T-DNA insert was a partial insert.

Table 3: Predicted and observed hybridizing bands on Southern blots of event DAS-06275-8 probed with *cry*1F, *bar*, *spc*, *tet,* outside LB and outside RB probes.

Probe	Restriction Enzyme	Figure	Predicted Fragment Size (bp)	Observed Fragment Size (bp)
5' & 3' mo*cry*1F	*Sac* I	Figure 3	> 6695	~ 5700
bar	*Sac* I	Figure 4	3887	~ 6000
5' & 3' mo*cry*1F	*Mfe* I	Figure 5	> 6695	~ 5700
bar	*Mfe* I	Figure 6	2260	2260
spc	*Sac* I	Figure 18	None	None
spc	*Mfe* I	Figure 19	None	None
tet	*Sac* I	Figure 20	None	None
tet	*Mfe* I	Figure 21	None	None
Outside LB	*Sac* I	Figure 22	None	None
Outside LB	*Mfe* I	Figure 23	None	None
Outside RB	*Sac* I	Figure 24	None	None
Outside RB	*Mfe* I	Figure 25	None	None

Figure 3: **Southern blot analysis of maize line 6275 – 5' and 3' mo*cry*1F probe.** DNA, isolated from leaves of plants containing event DAS-06275-8 (T1S1 and BC5 generations) and from leaves of PH09B, P38, and Hi-II unmodified corn (negative controls), was digested with *Sac* I and probed with combined 5'and 3' mo*cry*1F probes. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA. [Note: The DNA sample in lane 13 on the *Sac* I digestion gel was slightly overloaded and resulted in an exaggerated curvature of the hybridizing band. The same sample (DAS-06275-8-4 BC5) in lane 13 on the *Mfe* I gels was not overloaded and produced an identical hybridizing pattern compared to all other DAS-06275-8 samples.]

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Sac* I
2	Hi-II + 1 copies PHP12537	*Sac* I
3	Dig VII Marker	
4	Hi-II	*Sac* I
5	DAS-06275-8-9 (null) T1S1	*Sac* I
6	DAS-06275-8-5 T1S1	*Sac* I
7	DAS-06275-8-6 T1S1	*Sac* I
8	DAS-06275-8-7 T1S1	*Sac* I
9	DAS-06275-8-11 T1S1	*Sac* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Sac* I
12	DAS-06275-8-1 BC5	*Sac* I
13	DAS-06275-8-4 BC5	*Sac* I
14	DAS-06275-8-6 BC5	*Sac* I
15	DAS-06275-8-17 BC5	*Sac* I
16	P38	*Sac* I
17	PH09B	*Sac* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Sac* I
20	Hi-II + 5 copies PHP12537	*Sac* I

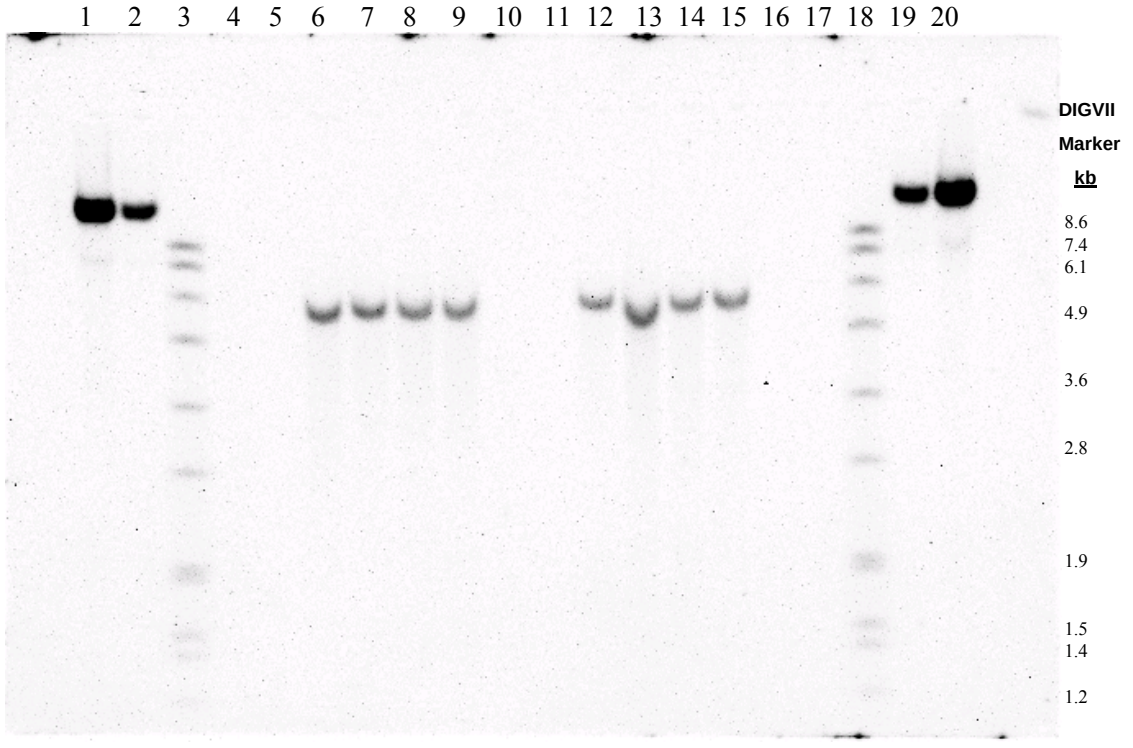


Figure 4: **Southern blot analysis of maize line 6275 – *bar* probe.** DNA, isolated from leaves containing event DAS-06275-8 (T1S1 and BC5 generations) and from leaves of PH09B, P38, and Hi-II unmodified corn (negative controls) was digested with *Sac* I and probed with the *bar* probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA. . [Note: The DNA sample in lane 13 on the *Sac* I digestion gel was slightly overloaded and resulted in an exaggerated curvature of the hybridizing band. The same sample (DAS-06275-8-4 BC5) in lane 13 on the *Mfe* I gels was not overloaded and produced an identical hybridizing pattern compared to all other DAS-06275-8 samples.]

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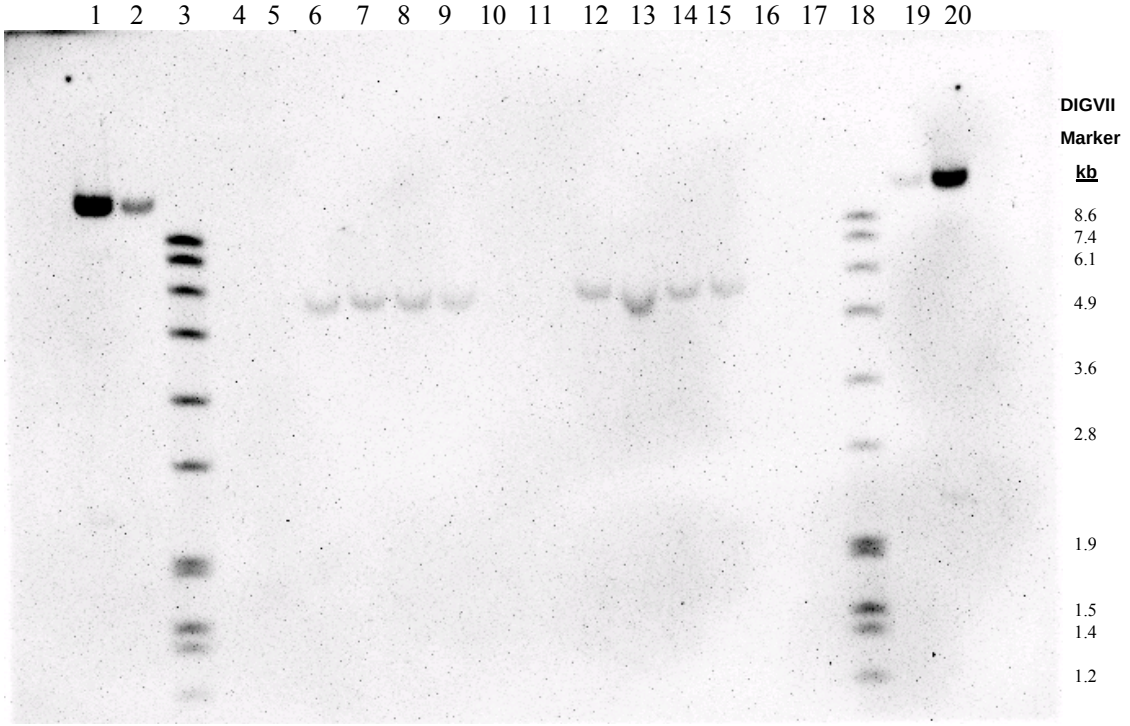
6	DAS-06275-8-5 T1S1	*Sac* I
7	DAS-06275-8-6 T1S1	*Sac* I
8	DAS-06275-8-7 T1S1	*Sac* I
9	DAS-06275-8-11 T1S1	*Sac* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Sac* I
12	DAS-06275-8-1 BC5	*Sac* I
13	DAS-06275-8-4 BC5	*Sac* I
14	DAS-06275-8-6 BC5	*Sac* I
15	DAS-06275-8-17 BC5	*Sac* I
16	P38	*Sac* I
17	PH09B	*Sac* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Sac* I
20	Hi-II + 5 copies PHP12537	*Sac* I



Analysis of the mocry1F and bar Genes and Integrity

In order to confirm the integrity of the two genes within the inserted T-DNA and to better characterize the partial insertion, DNA from *B.t.* moCry1F maize line 6275 plants, T1S1 and BC5 generations (4 DNA samples from plants expressing Cry1F and PAT and one null segregant per each generation), was digested with *Mfe* I and hybridized to the 5' and 3' mo*cry*1F and *bar* probes. Along with the set of event DAS-06275-8 samples, positive controls consisting of unmodified control corn DNA spiked with plasmid PHP12537 DNA at one and five approximate gene copy equivalents were digested with the same enzymes to allow for visualization on the Southern blot of the predicted size fragments produced from *Mfe* I enzyme digestion. Negative controls of the unmodified corn DNA were also digested and analyzed.

The T-DNA diagram in Figure 2 outlines the mo*cry*1F and *bar* gene and probe locations and the *Mfe* I restriction enzyme sites. The resulting fragments expected from the *Mfe* I restriction digest based upon the T-DNA sequence are shown in Table 3. Digestion with *Mfe* I releases one fragment of 3.887 kb from the T-DNA (Figure 2) that is predicted to hybridize to the 5' and 3' mo*cry*1F probe and a second fragment of 2.260 kb that is predicted to hybridize to the *bar* probe. Both of the *Mfe* I fragments contain the majority of the plant transcription unit for each gene. The predicted fragment sizes and the observed fragment sizes are listed in Table 3 along with the associated Southern blot figure number.

As shown in Figure 5, the 5' and 3' mo*cry*1F probe hybridized to a fragment of approximately 6.0 kb in *B.t.* moCry1F maize line DAS-06275-8, T1S1 and BC5 generations, in contrast to the expected size hybridizing fragment of 3.887 kb observed in the plasmid control lanes. Three to four very weakly hybridizing bands below the strong 6.0 kb band were visible in *B.t.* moCry1F maize line DAS-06275-8 samples digested with *Mfe* I and hybridized to 5' and 3' mo*cry*1F. These bands appeared to be the result of minor star activity (digestion at sites other than the predicted recognition site of the enzyme) of the *Mfe* I enzyme as these results were not consistent with the single hybridizing band evidenced in the *Sac* I digestion results. The *bar* probe hybridized to a single fragment of the expected size for the *Mfe* I digest in *B.t.* moCry1F maize line 6275 as shown in Figure 6 suggesting that the *Mfe* I sites located on the 5' and 3' ends of the *bar* transcription unit are intact (Figure 2). Taken together, the mo*cry*1F and *bar* hybridization results suggested that the larger than expected hybridizing band for the 5' and 3' mo*cry*1F probe was due to the loss of one *Mfe* I site upon integration of the T-DNA into the genome. Since the *Mfe* I sites surrounding the *bar* gene appeared to be intact, and the *Mfe* I site at the 5' end of the *bar* gene (*Mfe* I site at bp 4313 in Figure 2) is located at the 3' end of the mo*cry*1F gene transcription unit then the most likely *Mfe* I site to be absent in *B.t.* moCry1F maize line 6275 is the site located at the 5' end of the mo*cry*1F gene transcription unit (*Mfe* I site at bp 426 in Figure 2).

The results indicated that the complete *bar* gene transcription unit was inserted intact and the mo*cry*1F gene transcription unit was partially truncated at the 5' end, up to and including the *Mfe* I site located at bp 426 in Figure 2. The partial truncation of the mo*cry*1F transcription unit was consistent with the *Sac* I digestion results indicating insertion of a single partial copy of the T-DNA from plasmid PHP12537. A summary diagram of the DNA insertion in *B.t.* moCry1F maize line 6275 is shown in Figure 25.

Figure 5: **Southern blot analysis of event DAS-06275-8 – 5' and 3' mo*cry*1F probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Mfe* I and probed with combined 5'and 3' mo*cry*1F probes. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Mfe* I
2	Hi-II + 1 copies PHP12537	*Mfe* I
3	Dig VII Marker	
4	Hi-II	*Mfe* I
5	DAS-06275-8-9 (null) T1S1	*Mfe* I
6	DAS-06275-8-2 T1S1	*Mfe* I
7	DAS-06275-8-5 T1S1	*Mfe* I
8	DAS-06275-8-6 T1S1	*Mfe* I
9	DAS-06275-8-12 T1S1	*Mfe* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Mfe* I
12	DAS-06275-8-1 BC5	*Mfe* I
13	DAS-06275-8-4 BC5	*Mfe* I
14	DAS-06275-8-6 BC5	*Mfe* I
15	DAS-06275-8-17 BC5	*Mfe* I
16	P38	*Mfe* I
17	PH09B	*Mfe* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Mfe* I
20	Hi-II + 5 copies PHP12537	*Mfe* I

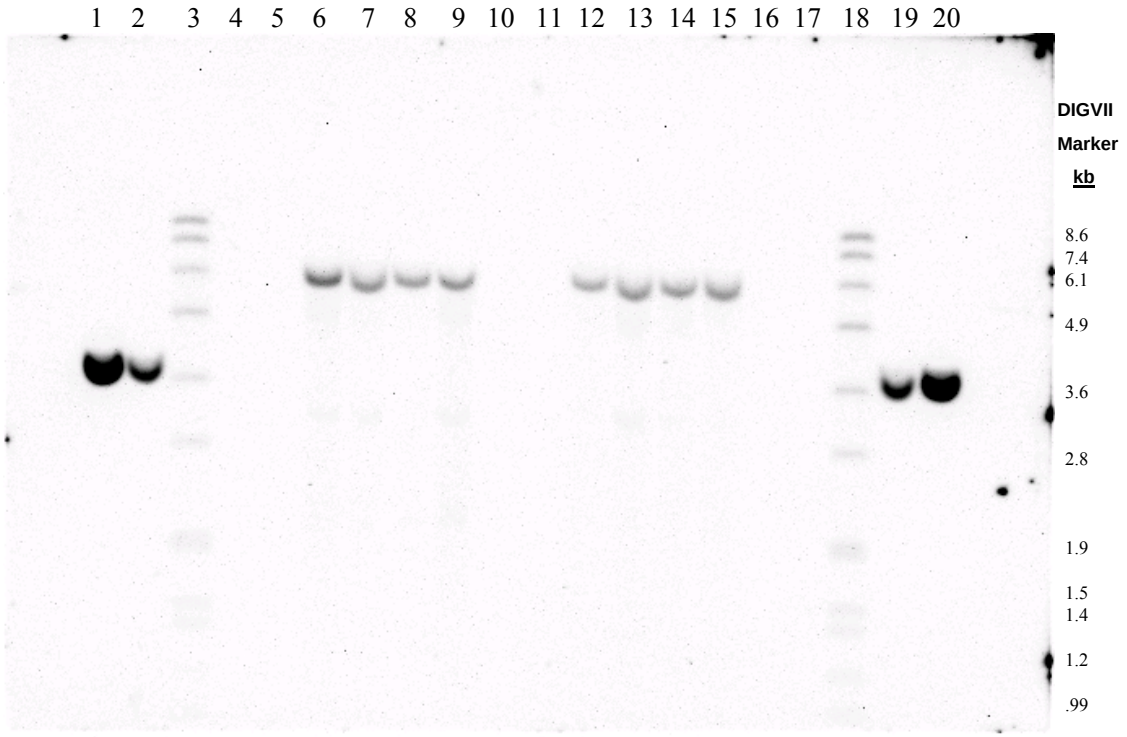
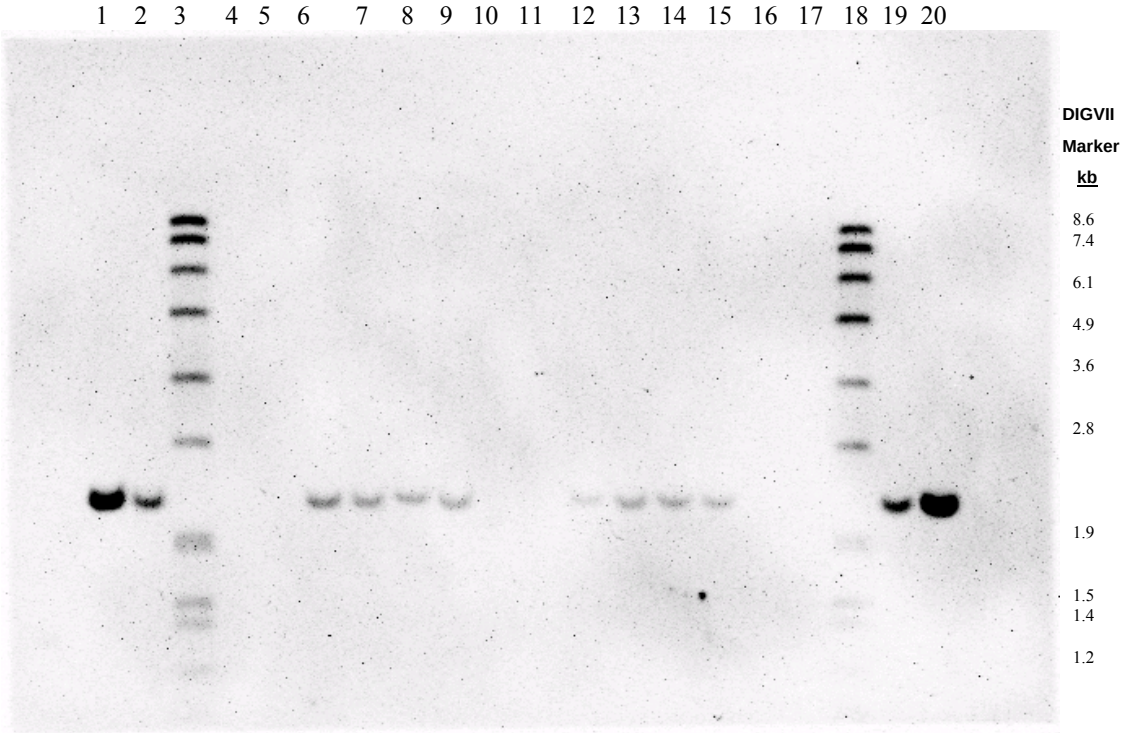


Figure 6: **Southern blot analysis of event DAS-06275-8 – *bar* probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Mfe* I and probed with the *bar* probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Mfe* I
2	Hi-II + 1 copies PHP12537	*Mfe* I
3	Dig VII Marker	
4	Hi-II	*Mfe* I
5	DAS-06275-8-9 (null) T1S1	*Mfe* I
6	DAS-06275-8-2 T1S1	*Mfe* I
7	DAS-06275-8-5 T1S1	*Mfe* I
8	DAS-06275-8-6 T1S1	*Mfe* I
9	DAS-06275-8-12 T1S1	*Mfe* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Mfe* I
12	DAS-06275-8-1 BC5	*Mfe* I
13	DAS-06275-8-4 BC5	*Mfe* I
14	DAS-06275-8-6 BC5	*Mfe* I
15	DAS-06275-8-17 BC5	*Mfe* I
16	P38	*Mfe* I
17	PH09B	*Mfe* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Mfe* I
20	Hi-II + 5 copies PHP12537	*Mfe* I



More detailed Southern analyses were carried out to confirm the confirm the presence of predicted intact copies of mo*cry*1F, *bar*, the adh intron, the PinII terminator and the 35S promoter, and a single truncated copy of the intron of the maize ubiquitin promoter (Green *et al.*, 2003; Appendix 2).

DNA samples from the BC4S1 generation of *B.t.* moCry1F maize line 6275 were cleaved with the restriction enzymes *Bam*H I, *Eco* RI, *Eco*RI/*Bam*H I and *Acc*65 I/*Nco* I to analyze the integrity of the insert containing the mo*cry*1F and *bar* genes. Digests were hybridized with probes specific to 3'mo*cry*1F, 5'mo*cry*1F, *bar*, the ubiquitin promoter, the ubiquitin intron, the 35S promoter, the adh intron and the Pin II terminator. A summary of the hybridization results with the various enzyme/probe combinations is given in Table 4 along with the expected hybridization results for an intact insert and the control plasmid. Figure 7 outlines the restriction sites on the PHP12537 plasmid and Figure 8 the restriction sites in the T-DNA insert.

Table 4. Expected and Observed Hybridizing Fragments for PHP12537 Plasmid and T-DNA Insert DNA on Southern blots probed with *cry*1F, *bar,* ubiquitin promoter, ubiquitin intron, 35S promoter, *adh* intron and PinII terminator

Probe	Enzyme	Figure	DAS-06275-8 Expected Fragment Size for an Intact T-DNA Insert (bp)	DAS-06275-8 Observed Fragment Size (bp)	PHP12537 Expected Fragment Size (bp)	PHP12537 Observed Fragment Size (bp)
mo*cry*1F 3'	*Eco*R I	9	5114	>8576; 7200; 5400[a]	5114	~5114
	*Bam*H I	9	3534	~3534	3534	~3534
	*Eco*R I + *Bam*H I	9	3534	~3534	3534	~3534
	Nco I + *Acc*65 I	9	4041	~4041	4041	~4041
mo*cry*1F 5'	*Eco*R I	10	5114	>8576; 7200; 5400[a]	5114	~5114
	*Bam*H I	10	3534	~3534	3534	~3534
	*Eco*R I + *Bam*H I	10	3534	~3534	3534	~3534
	Nco I + *Acc*65 I	10	4041	~4041	4041	~4041
bar	*Eco*R I	11	5114	>8576; 7200; 5400[a]	5114	~5114
	*Bam*H I	11	Left border (>1048)	3700; 2800[a]	27115	>8576
	*Eco*R I + *Bam*H I	11	967	~967	967	~967
	Nco I + *Acc*65 I	11	4041 + left border (>527)	~4041; ~3000	9202; 4041	~9202; ~4041
ubi promoter	*Eco*R I	12	Right border (>1584) + endogenous	Endogenous only	9185	>8576
	*Bam*H I	12	Right border (>2197) + endogenous	Endogenous only	9567	>8576
	*Eco*R I + *Bam*H I	12	Right border (>1584) + endogenous	Endogenous only	8954	>8576
	Nco I + *Acc*65 I	12	Right border (>1234) + endogenous	Endogenous only	4470	~4470
ubi intron	*Eco*R I	13	5114; right border (>1584) + endogenous	>8576; 7200; 5400[a] + endogenous	9185; 5114	>8576; ~5114
	*Bam*H I	13	Right border (>2197) + endogenous	>8576 + endogenous	9567	>8576
	*Eco*R I + *Bam*H I	13	613; right border (>1584) + endogenous	>8576 + endogenous	8954; 613	>8576[b]
	Nco I + *Acc*65 I	13	977; right border (>1234) + endog.	>8576 +endogenous	4470; 977	~4470; ~977

Table 4. (Cont.) Expected and Observed Hybridizing Fragments for PHP12537 Plasmid and T-DNA Insert DNA on Southern blots probed with *cry*1F, *bar,* ubiquitin promoter, ubiquitin intron, 35S promoter, *adh* intron and PinII terminator

Probe	Enzyme	Figure	DAS-06275-8 Expected Fragment Size for an Intact T-DNA Insert (bp)	DAS-06275-8 Observed Fragment Size (bp)	PHP12537 Expected Fragment Size (bp)	PHP12537 Observed Fragment Size (bp)
35S promoter	*Eco*R I	14	5114	NV[c]	5114	~5114
	*Bam*H I	14	3534	~3534	3534	~3534
	*Eco*R I + *Bam*H I	14	3534	~3534	3534	~3534
	Nco I + *Acc*65 I	14	4041	~4041	4041	~4041
ADH intron	*Eco*R I	15	5114 + endogenous	>8576; 7200; 5400[a] + endogenous	5114	~5114
	Bam HI	15	3534 + endogenous	~3534 + endogenous	3534	~3534
	*Eco*R I + *Bam*H I	15	3534 + endogenous	~3534 + endogenous	3534	~3534
	Nco I + *Acc*65 I	15	4041 + endogenous	~4041 + endogenous	4041	~4041
Pin II terminator	*Eco*R I	16	5114	NV[c]	5114	~5114
	*Bam*H I	16, 17	3534; left border (>1048)	3700; ~3534; 2800[a]	27115; 3534	~3534
	Eco RI + *Bam*H I	16, 17	3534; 967	~3534; 967	3534; 967	~3534; ~967
	Nco I + *Acc*65 I	16	4041; left border (>527)	~4041; ~3000	9202; 4041	>8576; ~4041

[a] Hybridization results indicate altered restriction enzyme digestion patterns due to the inability of *Eco*R I and *Bam*H I to digest methylated sites in plants. In the case of the *Eco*R I digestion the hybridization results could also be indicative of partial digestion.

[b] Presence of the 613 bp band cannot be confirmed because it was not retained on the gel.

[c] NV = no visible hybridization bands.

Figure 7. PHP12537 Plasmid Map with *Acc*65I, *Bam* HI, *Eco* RI, and *Nco* I Restriction Sites

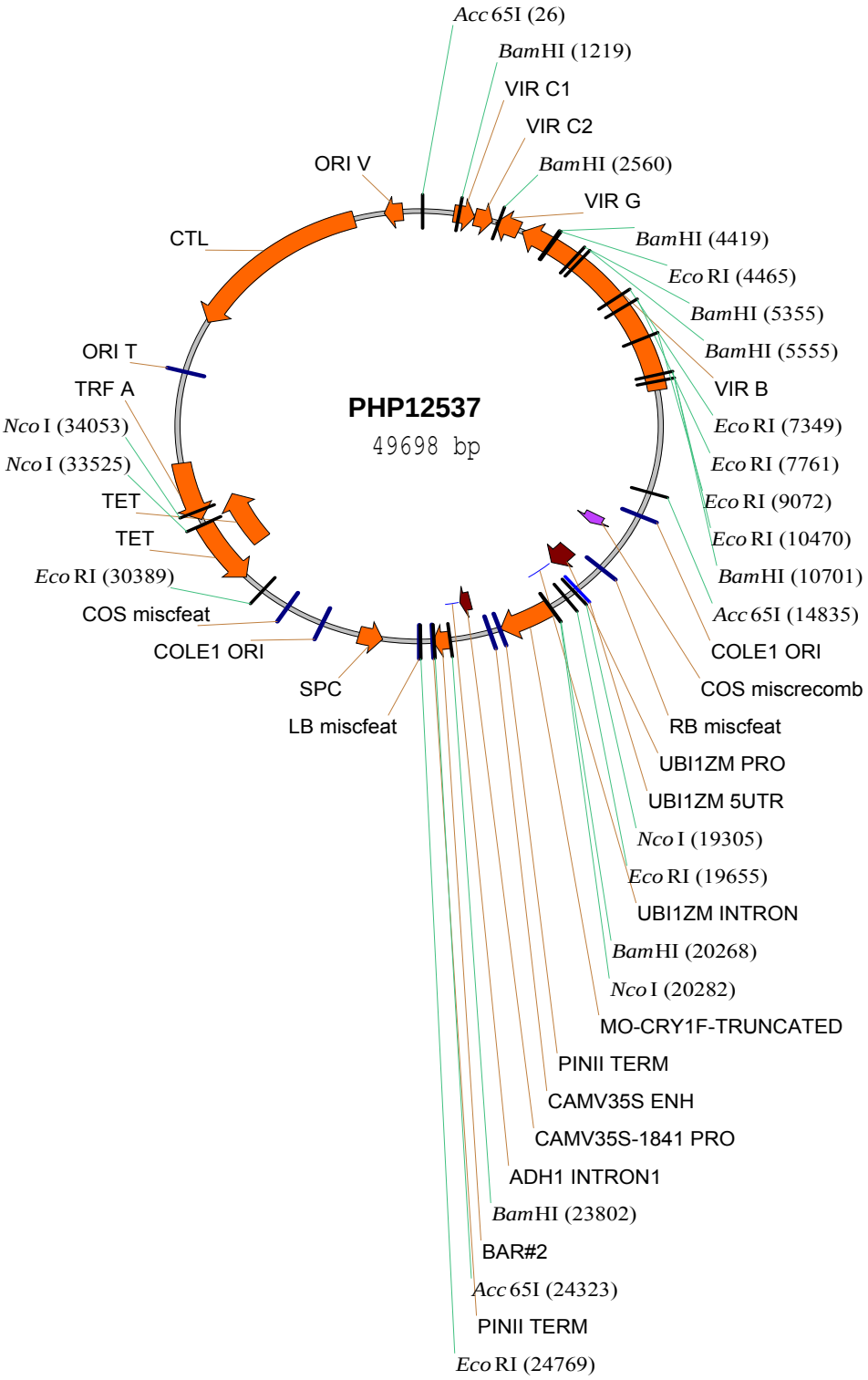
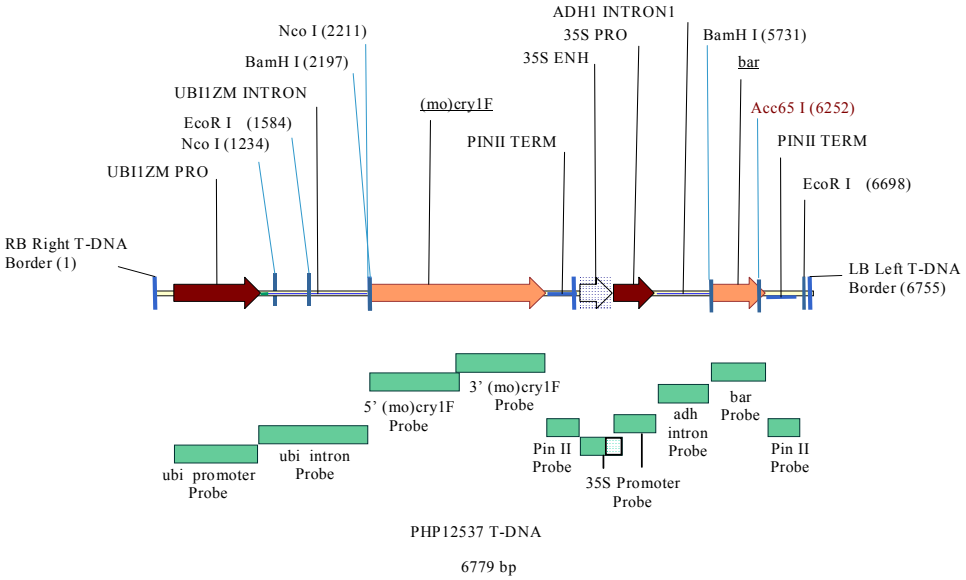
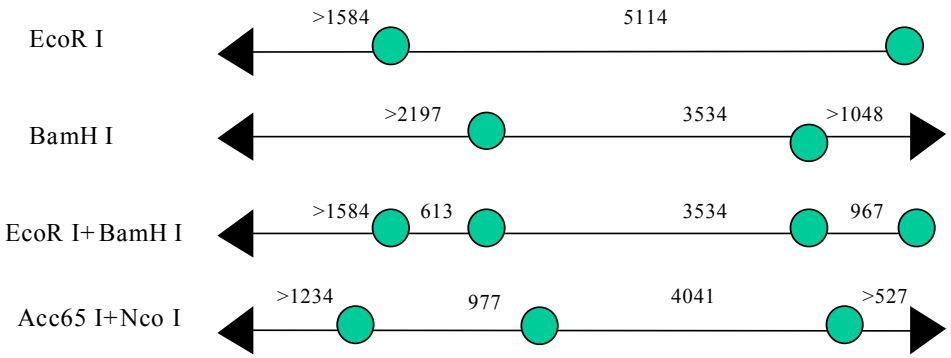


Figure 8. Plasmid PHP12537 T-DNA insert with *Acc*65I, *Bam*H1, *Eco*R1, *Nco*I and *Mfe*I restriction sites.





