Food and Feed Safety and Nutritional Assessment of MON 88017 Corn

Conclusion Based on Data and Information Evaluated According to FDA's Policy on Foods from New Plant Varieties

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FDA BNF 97

Monsanto # 04-CR-109F

Prepared by:

[redacted]

Contributors:

[redacted]

Submitted by:

[redacted]

Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

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CERTIFICATION

Monsanto Company is submitting this food and feed safety and nutritional assessment in compliance with the FDA's 1992 policy statement regarding foods derived from new plant varieties (57 FR 22984). At the agency's request, and where appropriate, this submission also complies with the recommendations contained in the proposed rule for Premarket Biotechnology Notice (PBN) Concerning Bioengineered Foods (66 FR 4706).

Specifically, as recommended in the proposed 21 CFR §192.25(a), the undersigned attests to the following:

1. It is the view of Monsanto Company (hereafter referred to as Monsanto) that: (i) MON 88017 is as safe and nutritious as other commercially available corn; and (ii) the intended uses of the food and feed derived from MON 88017 are in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.
2. Monsanto will make available to FDA, upon request, relevant data or other information not included in this submission, either during the course of FDA's evaluation of the submission, or for cause.
3. Monsanto will make relevant data or other information not included in this submission available to FDA either: (i) by allowing FDA to review and copy these data or information at Monsanto's offices in St. Louis, MO, during customary business hours; or (ii) by sending a copy of these data or information to FDA.
4. Monsanto makes no claim of confidentiality regarding either the existence of this submission, or any of the data or other information contained herein. However, Monsanto reserves the right to make a claim of confidentiality regarding any relevant data or other information not included in this submission, but requested by FDA, either in the course of its review of this submission, or for cause. Any such claim of confidentiality will be made at the time such data or information is provided, along with an explanation for the basis of the claim.
5. To the best of Monsanto's knowledge, this submission is representative and balanced, including information, unfavorable as well as favorable, pertinent to the evaluation of the safety, nutritional, or other regulatory issues that may be associated with MON 88017.

Signature:

______________________________ Date: ____________________

[redacted]
Director, North American Biotechnology Regulatory Affairs
Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

Release of Information

Monsanto is submitting the information in this assessment for review by the FDA as part of the regulatory process. By submitting this information, Monsanto does not authorize its release to any third party. In the event the FDA receives a Freedom of Information Act request, pursuant to 5 U.S.C., § 552, and 21 CFR Part 20, covering all or some of this information, Monsanto expects that, in advance of the release of the document(s), FDA will provide Monsanto with a copy of the material proposed to be released and the opportunity to object to the release of any information based on appropriate legal grounds, e.g., responsiveness, confidentiality, and/or competitive concerns. Monsanto understands that a copy of this information may be made available to the public in a reading room and by individual request, as part of a public comment period. Except in accordance with the foregoing, Monsanto does not authorize the release, publication or other distribution of this information (including website posting) without Monsanto's prior notice and consent.

Abbreviations[1] and Definitions

~	approximately
aa	amino acid
aad	bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7
ADF	acid detergent fiber
ALLERGEN3	allergen and gliadin protein sequence database, compiled by Monsanto Company
ALLPEPTIDES	Protein sequence database comprised of GenPept, PIR and SwissProt, as curated by Monsanto Company
avg	average
bp	base pair
B. subtilis	*Bacillis subtilis*
BSA	bovine serum albumin
B.t.	*Bacillus thuringiensis*
CaMV	cauliflower mosaic virus
CFR	Code of Federal Regulations
CI	confidence interval
CMS	cytoplasmic male sterile
CPB	Colorado potato beetle
CRW	corn rootworm
Cry3Bb1	a naturally-occurring wild type protein with activity against Coleopteran insects, produced by *Bacillus thuringiensis* (subsp. *kumamotoensis*)
Cry3Bb1.pvzmir39	a variant sharing an amino acid identity of >99% with the wild type Cry3Bb1 protein
cry3Bb1.pvzmir39	DNA sequence encoding the Cry3Bb1.pvzmir39 protein
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium sp.* strain CP4
cp4 epsps	*epsps* coding sequence from *Agrobacterium* sp. strain CP4
CTAB	cetyltrimethylammonium bromide
CTP	chloroplast transit peptide
CTP2	*Arabidopsis* EPSPS transit peptide
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DDE	daily dietary exposure
DTT	dithiothreitol
dwt	dry weight

[1] Standard abbreviations, e.g., units of measure, will be used according to the format described in 'Instructions to Authors' in the *Journal of Biological Chemistry*.

Abbreviations and Definitions (cont.)

E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
EU	European Union
FAS	Foreign Agricultural Service
FASTA	algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FMV	figwort mosaic virus
FR	Federal Register
FW	fresh weight
fwt	fresh weight of tissue
GenBank	a public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethseda, MD
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
kb	kilobase
kDa	kilodalton
LB	left border
LOD	limit of detection
LOQ	limit of quantitation
mA	milliampere
MAFF	Ministry of Agriculture, Forestry and Fisheries
MALDI-TOF MS	matrix assisted laser desorption ionization time of flight mass spectrometry
MHLW	Ministry of Health, Labor and Welfare
mM	millimolar
MO	monogerm
MOE	margin of exposure
MW	molecular weight
NASS	National Agricultural Statistics Services
NDF	neutral detergent fiber
NFDM	nonfat dry milk
NOEL	no effect level
OECD	Organization for Economic Co-operation and Development

Abbreviations and Definitions (cont.)

OD	optical density
ori-322	origin of replication for the ABI *Agrobacterium* derived from the broad host range plasmid RK2
ori-V	a segment of pBR322 that provides the origin of replication for maintenance of plasmids in *E. coli*
OSL	overseason leaf
OSR	overseason root
OSWP	overseason whole plant
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
P-FMV	35S promoter from a modified figwort mosaic virus (FMV)
PVDF	polyvinylidene difluoride
ract1 intron	intron from the rice actin gene
RB	right border
ROP	coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*
RB	right border
RNA	ribonucleic acid
RT	room temperature
rop	a segment of pBR322 that represses the formation of RNA primer and is critical to the maintenance and copy number control of plasmids in *E. coli*
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error of the mean
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
sp.	species
Taq	*Thermus aquaticus*, a thermophilic bacterium
tahsp17 3'	3' nontranslated region of the wheat heat shock protein 17.3 containing the polyadenylation sequence
TBA	tris-borate buffer with 0.25% (w/v) L-ascorbic acid
TBST	tris buffered saline with Tween
T/C/R	test/control/reference material
TDF	total detedgent fiber
T-DNA	transferred DNA of the Ti plasmid that integrates into the nuclear DNA of the plant during *Agrobacterium*-mediated transformation
TMB	3,3',5,5'-tetramethylbenzidene
Tris	tris(hydroxymethyl)-aminomethane
TE buffer