The four combinations of restriction digests used allowed for analysis of the genetic elements present in the insert both by size and integration pattern. Internal fragments, where both restriction sites are contained within the insert, were generated to confirm expected size. Border fragments, where one restriction site lies outside of the insert yielding fragments of unknown size that are unique to that event, were generated to examine the integration site. The plasmid control was used to confirm internal fragment sizes. The untransformed maize controls were used to identify endogenous sequences exhibiting background hybridization to the probes.

The *Bam*H I, *Eco*R I+*Bam*H I and *Nco* I+*Acc*65 I digests with the 3' and 5'mo*cry*1F probes gave hybridization signals of 3534 bp, 3534 bp and 4041 bp respectively, sizes consistent with those expected from an intact insert and full length mo*cry*1F gene (Table 4, Figures 9 and 10). The hybridization patterns of the *Eco* RI digests with the 3' and 5' mo*cry*1F probes were consistent with that of the proposed truncated insert (Figures 9 and 10).The *Eco* RI digest did not give the 5114 bp hybridization fragment expected for an intact insert with either the 3' or 5' mo*cry*1F probe. Truncation resulted in right border fragments due to the loss of an *Eco*R I site (bp 1584 in the T-DNA insert map in Figure 8). Multiple weak bands (approximately 3) were visible in the *EcoR* I digests when hybridized with the 3' and 5' mo*cry*1F probes (Lanes 7-8 in Figures 9 and 10). *Eco*R I sites can be methylated in plants and this methylation can result in the failure of endonuclease digestion or reduced efficiency of digestion by the enzyme. Multiple hybridization bands of relatively weak signal intensity and of increasingly larger sizes are indicative of methylation or partial digestion of the DNA by *Eco*R I. Thus, the three hybridizing bands actually result from the 3' and 5'mo*cry*1F probes hybridizing to the one mo*cry*1F gene.

Figure 9. Southern blot analysis of maize event DAS-06275-8 (3' mo*cry*1F probe). DNA isolated from moCry1F maize line 6275 (BC4S1) and unmodified corn was digested with *Eco* RI, *Bam* HI, *Eco* RI/*Bam* HI and Nco I/Acc 651 and probed with the 3'mo*cry*1F probe. Approximately 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lane 6 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	empty	
6	Negative control/ P38 *	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38	*Nco* I + *Acc*65 I
13	Negative control/ P38	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	

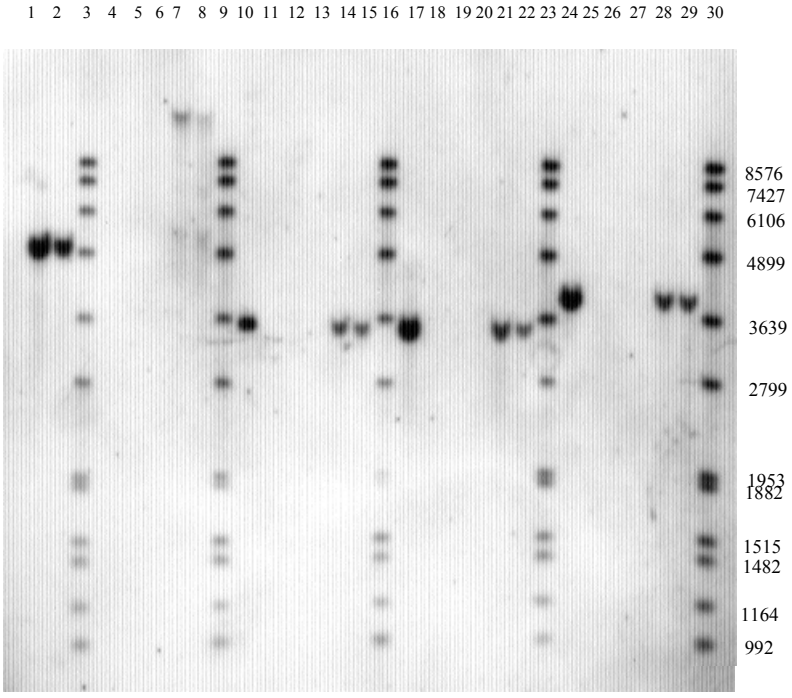
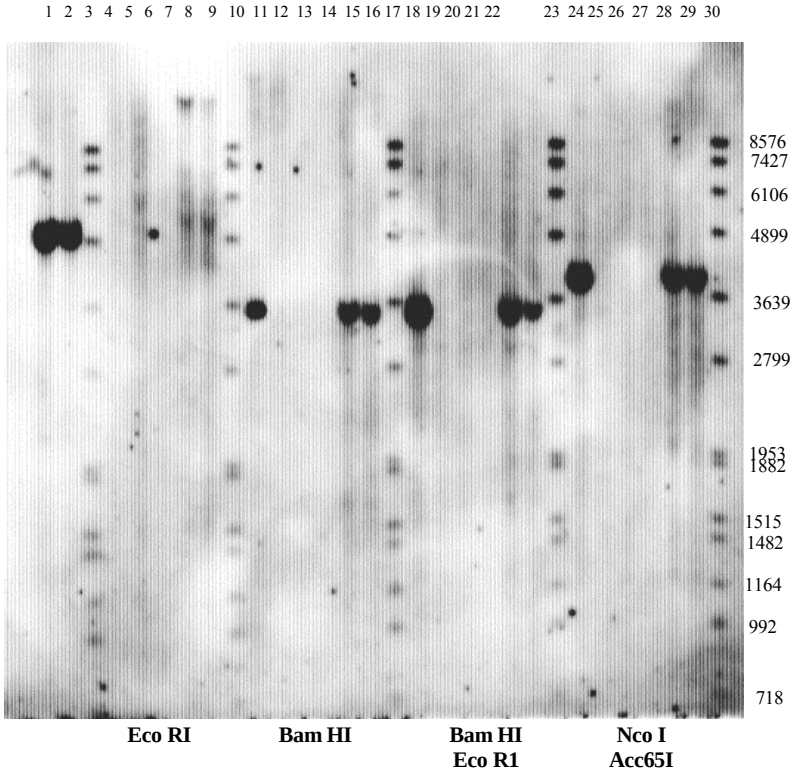


Figure 10. Southern blot analysis of maize event DAS-06275-8 (5' mo*cry*1F probe). DNA isolated from (mo)Cry1F maize line 6275 (BC4S1) and unmodified corn was digested with *Eco* RI, *Bam* HI, *Eco* RI/*Bam* HI and *Nco* I/ *Acc* 651 and probed with the 5'(mo)*cry*1F probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lane 6 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	empty	
6	Negative control/ P38 *	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38	*Nco* I + *Acc*65 I
13	Negative control/ P38	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	



Hybridization with the *bar* probe again indicated the insert contained the full length *bar* gene, and truncation on the 5' end of the T-DNA insert. The *Eco*R I digest did not give the 5114 bp fragment expected of an intact insert but rather a right border fragment (Figure 11, Lanes 7-8)). The *ba*r probe hybridized to the same *EcoR* I fragments as described above for the 3' and 5' mo*cry*1F probes. The *Eco*R I + *Bam*H I digest gave the expected 967 bp fragment (Figure 11, Lanes 21-22), and the *Nco* I + *Acc*65 I digest gave the expected 4041 bp and left border fragment >510 bp (Figure 11, Lanes 28-29). *Bar* probe hybridization to the *Bam*H I digest would be expected to produce a left border fragment of unpredicted size. As described for the *Eco*R I enzyme, *Bam*H I sites can be methylated in plants and *Bam*H I does not cleave methylated sites. Results of the *bar* hybridization suggest that the *Bam*H I sites in the surrounding genomic DNA were methylated. Two hybridizing fragments were detected in the *Bam*H I digests and the signals were weaker than expected, suggesting methylation or partial digestion (Figure 11, Lanes 14-15).

Southern blots of the digests probed with the ubiquitin promoter did not give hybridization signals for any of the sizes expected for an intact insert (Table 4) although hybridization was seen with maize endogenous ubiquitin sequences (Figure 12). The ubiquitin intron probe did not show any of the expected band sizes, but did have high molecular weight hybrization signals for each of the digests (Table 4, Figure 13). This indicates that at least some of the ubiquitin intron is present in the DAS-06275-8 insert, likely sequence 3' to the EcoRI site in the intron (bp 1584 in Figure 8).

Figure 11. Southern blot analysis of maize event DAS-06275-8 probed with the *bar* probe. DNA isolated from (mo)Cry1F maize line 6275 (BC4S1) and unmodified corn was digested with *Eco*R I, *Bam*H I, *Eco*R I+*Bam*H I and *Nco* I+*Acc*65 I and probed with the *bar* probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lanes 13, 20 and 27 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	Negative control/P38*	*Bam*H I + *Eco*R
6	Negative control/ P38	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38*	*Nco* I + *Acc*65 I
13	Negative control/ P38*	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	

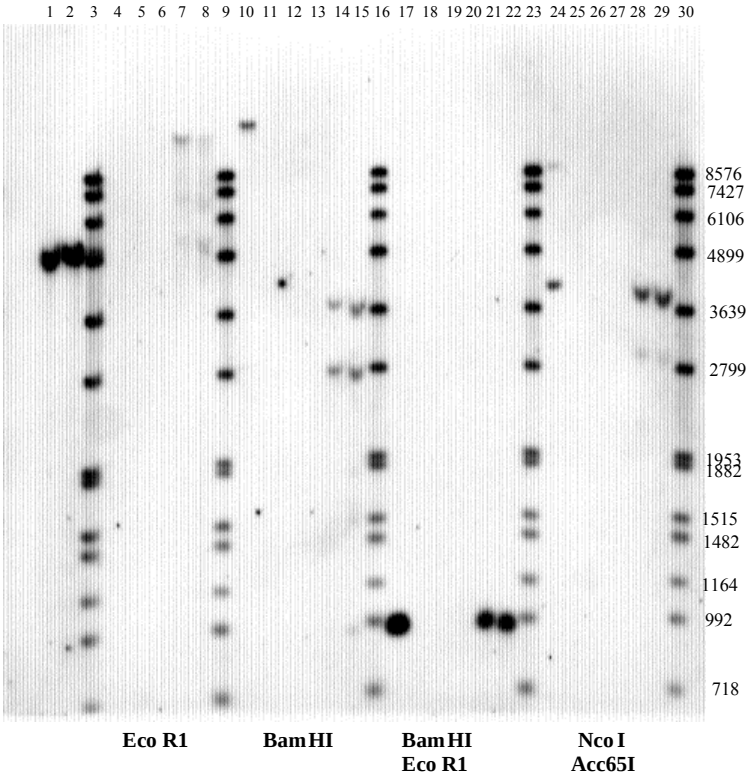


Figure 12. Southern blot analysis of maize event DAS-06275-8 probed with the ubiquitin promoter probe. DNA isolated from moCry1F maize line 6275 (BC4S1) and unmodified corn was digested with *Eco* RI, *Bam* HI, *Eco* RI/*Bam* HI and Nco I/Acc and probed with the ubiquitin promoter probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lanes 13, 20 and 27 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	Negative control/P38*	*Bam*H I + *Eco*R
6	Negative control/ P38	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38*	*Nco* I + *Acc*65 I
13	Negative control/ P38*	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	

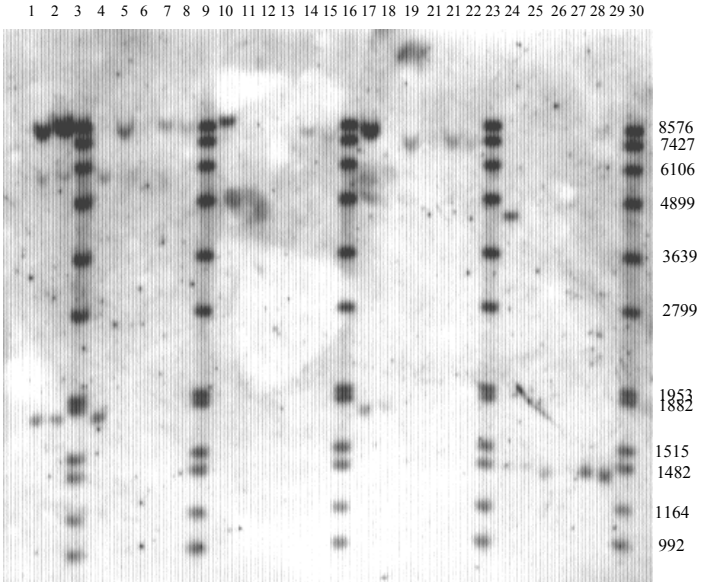
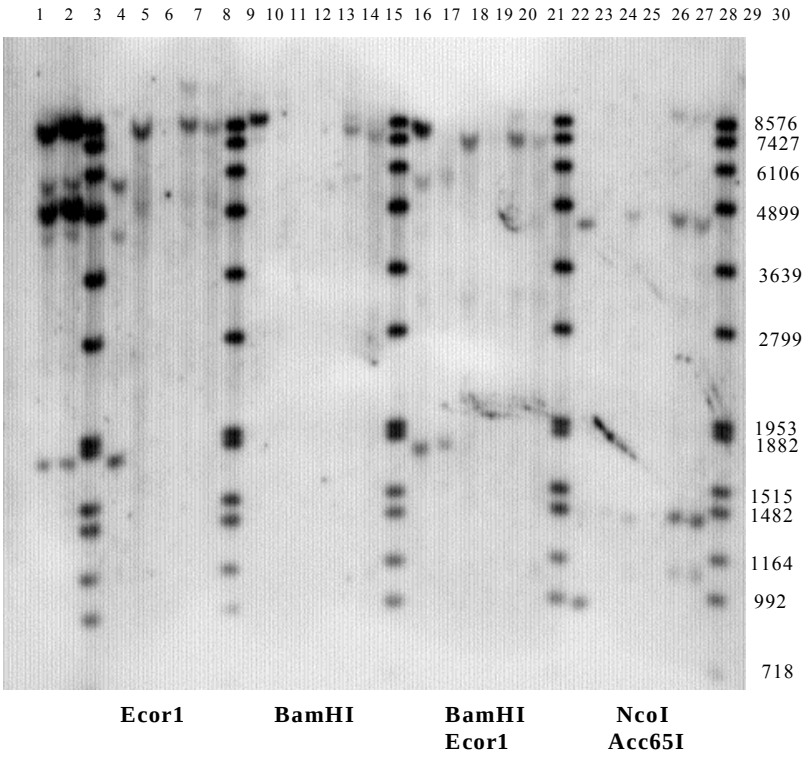


Figure 13. Southern analysis of maize event DAS-06275-8 probed with the ubiquitin intron probe.

DNA isolated from moCry1F maize line 6275(BC4S1) and unmodified corn was digested with *Eco* RI, *Bam* HI, *Eco* RI/*Bam* HI and Nco I/Acc and probed with the ubiquitin intron probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lanes 13, 20 and 27 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	Negative control/P38*	*Bam*H I + *Eco*R
6	Negative control/ P38	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38*	*Nco* I + *Acc*65 I
13	Negative control/ P38*	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	



The *Bam*H I, *Eco*R I+*Bam*H I and *Nco* I+*Acc*65 I digests with the PinII terminator, 35S promoter and adh intron probes (Figures 14,15,16, 17) gave the hybridization signals expected from an intact T-DNA insertion considering the described methylation for *Eco*R I and *Bam*H I sites in the surrounding genomic DNA (Table 4). As observed with the mo*cry*1F and *bar* probes, the *Eco* RI digests with these probes also did not give the 5114 bp hybridization fragment expected of an intact T-DNA insertion (Figures 14,15,16, 17).

Figure 14. Southern blot analysis of maize event DAS-06275-8 probed with the 35S promoter probe. DNA isolated from moCry1F maize line 6275 (BC4S1) and unmodified corn was digested with *Eco* RI, *Bam* HI, *Eco* RI/*Bam* HI and Nco I/Acc 651 and probed with the 35S promoter probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lane 6 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	empty	
6	Negative control/ P38 *	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38	*Nco* I + *Acc*65 I
13	Negative control/ P38	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	

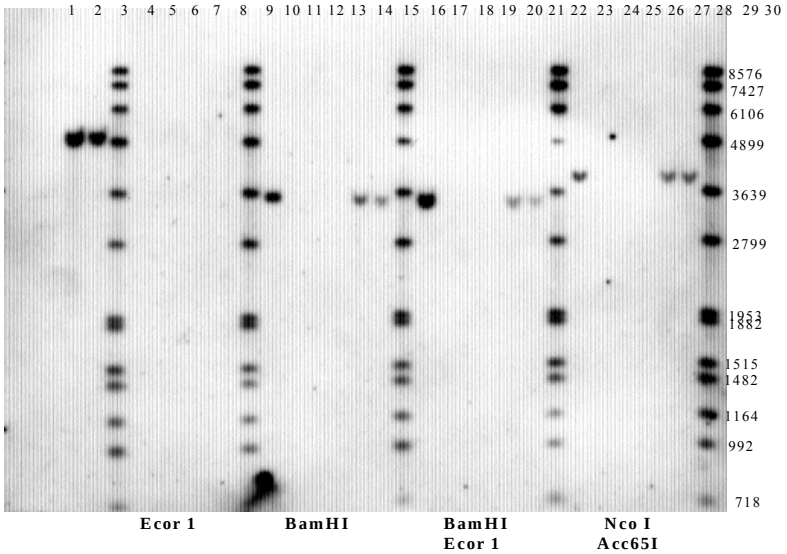


Figure 15. Southern blot analysis of event DAS-06275-8 probed with the adh intron probe. DNA isolated from moCry1F maize line 6275 (BC4S1) and unmodified corn was digested with *Eco* RI, *Bam* HI, *Eco* RI/*Bam* HI and Nco I/Acc651 and probed with the adh intron probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lanes 13, 20 and 27 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	Negative control/P38*	*Bam*H I + *Eco*R
6	Negative control/ P38	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38*	*Nco* I + *Acc*65 I
13	Negative control/ P38*	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	

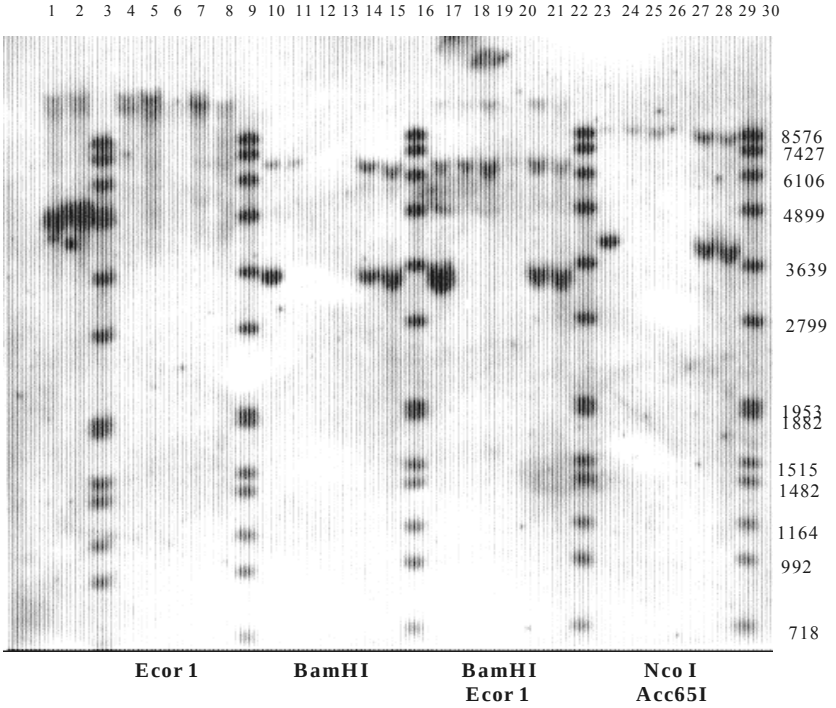


Figure 16. Southern blot analysis of event DAS-06275-8 probed with the PinII terminator probe. DNA isolated from moCry1F maize line 6275 (BC4S1) and unmodified corn was digested with *Eco* RI, *Bam* HI, *Eco* RI/*Bam* HI and Nco I/Acc651 and probed with the PinII terminator probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The DNA in lanes 13, 20 and 27 was slightly degraded. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	5X Plasmid control/ Hi-II	*Eco*R I	16	DIG VII size marker	
2	2X Plasmid control/ Hi-II	*Eco*R I	17	2X Plasmid control/Hi-II	*Bam*H I + *Eco*R
3	DIG VII size marker		18	Negative control/ Hi-II	*Bam*H I + *Eco*R
4	Negative control/ Hi-II	*Eco*R I	19	Negative control/ PH09B	*Bam*H I + *Eco*R
5	Negative control/ PH09B	*Eco*R I	20	Negative control/P38*	*Bam*H I + *Eco*R
6	Negative control/ P38	*Eco*R I	21	DAS-06275-8-20	*Bam*H I + *Eco*R
7	DAS-06275-8-20	*Eco*R I	22	DAS-06275-8-22	*Bam*H I + *Eco*R
8	DAS-06275-8-22	*Eco*R I	23	DIG VII size marker	
9	DIG VII size marker		24	2X Plasmid control/ Hi-II	*Nco* I + *Acc*65 I
10	2X Plasmid control/ Hi-II	*Bam*H I	25	Negative control/ Hi-II	*Nco* I + *Acc*65 I
11	Negative control/ Hi-II	*Bam*H I	26	Negative control/ PH09B	*Nco* I + *Acc*65 I
12	Negative control/ PH09B	*Bam*H I	27	Negative control/ P38*	*Nco* I + *Acc*65 I
13	Negative control/ P38*	*Bam*H I	28	DAS-06275-8-20	*Nco* I + *Acc*65 I
14	DAS-06275-8-20	*Bam*H I	29	DAS-06275-8-22	*Nco* I + *Acc*65 I
15	DAS-06275-8-22	*Bam*H I	30	DIG VII size marker	

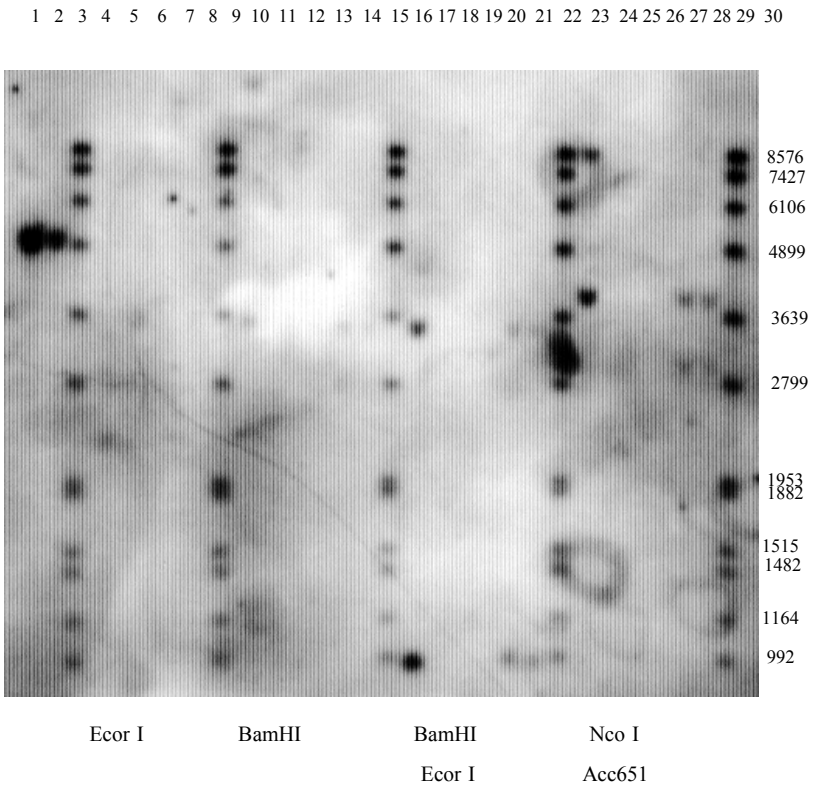
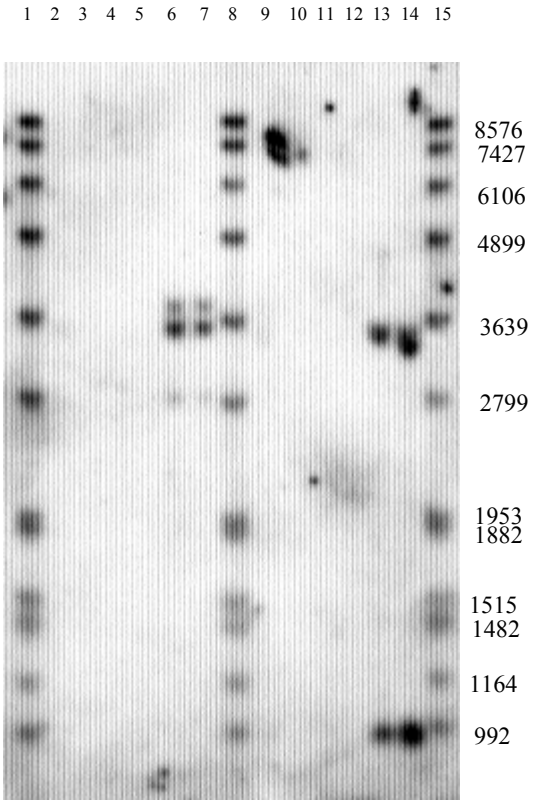


Figure 17. Southern Blot Analysis of Maize Event 6275/ PinII Terminator Probe. DNA isolated from (mo)Cry1F maize line 6275 (BC5 homozygous) and unmodified corn was digested with *Bam*H I and *Eco*R I+*Bam* HI and probed with the pinII terminator probe. Approx. 3μg of genomic DNA were digested and loaded per lane. Plasmid controls contained PHP12537 at the indicated gene copy number equivalent and 3 μg of unmodified control DNA. The lanes contained:

Lane	Sample	Digest	Lane	Sample	Digest
1	DIG VII size marker		9	1X plasmid control/Hi-II	*Eco*R I + *Bam*H I
2	1X plasmid control/Hi-II	*Ba*mH I	10	Negative control/ Hi-II	*Eco*R I + *Bam*H I
3	Negative control/ Hi-II	*Ba*mH I	11	Negative control/ PHO9B	*Eco*R I + *Bam*H I
4	Negative control/ PHO9B	*Ba*mH I	12	Negative control/ P38	*Eco*R I + *Bam*H I
5	Negative control/ P38	*Ba*mH I	13	DAS-06275-8-9 (BC5,	*Eco*R I + *Bam*H I
6	DAS-06275-8-4 (BC5,	*Ba*mH I	14	DAS-06275-8-9 (BC5,	*Eco*R I + *Bam*H I
7	DAS-06275-8-9 (BC5,	*Ba*mH I	15	DIG VII size marker	
8	DIG VII size marker				



The data indicate that the mo*cry*1F gene, *bar* gene, 35S promoter, adh intron, and PinII terminator are present as intact copies in the event DAS-06275-8 insert. The data also indicate the absence of the ubiquitin promoter sequence and truncation of the ubiquitin intron sequence 3' to the EcoR I site (Figure 25). These results are consistent with the previous study indicating truncation of the single intact insert on the 5' end involving portions of the ubiquitin promoter (Locke and Tyree, 2003; Appendix 2). The intron sequence of the ubiquitin promoter has been shown to have promoter-like features as well as a sequence homologous to the *opaque*-2 transcription factor binding box. This combination has been hypothesized to explain the promoter activity of the intron (Salgueiro, *et al.*, 2000). Therefore, even though event DAS-06275-8 appears not to contain the complete ubiquitin promoter, the presence of the truncated ubiquitin promoter intron may be driving the expression of the *cry*1F gene.

Absence of Tetracycline and Spectinomycin Resistance Genes and Absence of Regions Immediately Outside of the T-DNA Borders

DNA samples from *B.t.* moCry1F maize line DAS-06275-8, T1S1 and BC5 generations, and unmodified controls were digested with *Sac* I and *Mfe* I and probed with the *spc*, *tet*, Outside LB, and Outside RB probes to confirm the absence of the spectinomycin and tetracycline resistance genes and the absence of regions immediately outside of the T-DNA that were contained on the vector backbone of PHP12537. Positive controls consisting of unmodified control corn DNA spiked with plasmid PHP12537 DNA at one and five approximate gene copy equivalents was digested with *Mfe* I and *Sac* I in order to confirm successful probe hybridization and negative controls consisted of DNA from unmodified corn samples.

No hybridization signals were evident in any of the *B.t.* moCry1F maize line DAS-06275-8 samples or unmodified controls hybridized to the *spc, tet*, Outside LB or Outside RB probes (Figures 18 - 25). The positive control lanes contained the expected hybridizing bands showing that the probes were capable of hybridizing to any homologous DNA fragments if present in the samples. The data suggested that the insertion in *B.t.* moCry1F maize line 6275 did not go beyond the T-DNA borders of plasmid PHP12537 and did not insert either the spectinomycin or tetracycline resistance genes.

Figure 18. **Southern blot analysis of maize line 6275 – *spc* probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Sac* I and probed with the *spc* probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Sac* I
2	Hi-II + 1 copies PHP12537	*Sac* I
3	Dig VII Marker	
4	Hi-II	*Sac* I
5	DAS-06275-8-9 (null) T1S1	*Sac* I
6	DAS-06275-8-5 T1S1	*Sac* I
7	DAS-06275-8-6 T1S1	*Sac* I
8	DAS-06275-8-7 T1S1	*Sac* I
9	DAS-06275-8-11 T1S1	*Sac* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Sac* I
12	DAS-06275-8-1 BC5	*Sac* I
13	DAS-06275-8-4 BC5	*Sac* I
14	DAS-06275-8-6 BC5	*Sac* I
15	DAS-06275-8-17 BC5	*Sac* I
16	P38	*Sac* I
17	PH09B	*Sac* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Sac* I
20	Hi-II + 5 copies PHP12537	*Sac* I

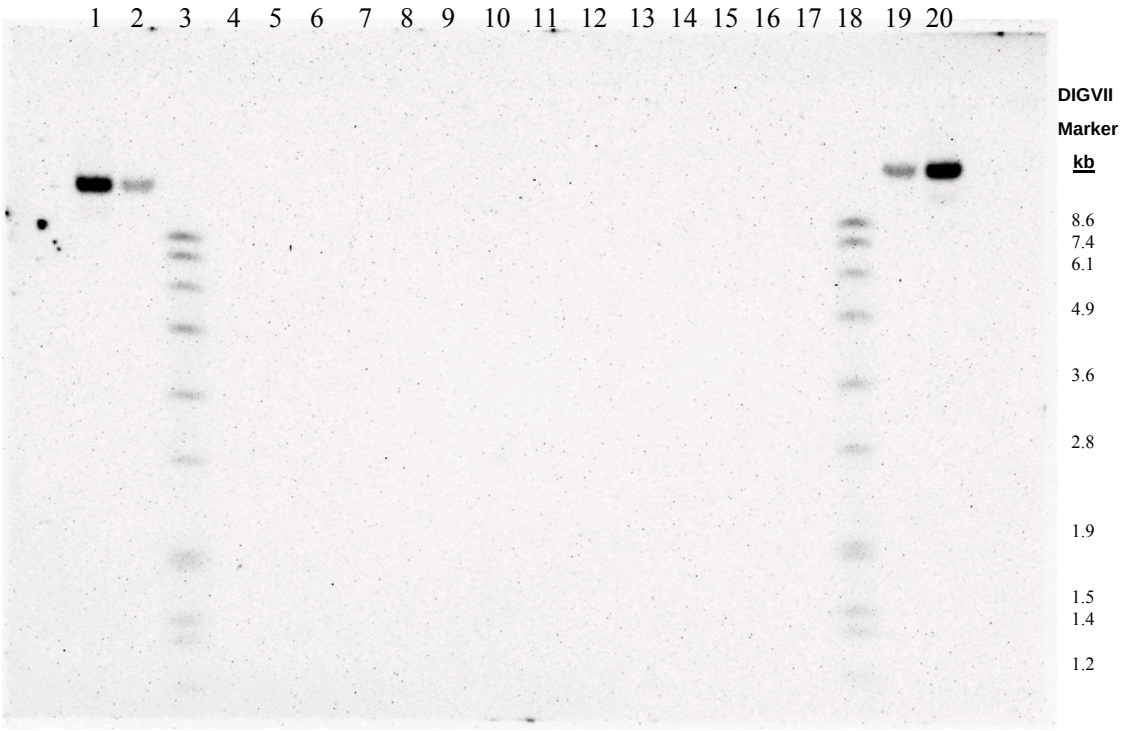


Figure 19. **Southern blot analysis of event DAS-06275-8 – *spc* probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Mfe* I and probed with the *spc* probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Mfe* I
2	Hi-II + 1 copies PHP12537	*Mfe* I
3	Dig VII Marker	
4	Hi-II	*Mfe* I
5	DAS-06275-8-9 (null) T1S1	*Mfe* I
6	DAS-06275-8-2 T1S1	*Mfe* I
7	DAS-06275-8-5 T1S1	*Mfe* I
8	DAS-06275-8-6 T1S1	*Mfe* I
9	DAS-06275-8-12 T1S1	*Mfe* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Mfe* I
12	DAS-06275-8-1 BC5	*Mfe* I
13	DAS-06275-8-4 BC5	*Mfe* I
14	DAS-06275-8-6 BC5	*Mfe* I
15	DAS-06275-8-17 BC5	*Mfe* I
16	P38	*Mfe* I
17	PH09B	*Mfe* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Mfe* I
20	Hi-II + 5 copies PHP12537	*Mfe* I

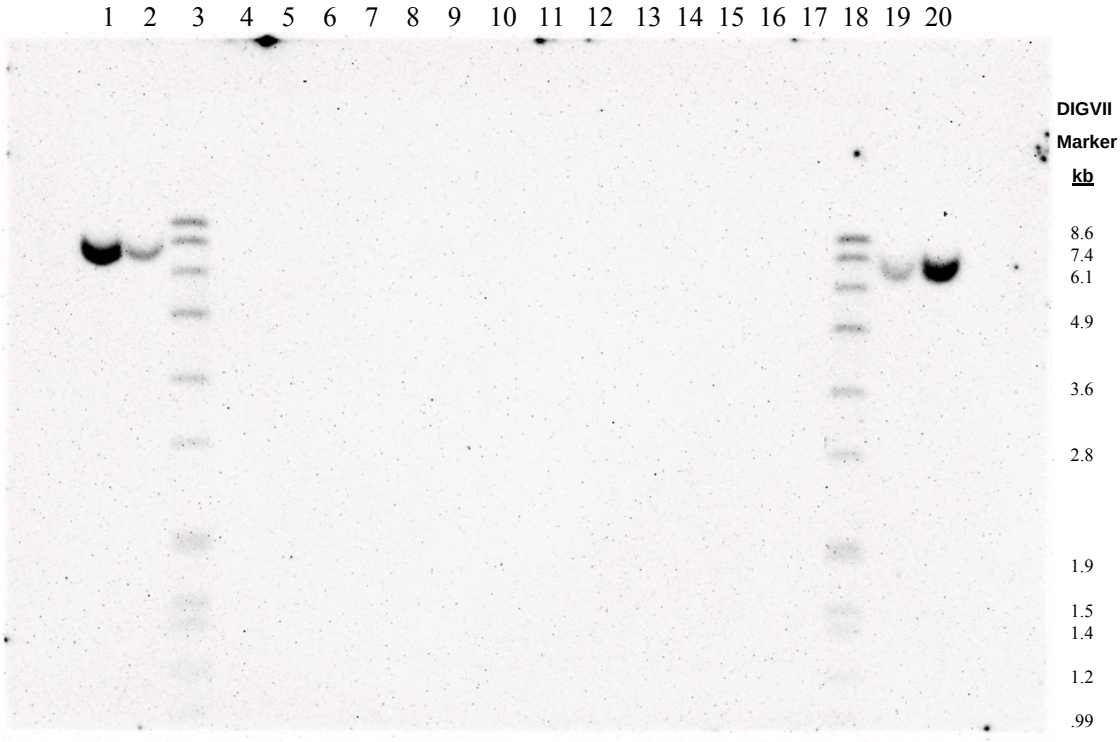


Figure 20. **Southern blot analysis of maize line 6275 – *tet* probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Sac* I and probed with the *tet* probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Sac* I
2	Hi-II + 1 copies PHP12537	*Sac* I
3	Dig VII Marker	
4	Hi-II	*Sac* I
5	DAS-06275-8-9 (null) T1S1	*Sac* I
6	DAS-06275-8-5 T1S1	*Sac* I
7	DAS-06275-8-6 T1S1	*Sac* I
8	DAS-06275-8-7 T1S1	*Sac* I
9	DAS-06275-8-11 T1S1	*Sac* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Sac* I
12	DAS-06275-8-1 BC5	*Sac* I
13	DAS-06275-8-4 BC5	*Sac* I
14	DAS-06275-8-6 BC5	*Sac* I
15	DAS-06275-8-17 BC5	*Sac* I
16	P38	*Sac* I
17	PH09B	*Sac* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Sac* I
20	Hi-II + 5 copies PHP12537	*Sac* I

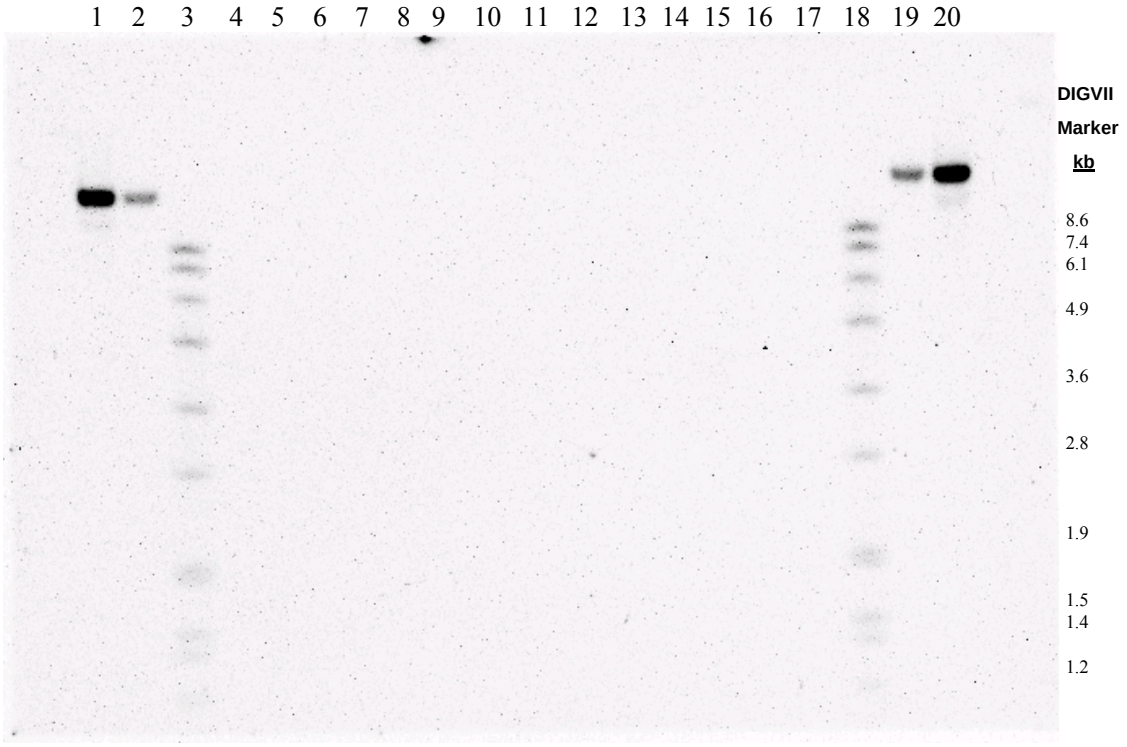


Figure 21. **Southern blot analysis of event DAS-06275-8 – *tet* probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Mfe* I and probed with the *tet* probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Mfe* I
2	Hi-II + 1 copies PHP12537	*Mfe* I
3	Dig VII Marker	
4	Hi-II	*Mfe* I
5	DAS-06275-8-9 (null) T1S1	*Mfe* I
6	DAS-06275-8-2 T1S1	*Mfe* I
7	DAS-06275-8-5 T1S1	*Mfe* I
8	DAS-06275-8-6 T1S1	*Mfe* I
9	DAS-06275-8-12 T1S1	*Mfe* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Mfe* I
12	DAS-06275-8-1 BC5	*Mfe* I
13	DAS-06275-8-4 BC5	*Mfe* I
14	DAS-06275-8-6 BC5	*Mfe* I
15	DAS-06275-8-17 BC5	*Mfe* I
16	P38	*Mfe* I
17	PH09B	*Mfe* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Mfe* I
20	Hi-II + 5 copies PHP12537	*Mfe* I

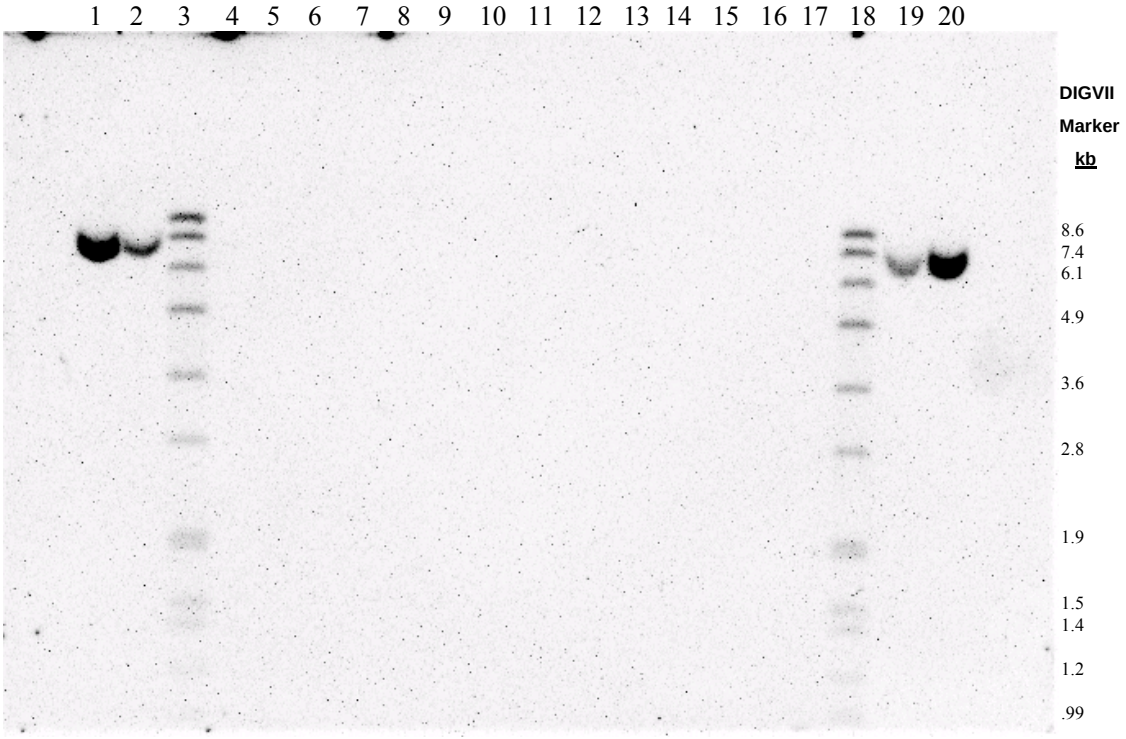


Figure 22. **Southern blot analysis of maize line 6275 – outside LB probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Sac* I and probed with the outside LB probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Sac* I
2	Hi-II + 1 copies PHP12537	*Sac* I
3	Dig VII Marker	
4	Hi-II	*Sac* I
5	DAS-06275-8-9 (null) T1S1	*Sac* I
6	DAS-06275-8-5 T1S1	*Sac* I
7	DAS-06275-8-6 T1S1	*Sac* I
8	DAS-06275-8-7 T1S1	*Sac* I
9	DAS-06275-8-11 T1S1	*Sac* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Sac* I
12	DAS-06275-8-1 BC5	*Sac* I
13	DAS-06275-8-4 BC5	*Sac* I
14	DAS-06275-8-6 BC5	*Sac* I
15	DAS-06275-8-17 BC5	*Sac* I
16	P38	*Sac* I
17	PH09B	*Sac* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Sac* I
20	Hi-II + 5 copies PHP12537	*Sac* I

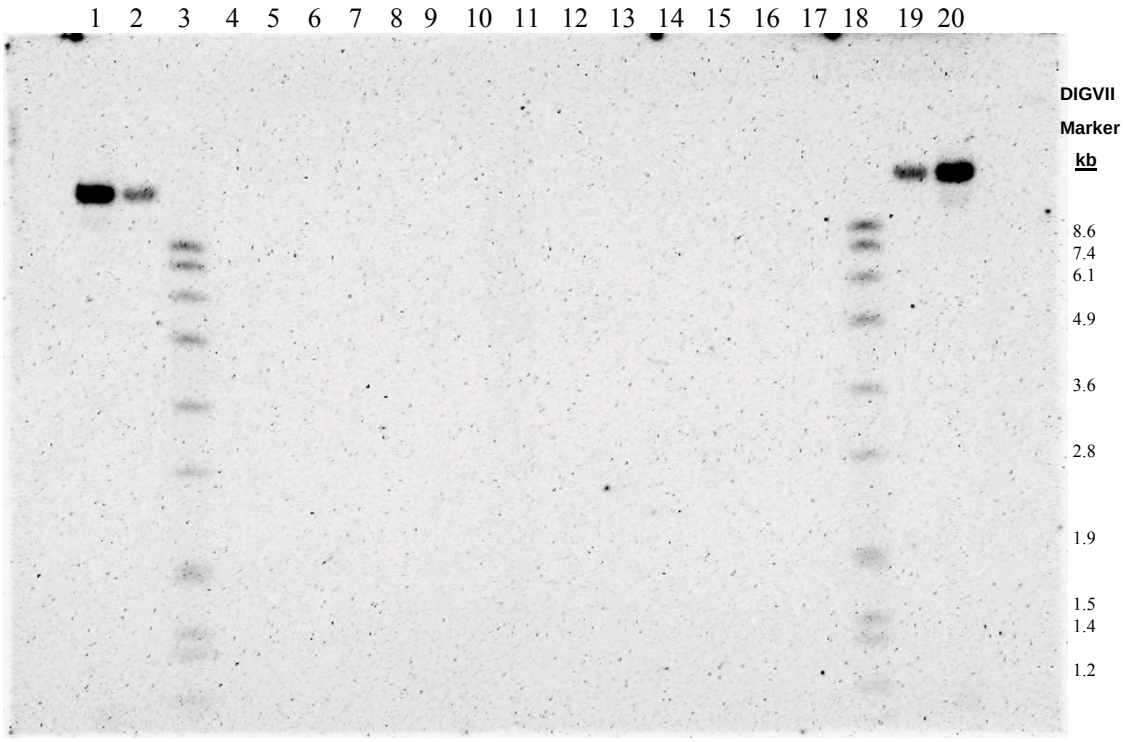


Figure 23. **Southern blot analysis of event DAS-06275-8 – outside LB probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Mfe* I and probed with the Outside LB probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Mfe* I
2	Hi-II + 1 copies PHP12537	*Mfe* I
3	Dig VII Marker	
4	Hi-II	*Mfe* I
5	DAS-06275-8-9 (null) T1S1	*Mfe* I
6	DAS-06275-8-2 T1S1	*Mfe* I
7	DAS-06275-8-5 T1S1	*Mfe* I
8	DAS-06275-8-6 T1S1	*Mfe* I
9	DAS-06275-8-12 T1S1	*Mfe* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Mfe* I
12	DAS-06275-8-1 BC5	*Mfe* I
13	DAS-06275-8-4 BC5	*Mfe* I
14	DAS-06275-8-6 BC5	*Mfe* I
15	DAS-06275-8-17 BC5	*Mfe* I
16	P38	*Mfe* I
17	PH09B	*Mfe* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Mfe* I
20	Hi-II + 5 copies PHP12537	*Mfe* I

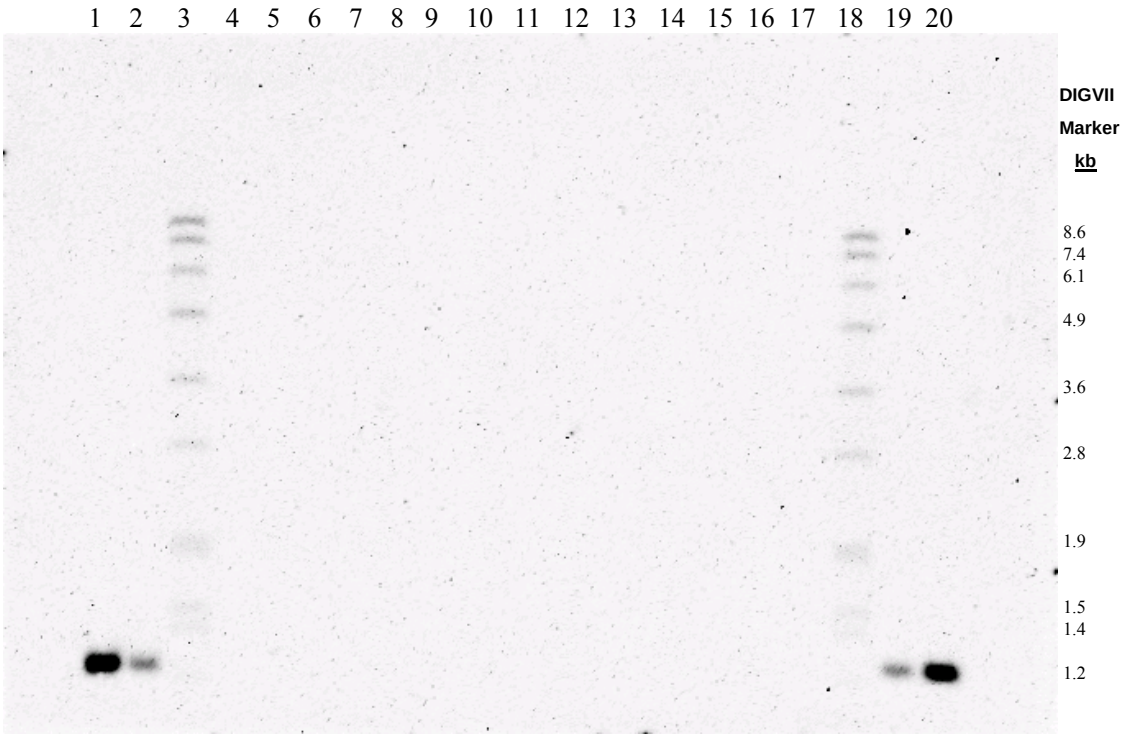


Figure 24. **Southern blot analysis of maize line 6275 – outside RB probe.** DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Sac* I and probed with the outside RB probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Sac* I
2	Hi-II + 1 copies PHP12537	*Sac* I
3	Dig VII Marker	
4	Hi-II	*Sac* I
5	DAS-06275-8-9 (null) T1S1	*Sac* I
6	DAS-06275-8-5 T1S1	*Sac* I
7	DAS-06275-8-6 T1S1	*Sac* I
8	DAS-06275-8-7 T1S1	*Sac* I
9	DAS-06275-8-11 T1S1	*Sac* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Sac* I
12	DAS-06275-8-1 BC5	*Sac* I
13	DAS-06275-8-4 BC5	*Sac* I
14	DAS-06275-8-6 BC5	*Sac* I
15	DAS-06275-8-17 BC5	*Sac* I
16	P38	*Sac* I
17	PH09B	*Sac* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Sac* I
20	Hi-II + 5 copies PHP12537	*Sac* I

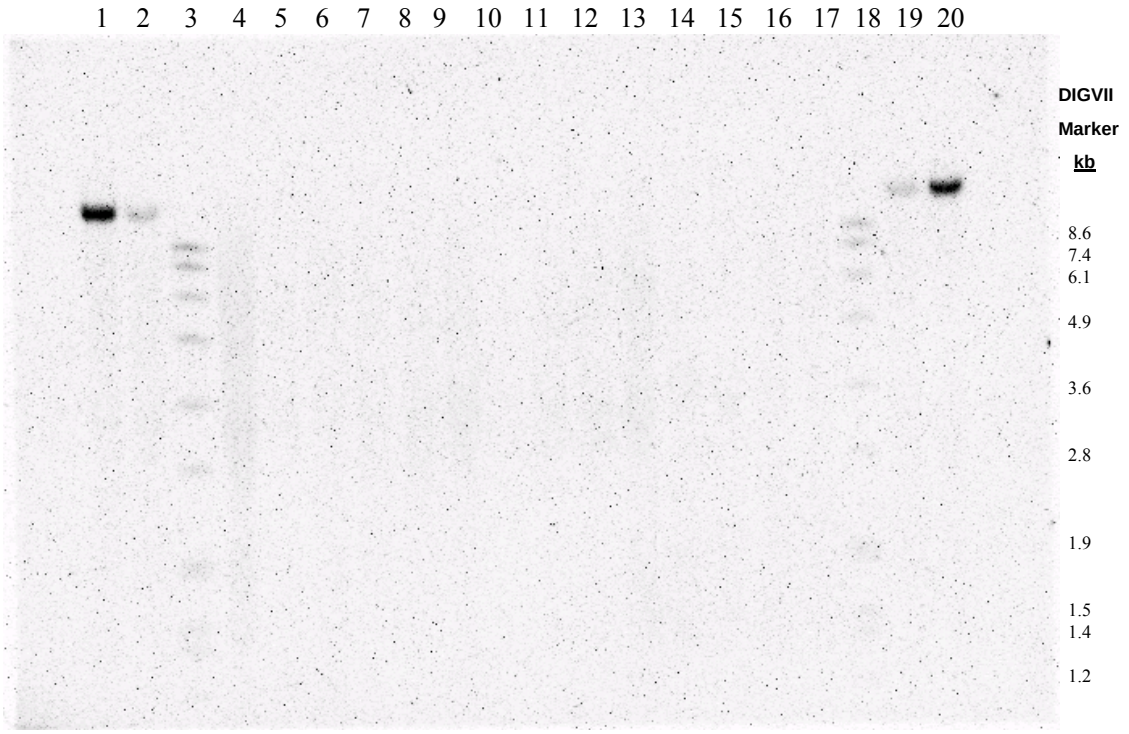
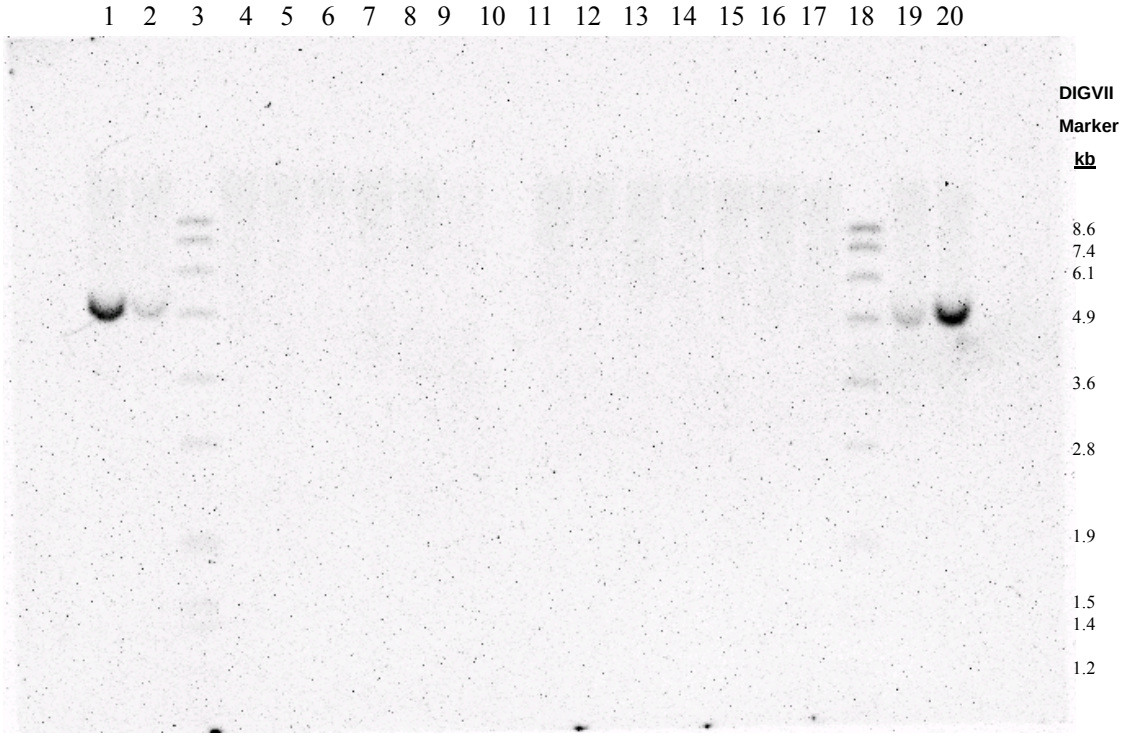


Figure 25. Southern blot analysis of event DAS-06275-8 – outside RB probe. DNA isolated from event DAS-06275-8 (T1S1 and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Mfe* I and probed with the outside RB probe. Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA.

Lane assignments:

Lane	Sample	DIGEST
1	Hi-II + 5 copies PHP12537	*Mfe* I
2	Hi-II + 1 copies PHP12537	*Mfe* I
3	Dig VII Marker	
4	Hi-II	*Mfe* I
5	DAS-06275-8-9 (null) T1S1	*Mfe* I
6	DAS-06275-8-2 T1S1	*Mfe* I
7	DAS-06275-8-5 T1S1	*Mfe* I
8	DAS-06275-8-6 T1S1	*Mfe* I
9	DAS-06275-8-12 T1S1	*Mfe* I
10	Empty	

Lane	Sample	Digest
11	DAS-06275-8-3 (null) BC5	*Mfe* I
12	DAS-06275-8-1 BC5	*Mfe* I
13	DAS-06275-8-4 BC5	*Mfe* I
14	DAS-06275-8-6 BC5	*Mfe* I
15	DAS-06275-8-17 BC5	*Mfe* I
16	P38	*Mfe* I
17	PH09B	*Mfe* I
18	Dig VII Marker	
19	Hi-II + 1 copies PHP12537	*Mfe* I
20	Hi-II + 5 copies PHP12537	*Mfe* I



V.B. STABILITY OF THE GENE INSERT

V.B.1. MOLECULAR CHARACTERIZATION OF INSERT STABILITY

Characterization of the inserted T-DNA in *B.t.* moCry1F maize line 6275 within a single generation of transgenic plants was analyzed by Southern blot techniques to determine the genetic equivalence of the inserted DNA in each plant within this generation (Locke and Dillon, 2003; Appendix 2).

Sixty (60) seeds from *B.t.* moCry1F maize line 6275, BC4S1 generation, were planted in individual pots. Of the 60 seeds planted, 47 seeds germinated and 13 seeds did not germinate. Plants from the *B.t.* moCry1F maize line 6275 seed that germinated were analyzed for expression of Cry1F (using a lateral flow immunochemistry test specific to Cry1F) and BAR (using leaf painting of glufosinate-ammonium). The results of the expression analysis indicated that of the 47 plants analyzed, 38 plants expressed both Cry1F and BAR and 9 plants did not express either protein. The BC4S1 was the resultant generation of the original transformation into Hi-II line followed by a cross of the T0 plant to the P38 inbred line followed by 4-5 crosses, an initial F1 cross and four backcrosses, to the recurrent inbred line, P38, with a final self pollination and had an expected segregation ratio for Cry1F and BAR expression of 3 to 1. The results of a binomial proportions test comparing 38 positives to 9 negatives to a hypothetical proportion of 0.75 (3:1) indicated no significant difference

DNA was prepared from leaf tissue harvested approximately 7-25 days after germination from 47 individual plants from one generation (BC4S1) of *B.t.* moCry1F maize line 6275 (also known as transgenic corn event DAS-06275-8).

The control substances used were DNA prepared from leaf tissue harvested at 7-25 days after germination from individual plants of each of the following unmodified corn varieties:

Hi-II: (unmodified corn for control)
P38: (unmodified inbred corn control)
PH09B: (unmodified inbred corn control)

These unmodified plants have a genetic background representative of the test substances, however, they do not contain the plant transcription units for the mo*cry*1F and *bar* genes.

Plasmid DNA from plasmid PHP12537 that was used for the transformation experiments to produce *B.t.* moCry1F maize line 6275 was added to DNA samples from unmodified corn plants to simulate approximate gene copy intensities during Southern analysis. In addition to the plasmid control, genomic DNA samples from *B.t.* moCry1F maize line 6275 (T1S1 and BC5 generations) were also included as reference substances for the Southern blot analysis.

DNA from leaf samples from 38 plants expressing Cry1F and BAR and from four (4) null segregants (not expressing Cry1F and BAR) were used for the Southern analysis. At least DNA from one leaf sample from individual plants for each of the three control substance lines was also used.

Genomic DNA samples from BC4S1 generation plants (38 DNA samples from plants expressing Cry1F and BAR and four (4) null segregant non-expressing plants), were cleaved with the restriction enzyme *Nco* I to determine the genetic equivalence of the insertion in individual plants when hybridized to mo*cry*1F and *bar* gene probes, separated electrophoretically on gels and then transferred to nylon membranes and probed using digoxigenin-labeled probes (Table 5) using Southern blot techniques (Locke and Dillon, 2003; Appendix 2). The *Nco* I enzyme has two

cleavage sites located within the T-DNA region of plasmid PHP12537 (Figures 26 and 27), one site located within the ubiquitin intron (bp 1234 in the T-DNA) and a second site located at the start codon for the Cry1F protein coding sequence (bp 2211 in the T-DNA). *B.t.* moCry1F maize line 6275 DNA digested with *Nco* I and hybridized to either the mo*cry*1F probe or the *bar* probe would be expected to result in one hybridizing band from cleavage of the *Nco* I site located at bp 2211 in the T-DNA and another site in the corn genome, flanking the insert. Since the site in the corn genome flanking the insert is unique to the site of insertion, each independent insertion of the T-DNA will have a unique fragment, generally termed a border fragment. In the case of T-DNA insertions, one hybridizing band produced from an enzyme that produces a border fragment usually indicates the presence of one copy of the T-DNA inserted at a single locus in the genome. Border fragments are typically larger than the predicted size of the T-DNA region included in the fragment due to the inclusion of genomic DNA in the fragment. The exact size of border fragments cannot be predicted in advance due to the unknown location of the cleavage site in the corn genome and the border fragments serve to provide a unique Southern hybridization pattern for each individual event.

Table 5. Description of DNA probes used to probe Southern blots for molecular stability.

Probe	Genetic Element	Plasmid Source	Position (bp)	Length (bp)
mo*cry*1F	5' end of mo*cry*1F gene (encodes Cry1F protein)	PHP12537	20284 – 21202	919
	3' end of mo*cry*1F gene (encodes Cry1F protein)	PHP12537	21174 – 22084	911
bar	*bar* gene (encodes BAR protein)	PHP12537	23810 – 24359	550

Figure 26. Plasmid map of PHP12537 indicating restriction enzyme sites for *Nco* I and coding regions for mo*cry*1F, *bar*, spectinomycin (*spc*), and tetracycline (*tet*).

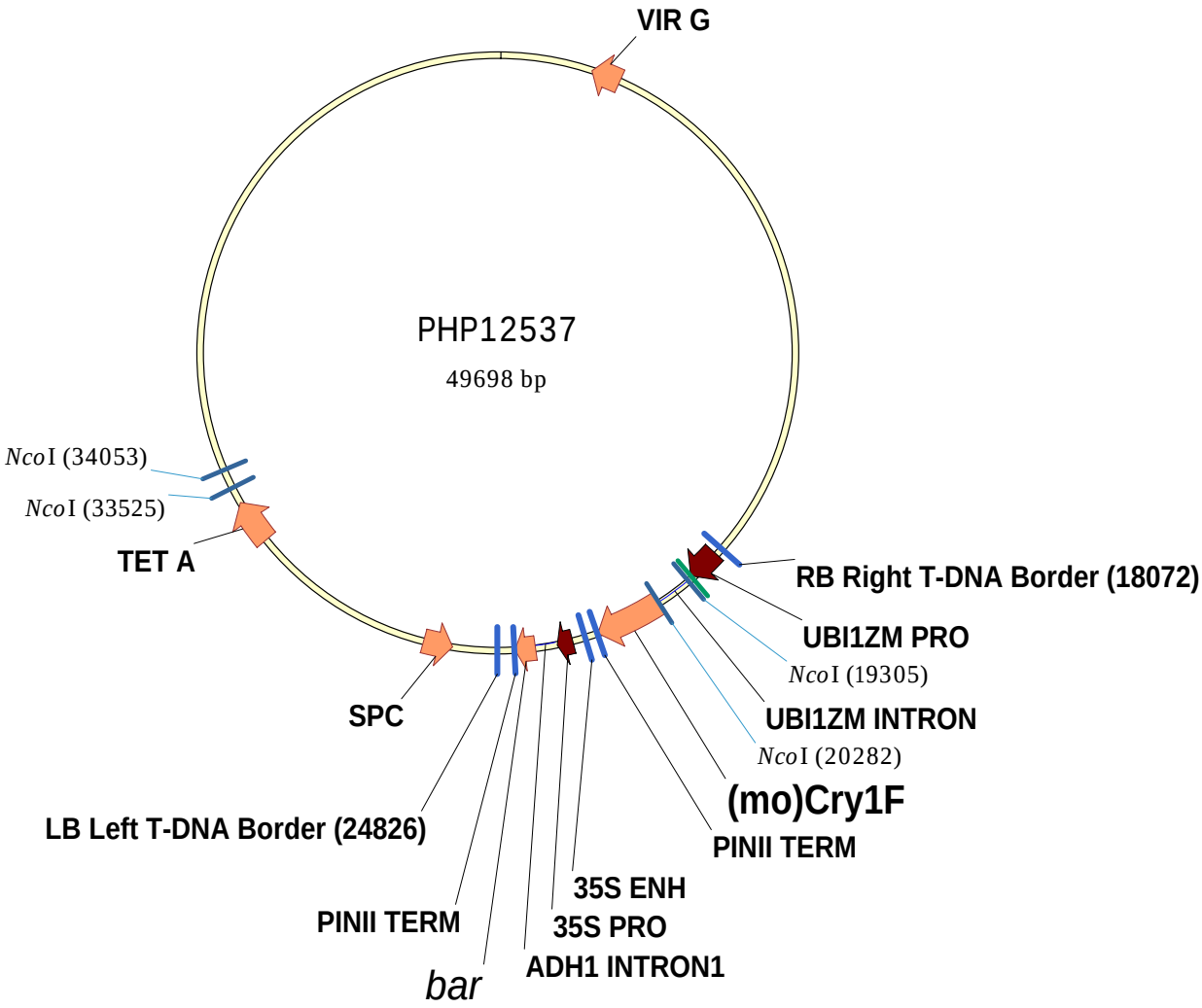
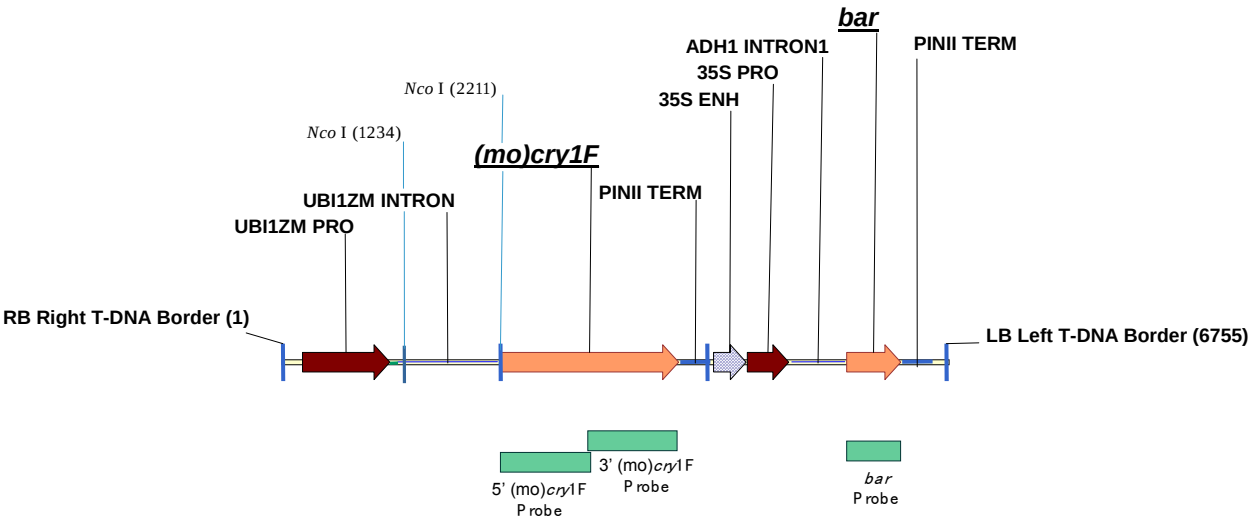


Figure 27. T-DNA region from plasmid PHP12537 indicating restriction enzyme sites for *Nco* I, and coding regions and probe locations for mo*cry*1F and *bar*.



PHP12537 T-DNA
6779 bp

Southern blot hybridization results from blots containing DNA digested with *Nco* I and hybridized with the mo*cry*1F probe are shown in Figures 28 and 29 and *bar* probe results are shown in Figures 30 and 31. The hybridizing band sizes detected on the Southern blots are reported in Table 6 along with the expected hybridizing fragment sizes based on the original T-DNA sequence from plasmid PHP12537. Tables 7 and 8 summarize the protein expression and Southern hybridization results for each gel. Hybridization with either the mo*cry*1F or the *bar* probe to DNA isolated from *B.t.* moCry1F maize line 6275 BC4S1 generation plants expressing Cry1F and BAR resulted in one hybridizing band with an approximate size of 7.0 kb. The size of the hybridizing band, ~7.0 kb, was larger than predicted from the T-DNA sequence as expected for a border fragment. All 38 expressing plants of *B.t.* moCry1F maize line 6275 BC4S1 generation exhibited identical Southern blot hybridization patterns, one unique border fragment of approximately 7.0 kb in size, providing evidence for genetic stability of the insert and linking the Southern blot hybridization pattern with the expected phenotype. In addition, two *B.t.* moCry1F maize line 6275 plants from two distinct generations, T1S1 and BC5, also resulted in one hybridizing band of approximately 7.0 kb, demonstrating genetic equivalence of the insert across three separate generations. Null segregants of *B.t.* moCry1F maize line 6275 BC4S1 generation did not hybridize to either probe as expected. One hybridizing band with the *Nco* I digest and the two probes strongly suggested that a single T-DNA insert was present in *B.t.* moCry1F maize line 6275. A summary diagram of the DNA insertion in *B.t.* moCry1F maize line 6275 is shown in Figure 2 (pages 11, 26 and 50 of this petition).

Table 6. Predicted and observed hybridizing bands on Southern blots of event *B.t.* moCry1F Maize Line 6275 Probed with mo*cry*1F and *bar* probes.

Probe	Restriction Enzyme	Figure	Predicted Fragment Size (bp)	Observed Fragment Size (bp)[1]
mo*cry*1F	*Nco* I	Figure 28	> 4569	~ 7000
mo*cry*1F	*Nco* I	Figure 29	> 4569	~ 7000
bar	*Nco* I	Figure 30	> 4569	~ 7000
bar	*Nco* I	Figure 31	> 4569	~ 7000

1. Observed fragment size for all *B.t.* moCry1F maize line 6275 samples that were expressing Cry1F and BAR in the BC4S1, T1S1, and BC5 generations.

Figure 28. Southern blot analysis of maize line 6275; Gel 1 – mo*cry*1F probe.
DNA isolated from *B.t.* moCry1F maize line 6275 (BC4S1, T1S1, and BC5 generations) and PH09B and P38 unmodified corn was digested with *Nco* I and probed with the mo*cry*1F probe [combined 5'and 3' mo*cry*1F probe]. Approximately 3 μg of genomic DNA were digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 3 μg of PH09B or P38 unmodified control DNA. [Note: Lane 2 contained only plasmid PHP12537 DNA without any unmodified control DNA.]

Lane	DNA Sample	Lane	DNA Sample
1	DNA Marker VII + ϕX174	16	6275-15
2	1 copy PHP12537	17	6275-16
3	P38 + 5 copies PHP12537	18	6275-19
4	PH09B	19	6275-20
5	6275-4	20	6275-21
6	6275-10	21	6275-22
7	6275-1	22	6275-24
8	6275-3	23	6275-26
9	6275-6	24	6275-27
10	6275-7	25	6275-29
11	6275-8	26	6275-30
12	6275-9	27	6275-6 T1S1
13	6275-12	28	6275-10 BC5
14	6275-13	29	P38
15	6275-14	30	PH09B + 5 copies PHP12537

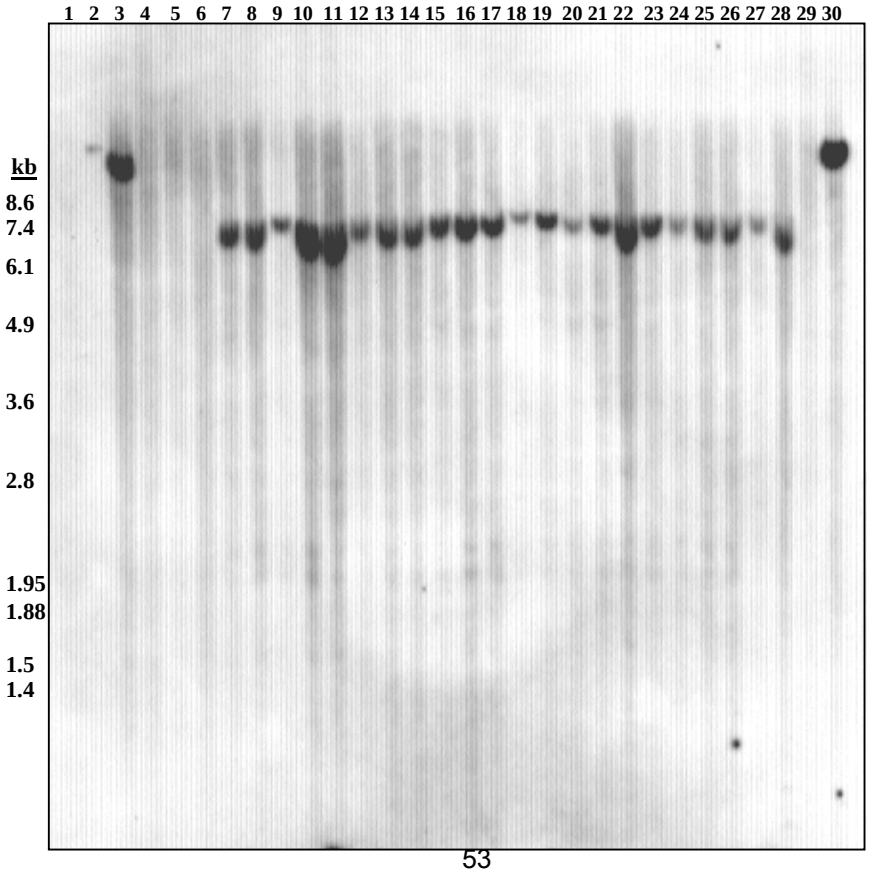


Figure 29. Southern blot analysis of maize line 6275; Gel 2 – mo*cry*1F probe.
DNA isolated from *B.t.* moCry1F maize line 6275 (BC4S1, T1S1, and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Nco* I and probed with the (mo)*cry*1F probe [combined 5'and 3' (mo)*cry*1F probe]. Approximately 3 μg of genomic DNA were digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 3 μg of PH09B, P38, or Hi-II unmodified control DNA.

Lane	DNA Sample	Lane	DNA Sample
1	DNA Marker VII + ϕX174	16	6275-47
2	Hi-II + 1 copy PHP12537	17	6275-50
3	P38 + 5 copies PHP12537	18	6275-51
4	PH09B	19	6275-52
5	6275-11	20	6275-53
6	6275-18	21	6275-55
7	6275-31	22	6275-57
8	6275-34	23	6275-58
9	6275-36	24	6275-6 T1S1
10	6275-38	25	6275-10 BC5
11	6275-39	26	Hi-II
12	6275-41	27	P38
13	6275-42	28	PH09B + 5 copies PHP12537
14	6275-43	29	6275-48
15	6275-45	30	DNA Marker VII

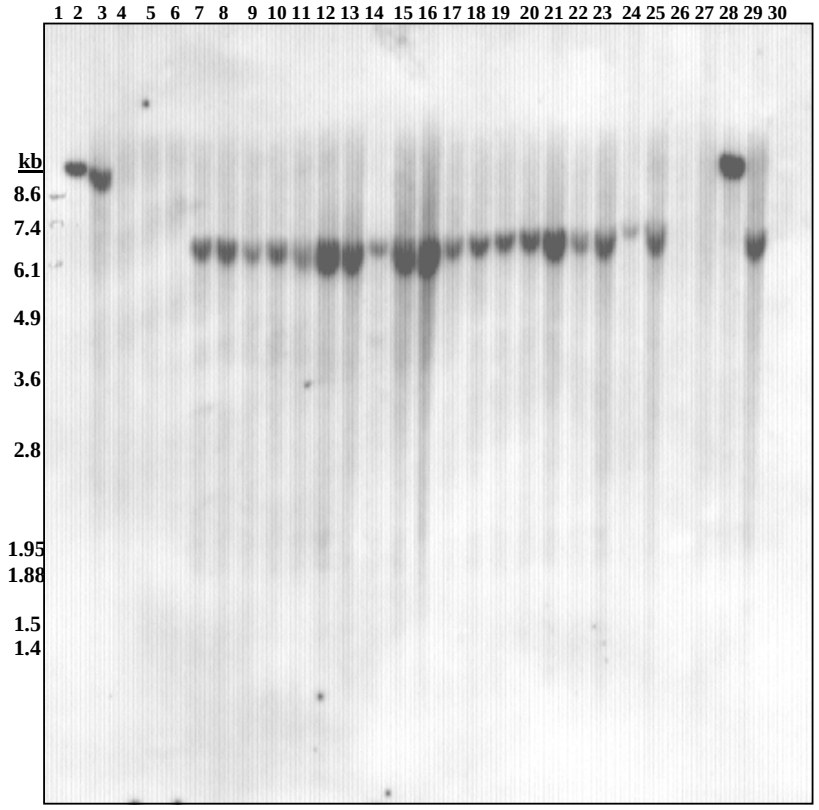


Figure 30. Southern blot analysis of maize line 6275; Gel 1 – *bar* probe.
DNA isolated from *B.t.* moCry1F maize line 6275 (BC4S1, T1S1, and BC5 generations) and PH09B and P38 unmodified corn was digested with *Nco* I and probed with the *bar* probe. Approximately 3 μg of genomic DNA were digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 3 μg of PH09B or P38 unmodified control DNA. [Note: Lane 2 contained only plasmid PHP12537 DNA without any unmodified control DNA.]

Lane	DNA Sample	Lane	DNA Sample
1	DNA Marker VII	16	6275-15
2	1 copy PHP12537	17	6275-16
3	P38 + 5 copies PHP12537	18	6275-19
4	PH09B	19	6275-20
5	6275-4	20	6275-21
6	6275-10	21	6275-22
7	6275-1	22	6275-24
8	6275-3	23	6275-26
9	6275-6	24	6275-27
10	6275-7	25	6275-29
11	6275-8	26	6275-30
12	6275-9	27	6275-6 T1S1
13	6275-12	28	6275-10 BC5
14	6275-13	29	P38
15	6275-14	30	PH09B + 5 copies PHP12537

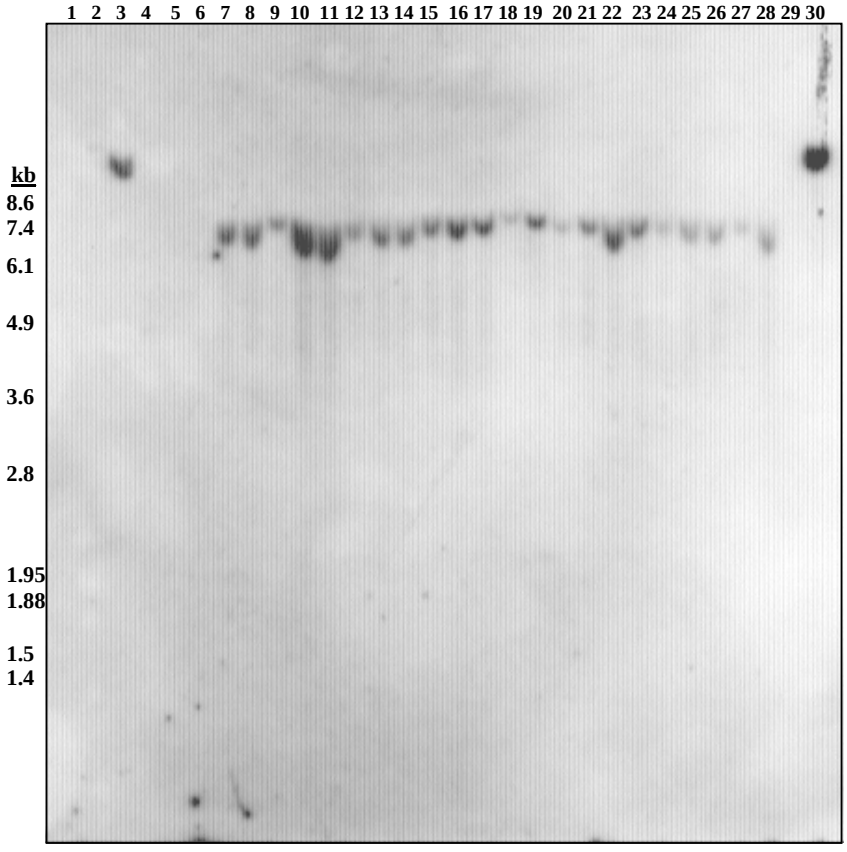


Figure 31. Southern blot analysis of maize line 6275; Gel 2 – *bar* probe.
DNA isolated from *B.t.* moCry1F maize line 6275 (BC4S1, T1S1, and BC5 generations) and PH09B, P38, and Hi-II unmodified corn was digested with *Nco* I and probed with the *bar* probe. Approximately 3 μg of genomic DNA were digested and loaded per lane. The gene copy number controls included plasmid PHP12537 at the indicated approximate gene copy number equivalents and 3 μg of PH09B, P38, or Hi-II unmodified control DNA.

.Lane	DNA Sample	Lane	DNA Sample
1	DNA Marker VII	16	6275-47
2	Hi-II + 1 copy PHP12537	17	6275-50
3	P38 + 5 copies PHP12537	18	6275-51
4	PH09B	19	6275-52
5	6275-11	20	6275-53
6	6275-18	21	6275-55
7	6275-31	22	6275-57
8	6275-34	23	6275-58
9	6275-36	24	6275-6 T1S1
10	6275-38	25	6275-10 BC5
11	6275-39	26	Hi-II
12	6275-41	27	P38
13	6275-42	28	PH09B + 5 copies PHP12537
14	6275-43	29	6275-48
15	6275-45	30	DNA Marker VII

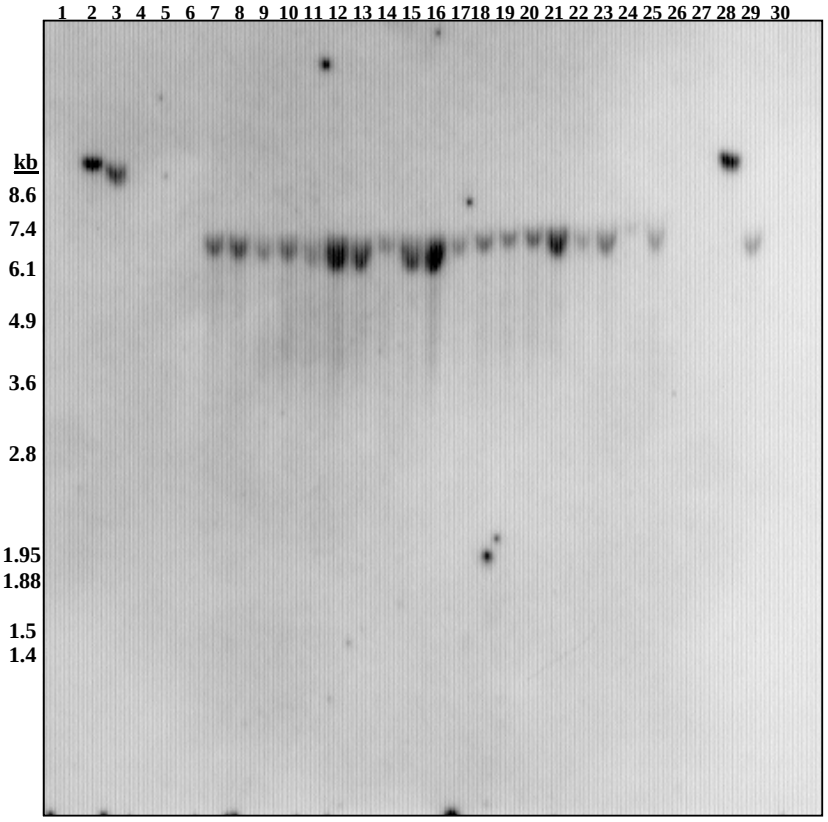


Table 7. Summary of Cry1F and BAR Protein Expression and SouthernHybridization Data for Gel 1 (Figure 28 with mo*cry*1F probe and Figure 30 with *bar* probe).

Lane	Sample	Cry1F and BAR Expression[1]	Southern Blot Figure 28 mo*cry*1F Probe[2]	Southern Blot Figure 30 *bar* Probe[2]
1	DNA Marker VII + ϕX174			
2	1 copy PHP12537	Plasmid Control	+	+
3	P38 + 5 copies PHP12537	Plasmid Control	+	+
4	PH09B	Negative/Control	-	-
5	6275-4	Negative	-	-
6	6275-10	Negative	-	-
7	6275-1	Positive	+	+
8	6275-3	Positive	+	+
9	6275-6	Positive	+	+
10	6275-7	Positive	+	+
11	6275-8	Positive	+	+
12	6275-9	Positive	+	+
13	6275-12	Positive	+	+
14	6275-13	Positive	+	+
15	6275-14	Positive	+	+
16	6275-15	Positive	+	+
17	6275-16	Positive	+	+
18	6275-19	Positive	+	+
19	6275-20	Positive	+	+
20	6275-21	Positive	+	+
21	6275-22	Positive	+	+
22	6275-24	Positive	+	+
23	6275-26	Positive	+	+
24	6275-27	Positive	+	+
25	6275-29	Positive	+	+
26	6275-30	Positive	+	+
27	6275-6 T1S1[3]	Positive/Control	+	+
28	6275-10 BC5[3]	Positive/Control	+	+
29	P38	Negative Control	-	-
30	PH09B + 5 copies PHP 12537	Plasmid Control	+	+

1. Positive Cry1F expression indicates detection of protein expression as determined by the immunoassay based lateral flow device specific for Cry1F protein detection. Negative indicates no detection of the Cry1F protein. Positive BAR expression indicates plants that exhibited resistance to the herbicide treatment and negative indicates plants that were sensitive to the herbicide.
2. + indicates hybridization signal on Southern Blot. - indicates no hybridization signal on Southern Blot.
3. DNA isolated from single plants from both the T1S1 and BC5 generations of *B.t.* moCry1F maize line 6275 that were characterized by Southern blot analysis in a previous study.

Table 8. Summary of Cry1F and BAR protein expression and Southern Hybridization data for Gel 2 (Figure 29 with mo*cry*1F probe and Figure 31 with *bar* probe).

Lane	Sample	Cry1F and BAR Expression[1]	Southern Blot Figure 29 mo*cry*1F Probe[2]	Southern Blot Figure 31 *bar* Probe[2]
1	DNA Marker VII + ϕX174			
2	Hi-II + 1 copy PHP12537	Plasmid Control	+	+
3	P38 + 5 copies PHP12537	Plasmid Control	+	+
4	PH09B	Negative Control	-	-
5	6275-11	Negative	-	-
6	6275-18	Negative	-	-
7	6275-31	Positive	+	+
8	6275-34	Positive	+	+
9	6275-36	Positive	+	+
10	6275-38	Positive	+	+
11	6275-39	Positive	+	+
12	6275-41	Positive	+	+
13	6275-42	Positive	+	+
14	6275-43	Positive	+	+
15	6275-45	Positive	+	+
16	6275-47	Positive	+	+
17	6275-50	Positive	+	+
18	6275-51	Positive	+	+
19	6275-52	Positive	+	+
20	6275-53	Positive	+	+
21	6275-55	Positive	+	+
22	6275-57	Positive	+	+
23	6275-58	Positive	+	+
24	6275-6 T1S1[3]	Positive/Control	+	+
25	6275-10 BC5[3]	Positive/Control	+	+
26	Hi-II	Negative Control	-	-
27	P38	Negative Control	-	-
28	PH09B + 5 copies PHP12537	Plasmid Control	+	+
29	6275-48	Positive	+	+
30	DNA Marker VII			

1. Positive Cry1F expression indicates detection of protein expression as determined by the immunoassay based lateral flow device specific for Cry1F protein detection. Negative indicates no detection of the Cry1F protein. Positive BAR expression indicates plants that exhibited resistance to the herbicide treatment and negative indicates plants that were sensitive to the herbicide.
2. + indicates hybridization signal on Southern Blot. - indicates no hybridization signal on Southern Blot.
3. DNA isolated from single plants from both the T1S1 and BC5 generations of *B.t.* moCry1F maize line 6275 that were characterized by Southern blot analysis in a previous

The data from these analyses suggest that *B.t.* moCry1F maize line 6275 contains a single integration of transgenic DNA containing the mo*cry*1F and *bar* genes and that the inheritance of mo*cry*1F and *bar* genes is stable within a segregating generation (BC4S1). Furthermore, the genes were found to exhibit the expected segregation ratios. Genotypic segregation was linked to phenotypic segregation in the BC4S1 generation through comparison of Southern blot hybridization patterns to expression data for the Cry1F and BAR proteins. In addition to the BC4S1 data, identical Southern blot hybridization patterns were observed in plants from two distinct generations of *B.t.* moCry1F maize line 6275 (T1S1 and BC5 generations), indicating stability of inheritance across three generations.

V.C. MENDELIAN INHERITANCE

Analysis of the segregation of one or more traits in offspring from a cross with a particular parental line is used to evaluate Mendelian inheritance of each trait and the linkage of the respective traits. Evaluation of Mendelian inheritance also provides evidence of the stable inheritance of newly introduced genetic material.

The Mendelian segregation of the *B.t.* Cry1F maize line 6275 was recorded and analyzed, using Chi-square analysis, at six stages (Table 9, Figure 32). Spraying at each generation with glufosinate-ammonium eliminated herbicide-susceptible plants and resulted in hemizygous seed.

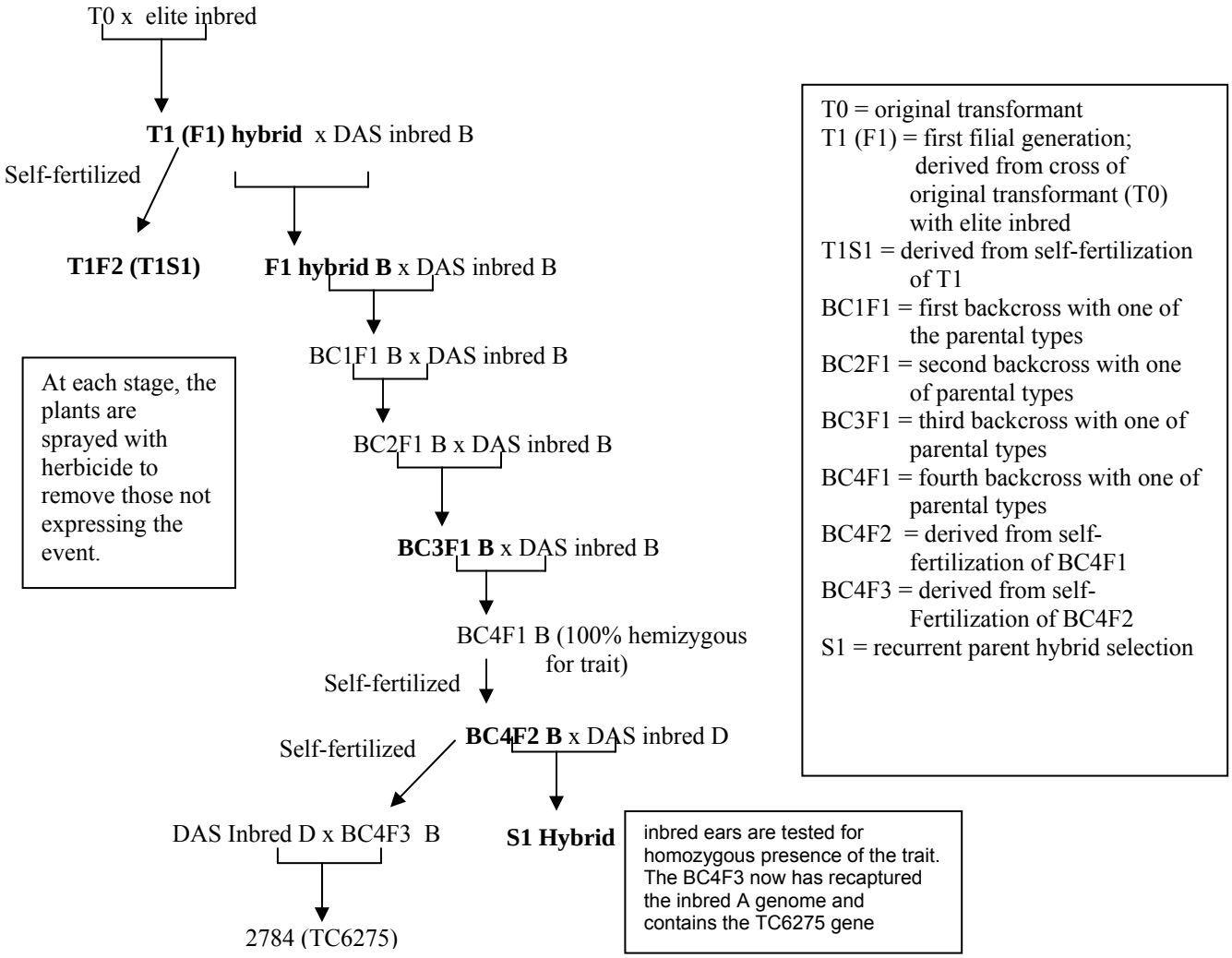
After the S1 hybrids were sprayed with glufosinate-ammonium and scored for tolerance, 200 neonate ECB larvae were used to infest each S1 plant that exhibited herbicide tolerance. All of the plants determined to be herbicide tolerant were also found to be resistant to ECB infestation. These results support the conclusion that event DAS-06275-8 is a stable insertion event and is inherited as a Mendelian dominant gene.

Table 9. Mendelian segregation of *B.t.* Cry1F maize line 6275.

Generation	Expected Ratio*	Observed Herbicide Tolerant	Observed Susceptible to Herbicide	Significance
F1 (T1)	18.5: 18.5 (1:1)	19	18	NS**
F1	23 : 23 (1:1)	21	25	NS
T1F2 (T1S1)	36 : 12 (3:1)	35	13	NS
BC1F1	71.5 : 71.5 (1:1)	67	76	NS
BC3F1	368 : 368 (1:1)	390	346	NS
BC4F2	2118.75 : 706.25 (3:1)	2112	713	NS
S1 Hybrid	357: 179 (2:1)	338	198	NS

*Data expressed as number of plants expected to be resistant to glufosinate : number of plants expected to be susceptible to glufosinate. Numbers in ( ) = expected ratio.
**NS = non-significant at 0.05.

Figure 32. Breeding schematic indicating the generations tested for Mendelian inheritance in maize line 6275. The bolded text designates the generations listed in Table 9.



V.D. CHARACTERISTICS OF INSERTED PROTEINS

The synthetic *cry*1F gene contained in *B.t.* Cry1F maize line 6275 encodes the same Cry1F protein as the synthetic *cry*1F gene contained in the previously deregulated *B.t.* Cry1F maize line 1507. Tests were done to confirm the equivalency of the Cry1F protein expressed *in planta* in *B.t.* Cry1F maize line 6275 with the microbially-produced Cry1F (via *Pseudomonas fluorescens (Pf))* protein test material (MR872) used in the non-target organism tests.

Characterization of the biochemical properties of the microbially-produced and plant proteins was accomplished through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), glycoprotein detection methods, western blot, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and N-terminal sequence analysis. Utilizing these methods the Cry1F protein from *Pf* and transgenic maize (event DAS-06275-8) were shown to be biochemically equivalent. These data support the use of the microbial protein for use in non-target organism testing.

V.D.1. CHACTERISTICS OF THE CRY1F PROTEIN

Cry1F as expressed in event DAS-06275-8 is identical in sequence to amino acids 1-605 of the native, 1174 amino acid residue Cry1F protein except for a single amino acid residue substitution ($F_{604}L$). This change in coding sequence was made to introduce an *Xho*I restriction site for fusion of sequences encoding the C-terminal protoxin domain. The codons for the C-terminal 569 amino acids of the full-length protoxin, approximately those removed by alkaline proteases in the insect gut during formation of the active Cry1F toxin, were omitted from the transgene sequence. The peptide sequence of Cry1F is shown in Figure 33.

Engineering *B.t.* δ-endotoxin genes for nuclear expression in plants was first reported in the scientific literature using native bacterial genes encoding the entire protoxin (Adang *et al*., 1987). In such instances, *B.t.* δ-endotoxin expression at the RNA or protein level was undetectable or very low; however, insect toxicity was observed. Expression of native *B.t.* genes was measurable at the RNA or protein level when the genes were engineered to encode only the protease-resistant core toxin (Barton *et al*., 1987; Fischhoff *et al*., 1987; Vaeck *et al*., 1987). Examination of mRNA in plants transformed with native gene sequences has revealed the presence of many species shorter than the expected full-length size, suggesting improper postranscriptional processing or rapid mRNA turnover (Barton *et al*., 1987; Adang *et al*., 1987). Evidence favoring the latter mechanism was provided in a study by Murray *et al*. (1991).

Since these early studies, numerous reports have demonstrated that more efficient nuclear expression of *B.t.* proteins in plants can be achieved using synthetic transgenes in which the relatively low G+C content of native bacterial δ-endotoxin genes is altered to more closely approximate the higher G+C content typically found in plant genes. Design of synthetic *B.t*. transgenes involves substitutions of alternative codons to bias the codon usage to that preferred by the target plant, and to remove certain deleterious A+T rich sequence elements responsible for mRNA destablization such as RNA cleavage signals, inappropriate polyadenylation sequences, intron splice sites, hairpin sequences, and transcription termination signals. Transgenes encoding Cry1F were optimized for maize expression using this general approach.

Figure 33. Alignment of the polypeptide sequences of the insecticidal crystal proteins comparing the chimeric Cry1F/Cry1Ab (MR872; microbially-produced) toxin, transgenic moCry1F toxin (Cry1Fsyn) and native Cry1F protein.

The consensus sequence represents identical residues among all three genetic versions encoding the Cry1F toxin. The positions of putative protease cleavage sites at the start (about residue 28 or 31) and end (about residue 612 or 615) of the active core toxin are marked with a ↓. Note the single $F_{604}L$ substitution in the transgenic polypeptide. This difference is due to codon changes resulting from the introduction of a restriction enzyme site that enables fusion of the alternative C-terminal half of the protoxin.

```
     1           ↓ ↓       50
MR872       MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP
Cry1Fsyn    MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP
Cry1F       MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP
Consensus   MENNIQNQCV PYNCLNNPEV EILNEERSTG RLPLDISLSL TRFLLSEFVP

           51                                                  100
MR872       GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG
Cry1Fsyn    GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG
Cry1F       GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG
Consensus   GVGVAFGLFD LIWGFITPSD WSLFLLQIEQ LIEQRIETLE RNRAITTLRG

          101                                                  150
MR872       LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS
Cry1Fsyn    LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS
Cry1F       LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS
Consensus   LADSYEIYIE ALREWEANPN NAQLREDVRI RFANTDDALI TAINNFTLTS

          151                                                  200
MR872       FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR
Cry1Fsyn    FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR
Cry1F       FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR
Consensus   FEIPLLSVYV QAANLHLSLL RDAVSFGQGW GLDIATVNNH YNRLINLIHR

          201                                                  250
MR872       YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV
Cry1Fsyn    YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV
Cry1F       YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV
Consensus   YTKHCLDTYN QGLENLRGTN TRQWARFNQF RRDLTLTVLD IVALFPNYDV

          251                                                  300
MR872       RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
Cry1Fsyn    RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
Cry1F       RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM
Consensus   RTYPIQTSSQ LTREIYTSSV IEDSPVSANI PNGFNRAEFG VRPPHLMDFM

          301                                                  350
MR872       NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE
Cry1Fsyn    NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE
Cry1F       NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE
Consensus   NSLFVTAETV RSQTVWGGHL VSSRNTAGNR INFPSYGVFN PGGAIWIADE

          351                                                  400
MR872       DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI
Cry1Fsyn    DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI
Cry1F       DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI
Consensus   DPRPFYRTLS DPVFVRGGFG NPHYVLGLRG VAFQQTGTNH TRTFRNSGTI

          401                                                  450
MR872       DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
Cry1Fsyn    DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
Cry1F       DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
Consensus   DSLDEIPPQD NSGAPWNDYS HVLNHVTFVR WPGEISGSDS WRAPMFSWTH
```

```
          451                                                        500
MR872       RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP
Cry1Fsyn    RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP
Cry1F       RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP
Consensus   RSATPTNTID PERITQIPLV KAHTLQSGTT VVRGPGFTGG DILRRTSGGP

          501                                                        550
MR872       FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
Cry1Fsyn    FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
Cry1F       FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT
Consensus   FAYTIVNING QLPQRYRARI RYASTTNLRI YVTVAGERIF AGQFNKTMDT

          551                                                        600
MR872       GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
Cry1Fsyn    GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
Cry1F       GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV
Consensus   GDPLTFQSFS YATINTAFTF PMSQSSFTVG ADTFSSGNEV YIDRFELIPV

          601           ↓ ↓                                          650
MR872       TATFEAEYDL ERAQKAVNAL FTSINQIGIK TDVTDYHIDR VSNLVECLSD
Cry1Fsyn    TATLE*.... .......... .......... .......... ..........
Cry1F       TATFEAEYDL ERAQKAVNAL FTSINQIGIK TDVTDYHIDQ VSNLVDCLSD
Consensus   TAT-E----- ---------- ---------- ---------- ----------

          651                                                        700
MR872       EFCLDEKKEL SEKVKHAKRL SDERNLLQDP NFRGINRQLD RGWRGSTDIT
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       EFCLDEKREL SEKVKHAKRL SDERNLLQDP NFKGINRQLD RGWRGSTDIT
Consensus   ---------- ---------- ---------- ---------- ----------

          701                                                        750
MR872       IQGGDDVFKE NYVTLLGTFD ECYLTYLYQK IDESKLKAYT RYQLRGYIED
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       IQRGDDVFKE NYVTLPGTFD ECYPTYLYQK IDESKLKPYT RYQLRGYIED
Consensus   ---------- ---------- ---------- ---------- ----------

          751                                                        800
MR872       SQDLEIYLIR YNAKHETVNV PGTGSLWRLS APSPI..... ..........
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       SQDLEIYLIR YNAKHETVNV LGTGSLWPLS VQSPIRKCGE PNRCAPHLEW
Consensus   ---------- ---------- ---------- ---------- ----------

          801                                                        850
MR872       .......... .GKCAHHSHH FSLDIDVGCT DLNEDLGVWV IFKIKTQDGH
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       NPDLDCSCRD GEKCAHHSHH FSLDIDVGCT DLNEDLDVWV IFKIKTQDGH
Consensus   ---------- ---------- ---------- ---------- ----------

          851                                                        900
MR872       ARLGNLEFLE EKPLVGEALA RVKRAEKKWR DKREKLEWET NIVYKEAKES
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       ARLGNLEFLE EKPLVGEALA RVKRAEKKWR DKREKLELET NIVYKEAKES
Consensus   ---------- ---------- ---------- ---------- ----------

          901                                                        950
MR872       VDALFVNSQY DRLQADTNIA MIHAADKRVH SIREAYLPEL SVIPGVNAAI
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       VDALFVNSQY DQLQADTNIA MIHAADKRVH RIREAYLPEL SVIPGVNVDI
Consensus   ---------- ---------- ---------- ---------- ----------

          951                                                       1000
MR872       FEELEGRIFT AFSLYDARNV IKNGDFNNGL SCWNVKGHVD VEEQNNHRSV
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       FEELKGRIFT AFFLYDARNV IKNGDFNNGL SCWNVKGHVD VEEQNNHRSV
Consensus   ---------- ---------- ---------- ---------- ----------

         1001                                                       1050
MR872       LVVPEWEAEV SQEVRVCPGR GYILRVTAYK EGYGEGCVTI HEIENNTDEL
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F       LVVPEWEAEV SQEVRVCPGR GYILRVTAYK EGYGEGCVTI HEIENNTDEL
```

```
Consensus   ---------- ---------- ---------- ---------- ----------

         1051                                                1100
MR872       KFSNCVEEEV YPNNTVTCND YTATQEEYEG TYTSRNRGYD GAYESNSSVP
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F         KFSNCVEEEV YPNNTVTCND YTANQEEYGG AYTSRNRGYD ETYGSNSSVP
Consensus   ---------- ---------- ---------- ---------- ----------

         1101                                                1150
MR872       ADYASAYEEK AYTDGRRDNP CESNRGYGDY TPLPAGYVTK ELEYFPETDK
Cry1Fsyn    .......... .......... .......... .......... ..........
Cry1F         ADYASVYEEK SYTDGRRDNP CESNRGYGDY TPLPAGYVTK ELEYFPETDK
Consensus   ---------- ---------- ---------- ---------- ----------

         1151                     1175
MR872       VWIEIGETEG TFIVDSVELL LMEE*
Cry1Fsyn    .......... .......... .....
Cry1F         VWIEIGETEG TFIVDSVELL LMEE*
Consensus   ---------- ---------- -----
```

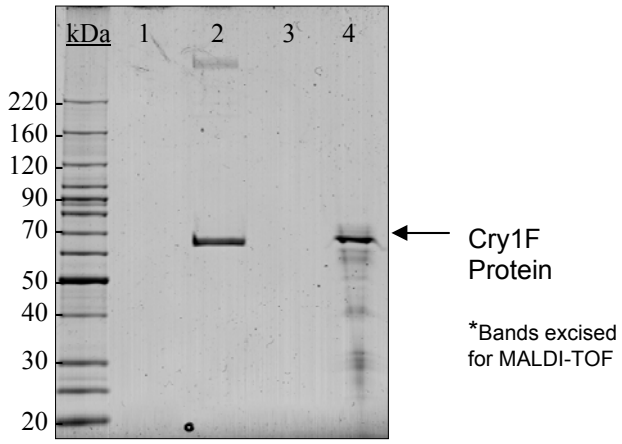
SDS-PAGE and Western Analyses

SDS-PAGE was performed with crude leaf extracts, immuno-purified Cry1F fractions from leaf tissue and microbe-derived Cry1F protein from *Pseudomonas fluorescens* (Schafer, 2003; Appendix 2). Following electrophoresis, the proteins were transferred to a nylon membrane and probed with a Cry1F specific polyclonal rabbit antibody.

In the microbial preparation from *P. fluorescens*, the major Cry1F band, as visualized on Coomassie stained SDS-PAGE gels, was approximately 65 kDa. As expected, the corresponding DAS-06275-8 maize-derived Cry1F was nearly identical (Figures 34 and 35A). This is consistent with the previous findings for the DAS-01507-1 maize-derived poCry1F (Shanahan & Stauffer, 2000). Predictably, the plant purified fractions contained a minor amount of proteolytic products in addition to the intact protein. This could be accounted for by the need to highly concentrate the partially purified fractions for visualization on SDS-PAGE. This process may have contributed to some protein degradation. The immunoaffinity column was prepared with anti-Cry1F polyclonal antibodies, and therefore, all available epitopes were most likely captured and co-purified with the intact Cry1F core protein. These truncation products were minor components of the final purified preparation.

Figure 34. SDS-PAGE of microbe-derived Cry1F and immuno-purified Cry1F from moCry1F maize leaf tissue. SDS-PAGE was performed with a Bio-Rad Ready Gel fitted into a Mini-Protean II gel module. Immuno-purified Cry1F and microbe-derived Cry1F (TSN103620) were mixed 1:1 with Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and boiled for 5 minutes at 100 °C. The electrophoresis was conducted at a constant amperage of 25 mA per gel for 60 minutes using Tris/glycine/SDS buffer. After separation the gel was stained with Pierce GelCode Blue protein stain. The bands noted by the arrow were excised from the gel and used for peptide mass fingerprint analysis. For clarity not all of the molecular weight markers were labeled. The lanes contained:

Lane kDa	Benchmark Molecular Weight Markers – 5 μL
Lane 1	Blank
Lane 2	Microbe-Derived Cry1F (TSN103620) – 740 ng (25 μL)
Lane 3	Blank
Lane 4	Immuno-purified moCry1F from Maize Leaf Tissue – 480 ng (40 μL)



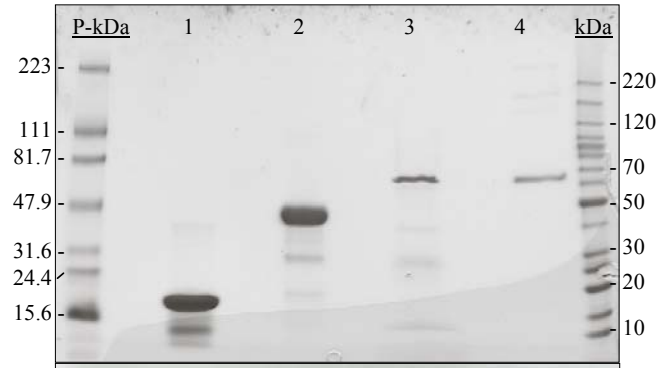
Glycosylation Analysis, Tryptic Mass Fingerprinting, N-Terminal Sequencing

Detection of carbohydrates possibly covalently linked to Cry1F (microbe- and maize-derived) was assessed by the GelCode Glycoprotein Staining Kit from Pierce. Microbe-derived and the immunoaffinity-purified Cry1F proteins were electrophoresed simultaneously. A glycoprotein, horseradish peroxidase, was loaded as a positive indicator for glycosylation and a non-glycoprotein, soybean trypsin inhibitor, was employed as a negative control. The results showed (Figure 35) that both the maize- and microbe-derived Cry1F proteins had no detectable carbohydrates (Schafer, 2002).

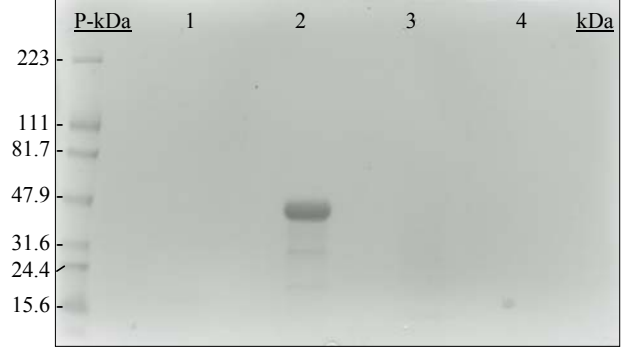
Figure 35. SDS-PAGE gels stained with GelCode Blue Stain (Panel A) and GelCode Glycoprotein stain (Panel B). SDS-PAGE was performed with two identically prepared Bio-Rad Ready Gels fitted in a Mini-Protean II gel module. The proteins were mixed 1:1 with Laemmli sample buffer (containing 5% freshly added 2-mercaptoetahanol) and boiled for 5 minutes at 100 °C. The electrophoresis was conducted at a constant amperage of 20 mA per gel for 60 minutes using Tris/glycine/SDS buffer. After separation, one gel was stained with Pierce GelCode Blue total protein stain and the second gel was stained with Pierce GelCode Glycoprotein Stain according to the manufactures' protocol. For clarity not all of the Benchmark molecular weight markers were labeled in Panel A. The Benchmark molecular weight markers are not glycoproteins and were therefore not visualized in the gel stained with the glycoprotein stain (Panel B). The lanes contained:

Lane P-kDa	TriChromRanger Prestained Molecular Weight Markers – 5 μL
Lane 1	Soybean Trypsin Inhibitor – 5 μg
Lane 2	Horseradish Peroxidase (known glycoprotein) – 5 μg
Lane 3	Immuno-purified moCry1F from Maize Leaf Tissue – 600 ng
Lane 4	Microbe-Derived Cry1F (TSN103620) – 600 ng
Lane 5	Benchmark Molecular Weight Markers – 5 μL

Panel A



Panel B



In the tryptic mass fingerprinting, 19 peptides were identified in the trypsin digest of the microbial Cry1F, whereas in the plant-derived Cry1F protein, 22 peptides were identified that matched the theoretical deduced peptide masses (Table 10A). These results indicate identity with the predicted Cry1F sequence for both protein sources. The differences in tryptic mass fingerprints between the protein and microbial protein samples are not unexpected and may be caused by differential digestion and ionization (Schafer, 2002; Appendix 2). N-terminal sequencing was performed using the Edman degradation reaction. The 15 N-terminal amino acid residues determined from the truncated Cry1F of both *P. fluorescens* and transgenic corn were the same and were found to correspond to residues #28 to #42 of the moCry1F (Table 10B; Schafer, 2002).

Table 10. A) Tryptic peptide mass data (m/z $[M+H]^+$) obtained by MALDI-TOF MS and B) N-terminal sequence of Cry1F proteins.

A

Cry1F residue number	Theoretical mass[a] (*m/z*)	*P. fluorescens* Cry1F [M+H]	DAS-06275-8 Maize Cry1F [M+H]
32-42	1227.73	1227.70	1227.68
86-91	760.42	ND	760.40
94-99	674.42	ND	674.39
100-113	1612.82	1612.81	1612.82
114-125	1441.68	1441.66	1441.67
194-200	878.56	878.53	878.54
223-226	560.29	ND	560.27
227-231	711.36	711.32	711.34
252-263	1394.73	1394.71	1394.72
264-286	2509.22	2509.20	2509.21
312-324	1413.72	1413.72	1413.72
358-366	1033.57	1033.54	1033.55
367-379	1386.73	1386.71	1386.72
380-392	1416.70	1416.68	1416.70
431-442	1376.62	1376.60	1376.62
452-463	1301.63	1301.61	1301.63
464-471	911.59	ND	911.55
472-483	1269.69	1269.66	1269.69
484-494	1089.57	1089.54	1089.56
522-529	925.47	925.44	925.47
530-538	1007.55	1007.53	1007.54
539-546	924.49	924.46	924.47
595-612	2143.06	2143.07	Due to slight differences at the C-terminus, this peptide is not present in the plant expressed protein[b, c]

[a] Two digit decimals were used for mass data in this table although raw data obtained from the MALDI-TOF-MS spectrometer were shown in 4 digit decimals. A peptide was considered a match if its *m/z* is within +/- 0.1 error range of its theoretical *m/z*.

[b] ND: not detected. NP: peptide fragment not present in both proteins.

[c] The C-terminus of the microbe-derived Cry1F is 7 amino acids longer.

B

1	$M^{1}ENNIQNQCVPYNCLNNPEVEILNEER^{27}$↓	$S\ T\ G\ R\ L\ P\ L\ D\ I\ S\ L\ S\ L\ T\ R^{42}$
2		$^{1}S\ T\ G\ R\ L\ P\ L\ D\ I\ S\ L\ S\ L\ T\ R^{15}$
3		$^{1}S\ T\ G\ R\ L\ P\ L\ D\ I\ S\ L\ S\ L\ T\ R^{15}$
4		$^{1}S\ T\ X\ R\ L\ P\ L\ D\ I\ S\ L\ S\ L\ T\ X^{15}$

1: Expected N-terminal sequence of the first 42 amino acid residues of maize-derived Cry1F
2: Expected N-terminal sequence of *P. fluorescens*-derived truncated Cry1F;
3: Detected N-terminal sequence of *P. fluorescens*-derived truncated Cry1F;
4: Detected N-terminal sequence of the immuno-affinity purified moCry1F.
↓ indicates a trypsin cleavage site.
Numbers in superscript indicate amino acid residue numbers in the sequence.
Amino acid residues:

C:	cysteine
D:	aspartic acid
E:	glutamic acid
G:	glycine
I	isoleucine
L:	leucine
M:	methionine
N:	asparagine
P:	proline
Q:	glutamine
R:	arginine
S:	serine
T:	threonine
V:	valine
X:	an uncertain assignment in the Edman reaction
Y:	tyrosine

V.D.2. CHARACTERISTICS OF THE BAR PROTEIN

The *bar* gene from *Streptomyces hygroscopicus* contained in *B.t.* Cry1F maize line 6275 expresses the phosphinothricin acetyltransferase (PAT) protein. Hereafter, the PAT protein expressed by the *bar* gene will be referred to as the BAR protein to delineate it from the PAT protein expressed by the *pat* gene. Extensive literature exists which shows that the BAR protein is very similar to the PAT protein encoded by the *pat* gene from *Streptomyces viridochomogenes* which is contained in the *B.t.* Cry1F maize line 1507 (00-136-01p) (Wehrmann, *et al*, 1996).

Figure 36. PAT (BAR) peptide sequence.

```
  1                                                    50
  MSPERRPADI RRATEADMPA VCTIVNHYIE TSTVNFRTEP QEPQEWTDDL
 51                                                   100
  VRLRERYPWL VAEVDGEVAG IAYAGPWKAR NAYDWTAEST VYVSPRHQRT
101                                                   150
  GLGSTLYTHL LKSLEAQGFK SVVAVIGLPN DPSVRMHEAL GYAPRGMLRA
151                                  183
  AGFKHGNWHD VGFWQLDFSL PVPPRPVLPV TEI
```

Characterization of the biochemical properties of the microbially-produced and plant-produced BAR proteins was accomplished through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), glycoprotein detection methods, western blot, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and N-terminal sequence analysis (Gao and Korjagin, 2002, Appendix 2). Utilizing these methods, the BAR protein from the microbial preparation and the transgenic maize (event DAS-06275-8) were shown to be biochemically equivalent.

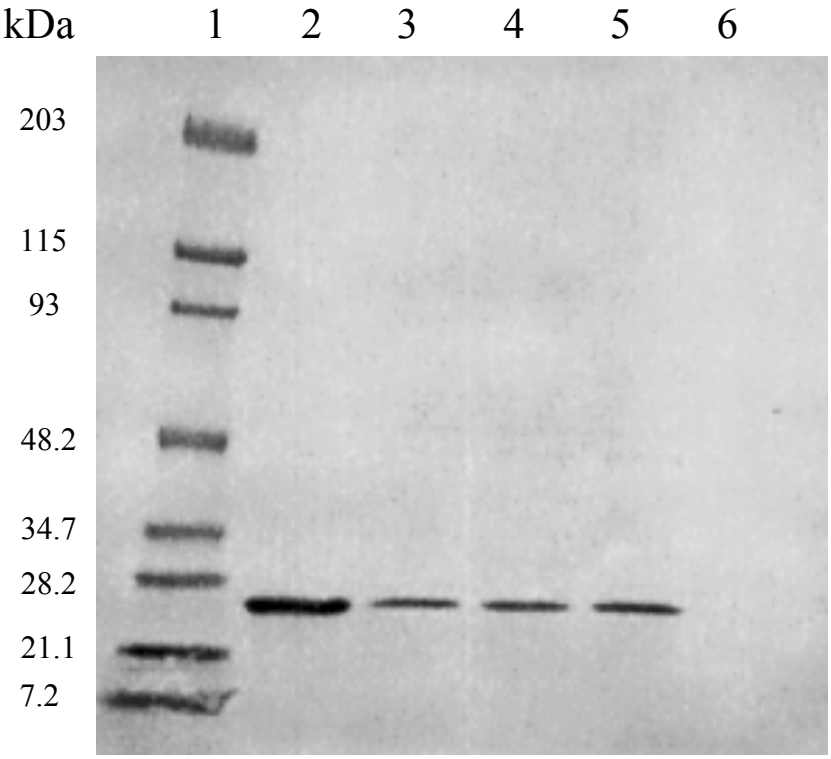
SDS PAGE and Western Analyses

SDS-PAGE and western blot analyses were conducted on the *E. coli*-produced BAR (*E.coli* strain MR1513) and leaf extracts from maize line 6275 plants grown in the greenhouse (Gao and Korjagin, 2002, Appendix 2). For immunodetection, a specific rabbit polyclonal antibody against BAR protein was used as the primary antibody.

The molecular weight (MW) of the BAR protein is 20.6 kDa based on its amino acid sequence. Figure 37 shows that the apparent MW of the *E. coli* derived recombinant BAR protein was approximately 21 kDa, matching the theoretical MW. It should be noted that the mass accuracy of the Invitrogen Benchmark protein ladder is superior to the pre-stained MW markers of Bio-Rad. The Western blot analysis showed that the transgenic event 6275 corn contained a BAR protein with the same MW as the microbial BAR (Figure 37). Both the microbial BAR protein and the transgenic corn BAR protein were immunoreactive to the rabbit polyclonal antibodies (PAb) against BAR.

Figure 37. Western blot of *E. coli* produced and corn derived BAR protein. The protein samples were mixed with Laemmli sample buffer containing 5% freshly added β-mercaptoethanol, and boiled for 5 min at 100 °C. The electrophoresis was conducted at a constant voltage of 200 V for approximately 30 min. After separation, the gel was electro-blotted to a nitrocellulose membrane with a Mini Trans-Blot electrophoretic transfer cell for 60 min under a constant voltage of 100 V. For immunodetection, a specific rabbit polyclonal antibody against BAR protein was used as the primary antibody. The lanes contained:

Lane 1: Pre-stained molecular weight markers (Bio-Rad, broad range), 7 µL
Lane 2: *E. coli*-derived BAR protein, 0.9 µg
Lane 3: Transgenic corn leaf extract, plant #21, 8 µL
Lane 4: Transgenic corn leaf extract, plant #22, 8 µL
Lane 5: Transgenic corn leaf extract, plant #23, 8 µL
Lane 6: Non-transgenic corn leaf extract, 8 µL

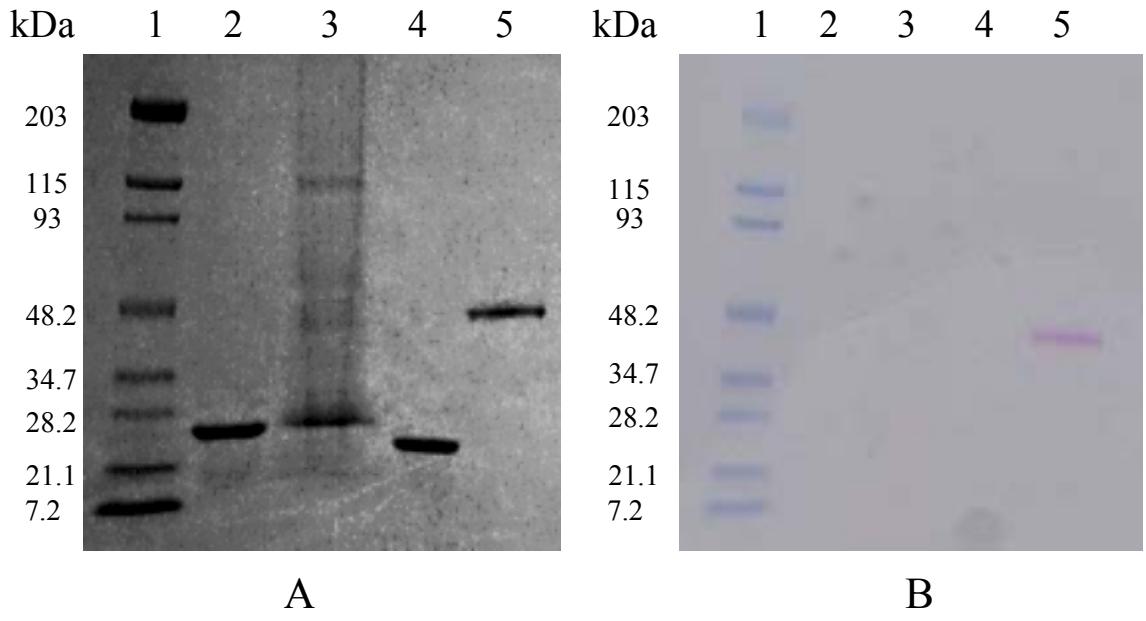


Glycosylation, Mass Peptide Fingerprinting and N-Terminal Sequencing Analyses

The potential presence of carbohydrates covalently linked to the BAR protein was assessed by the GelCode Glycoprotein Staining Kit from Pierce. *E. coli*-derived and the immunoaffinity-purified transgenic corn BAR protein preparations were electrophoresed simultaneously. A glycoprotein, horseradish peroxidase, was loaded as a positive control for glycosylation. A non-glycoprotein, soybean trypsin inhibitor, was employed as a negative control. The results showed that both the corn- and *E. coli*-derived BAR protein had no detectable carbohydrates (Figure 38).

Figure 38. SDS-PAGE (4-15%) stained by Coomassie Brilliant Blue (A) or by Pierce GelCode Glycoprotein Stain (B). The protein samples were mixed with Laemmli sample buffer containing 5% freshly added β-mercaptoethanol (β-ME), and boiled for 5 min at 100 °C. The electrophoresis was conducted at a constant voltage of 200 V for approximately 30 min. The lanes contained:

Lane 1: Pre-stained molecular weight markers (Bio-Rad)
Lane 2: *E. coli*-derived BAR protein, 2 µg
Lane 3: Corn-derived BAR protein (immunoaffinity purified fractions #3-6)
Lane 4: Soybean trypsin inhibitor, 2 µg
Lane 5: Horseradish peroxidase, 2 µg



The amino acid residues at the N-termini of the BAR protein samples (the recombinant microbial BAR from *E. coli,* and the affinity chromatography purified corn BAR) were sequenced. The Edman degradation reaction was performed for the first 15 cycles. The results are summarized in Table 11. Based on the *bar* gene sequence, the expected N-terminal sequence of the first 15 amino acid residues of the BAR protein is M^{1} A^{2} P^{3} E^{4} R^{5} R^{6} P^{7} A^{8} D^{9} I^{10} R^{11} R^{12} A^{13} T^{14} E^{15}. The amino acid residues determined from both *E. coli* BAR and corn BAR matched the expected sequence (Table 11). Removal of the N-terminal methionine (M) was observed for both the microbial and corn BAR protein. For *E. coli*-produced BAR, both the methionine-intact and methionine-removed forms were present (Table 11, rows 2 and 3). For the BAR protein purified from the transgenic corn, only the methionine-removed form was detected in N-terminal sequence analysis (Table 11, row 4). However, through the use of more sensitive MALDI-TOF-MS, it was demonstrated that the methionine-intact form was also present in the corn-derived BAR. As shown in Table 12, in the trypsin digest of corn BAR protein, the peptide with residues #1-11 was detected. Our results clearly indicate that the N-terminal methionine of the recombinant BAR protein could be cleaved in both the transgenic corn plants and *E. coli*. The results are not uncommon, as it is known that N-terminal methionine can be cleaved by methionine aminopeptidases in both prokaryotic and eukaryotic systems (Bradshaw *et al*. 1998).

Table 11. N-terminal sequence of BAR protein.

1	M	A	P	E	R	R	P	A	D	I	R	R	A	T	E
2	M	A	P	E	R	R	P	A	D	I	R	R	A	T	E
3		A	P	E	R	R	P	A	D	I	R	R	A	T	E
4		X	P	E	R	R	P	A	D	I	R	R	A	T	E

1: Expected N-terminal sequence of the first 15 amino acid residues of BAR protein;
2 and 3: Detected N-terminal sequence of *E. coli*-produced BAR protein;
4: Detected N-terminal sequence of the transgenic corn-derived BAR protein.
Amino acid residues:

- A: alanine
- D: aspartic acid
- E: glutamic acid
- I Isoleucine
- M: methionine
- P: proline
- R: arginine
- T: threonine
- X: an uncertain assignment in the Edman reaction

Table 12. Tryptic peptide mass data (*m/z* [M+H]$^+$) of the BAR protein obtained by MALDI-TOF MS.

Amino acid residues #	Theoretical mass (*m/z*)	*E. coli* BAR[a]	Corn BAR[a]
1-11	1311.69	1311.66	1311.84
1-12	1467.79	1467.73	ND[b]
38-52	1842.84	1842.82	1843.07
81-96	1858.85	1858.82	1859.07
100-112	1403.78	1403.74	1403.94
121-135	1522.85	1522.83	1523.03
136-145	1144.55	1144.52	1144.67

[a] Two digit decimals were used for mass data in this table although raw data obtained from the MALDI-TOF-MS spectrometer were shown in 4 digit decimals. A peptide was considered a match if its *m/z* was within 500 ppm error range of its theoretical *m/z*.

[b] ND = not detected.

The BAR protein derived from both *E. coli* and transgenic corn event 6275 was further separated from impurities by SDS-PAGE, and the respective bands were excised and subjected to in-gel digestion by trypsin. The resulting peptide mixture was analyzed by MALDI-TOF MS. The masses of the detected peptides were compared to those deduced based on trypsin cleavage sites in the sequence of the BAR protein. Figure 39 illustrates the theoretical cleavage generated *in silico* using PAWS (Protein Analysis Worksheet) software from Proteometrics Inc. In the trypsin digest of *E. coli*-derived BAR protein, 7 peptides were identified matching the theoretical deduced peptide masses of BAR (Table 12). In the trypsin digest of the transgenic corn-derived BAR protein, 6 peptides were identified matching the deduced theoretical peptide masses (Table 12). These peptides together cover 43% of the BAR protein sequence. It should be noted that peptides smaller than 710 Da or higher than 5000 Da were excluded from the analysis because of the setting of the MALDI-TOF MS spectrometer. There were some unidentified peptides detected with the MALDI-TOF-MS analysis. Many factors could contribute to the formation of the unidentified peptides, such as over digestion (which results in non-specific cleavage), self-digestion products of trypsin, and random breakage of peptides during ionization.

Figure 39. Potential peptides after trypsin digestion of the amino acid sequence of the BAR protein. Alternating blocks of upper and lower case letters within the amino acid sequence are used to differentiate the potential peptides after trypsin digestion digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

1	MAPERrpadirRateadmpavctivnhyie	30
31	tstvnfrTEPQEPQEWTDDLVRIrERypwl	60
61	vaevdgevagiayagpwkARnaydwtaest	90
91	vyvsprHQRtglgstlythllkSLEAQGFK	120
121	svvavigIpndpsvrMHEALGYAPRgmIrA	150
151	AGFKhgnwhdvgfwqldfslpvppprpvlpv	180
181	tei	183

V.D.3. CRY1F EXPRESSION IN PLANT TISSUES

A field expression study was conducted in Chile in 2001-2002 which consisted of six (6) separate field trials in Buin, Maipo and Viluco in which the genetically modified hybrid line 6275 and its near isoline control CHPH09B/2MW were grown. Expression levels of the Cry1Fprotein were analyzed from six (6) tissues at various growth stages from the transgenic hybrid line along with the appropriate control line.

The Cry1F ELISA kit (from Strategic Diagnostics, Inc.) is an immunoassay system based on the specific interaction between the antibodies and the Cry1F protein (Essner, 2003). It was used to determine the concentration of the Cry1F protein in the transgenic crop plants. Samples consisting of extracts from transformed plants, non-transformed plants (controls) and truncated Cry1F protein as a standard were incubated with enzyme-conjugated (horse radish peroxidase) antibody (monoclonal) and anti-Cry1F antibodies (polyclonal) coated in the wells of a microtiter plate. At the end of the incubation period, the unbound reagents were removed from the microtiter plate by washing. An enzyme substrate solution (tetramethylbenzidine) was added to the microtiter plate, which generates a blue colored product. The enzyme reaction was stopped with a dilute acid solution which causes a color change to yellow and the absorbance at 450nm was measured in each well on the microtiter plate using a microtiter plate reader. Since the protein is bound in the antibody sandwich, the level of color development is proportional to the concentration of Cry1F protein in the sample (i.e., lower protein concentrations result in lower color development and less absorbance at 450nm). A calibration curve was generated with the Cry1F protein standard and the Cry1F protein concentration in the plant sample extracts was calculated using a regression equation. Results from the ELISA are shown in Table 13.

Leaf, root, whole plant, grain, pollen, and stalk samples from all locations expressed the Cry1F protein at measurable levels, except for leaf and root samples at the V9 stage and at senescence for leaf at one location. The location at Buin had one sample (of six total) that had no detectable

expression of Cry1F in leaf and root from the V9 and senescent leaf stages. Mean expression levels for the hybrid 6275 on a tissue dry weight basis ranged from 0.71 ng/mg tissue dry weight for leaf senescent tissue to 44.8 ng/mg tissue dry weight for R4 leaf tissue. None of the control tissues expressed the Cry1F protein.

Table 13. Summary of expression levels of Cry1F protein (ng/mg tissue dry weight) measured in tissues collected from maize hybrid line 6275H and control line CHPH09B/2MW by ELISA. Samples were analyzed using a Cry1F ELISA Kit (Strategic Diagnostics, Inc.). The plant samples were obtained from the field trials conducted in Chile in 2001-2002.

Hybrid	Tissue	Growth Stage	Mean (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range (ng/mg Tissue Dry Weight)	Number of Samples[2]
6275H **CHPH09B/2MW**	Leaf	V9	16.7 0	4.60 0	0 - 23.8 0 - 0	30/1 6/6
6275H **CHPH09B/2MW**	Root	V9	6.14 0	1.87 0	0 - 8.14 0-0	18/1 6/6
6275H **CHPH09B/2MW**	Whole Plant	V9	6.22 0	1.16 0	4.98 – 7.87 0 - 0	6/0 6/6
6275H **CHPH09B/2MW**	Leaf	R1	28.5 0	5.38 0	16.5 - 36.7 0 - 0	30/0 6/6
6275H **CHPH09B/2MW**	Root	R1	6.60 0	1.98 0	3.14 - 10.9 0 - 0	30/0 6/6
6275H **CHPH09B/2MW**	Whole Plant	R1	7.16 0	1.45 0	5.32 - 9.57 0 - 0	6/0 6/6
6275H **CHPH09B/2MW**	Pollen	R1	3.67 0	0.34 0	3.09 - 4.60 0 - 0	30/0 6/6
6275H **CHPH09B/2MW**	Stalk	R1	11.0 0	2.67 0	6.77 - 16.4 0 - 0	30/0 6/6
6275H **CHPH09B/2MW**	Leaf	R4	44.8 0	16.8 0	35.8 - 109.2 0 - 0	18/0 6/6
6275H **CHPH09B/2MW**	Root	R4	5.99 0	1.89 0	2.35 – 9.26 0 - 0	18/0 6/6
6275H **CHPH09B/2MW**	Forage	R4	6.26 0	1.09 0	5.05- 7.77 0 – 0	6/0 6/6

Hybrid	Tissue	Growth Stage	Mean (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range (ng/mg Tissue Dry Weight)	Number of Samples[2]
6275H CHPH09B/2MW	Grain	Maturity	1.14 0	0.27 0	0.62 - 1.68 0 - 0	30/0 6/6
6275H CHPH09B/2MW	Leaf	Senescence	0.71 0	1.14 0	0 - 3.09 0 - 0	18/10 6/6
6275H CHPH09B/2MW	Root	Senescence	1.97 0	2.03 0	0.29 - 6.91 0 - 0	18/0 6/6
6275H CHPH09B/2MW	Whole Plant	Senescence	2.47 0	0.41 0	1.95 - 3.07 0 - 0	6/0 6/6

[1] Number of Samples: number of samples analyzed/number of samples < sample LLOQ
[2] For values below sample LLOQ a value of zero was assigned for calculation purposes

Table 14 compares Cry1F events DAS-06275-8 and DAS-01507-1 in terms of tissue expression of Cry1F protein in plant parts at various stages of growth. DAS-06275-8 is clearly distinguished from DAS-01507-1 in terms of the quantities of protein expressed in plants over time. Significantly increased content of Cry1F protein in DAS-06275-8 stalks sampled at flowering (R1) corresponds to similar trends for increased Cry1F in leaves, whole plants, and forage of DAS-06275-8 in comparison to DAS-01507-1. Increased stalk expression of Cry1F protein toxin in DAS-06275-8 is a clear benefit resulting in reduced risk of resistance development by borers. There is additionally significantly decreased grain and pollen expression of Cry1F in DAS-06275-8 as compared to DAS-01507-1. The 6-fold reduction in pollen expression and 2-fold reduction in grain expression has positive environmental and human health impact as these tissues are likely sources of non-target exposure to Cry1F protein expressed in corn.

Table 14. Comparison of Cry1F events DAS-06275-8 and DAS-01507-1 in Terms of Tissue Expression in Plant Parts at Various Growth Stages. Data for event DAS-06275-8 is given in Table 13 of this extension petition. Data for event DAS-01507-1 is taken from USDA petition #00-136-01p.

		DAS-06275-8 Hybrid			DAS-01507-1 Hybrid		
Tissue	***Growth Stage***	***Mean (ng/mg tissue dry wt.***	***Std. Deviation***	***Min/Max (ng/mg tissue dry wt.***	***Mean (ng/mg tissue dry wt.***	***Std. Deviation***	***Min/Max (ng/mg tissue dry wt.***
Leaf	V9	16.7	4.6	0 - 23.8	12.1	6.2	0 - 24
Root	V9	6.14	1.87	0 - 8.14			
Whole Plant	V9	6.22	1.16	4.98 - 7.87	5.2	1.9	2.6 - 6.8
Leaf	R1	28.5	5.38	16.5 - 36.7			
Root	R1	6.6	1.98	3.14 - 10.9			
Whole Plant	R1	7.16	1.45	5.32 - 9.57	3.6	1.1	2.5 - 4.7
Pollen	R1	3.67	0.34	3.09 - 4.6	21.9	2.9	16.4 - 27.2
Stalk	R1	11	2.67	6.77 - 16.4	5.8	1.7	3.3 - 10.3
Leaf	R4	44.8	16.8	35.8 - 109.2			
Root	R4	5.99	1.89	2.35 - 9.26			
Forage	R4	6.26	1.09	5.05 - 7.77	1.7	1.1	0 - 3.2
Leaf	Senescence	0.71	1.14	0 - 3.09			
Root	Senescence	1.97	2.03	0.29 - 6.91			
Whole Plant	Senescence	2.47	0.41	1.95 - 3.07	1.6	0.6	0.9 - 2.4

V.D.4. BAR PROTEIN EXPRESSION IN PLANT TISSUES

A specific sandwich ELISA Kit from EnviroLogix, Inc. was used to quantify the BAR protein in genetically modified maize tissues (Essner, 2003). The BAR protein is extracted from maize tissues using a phosphate buffered saline solution containing the detergent Tween –20 (PBST). The extract is centrifuged, the supernatant collected and analyzed for total protein concentration. The supernatant is then diluted and assayed using the BAR enzyme-linked immunsorbent assay (ELISA) kit. Sample extracts are added to test wells coated with antibodies raised against Phosphinothricin Acetyltransferase protein from the *bar* gene (BAR). The BAR protein present in the sample extract binds to the antibodies, and is then detected by addition of enzyme (horseradish peroxidase)-labeled BAR antibody. Unbound substances are washed from the plate and the detection of the bound complex is accomplished through the addition of an enzyme substrate, generating a colored product. The resultant color intensity, measured as optical density (OD), is related by a quadratic equation to the amount of BAR protein present in the unknown extracts.

The BAR protein was found to be expressed in leaf, root, whole plant, grain, pollen and stalk samples from all locations and developmental stages (Table 15). One sample each from both leaf and root

from the V9 stage was below detectable levels. BAR protein expression in pollen at the R1 stage was low and 27 of 30 6275 hybrid samples were below detectable limits.

Table 15. Summary of expression levels of BAR protein (ng/mg tissue dry weight) measured in tissues collected from maize hybrid line 6275H and control line CHPH09B/2MW by ELISA. The samples were obtained from the field trials grown in Chile in 2001-2002. The samples were analyzed for BAR protein expression using a BAR specific ELISA Kit (Envirologix, Inc.).

Hybrid	Tissue	Growth Stage	Mean (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range (ng/mg Tissue Dry Weight)	Number of Samples [1,2]
6275H **CHPH09B/2MW**	Leaf	V9	323 0	91.0 0	0 – 538 0– 0	30/1 6/6
6275H **CHPH09B/2MW**	Root	V9	112 0	35.3 0.147	0 – 170 0 - 0	18/1 6/6
6275H **CHPH09B/2MW**	Whole Plant	V9	5 0	3.50 0	1 – 11 0 - 0	6/0 6/6
6275H **CHPH09B/2MW**	Leaf	R1	674 0	98.1 0	539 – 935 0 - 0	30/0 6/6
6275H **CHPH09B/2MW**	Root	R1	253 0	162 0	61 – 673 0 – 0	30/0 6/6
6275H **CHPH09B/2MW**	Whole Plant	R1	72 0	32.9 0	35 – 108 0 - 0	6/0 6/6
6275H **CHPH09B/2MW**	Pollen	R1	0 0	0.766 0	0 – 4.07 0 - 0	30/27 6/6
6275H **CHPH09B/2MW**	Stalk	R1	282 0	68.5 0	177 – 475 0 – 0	30/0 6/6
6275H **CHPH09B/2MW**	Leaf	R4	682 0	254 0	451 – 1584 0 - 0	18/0 6/6

Hybrid	Tissue	Growth Stage	Mean (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range (ng/mg Tissue Dry Weight)	Number of Samples [1,2]
6275H **CHPH09B/2MW**	Root	R4	223 0	105 0.308	85 – 511 0 - 1	18/0 6/5
6275H **CHPH09B/2MW**	Forage	R4	7 0	7.05 0	1 – 19 0 – 0	6/0 6/6
6275H **CHPH09B/2MW**	Grain	Maturity	23 0	6.33 0	13 – 33 0 - 0	30/0 6/6
6275H **CHPH09B/2MW**	Leaf	Senescence	0 0	0.461 0	0 – 1 0 – 0	18/13 6/6
6275H **CHPH09B/2MW**	Root	Senescence	41 0	49.5 0	0 – 148 0 - 0	18/4 6/6
6275H **CHPH09B/2MW**	Whole Plant	Senescence	18 0	5.27 0	9 – 23 0 - 0	6/0 6/6

[1] Number of Samples: number of samples analyzed/number of samples < sample LLOQ
[2] For values below sample LLOQ a value of zero was assigned for calculation purposes.

V.E. GRAIN COMPOSITION

Grain and forage samples taken from the 2001/2002 field study conducted in Chile were also analyzed for their proximate content (Essner, 2003;Table 16). The study consisted of six separate field trials located in Chile in which the genetically modified hybrid line 6275 (referred to hereafter as 6275H) and its near isoline control CHPH09B/2MW were grown. Nutrient composition analysis was conducted at EPL Bio-Analytical Services, (Harristown, IL). Data were statistically analyzed using least-squares means of entries. The differences between locations and entries were tested at a significance level of 5%. The results are presented in Table 16 and Table 17.

The summary analysis values for proximates in grain for hybrid line 6275 and the control hybrid line were within reported literature ranges except for ADF from hybrid line 6275. The individual values for ADF hybrid 6275 were all below reported literature ranges at all locations and mean ADF of 6725H was lower than that of the near iosgenic control. A corresponding difference in mean fiber content was not observed consistently across locations.

Table 16. Summary of proximates analysis in grain for hybrid line 6275 and the control hybrid line (near isoline CHPH09B/2MW). Samples were collected from the field trial conducted in the maize growing regions of Chile during 2001/2002. The values are averages across the six locations.

Analyte[1]	Range of Values in Literature[2]	6275H (Mean[3])	CH (Mean[3])	Standard Error of the Mean
Fat	1.2 - 18.8[4]	4.62	4.80	0.15
Protein	8 - 14[4]	9.88	9.66	0.13
Fiber (crude)	2.0 – 5.5 [4]	2.0	2.2	0.06
ADF	3.0 – 4.3 [4]	2.7	3.5	0.11
NDF	8.3 – 11.9	10.0	10.7	0.45
Ash	1.1 – 3.9	1.16	1.16	0.04
Carbohydrates[5]	78.4 – 89.8	84.3	84.4	0.23

[1]Percent of dry weight
[2]Watson, 1987
[3]Least square means
[4] Watson, 1982
[5]Carbohydrates are calculated as the percentage of dry weight = 100% - % protein - % fat - % ash

The summary analysis values (Table17) for proximates in forage for the hybrid line 6275 and the control line were all within the reported literature ranges for corn forage. Statistical differences were observed at individual locations between the hybrid line 6275 and the control line for fat, crude fiber, ash and carbohydrates. These differences were isolated and were not consistent across the locations.

Table 17. Summary analysis of proximates in forage for hybrid line 6275 (6275H) and the control hybrid line (CH; near isoline CHPH09B/2MW). The samples were collected from the field trial grown in the maize growing regions of Chile in 2001/2002. The values are averages across the six locations.

Analyte[1]	Literature Range[2]	6275H Mean[3]	CH Mean[3]	Standard Error
Fat	0.7 – 6.7	1.95	2.78	0.08
Protein	3.5 – 15.9	6.85	6.96	0.13
Crude Fiber	19 - 42	23.9	23.3	0.33
ADF	30 (mean)	30.3	28.7	0.59
NDF	51 (mean)	49.3	49.3	0.54
Ash	1.3 – 10.5	5.00	4.76	0.06
Carbohydrates[4]	66.9 – 94.5	86.1	85.5	0.20

[1]Percent of dry weight
[2]Watson, 1982
[3]Least square means
[4]Carbohydrates are calculated using the following formula = 100% - % protein - % fat - % ash

Table 18 summarizes all the data that will be presented to the US Food and Drug Administration (FDA) for the pre-market biotechnology notification (PBN) of line 6275.

Table 18. Summary of compositional analytes measured for grain and forage of Line 6275.

Grain			Forage
Proximates	***Vitamins***	***Amino Acids (Essential)***	***Proximates***
Fat	*Vitamin A*	*Methionine*	*Fat*
Protein	*Vitamin B1*	*Cysteine*	*Protein*
Crude Fiber	*Vitamin B2*	*Lysine*	*Crude Fiber*
ADF	*Folic Acid*	*Tryptophan*	*ADF*
NDF	*Vitamin E*	*Threonine*	*NDF*
Ash	*Tocopherols (total)*	*Isoleucine*	*Ash*
Carbohydrates	***Secondary Metabolites***	*Histidine*	*Carbohydrates*
Minerals	*Inositol*	*Valine*	***Minerals***
Calcium	*Raffinose*	*Leucine*	*Calcium*
Phosphorus	*Furfural*	*Arginine*	*Phosphorus*
Copper	*P-Coumaric acid*	*Phenylalanine*	
Iron	*Ferulic acid*	*Glycine*	
Magnesium	***Anti-Nutrients***		
Manganese	*Phytic acid*		
Potassium	*Trypsin Inhibitor*		
Sodium	***Amino Acids (Non-essential)***		
Zinc	*Alanine*		
Fatty Acids	*Aspartic acid*		
Palmitic acid	*Glutamic acid*		
Stearic acid	*Proline*		
Oleic acid	*Serine*		
Linoleic acid	*Tyrosine*		
Linolenic acid			

V.E. AGRONOMIC, DISEASE AND PEST CHARACTERISTICS

B.t. moCry1F Maize line 6275 was evaluated in the field in 1999-2003 in California, Iowa, Illinois, Indiana, Kentucky, Missouri, Mississippi, North Dakota, Ohio, Wisconsin, Minnesota, Hawaii, Michigan, Nebraska and the territory of Puerto Rico under authorizations granted by APHIS (Attachment 1). The purpose of the trials was to evaluate agronomic performance, efficacy against the target pests, increase seed for research purposes, advance generations and/or to evaluate segregation ratios of the event. The field trials were monitored and observed by personnel familiar with corn cultivation practices (breeders, field station managers, field agronomists, field associates).

The personnel conducting the field tests visually monitored plant disease (Northern corn leaf blight, Southern corn leaf blight, Southern rust, grey leaf spot, Stewart's wilt, smut) and pest resistance (European corn borer, corn earworm, fall armyworm, thrips, aphids, red spider mites) characteristics of *B.t.* Cry1F maize line 6275 and non-modified maize lines. There were no differences reported in severity of disease symptoms or insect damage (other than the targeted organisms susceptible to the Cry1F protein) between the transgenic plants and the non-modified plants.

V.E.1. EFFICACY OF EVENT DAS-06275-8 AGAINST LEPIDOPTERAN CORN PESTS

Event DAS-06275-8 was highly effective at controlling infestation from European corn borer (ECB). The level of protection afforded hybrids containing this event was similar to the protection level provided by the previously deregulated Cry1F Event DAS-01507-1. Replicated small plot efficacy trials were conducted at two locations in 2002 (Kentucky and Indiana) to demonstrate ECB efficacy by Event DAS-06275-8. In 2001 a large-scale trial (Indiana) designed to demonstrate high dose efficacy of Event DAS-06275-8 was conducted.

All of these trials were infested at 1st and or 2nd generation timing or in some cases were infested with naturally occurring ECB. Artificial infestations were conducted using the bazooka method (Guthrie and Barry, 1987). In all cases, damage was evaluated for each plot by selecting multiple plants from each plot at random and evaluating for injury. About one week after the last infestation simulating a 1st generation infestation of ECB, (four weeks after the first infestation) or when significant foliar feeding damage was obvious in the non-Bt isogenic plots, larval feeding damage was measured using a foliar damage scale developed by Guthrie et. al. (1960) and described in Table 19.

Table 19. First generation ECB feeding damage scale for corn.

Rating	Description
1	No visible damage.
2	Small amount of shot-hole type lesions on a few leaves.
3	Shot-hole injury common on several leaves.
4	Several leaves with shot-hole and elongated lesions.
5	Several leaves with elongated lesions.
6	Several leaves with elongated lesions about 1 inch (2.5 cm) long.
7	Long lesions common on about half (1/2) of the leaves.
8	Long lesions common on about two-thirds (2/3) of the leaves
9	Long lesions common on most leaves

Plants were artificially infested with neonate ECB beginning at tasseling to simulate 2nd generation ECB infestation. Each plant was infested a minimum of 3 times with at least 2 days between infestations. 2nd generation feeding damage was evaluated near harvest. Stalks were split and the number of tunnels in each plant was recorded. Also, the presence of live larvae and total length of tunnels per plant were recorded.

Highly significant levels of resistance to ECB feeding at 1st and 2nd generation timing were measured (Figures 40 through 43). The levels of protection associated with Event DAS-06275-8 was identical to the levels provided by Event DAS-01507-1. Both events prevented feeding damage to the foliage (Figure 40) and to stalks (Figures 41 and 42). Additionally, larvae were rarely recovered from the events (Figure 43). Collectively, these results show that Event DAS-06275-8 delivers high levels of protection from feeding damage from European corn borer at all times during the different growing phases of the plant.

Figure 40. Efficacy of Event DAS-06275-8 and Event DAS-01507-1 for the control of first generation ECB feeding damage as measured by foliar injury ratings. Summary of 2001 and 2002 data. Data were obtained from trials conducted under notifications 02-060-17n in 2002 and 01-11607n in 2001.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
reps	4	0.9079	0.2605	0.0651	0.41	0.796
Gen Name	2	5.6065	5.6065	2.8032	17.76	0.000
Error	11	1.7366	1.7366	0.1579		
Total	17	8.2510				

CLEVEL	95.0000	SIGMA	0.397335
LSD_T	2.20099	LSD_TSIG	0.874528

CALCULATE LSD = LSD_TSIG * SQRT(1/Ni + 1/Nj)

Row	TRTLABEL	LSMEAN	TRTCNT	SIGNIF	COMPARE
1	Non-Bt Iso-hybrid	2.27478	5	A	X
2	Herculex I	1.00000	4	B	X*
3	Mo Cry1F (TC6275)	1.00000	9	B	*X

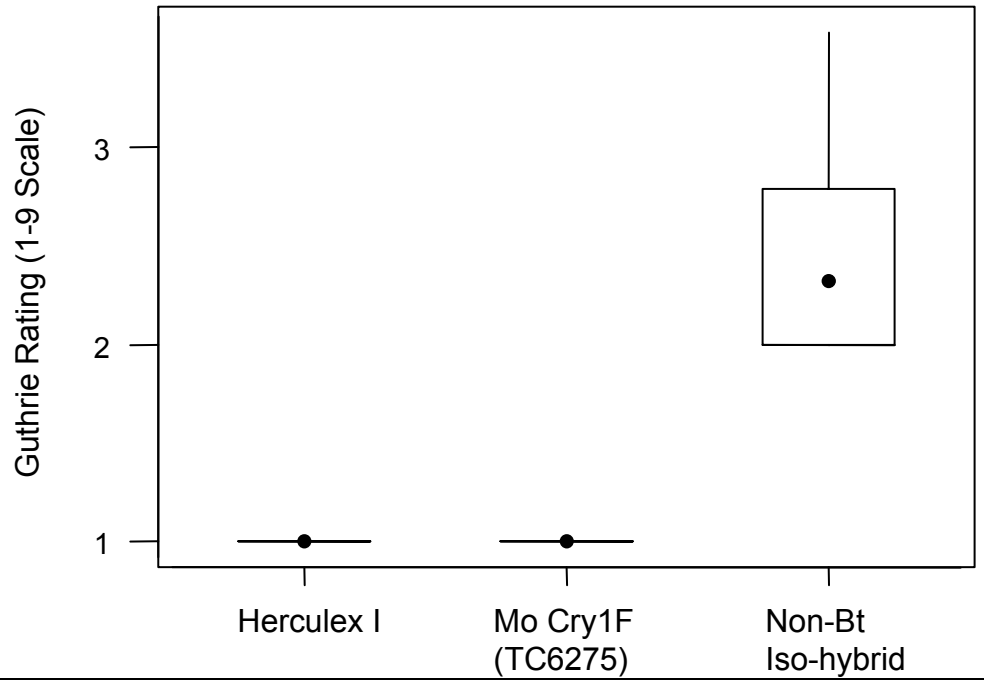


Figure 41. Efficacy of Event DAS-06275-8 and Event DAS-01507-1 for the control of 2nd generation ECB stalk tunneling damage (cm/stalk). Summary of 2001 and 2002 data. Data were obtained from trials conducted under notifications 02-060-17n in 2002 and 01-11607n in 2001.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
reps	4	87.90	50.89	12.72	1.06	0.395
Gen Name	2	1573.79	1573.79	786.90	65.74	0.000
Error	26	311.22	311.22	11.97		
Total	32	1972.91				

CLEVEL	95.0000	SIGMA	3.45975
LSD_T	2.05553	LSD_TSIG	7.11161

CALCULATE LSD = LSD_TSIG * SQRT(1/Ni + 1/Nj)

Row	TRTLABEL	LSMEAN	TRTCNT	SIGNIF	COMPARE
1	Non-Bt Iso-hybrid	15.1327	12	A	X
2	Herculex I	0.8439	8	B	X*
3	Mo Cry1F (TC6275)	0.5193	13	B	*X

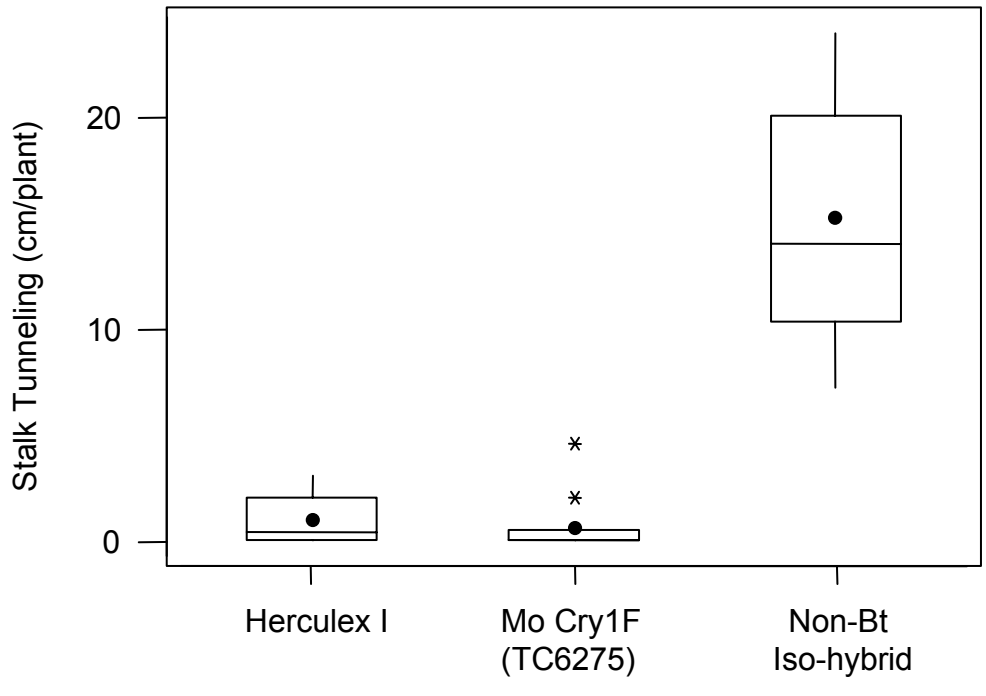


Figure 42. Efficacy of Event DAS-06275-8 and Event DAS-01507-1 for the control of 2nd generation ECB stalk tunneling damage (tunnels/stalk). Summary of 2001 and 2002 data. Data were obtained from trials conducted under notifications 02-060-17n in 2002 and 01-11607n in 2001.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
reps	4	2.502	1.028	0.257	0.77	0.556
Gen Name	2	64.766	64.766	32.383	96.79	0.000
Error	26	8.699	8.699	0.335		
Total	32	75.967				

CLEVEL 95.0000 SIGMA 0.578410
LSD_T 2.05553 LSD_TSIG 1.18894

CALCULATE LSD = LSD_TSIG * SQRT(1/Ni + 1/Nj)

Row	TRTLABEL	LSMEAN	TRTCNT	SIGNIF	COMPARE
1	Non-Bt Iso-hybrid	3.05217	12	A	X
2	Herculex I	0.15300	8	B	X*
3	Mo Cry1F (TC6275)	0.08800	13	B	*X

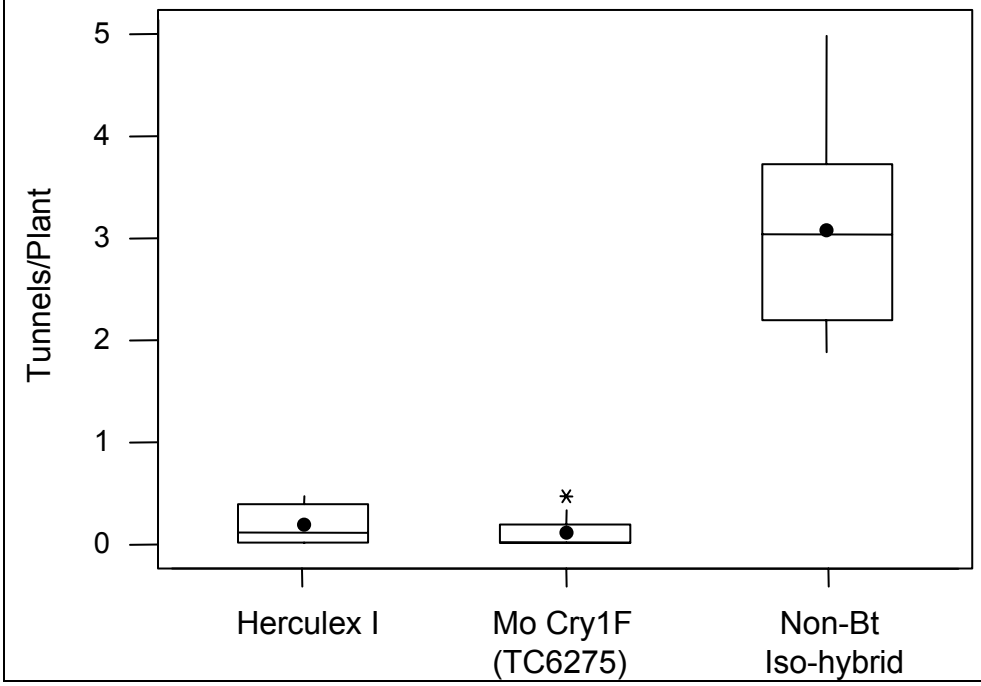


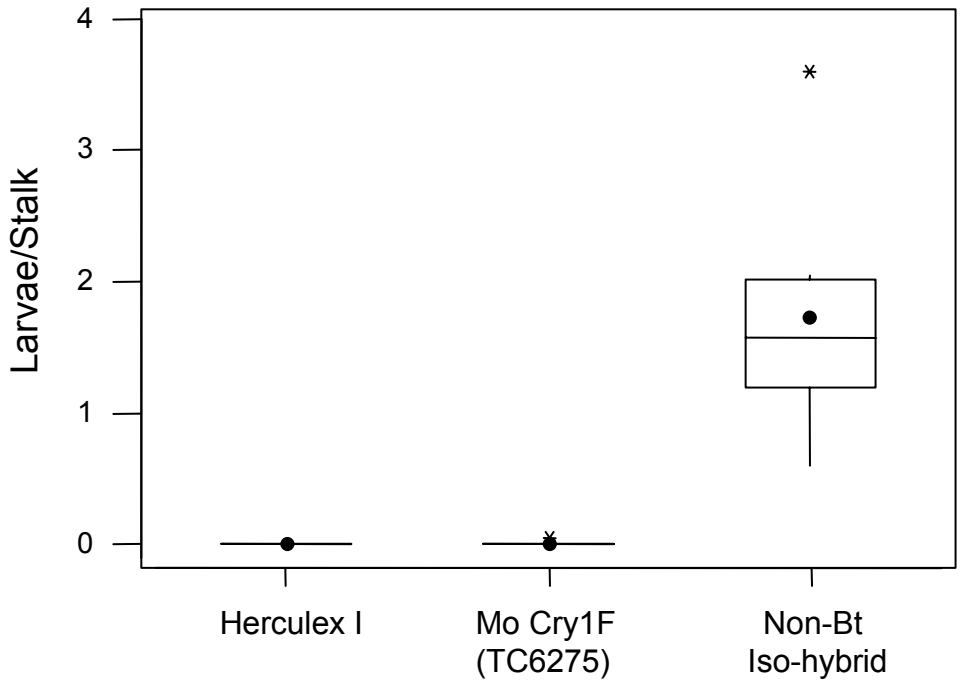
Figure 43. Efficacy of Event DAS-06275-8 and Event DAS-01507-1 for the control of 2nd generation ECB infesting stalks (Larvae/Stalk). Summary of 2001 and 2002 data. Data were obtained from trials conducted under notifications 02-060-17n in 2002 and 01-11607n in 2001.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
reps	4	1.5784	1.1250	0.2813	0.90	0.488
Gen Name	2	14.1111	14.1111	7.0556	22.70	0.000
Error	14	4.3519	4.3519	0.3108		
Total	20	20.0414				

CLEVEL	95.0000	SIGMA	0.557538
LSD_T	2.14479	LSD_TSIG	1.19580

CALCULATE LSD = LSD_TSIG * SQRT(1/Ni + 1/Nj)

Row	TRTLABEL	LSMEAN	TRTCNT	SIGNIF	COMPARE
1	Non-Bt Iso-hybrid	1.71750	8	A	X
2	Mo Cry1F (TC6275)	0.00500	9	B	X*
3	Herculex I	-0.00125	4	B	*X



In 2002, a replicated research trial was conducted at the Dow AgroSciences Coastal Research Center in Wayside MS. This trial was designed to evaluate the full season efficacy of Event DAS-06275-8 under conditions of high southwestern corn borer feeding pressure. Artificial infestations were employed to create conditions of high insect pressure (Table 20). Native flights of SWCB and corn earworm CEW (*Heliothis zea*) were also present in the plot and likely also contributed to the overall insect pressure in the trial.

Table 20. Infestation density and timing of southwestern corn borer onto plants used to evaluate Event DAS-06275-8 efficacy compared to Event DAS-01507-1 and a non-Bt isogenic hybrid. Data were obtained from a trial conducted under notification 02-060-17n in 2002.

Infestation Date	Crop Growth Stage	Infested Life Stage	Infestation Density per Plant	Infestation Method
22May02	V1	Egg	90	Egg Mass on Wax Paper
24May02	V2	Egg	90	Egg Mass on Wax Paper
28May02	V4	Egg	90	Egg Mass on Wax Paper
31May02	V5	Egg	90	Egg Mass on Wax Paper
28Jun02	Tassel	Egg	90	Egg Mass on Wax Paper
01Jul02	Tassel	Neonates	90	Neonates in corn grits
07Jul02	Tassel	Neonates	90	Neonates in corn grits

Insect feeding damage was evaluated first on June 19 when foliar injury evaluations were made. Ten plants from each plot were evaluated based on the Davis Scale (Davis et. al., 1992) which assigns a feeding score based on a 0-9 scale (Table 21). Evaluations of stalk tunneling were conducted for 1st generation on the 26th of June and for 2nd generation on the 19th of July. At each sampling interval, ten stalks from each plot were selected at random and split in half to reveal any damage. The number of tunnels per stalk, the length of those stalks and the presence of larvae in the stalks was recorded. The number of larvae in ears was also recorded as well as the area of ear feeding damage. The area of damage in the ears was not solely from SWCB feeding. Heliothis zea (corn earworm; CEW) were also present in ears and contributed significantly to the damage. All subsamples within each plot were averaged and the average score or measurement was used for analysis of variance and means separation.

Table 21. Davis rating scale for describing SWCB feeding damage to whorl stage corn.

RATING	DESCRIPTION
0	No visible damage
1	Only pinhole lesions present on whorl leaves
2	Pinhole and small circular lesions present on whorl leaves
3	Small circular lesions and a few small elongated (rectangular-shaped) lesions of up to 1.3 cm in length present on whorl and furl leaves
4	Several small to mid-sized 1.3 -2.5 cm in length elongated lesions present on a few whorl and furl leaves
5	Several large elongated lesions greater than 2.5 cm in length present on a few whorl and furl leaves and/or a few small to mid sized uniform to irregular shaped holes (basement membrane consumed) eaten from the whorl and/or furl leaves
6	Several large elongated lesions present on several whorl and furl leaves and/or several large uniform to irregular shaped holes eaten from the whorl or furl leaves
7	Many elongated lesions of all sizes present on several whorl and furl leaves plus several large uniform to irregular shaped holes eaten from the whorl and furl leaves
8	Many elongated lesions of all sizes present on most whorl and furl leaves plus many mid large sized uniform to irregular shaped holes eaten from the whorl and furl leaves.

9	Whorl and furl leaves almost totally destroyed

Event DAS-06275-8 demonstrated high levels of resistance to SWCB feeding damage at 1st and 2nd generation infestation timings (Table 22). Only one data set was available for analysis, however, these results suggest that the performance of DAS-06275-8 is comparable to Event DAS-01507-1 for the control of this pest. Highly significant protection from whorl feeding was measured. The average tunneling damage measurements (cm/stalk) also support significant differences between the isogenic non-Bt hybrid and the Event DAS-06275-8 hybrid. Damage recorded at 2nd generation timing also supports significant differences between the non-Bt isogenic hybrid and the Event DAS-06275-8 and the Event DAS-01507-1 hybrid. Significant parameters were: total tunnel length (cm/stalk), tunnels per stalk, and SWCB larvae per stalk. The parameters of SWCB larvae per ear and ear damage (cm^2 per ear) were not significant, possibly as the result of larval movement and or feeding from other species (CEW contributed to ear damage estimates).

Table 22. Efficacy of Event DAS-06275-8 for the control of southwestern corn borer infesting corn in a season long artificially infested field trial. Data were obtained from a trial conducted under notification 02-060-17n in 2002.

	1st generation			2nd Generation				
Treatment	**Guthrie Rating (0-9 Scale)**	**Total Tunnel Length (cm)**	**Tunnels per Plant**	**Total Tunnel Length (cm)**	**Tunnels per Plant**	**SWCB Larvae per Stalk**	**SWCB Larvae per Ear**	**Ear Damage (cm^2)***
Non-Bt Isogenic	8.4 a	17.7 a	2.17 a	7.2 a	2.35 a	1.4 a	.23 a	7.6 a
DAS-06275-8	0.2 b	0 b	0.5 a	0.07 b	0.0 b	0.025 b	0.0 a	1.9 a
Heculex I	0.2 b	0 b	0.0 a	0.00 b	0.025 b	0.0 b	0.125 a	1.6 a
F Test (p=)	0.000	0.000	0.114	0.008	0.000	0.002	0.37	0.034

*Also includes feeding damage from corn earworm

Three trials were conducted in 2002 (one in Indiana and two in Mississippi) to evaluate performance of FAW resistance in Event DAS-06275-8 compared to Event DAS-01507-1 and non-Bt isogenic hybrids. Replicate data were available for the trial in Indiana and one of the trials in Mississippie, however, only treatment averages were available for the trial conducted at the Brown Loam Branch Experiment Station in Mississippi. The trials were artificially infested as well as being exposed to naturally occurring populations of FAW. Plant resistance was determined for each plot by estimating the amount of foliar feeding injury based on the Davis scale described in Table 21. Replicate data from two locations and the average data from the third location was pooled and analyzed using analysis of variance and a Fisher's protected LSD test at the 0.05 confidence level.

The analysis of foliar injury data from three locations indicates that there were no statistically significant differences between the DAS-01507-1 and DAS-06275-8 events, however, both of these events conferred a statistically significant level of resistance to damage compared to the non-Bt isogenic hybrid (Figure 44).

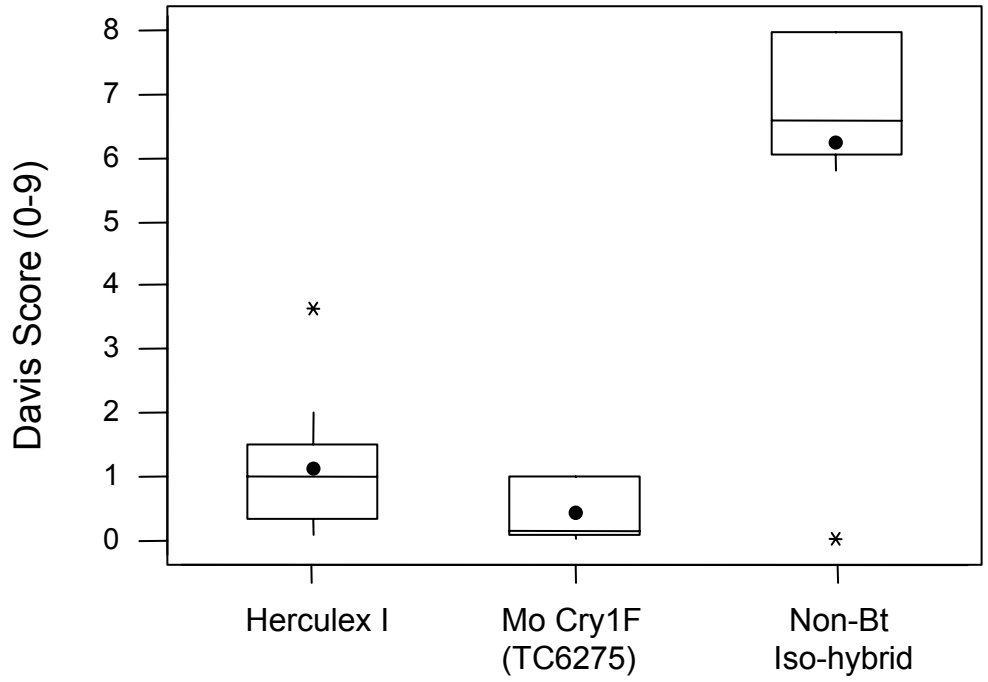
There were numerical differences between Event DAS-01507-1 and Event DAS-06275-8 with Event DAS-06275-8 having on average less damage. Overall pressure in these trials was high with an average Davis rating of 6.47 on the 0-9 scale and indicates that significant feeding pressure was present in these trials.

Figure 44. Efficacy of Event DAS-06275-8 for the control of FAW feeding damage to whorl stage corn compared to Event DAS-01507-1 and the non-Bt isogenic hybrids. Summary of 3 trials (9 reps). Data were obtained from trials conducted under notification 02-020-17n in 2002.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
REP	3	9.482	2.749	0.916	0.33	0.801
Gen Name	2	175.612	175.612	87.806	31.93	0.000
Error	21	57.747	57.747	2.750		
Total	26	242.841				

CLEVEL	95.0000	SIGMA	1.65827
LSD_T	2.07961	LSD	1.62567

Row	TRTLABEL	LSMEAN	TRTCNT	SIGNIF	COMPARE
1	Non-Bt Iso	6.47017	9	A	X
2	Herculex I	1.29811	9	B	X*
3	Mo Cry1F	0.64350	9	B	*X



A large plot black cutworm (BCW; *Agrotis ipsilon*) protocol was conducted at 9 locations in 2002 (3 trials in Indiana; 1 trial in Kentucky; 1 trial in Ohio; 3 trials in Missouri; and 1 trial in Iowa). The trials were designed to simulate commercial type plantings of corn infested with BCW. Plot locations were identified that might be attractive to natural populations of BCW and these locations were planted with the trials. Each trial was conducted as a randomized complete block design with 4 replicates. Plots were 8 rows wide and 40 ft long. Although these trials were targeted for areas that might be naturally infested with BCW, all of the locations received artificial infestations of BCW larvae to supplement any natural infestation that was present. Cut plants were measured during the course of the trial and at the end of the trial the number of cut plants or plants lost from BCW feeding damage was expressed as a percent. These data were pooled across locations and subject to analysis of variance. Means were separated using a Fishers protected LSD (p=0.05).

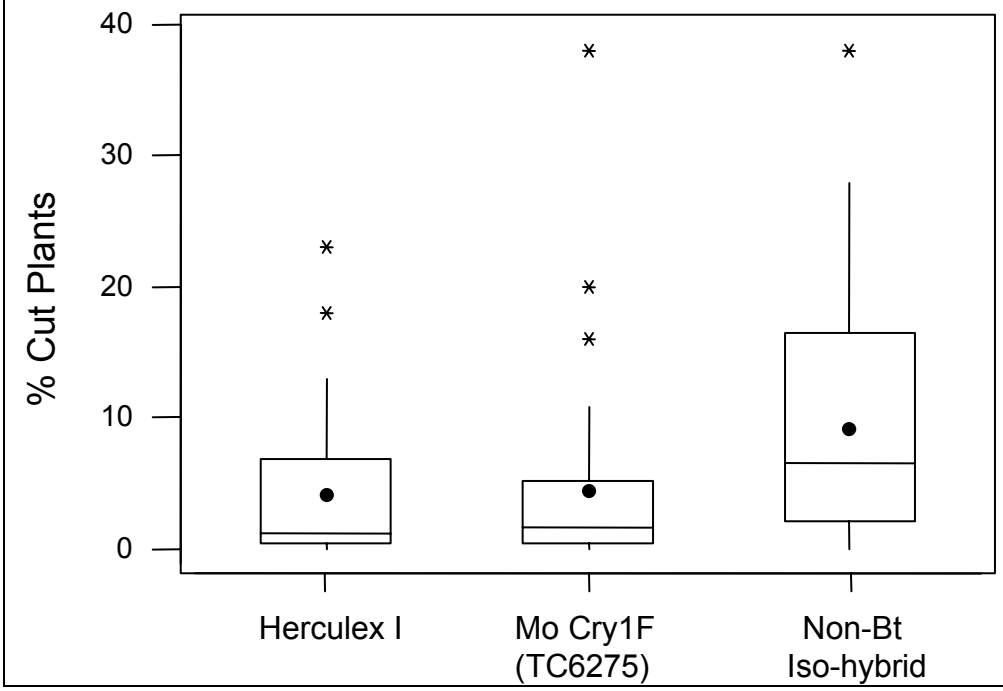
Event DAS-06275-8 was statistically less damaged than were plots planted to the non-Bt isogenic hybrid (Figure 45). Event DAS-01507-1 provided protection from stand loss that was significantly superior to the non-Bt isogenic hybrid and equal to the hybrids containing Event DAS-06275-8. These results suggest that Event DAS-06275-8 will match the benefits provided by Herculex I for the prevention of stand loss from BCW feeding damage.

Figure 45. Performance of Event DAS-06275-8 and DAS-01507-1 for the prevention of BCW mediated stand reduction in 2002 trials. Data were obtained from trials conducted under notification 02-060-17n in 2002.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
reps	3	2.19	2.19	0.73	0.01	0.998
Desc	2	557.30	557.30	278.65	4.54	0.013
Error	99	6079.30	6079.30	61.41		
Total	104	6638.79				

CLEVEL	95.0000	SIGMA	7.83627
LSD_T	1.98422	LSD	3.71689

Row	TRTLABEL	LSMEAN	TRTCNT	SIGNIF	COMPARE
1	Non-Bt Isogenic	9.14786	35	A	X
2	Mo Cry1F	4.40472	35	B	X*
3	Herculex I	4.12843	35	B	*X



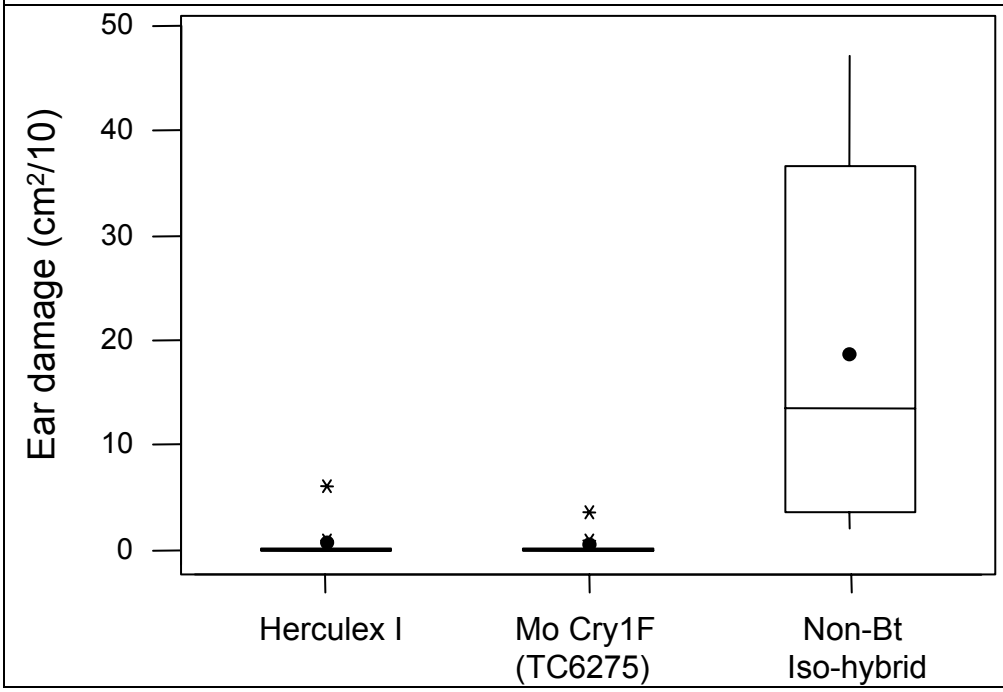
Event DAS-06275-8 was evaluated at two locations in 2002 (Nebraska) for protection. Both locations were infested with western bean cutworm WBCW (*Richia albicosta*). As a result of these infestations, statistically significant differentiation between treatment entries was noted with both DAS-06275-8 and Event DAS-01507-1 providing high levels of protection from ear feeding damage (Figure 46). At one location, the number of WBCW larvae per 10 ears was recorded for each plot and these data indicated that both Event DAS-06275-8 and Event DAS-01507-1 were significantly less infested than was the non-Bt isogenic hybrid. These results indicate that the efficacy of Event DAS-06275-8 is comparable to that of DAS-06275-8 and provides high levels of resistance to WBCW feeding damage.

Figure 46. Efficacy of DAS-06275-8 compared to Event DAS-01507-1 and a non-Bt isogenic hybrid for the prevention of western bean cutworm ear feeding. Data were obtained from trials conducted under notification 02-060-17n in 2002.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
reps	3	118.9	118.9	39.6	0.35	0.787
Gen Name	2	2169.9	2169.9	1084.9	9.68	0.001
Error	24	2689.3	2689.3	112.1		
Total	29	4978.1				

CLEVEL	95.0000	SIGMA	10.5856
LSD_T	2.06390	LSD	9.77056

Row	TRTLABEL	LSMEAN	TRTCNT	SIGNIF	COMPARE
1	Non-Bt Isogenic	18.2767	10	A	X
2	Hercuelx I	0.3567	10	B	X*
3	Mo Cry1F	0.1167	10	B	*X



Event DAS-06275-8 was evaluated at two locations in three trials in 2002 (2 trials in California and 1 in Mississippi). The California location was infested by both naturally occurring and artificially infested corn earworm CEW (*Heliothis zea*). The Mississippi location was infested by naturally occurring CEW. Small increases in resistance to damage from CEW were apparent between the Cry1F containing hybrids (Event DAS-01507-1 and Event DAS-06275-8) and the non-Bt isogenic hybrids, however, these differences were typically not statistically significant. The level of resistance to CEW feeding damage is consistent with the intermediate levels of resistance to CEW feeding damage provided by DAS-01507-1 hybrids (Table 23).

The level of resistance to CEW by Event DAS-06275-8 was numerically consistent with that described for Event DAS-01507-1. Event DAS-01507-1 is considered to provide suppression of CEW feeding damage and this categorization is also appropriate for describing the activity provided by Event DAS-06275-8.

Table 23. Efficacy of Event DAS-06275-8 for prevention of CEW feeding damage and ear infestations compared to Event DAS-01507-1 and a non-Bt isogenic hybrid. Data were obtained from trials conducted under notification 02-060-16n in 2002.

Hybrid Entry	Damage (cm^2)/ear	% Infested Ears	Damage Rating Scale 0-3	Small Larvae (L1-L2)/ear	Large Larvae (L3-L5)/ear
Trials with Data	*Mississippi*	*Mississippi*	*California*	*California* *Mississippi*	*California* *Mississippi*
Non-Bt Isogenic	5.3	82	2.03 a	0.25	0.64
Herculex I	2.9	56	1.39 b	0.39	0.56
DAS-06275-8	3.9	68	1.17 b	0.41	0.37
F Stat. (P=)	0.36	0.11	0.002	0.27	0.14

Therefore, The performance of Event DAS-06275-8 is statistically equivalent to that delivered by Event DAS-01507-1 (plant-optimized Cry1F, DAS-01507-1) for all pests evaluated.

V.E.2. AGRONOMIC CHARACTERISTICS

Two agronomic trials, encompassing 15 and 11 locations, respectively, in the US Corn Belt, were conducted in 2002. The first trial, designated Traits315, was a 113-day relative maturity trial and the second trial, designated Traits313, was a 100-day relative maturity trial. A list of trial locations is given in Table 24.

Table 24. Agronomic Performance Trials – conducted in 2002 in the US corn belt. Data were obtained from trials conducted under notifications 02-060-17n, 02-020-16n and 02-060-14n in 2002.

Trial Name	Affiliation	Location	Trial Type	ECB Pressure*
Traits315	Mycogen Seeds	DECATUR, IL	Agronomic	Low
Traits315	Mycogen Seeds	PONTIAC, IL	Agronomic	Low
Traits315	Mycogen Seeds	MACOMB, IL	Agronomic	Moderate
Traits315	Mycogen Seeds	WALKERTON, IN	Agronomic	Low*
Traits315	Mycogen Seeds	YORK, NE	Agronomic	Low
Traits315	Mycogen Seeds	ATLANTIC, IA	Agronomic	Low
Traits315	Mycogen Seeds	FOWLER, IN	Agronomic	Low
Traits315	Mycogen Seeds	SCHALLER, IA	Agronomic	Moderate
Traits315	Mycogen Seeds	SEYMOUR, IL	Agronomic	Low
Traits315	Mycogen Seeds	SULLIVAN, IN	Agronomic	Low
Traits315	Mycogen Seeds	COVINGTON, OH	Agronomic	Low
Traits315	Mycogen Seeds	DAVENPORT, IA	Agronomic	Low
Traits315	Mycogen Seeds	DAVID CITY, NE	Agronomic	Moderate
Traits315	Mycogen Seeds	HOLDREGE, NE	Agronomic	Low
Traits315	Mycogen Seeds	HUXLEY, A	Agronomic	Moderate
Traits313	Mycogen Seeds	AUSTIN, MN	Agronomic	Moderate
Traits313	Mycogen Seeds	JACKSON, MN	Agronomic	Low
Traits313	Mycogen Seeds	WATERTOWN, WI	Agronomic	Moderate
Traits313	Mycogen Seeds	KANAWHA, IA	Agronomic	Moderate
Traits313	Mycogen Seeds	MANTADOR, ND	Agronomic	Moderate
Traits313	Mycogen Seeds	OLIVIA , MN	Agronomic	Moderate
Traits313	Mycogen Seeds	SAUK CENTRE, MN	Agronomic	Low
Traits313	Mycogen Seeds	SCHALLER, IA	Agronomic	Moderate
Traits313	Mycogen Seeds	ARLINGTON, WI	Agronomic	Low
Traits313	Mycogen Seeds	ROCHELLE, IL	Agronomic	Moderate
Traits313	Mycogen Seeds	WALKERTON, IN	Agronomic	Low

* ECB Pressure based either on plot observation or stalk splitting data.

The design of the experiments was a random complete block (RCB) with two replications per location and two row plots. Row length was 20 feet and each row was seeded at 37 seeds/row, with subsequent thinning to a maximum of 32 plants/row. Standard regional agronomic practices were utilized and consistently applied across locations in both trials.

Entries in each trial included a DAS-06275-8 hybrid, a Cry1F DAS-01507-1 isogenic hybrid and a non-trangenic isogenic hybrid of either 113-day or 100-day relative maturity. All three entries (hybrids) had the same female parent within a given maturity (113-day or 100-day). The male parent was the same genetic background except when it carried either DAS-06275-8 or DAS-01507-1 events. The trait bearing inbreds were all homozygous for their respective trait. Both the DAS-01507-1 hybrids and the non-transgenic hybrids are commercial hybrids in the US. Data which were collected are representative of the type of data used by commercial corn seed companies to develop elite hybrids.

The thirteen agronomic characteristics on which data were taken and analyzed for each trial are listed in Tables 25 and 26. Generally, the parameters of yield (bushels/acre adjusted for moisture) and grain density (weight in pounds of a bushel of corn adjusted to 15.5% moisture) define reproductive capabililty of the hybrid. Percent moisture at harvest, accumulated growing degree-days to 50% pollen shed and silking (base 50) define the maturity of the hybrid. The agronomic characteristics of percent stalk, root lodging, dropped ears and top integrity determine the stability of a hybrid. Stand count and emergence vigor determine a measure of seed quality and early season growing conditions that may affect yield later in the season. Plant height, ear height and to some degree top integrity provide information about the appearance of the hybrids.

Table 25. Traits 315 trial: Agronomic characteristics of a 113-day relative maturity DAS-06275-8 hybrid as compared to the DAS-01507-1 and non-transgenic isogenic hybrids. Data were obtained from trials conducted under notifications 02-060-17n, 02-020-16n and 02-060-14n in 2002.

Trait	Late Hybrid Maize DAS-06275-8	Late Hybrid Maize DAS-01507-1 Near Iso	Late Near-isogenic hybrid	Number of locations	Number of reps	LSD[1]
Yield (bushels per acre)	184.6	186.1	183.4	15	30	11.7
Moisture (%)	20.4	19.7	19.8	15	30	0.7
Accumulated maize growing degree units to reach 50% pollen shed	1413.5	1433	1413.5	1	2	21.1
Accumulated maize growing degree days to reach 50% silking	1413.5	1443	1423.5	1	2	40.3
Grain density[2]	58.2	57.6	57.1	11	22	1.6
Plant height (inches)	89.6	92.1	91.3	10	20	2.9
Ear height (inches)	42.8	43.5	42.7	10	20	3
Final stand count (average number of plants per acre in thousands)	30.1	29.8	30.9	15	30	4.7
Visual rating of emergence vigor from spike to one-leaf stage[3]	7.0	7.0	7.0	1	2	0
Stalk lodging[4]	2.9	0.3	1.7	15	30	1.5
Root lodging[4]	2	2.9	2.2	15	30	4.3
Dropped ears per plot	0.0	0.0	0.0	11	22	0.1
Top integrity[5]	7.9	7.9	6.5	10	20	0.5

1 Least Significant Difference at the 0.05 level

2 Weight (in pounds) of a bushel of grain at 15.5% moisture
3 1-9 visual scale, with 9 being best
4 Percent of plants per plot that showed lodging of the specified type
5 1-9 visual scale that describes how well the stalks remain intact above the ear, with 9 being best

Table 26. Traits313 trial: Agronomic characteristics of a 100-day relative maturity DAS-06275-8 hybrid as compared to the DAS-01507-1 and non-transgenic isogenic hybrids. Data were obtained from trials conducted under notifications 02-060-17n, 02-020-16n and 02-060-14n in 2002.

Trait	Early Hybrid Maize DAS-06275-8	Early Hybrid Maize DAS-01507-1 Near Iso	Early Near-isogenic hybrid	Number of locations	Number of reps	LSD[1]
Yield (bushels per acre)	179.1	179.3	157.3	11	22	11.3
Moisture (%)	20.5	20.3	20.2	11	22	0.8
Accumulated maize growing degree units to reach 50% pollen shed	1279.6	1288.4	1276.1	4	7	11.5
Accumulated maize growing degree days to reach 50% silking	1314.3	1325.4	1298.6	4	7	11.0
Grain density[2]	55.5	55.9	56.2	11	22	0.8
Plant height (inches)	79.5	83	78.3	4	8	4.7
Ear height (inches)	33.9	35.1	34.6	4	8	4
Final stand count (average number of plants per acre in thousands)	31.6	31.1	29.4	11	22	1.7
Visual rating of emergence vigor from spike to one-leaf stage[3]	6.75	6.5	5.75	2	4	0.48
Stalk lodging[4]	0.4	1.5	3	11	22	1.3
Root lodging[4]	11.5	10.8	9.3	11	22	4.6
Dropped ears per plot	0	0.3	0.4	9	18	0.3
Top integrity[5]	6.4	5.7	5.7	11	22	0.5

[1]Least Significant Difference at the 0.05 level
[2] Weight (in pounds) of a bushel of grain at 15.5% moisture
[3] 1-9 visual scale, with 9 being best
[4] Percent of plants per plot that showed lodging of the specified type
[5] 1-9 visual scale that describes how well the stalks remain intact above the ear, with 9 being best

In both trials, the primary yield comparison was between DAS-06275-8 hybrid and the DAS-01507-1 hybrid. This comparison factors out the influence of the European corn borer (ECB)

feeding on yield. ECB pressure across most the Traits315 trial locations in 2002 was light, with only a few trial locations receiving moderate damage (Table 24). Because of the light ECB pressure, no statistical difference (via least significance difference (LSD) calculations at the 0.05 level) in yield was observed between all three hybrids (DAS-06275-8, DAS-01507-1, non-transgenic) in the Traits315 trial (Table 25). In the Traits313 trial, the DAS-06275-8 hybrid was not significantly different from the DAS-01507-1 isogenic hybrid, however, both transgenic hybrids (DAS-06275-8 and DAS-01507-1) were significantly different from the non-transgenic control hybrid (Table 26). The reason for this difference was due to the protection that the Cry1F protein provided against ECB damage in the Traits313 trial (Table 24). There were no significant differences in either trial between the three hybrids in test weight indicating that grain density was not changed by the presence of event DAS-06275-8 in the hybrid.

Maturity was best assessed by the comparison between the isogenic hybrid and DAS-06275-8 hybrid. No significant differences were detected in either trial, however, there was a trend of the DAS-06275-8 hybrid being a slightly later maturity than the non-transgenic hybrid in the Traits315 trial. This trend was not seen in the Traits313 trial. The DAS-06275-8 hybrid had significantly more moisture than the DAS-01507-1 hybrid in the Traits315 trial (Table 25) but not in the Traits313 trial (Table 26). These trends are most likely associated with slight genetic differences in dry down and are not of consequence to hybrid commercial use patterns. Maturity is calculated by gathering data on growing degree units to 50% pollen shed and silking. There were no significant changes in flowering between any of the three late maturity hybrids (Traits315 trial; Table 25). The early maturity DAS-06275-8 hybrid did not significantly differ in time to pollen shed in comparison to either the DAS-01507-1 hybrid or the non-transgenic hybrid. There was a trend in Traits313 trial for the DAS-06275-8 hybrid to flower later than the non-transgenic hybrid. The DAS-01507-1 hybrid also appeared to flower later than the DAS-06275-8 hybrid in this trial (Table 26). These trends, while statistically significant, are minor and since percent moisture did not differ between the hybrids, there would be no change in the use pattern of the commercial hybrids.

There were no significant differences observed in the final stand and emergence vigor between hybrids in the Traits315 trial. There were significantly fewer non-transgenic plants than transgenic plants in the Traits313 trial. There were also significantly higher early vigor ratings between the transgenic hybrids and the non-transgenic hybrid in this trial. The populations in both trials contained more than 29,000 plants per acre, which indicated that, almost all of the seeds germinated at all locations. The early vigor ratings were within a range that was indicative of plants that were thrifty upon emergence.

No significant differences were observed between the three hybrids in both trials for plant height and ear height, indicating that the three hybrids had a similar appearance. Percent stalk lodging, root lodging, dropped ears and top integrity are all measurements of a hybrid's ability to be harvested. No significant difference was found in either trial for root lodging and dropped ears between the three hybrids. In the Traits315 trial, there was significantly more stalk lodging in the DAS-06275-8 hybrid than was observed in the DAS-01507-1 hybrid, however, no significant differences were observed between the DAS-06275-8 hybrid and non-transgenic hybrid (Table 25). Significant differences in stalk lodging in the Traits313 trial were observed between the non-transgenic and the transgenic hybrids. No differences in stalk lodging were observed between the hybrids in the Traits313 trial (early maturity). These differences in stalk lodging are most likely caused by slight genetic differences in trait bearing inbreds resulting from the conversion process. The differences do no appear to be related to the transgenic events, and from a commercial perspective, these differences are small and would not affect how the hybrid is used. In both trials, DAS-06275-8 hybrids had significantly better top integrity than the non-transgenic isogenic hybrid. This is what would be expected from a gene that provides full season control of ECB. The overall top integrity is better in Triats315 hybrids than in the Traits313 hybrids. These differences are known to exist in the non-transgenic hybrid, and are seen in both transgenic and non-transgenic comparisons (Tables 25 and 26).

None of the statistically significant differences observed between the three hybrids in either of these trials (Traits315 and Traits313) are likely caused by the presence of the *cry1F* gene inevents DAS-06275-8 or DAS-01507-1, with the exception of top integrity. Furthermore, none of the differences are biologically or commerically significant. The overall range of values for the measured parameters are all within the range of values obtained for traditional maize hybrids and would not lead to a conlcusion of increased weediness.

B.t. Cry1F maize line 6275 was also tested for germination under both cold and warm growing conditions. The cold test is a stress test on seed simulating early spring planting conditions in the Midwestern US. Field soil is spread in a thin layer over a wet germination towel and chilled overnight. The following day, hybrid test seeds (e.g., *B.t.* Cry1F maize line 6275) are placed into the soil. The test seed is placed in a 10°C (50°F) chamber for seven (7) days. After the incubation period, the test seed is moved to a 25°C (78°F) chamber for a three-day growout period. The average percentage of germination of the test seed is compared to seed from four hybrids grown under the same conditions. The warm germination test is a germination test required by federal regulations governing seed quality and the test is conducted under optimal growing conditions. Test seed is placed between sheets of germination toweling. The toweling is then rolled inside a sheet of polyethylene coated paper. The rolls are placed at 25°C (78°F) chamber at 90% relative humidity for five (5) days. After the incubation period, the average percentage of germination is calculated and compared to standard germination percentages for maize.

The results of the cold and warm germination tests are given in Table 27. The results demonstrated that *B.t.* Cry1F maize line 6275 had germination percentages that were very similar to the control hybrid seed. The results show that the *B.t.* Cry1F maize line 6275 is comparable to other maize hybrids in seed germination characteristics.

Table 27. Germination of seed from *B.t.* Cry1F maize line 6275.

Cold Germination	Warm Germination
Maize Line 6275 91	**Maize Line 6275** 99
Control Hybrids 94	**Expected Range** 92% to 100%

V.F. SECONDARY METABOLITES AND ALLERGENIC POTENTIAL OF PROTEINS IN LINE 6275

Since the Cry1F protein expressed in line 6275 is the same protein (determined by sequence analysis) as expressed in the previously deregulated event (00-136-01p), the studies conducted to ascertain allergenic potential for the previous event pertain to event DAS-06275-8. The results of those studies were 1) there was no sequence homology to known allergens; 2) the Cry1F protein rapidly digested in simulated gastric fluid; and 3) the Cry1F protein was not biologically active following exposure to elevated temperature (>75°C). For the BAR protein expressed in line 6275, a sequence homology evaluation scheme was used to assess the similarity of the BAR protein to known protein allergen sequences contained in several widely accepted databases (Song, 2002; Appendix 2). No immunologically significant sequence identity (a match of at least eight contiguous identical amino acids) was detected indicating no homology to known allergens. *In vitro* simulated gastric fluid (SGF) digestibility study indicated that BAR was rapidly digested (<0.5 minutes) as demonstrated by SDS-PAGE and western blot analyses (Korjagin, 2003; Appendix 2). Neutralized BAR protein was readily visible on the SDS-PAGE and western blot at less than 2% of the amount of the BAR protein loaded in the digestion samples. Therefore, the point at which >98% of the protein is degraded is <0.5 minutes (DT_{98} = <0.5 minutes).

The results of the previous studies conducted for Cry1F (in petition 00-136-01p) and the results of the studies conducted on the BAR protein indicate that the Cry1F and PAT proteins do not exhibit characteristics commonly attributed to allergenic proteins.

Grain was also measured for the content of known anti-nutrients of corn—phytic acid and trypsin inhibitor. The results are presented in Table 28. The values of phytic acid and trypsin inhibitor were within the reported literature ranges, however, there was a statistical difference noted between the hybrid line 6275 and the control line. This was probably due to differences noted between several locations. However, these differences were isolated and were not observed across all locations.

Table 28. Summary analysis of anti-nutrients from hybrid line 6275 and the control hybrid line (near isoline CHPH09B/2MW). The samples were collected from the field trial conducted in maize growing regions of Chile in 2001/2002.

Analyte[1,2]	Literature Range[3]	6275H Mean[4]	CH Mean[4]	Standard Error
Phytic acid	0.45 – 1.0[6]	0.561	0.536	0.02
Trypsin Inhibitor (TIU/g)	NR[5]	1.82	2.07	0.07

[1] Percent of dry weight
[2] Abbreviation: TIU, trypsin inhibitor units
[3] Watson, 1982
[4] Least square means
[5] NR: Not reported
[6] Organisation for Economic Cooperation and Development

VI. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION

Field tests of *B.t.* moCry1Fmaize line 6275 and non-modified hybrid lines demonstrated no significant differences apart from the intended change, i.e., resistance to the European corn borer and certain other Lepidopterous pests. No differences in morphology, disease or pest resistance between *B.t.* moCry1F line 6275 and previously considered *B.t.* Cry1F-containing maize lines were noted. Thus, there is no reason to believe that the cultivation of *B.t.* moCry1F maize line 6275 and its progeny will have any environmental effects different from cultivation of other *B.t.* Cry1F-containing maize lines, which have already been considered by APHIS (Shanahan & Stauffer, 2000).

VI.A. ESTIMATED ENVIRONMENTAL CONCENTRATION

Expression data indicate that *B.t.* Cry1F maize line 6275 plants express the Cry1F protein at levels in the pollen and grain which are one-sixth (1/6) and one-half (½) of the respective concentrations in *B.t.* Cry1F maize line 1507 plants. Thus, the estimated environmental concentrations for Cry1F *B.t.* event DAS-01507-1 (in petition 00-136-01p) conservatively represent the estimated environmental concentrations for DAS-06275-8.

VI.B. EXPOSURE TO NON-TARGET ORGANISMS AND HUMANS

Exposure to non-target organisms

The mo*cry*1F (maize optimized) gene encodes for the identical truncated Cry1F protein as that expressed by maize plants containing the po*cry*1F (plant optimized) gene (USDA 00-136-01p). Codon changes were made to the gene to improve expression in moCry1F maize plants, but these changes did not alter the amino acid sequence of the protein as compared to that expressed in poCry1F maize plants (Duck and Coats, 2000; Schafer, 2002; Appendix 2).

Based on the above, the specificity (i.e., toxicity toward target pests) of the Cry1F protein expressed in moCry1F and poCry1F plants is expected to be similar. Expression data indicate that *B.t.* Cry1F maize line 6275 plants express the Cry1F protein at levels in the pollen and grain which are one-sixth (1/6) and one-half (½) of the respective concentrations in *B.t.* Cry1F maize line 1507 plants. Testing with microbially-derived Cry1F protein at levels greatly exceeding the expression levels found in Cry1F transgenic plants resulted in no effects on several beneficial species (Shanahan and Stauffer, 2000). Therefore, the data presented in the previous petition (00-136-01p) adequately addresses toxicity to humans and non-target organisms for *B.t.* Cry1F maize line 6275 plants.

Effects on endangered species

No change in the rationale from the previous petition (00-136-01p). Since pollen and grain expression for *B.t.* Cry1F maize line 6275 is substantially lower than considered in petition 00-136-01p, there are extra margins of safety for *B.t.* Cry1F maize line 6275 over that considered previously.

Dietary exposure

The estimated worst case 95th percentile per capita exposure for acute dietary consumption of maize-optimized (mo) Cry1F protein in corn and corn products is 0.022 mg (kg BW)$^{-1}$ for the most sensitive population (non-nursing infants, less than 1 year old) (Wolt, 2003; Appendix 2). This exposure estimate is based on the maximum measured residue level for Cry1F protein in line 6275 corn grain (1.68 mg kg^{-1}, range 0.62 – 1.68 mg kg^{-1}).

This screening level assessment is very conservative in that it uses maximum anticipated residue levels in all corn and corn products, it considers 100% of crop treated (that is Cry1F protein occurrence in all corn and corn products), and it includes no factors to account for food processing. As an example of the conservatism of the worst case estimate, if high fructose corn syrup and corn oil are excluded from analysis, since these products will not contain protein, the 95th percentile per capita exposure estimate decreases to 0.0043 mg (kg BW)$^{-1}$ for non-nursing infants, less than 1 year old.

In the acute oral toxicity study for Cry1F protein, the gavage dose to mouse was greater than 5050 mg microbial protein (kg BW)$^{-1}$. The dose level in the acute gavage study was restricted due to protein purity and dose volume limitations. When adjusted for purity of the test material (11.4%), the acute dose level in this study was 576 mg (kg BW)$^{-1}$. At this dose level, no LD_{50} was demonstrated. The dose represents a value 26,736 × greater than estimated worst case 95th percentile dietary exposure and can be used as a conservative estimate of the margin of exposure. Therefore, an adequate dose level was attained in the acute mouse study to conservatively determine an acute effect level for Cry1F protein.

VI.C. WEEDINESS OF *B.T.* CRY1F MAIZE LINE 6275

No change in the rationale from the previous petition (00-136-01p).

VI.D. PROPOSED INSECT RESISTANCE MANAGEMENT PLAN

The mo*cry*1F (maize optimized) gene encodes for the identical truncated Cry1F protein as that expressed by maize plants containing the po*cry*1F (plant optimized) gene (Petition 00-136-01p). Codon changes were made to the gene to improve expression in moCry1F maize plants, but these changes do not alter the amino acid sequence of the protein as compared to that expressed in poCry1F maize plants (Duck and Coats, 2000; Appendix 2).

Based on the above, the specificity (i.e., toxicity toward target pests) of the Cry1F protein expressed in moCry1F and poCry1F plants is expected to be similar. Expression data show concentrations of Cry1F in the tissue primarily consumed by corn borers (i.e., stalks) are up to 50% greater in plants from moCry1F event DAS-06275-8 than in plants from poCry1F event DAS-01507-1 (Zabik *et al.,* 2003). Expression in other relevant tissues is similar for the two events. Furthermore, field data indicate that the efficacy of moCry1F against the target pests is equal to or greater than that for poCry1F event DAS-01507-1 (Babcock and Bing, 2003). The poCry1F event DAS-01507-1 has been shown to be expressed at high dose against the key target pests by two methods (MRID # 45131101 and # 45307701). Babcock et al. (2003) provides data indicating that moCry1F event DAS-06275-8 is also a high dose event against the European corn borer (*Ostrinia nubilalis*).

There is, therefore, sufficient information to support the implementation of the same product durability plan for moCry1F event DAS-06275-8 as for poCry1F event DAS-01507-1 (MRID #45020116), which is the Industry Unified Plan. The plan consists of a 20% non-Bt refuge (50% in certain cotton-growing counties), which must be planted within ½ mile of the Bt corn, and may be treated with insecticides for control of European corn borer, southwestern corn borer, fall armyworm and black cutworm if an economic threshold is reached. Other insect resistance management-related elements of the conditions of registration for DAS-01507-1 (EPA Registration 68467-2) are equally applicable to insect resistance management for DAS-06275-8.

VI.E. POTENTIAL CHANGES IN AGRICULTURAL PRACTICES ASSOCIATED WITH THE USE OF HERBICIDE TOLERANT MAIZE LINES

Approximately 80 million acres of maize are planted annually in the United States, and nearly all of these acres are treated with herbicide. Products are applied pre-plant, pre-emergence and post-emergence. In general, maize receives a soil applied herbicide application followed by a post-emergence application. Maize line 6275 is glufosinate tolerant, and as such, provides an alternative weed management tool to growers. Glufosinate-ammonium is a broad spectrum, post-emergence herbicide. Such an herbicide could provide growers the opportunity to move away from pre-emergence, residually active compounds and could increase the amount of conservation and no-till acres of maize planted in the United States.

The only other glufosinate-ammonium tolerant agricultural crop currently on the market is canola, which is not typically a rotational crop with maize. Maize line 6275 is still susceptible to other herbicides normally used to control maize should it appear as a volunteer weed in other crops. For example, in soybean, the crops most commonly rotated with maize, herbicides based on sulfonylurea, lipid biosynthesis inhibitors or Fluazifop/fomesafen could be used to control maize volunteers.

It is estimated that approximately 10 million acres of herbicide tolerant maize will planted in 2000-2001. This includes other glufosinate- and glyphosate-tolerant maize developed through

recombinant technology as well as imidizolinone tolerant maize developed through selected mutagenesis and traditional plant breeding. If line 6275 crosses with corn lines expressing resistance to herbicides with different modes of action, corn volunteers with multiple herbicide resistance may emerge. However, the competition from the pollen load within a given field would keep the incidence of this very low. Additionally, various agronomic practices including appropriate variety selections and crop rotation, and rotation of herbicides with different modes of action can be used to avoid or manage volunteer corn resistant to one or a few herbicides.

Maize lines that contain both *B.t.* and herbicide tolerance have been on the market since 1997 and have had a positive impact on agricultural practices. These products have provided simple, inexpensive yet highly effective means of controlling lepdopteran pests; have been environmentally benign so they preserve beneficial insects, decrease cultivation needs, and require less total pounds of active pesticidal compounds (both insecticidal and herbicidal).

VI.F. VERTICAL TRANSFER OF THE INTRODUCED GENETIC MATERIAL

No change in the rationale from the previous petition (00-136-01p).

VI.G. HORIZONTAL TRANSFER OF THE INTRODUCED GENETIC MATERIAL

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; Redenbaugh *et al.*, 1994). Even if such a transfer were to take place, transfer of the *cry*1F or *bar* gene from *B.t.* Cry1F maize line 6275 would not present a human health or plant pest risk. Genes encoding the BAR protein and similar acetyl transferases are found in nature. Similarly, the *cry*1F gene in *B.t.* Cry1F maize line 6275 was isolated from *Bacillus thuringiensis* var. *aizawai,* which is a ubiquitous soil bacterium that produces a variety of Cry proteins including Cry1F protein. Recipient *Bacillus thuringiensis* species would therefore not pose a greater plant pest risk than the wild type microbes from which the genes originated.

The *spc* and *tet* genes coding for resistance to the antibiotics spectinomycin and tetracycline, respectively, were demonstrated to not be present in *B.t.* Cry1F maize line 6275. The T-DNA fragment (designated insert PHP12537) used to transform *B.t.* Cry1F maize line 6275 did not contain the *spc* or *tet* genes, although these genes were present on the original plasmid vector PHP12537. Therefore, there are no concerns for occurrence and effects of antibiotic resistance markers in the environment.

VII. ADVERSE CONSEQUENCES OF INTRODUCTION

The evidence provided in this petition supports the conclusion that *B.t*. Cry1F maize line 6275 presents low risk to human health and the environment and does not present a plant pest risk. In 2001, EPA issued a final rule exempting Cry1F from the requirement of a tolerance in field corn, sweet corn and popcorn when used as a plant incorporated protectant (PIP) [Federal Register: June 6, 2001 (Volume 66, Number 109)] [Rules and Regulations] [Page 30321-30325]. In exempting Cry1F, EPA concluded that ``there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information."

Data that were submitted demonstrated the lack of mammalian toxicity at high levels of exposure to the pure Cry1F protein. These data demonstrated the safety of the products at levels well above maximum possible exposure levels that are reasonably anticipated in the crops. The acute oral toxicity data submitted supported the prediction that the Cry1F protein would be non-toxic to humans. Male and female mice (5 of each) were dosed with 15% (w/v) of the test substance, which consisted of Bacillus thuringiensis var. aizawai Cry1F protein at a net concentration of 11.4%. Two doses were administered approximately an hour apart to achieve the dose totaling

33.7 mL/kg body weight. Outward clinical signs and body weights were observed and recorded throughout the 14 day study. Gross necropsies performed at the end of the study indicated no findings of toxicity. No mortality or clinical signs were noted during the study. An LD_{50} was estimated at >5,050 mg/ kg body weight of this microbially produced test material. The actual dose administered contained 576 mg Cry1F protein/kg body weight. At this dose, no LD_{50} was demonstrated as no toxicity was observed. Grain of *B.t*. Cry1F maize line 6275 contains a maximum of 1.68 μg of Cry1F/gram of corn kernel tissue. On the basis of worst case estimates for Cry1F residues in corn and corn products, the dose used in the acute mouse study conservatively represents a margin of exposure 26,736 × greater than the 95th percentile dietary exposure for the most sensitive US population (Wolt, 2003; See Appendix 1 for calculations).

Further, amino acid sequence comparisons submitted to EPA showed no similarity between Cry1F protein to known toxic proteins available in public protein databases. Data were also submitted demonstrating that the Cry1F protein was rapidly degraded by gastric fluid *in vitro* and was non-glycosylated. In a solution of Cry1F:pepsin at a molar ratio of 1:100, 98% degradation of 98% Cry1F to amino acids and small peptides occurred in less than 0.5 minutes, and complete degradation occurred within 5 minutes. A heat lability study demonstrated the loss of bioactivity of Cry1F protein to neonate tobacco budworm larvae after 30 minutes at 75^{o}C. Thus, EPA concluded that the potential for the Cry1F protein to be a food allergen is minimal.

Based on exposure estimates and the results of toxicological studies, there is low risk to non-target organisms and beneficial insects from expression of the Cry1F protein in maize line 6275. *B.t*. Cry1F maize line 6275 exhibits typical agronomics and normal Mendelian inheritance of the introduced genetic material. There has been no evidence of increased susceptibility to insect pests or disease in *B.t.*Cry1F maize line 6275 when compared to conventional maize hybrids.

The BAR (PAT) protein present in maize line 6275 is the expression product of the *bar* gene. The PAT protein from both the *bar* and *pat* genes has been studied extensively and has been found to be safe for consumption in food or feed (OECD, 1999). In the US, the USEPA established an exemption from the requirement of a tolerance for residues of the plant-pesticide ingredients phosphinothricin acetyltransferase (PAT) and the genetic material necessary for its production in all plants (USEPA, 1997).

The commercial introduction of transgenic maize expressing the *B.t.* toxin has provided growers with a simple, cheap, highly effective, and environmentally benign means of controlling the European corn borer and other pests. This in turn has lead to a rapid adoption of this technology by the agricultural community. The high rate of adoption has resulted in fears that ECB will develop resistance to *B. thuringiensis* proteins when expressed in plants, despite the fact that ECB has not developed field resistance to any class of insecticides. Stable laboratory resistance to *B.t.* proteins at levels comparable to plant expression also has not been reported, despite many efforts to achieve this.

Hybrids derived from *B.t*. Cry1F maize line 6275 will be the second maize hybrids to use an insect control protein derived from *Bacillus thuringiensis* var. *aizawai*. These hybrids demonstrate a different spectrum of activity against target Lepidopteran pests than do commercial corn hybrids expressing other *B.t.* proteins. Field research trials with *B.t.*Cry1F maize line 6275 have indicated that Cry1F can control maize pests, such as fall armyworm, southwestern corn borer, and black cutworm, that have not previously been well-controlled by other *B.t.* proteins expressed in plants.

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X. APPENDICES

1. DIETARY EXPOSURE CALCULATIONS

2. EPA SECTION 3 REGISTRATION SUBMITTED STUDIES [CBI]

Appendix 1. MAIZE-OPTIMIZED CRY1F: ESTIMATE OF ACUTE DIETARY MARGIN OF EXPOSURE

April 30, 2003

The estimated worst case 95th percentile per capita exposure for acute dietary consumption of maize-optimized (mo) Cry1F protein in corn and corn products is 0.022 mg (kg BW)$^{-1}$ for the most sensitive population (non-nursing infants, less than 1 year old). This exposure estimate is based on the maximum measured residue level for Cry1F protein in moCry1Fcorn grain (1.68 mg kg^{-1}, range 0.62 – 1.68 mg kg^{-1}).

This screening level assessment is very conservative in that it uses maximum anticipated residue levels in all corn and corn products, it considers 100% of crop treated (that is Cry1F protein occurrence in all corn and corn products), and it includes no factors to account for food processing. As an example of the conservatism of the worst case estimate, if high fructose corn syrup and corn oil are excluded from analysis, since these products will not contain protein, the 95th percentile per capita exposure estimate decreases to 0.0043 mg (kg BW)$^{-1}$ for non-nursing infants, less than 1 year old.

In the acute oral toxicity study for Cry1F protein, the gavage dose to mouse was greater than 5050 mg microbial protein (kg BW)$^{-1}$. The dose level in the acute gavage study was restricted due to protein purity and dose volume limitations. When adjusted for purity of the test material (11.4%), the acute dose level in this study was 576 mg (kg BW)$^{-1}$. At this dose level, no LD_{50} was demonstrated. The dose represents a value 26,736 × greater than estimated worst case 95th percentile dietary exposure and can be used as a conservative estimate of the margin of exposure. Therefore, an adequate dose level was attained in the acute mouse study to conservatively determine an acute effect level for Cry1F protein.

Jeffrey D. Wolt, PhD

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Filename: L:\My Documents\DEEM data\moCry1F.RS7          Chemical: moCry1F corn
RfD(Chronic): 0 mg/kg bw/day  NOEL(Chronic): 0 mg/kg bw/day
RfD(Acute): 0 mg/kg bw/day  NOEL(Acute):  576 mg/kg bw/day
Date created/last modified: 04-30-2003/08:52:01/7          Program ver. 7.81
Comment: maximum grain expression for all corn and corn products
```

Food Code	Crop Grp	Food Name	Def Res (ppm)	Adj.Factors #1	#2	Comment
267	15	Corn grain-bran	1.680000	1.000	1.000	worst
		Full comment: worst case grain expression				
266	15	Corn grain-endosperm	1.680000	1.000	1.000	
289	15	Corn grain-oil	1.680000	1.000	1.000	
268	15	Corn grain/sugar/hfcs	1.680000	1.500	1.000	
388	15	Corn grain/sugar-molasses	1.680000	1.500	1.000	
237	15	Corn/pop	1.680000	1.000	1.000	
238	15	Corn/sweet	1.680000	1.000	1.000	

Case 1: Maximum grain expression in all corn and corn products.

```
Dow AgroSciences                                                    Ver. 7.81
DEEM ACUTE Analysis for MOCRY1F CORN                          (1994-96 data)
Residue file: moCry1F.RS7                    Adjustment factor #2 NOT used.
Analysis Date: 04-30-2003/08:45:05    Residue file dated: 04-30-2003/08:43:07/7
NOEL (Acute) = 576.000000 mg/kg body-wt/day
Daily totals for food and foodform consumption used.
Run Comment: "maximum grain expression for all corn and corn products"
================================================================================

Summary calculations (per capita):
```

	95th Percentile Exposure	95th Percentile MOE	99th Percentile Exposure	99th Percentile MOE	99.9th Percentile Exposure	99.9th Percentile MOE
U.S. Population:	0.009073	63483	0.015980	36045	0.030035	19177
All infants:	0.020624	27928	0.043574	13218	0.068140	8453
Nursing infants (<1 yr old):	0.009549	60318	0.026599	21655	0.026753	21530
Non-nursing infants (<1 yr old):	0.021544	26736	0.044451	12958	0.068104	8457
Children 1-6 yrs:	0.017440	33027	0.026115	22055	0.044668	12895
Children 7-12 yrs:	0.012727	45256	0.020818	27668	0.038276	15048
Females 13+ (preg/not nursing):	0.006953	82845	0.008922	64561	0.009222	62460
Females 13+ (nursing):	0.006009	95852	0.009196	62632	0.010512	54793

Case 2: Maximum grain expression in corn and corn products, exclusive of HFCS and oil.

```
Dow AgroSciences                                                    Ver. 7.81
DEEM ACUTE Analysis for MOCRY1F CORN                          (1994-96 data)
Residue file: moCry1F.RS7                        Adjustment factor #2 NOT used.
Analysis Date: 04-30-2003/08:54:53    Residue file dated: 04-30-2003/08:52:01/7
NOEL (Acute) = 576.000000 mg/kg body-wt/day
Daily totals for food and foodform consumption used.
Run Comment: "maximum grain expression for corn and corn products exclusive of
 oil anf HFCS"
================================================================================
```

Summary calculations (per capita):

	95th Percentile		99th Percentile		99.9th Percentile	
	Exposure	MOE	Exposure	MOE	Exposure	MOE
U.S. Population:						
	0.003489	165099	0.007464	77170	0.016089	35800
All infants:						
	0.003862	149142	0.010199	56476	0.013666	42148
Nursing infants (<1 yr old):						
	0.000563	>1000000	0.002865	201068	0.013594	42373
Non-nursing infants (<1 yr old):						
	0.004389	131247	0.010184	56561	0.013706	42025
Children 1-6 yrs:						
	0.007957	72392	0.014670	39264	0.029809	19322
Children 7-12 yrs:						
	0.005522	104304	0.011007	52330	0.020528	28059
Females 13+ (preg/not nursing):						
	0.002502	230221	0.005039	114301	0.005049	114076
Females 13+ (nursing):						
	0.002697	213601	0.004319	133376	0.004335	132876

Appendix 2. Study Reports Supporting Regulatory Approval of *B.t.* Cry1F Maize Line 6275.

CBI DELETED COPY

All study reports contained in this appendix are <u>Confidential Business Information.</u>

Appendix 2 – Section 1

[CBI-Deleted]

Bridging Document: Product Characterization (Equivalency of Microbial- and Maize-Expressed Proteins)

Bridging Document: Product Characterization (Maize-Insect-Pest Susceptibility)

Bridging Document: Toxicology Data

Bridging Document: Product Performance and Product Durability Plan

Bridging Document: Ecological Risk Assessment

Bridging Document: Product Durability Plan

Bridging Document: Analytical Method and Independent Laboratory Validation

Appendix 2 – Section 2

[CBI-Deleted]

Characterization of DNA Inserted into Transgenic Corn Event TC6275

Locke, M., et al February 6, 2003

Study ID: PHI-2002-002

Pages: 1-37

Appendix 2 – Section 3

[CBI-Deleted]

Detailed Molecular Characterization of the DNA Inserted into the Transgenic Corn Event TC6275
Green, S.B., et al. June 6, 2003
Study ID: PHI-2002-028
Pages: 1-36

Appendix 2 – Section 4

[CBI-Deleted]

Nutrient Composition and/or Quantitative Analysis of Cry1F and BAR Protein Expression Levels of Maize Hybrid, Inbred and Progenitor Lines Containing Event TC6275

Essner, R. April 28, 2003

Study ID: PHI-2001-057

Pages: 1-1119

Appendix 2 – Section 5

[CBI-Deleted]

Characterization of Cry1F Protein Derived from Pseudomonas fluorescens and Transgenic Corn
Schafer, B.W., et al February 5, 2003
Study ID: GH-C 5629 (020053)
Pages: 1-33

Appendix 2 – Section 6

[CBI-Deleted]

Characterization of Phosphinothricin Acetyltransferase (BAR) Derived from Recombinant E. coli and Transgenic Corn

Gao, Y., et al November 21, 2002
Study ID: GH-C 5560 (020043)
Pages: 1-72

Appendix 2 – Section 7

[CBI-Deleted]

Cry1F Insecticidal Crystal Protein: An 8-Day Dietary Toxicity Study with the Rainbow Trout, Oncorhynchus mykiss, Walbaum

Marino, T.A. and Yaroch, A.M
Study ID: 021072R
Pages: 1-23

September 18, 2002
Revised: October 28, 2003

Appendix 2 – Section 8

[CBI-Deleted]

Bialaphos Resistant Protein: Acute Oral Toxicity Study in CD-1 Mice
Wilson, D.M., et al — September 12, 2002
Study ID: 021073
Pages: 1-41

Appendix 2 – Section 9

[CBI-Deleted]

In Vitro Simulated Gastric Fluid Digestibility Study of Recombinant Phosphinothricin Acetyltransferase (BAR)

Korjagin, V.A. March 7, 2003
Study ID: 020092
Pages: 1-52

Appendix 2 – Section 10

[CBI-Deleted]

Comparison of Amino Acid Sequence of the Phosphinothricin Acetyltransferase Protein (BAR) to Known Protein Allergens

Song, P. September 26, 2002
Study ID: GH-C 5536
Pages: 1-33

Appendix 2 – Section 11

[CBI-Deleted]

Field Efficacy of Maize-Optimized Cry1F (TC6275) for the Control of Lepidoptera Pests of Corn
Babcock, J.M. and Bing, J. May 2, 2003
Study ID: GH-C 5649
Pages: 1-35

Appendix 2 – Section 12

[CBI-Deleted]

Demonstration of High Dose Activity against European Corn Borer in Maize-Optimized Cry1F Events TC6228 and TC6275

Babcock, J.M., et al May 7, 2003
Study ID: GH-C 5652
Pages: 1-43

ATTACHMENT

1. USDA APHIS release notifications relevant to the field testing of *B.t.* Cry1F maize line 6275

Attachment 1. USDA APHIS release notifications relevant to the field testing of *B.t.* Cry1F maize line 6275.

Internal ID	USDA #	County and State of Release	State Where No Release of TC6275 (DAS-06275-8) Occurred*
278-LE-CRN-R	03-052-08n	Freeborn, MN, Jackson, MN, Otter Tail, MN, Pipestone, MN, Renville, MN, Stearns, MN, Wilkin, MN	N/A
277-LE-CRN-R	03-031-04n	Trials in progress	Trials in progress
276-LE-CRN-MR	03-031-03n	Cass, IA, Sac, IA, Scott, IA, Story, IA, Washington, IA, Wright, IA, DeKalb, IL, Macon, IL, McDonough, IL, Piatt, IL, Wayne, IL, Woodford, IL, Benton, IN (2), Marshall, IN, Sullivan, IN, Tipton, IN, Vanderburg, IN, Fayette, KY, Franklin Parish, LA, St. Landry Parish, LA, Richland, ND, Butler, NE, Lincoln, NE, Perkins, NE, Phelps, NE, York, NE, Clark, OH, Miami, OH, Columbia, WI, Jefferson, WI	CA, MS, PR, SD
MS249	02-077-06n	Buena Vista., IA	N/A
MS240	02-060-17n	Fresno, CA, Scott, IA, Hancock, IA, Madison, IA (3), Story, IA, Washington, IA, Cass, IA, Wayne, IL, McDonough, IL, DeKalb, IL, Woodford, IL, Piatt, IL, Macon, IL, Sullivan, IN, Gibson, IN, Benton, IN (7), La Porte, IN, Tipton, IN, Fayette, KY (3), Callaway, MO (2), Cass, MO, Holt, MO (2), Boone, MO, Hinds, MS, Washington, MS (3), Richland, ND, Clark, OH (2), Miami, OH, Santa Isabel, PR, Dane, WI, Columbia, WI, Jefferson, WI	KS
MS239	02-060-16n	York, NE, Phelps, NE, Butler, NE, Perkins, NE, Lincoln, NE	N/A
MS238	02-060-15n	Maui, HI	N/A
MS237	02-060-14n	Otter Tail, MN, Freeborn, MN, Jackson, MN, Pipestone, MN, Sterns, MN, Renville, MN	N/A
MS200	01-116-07n	Benton, IN	IA, IL, NE, WI
MS192	01-092-18n	Wilikin, MN, Jackson, MN, Renville, MN, Brown, MN	N/A
MS189	01-092-16n	Madison, IA, Wright, IA, Story, IA, Plymouth, iA, Sac, IA, Logan, IL, DeKalb, IL, Champaign, IL, Decatur, IN, Posey, IN, Lenawee, MI, Butler, NE, York, NE, Columbia, WI, Pierce, WI, Jefferson, WI	CO, HI, KS, KY, MD, MO, MS, ND, NM, NY, OH, OK, PA, PR, SD, TX
MS179	01-047-21n	Maui, HI, Story, IA, Tipton, IN, Santa Isabel, PR	N/A
MS160	00-224-02n	no release	IA, PR
MS136	00-097-03n	Renville, MN	N/A
MS131	00-083-03n	Story, IA, Benton, IN	PR, WI
MS135	00-097-02n	Sac, IA, Page, IA, Logan, IL, Benton, IN, Lenawee, MI, York, NE, Columbia, WI	PR
MS113	99-357-08n	Santa Isabel, PR	IA, IN, WI
MS095	99-274-10n	no release	IA, IN, PR, WI
MS082	99-078-10n	Story, IA, Benton, IN,	PR, WI
MS068	98-296-07n	no release	PR
MS066	98-288-18n	Santa Isabel, PR	N/A

* Locations not planted are listed on the final termination report and/or a separate letter to USDA describing location not planted under release notifications.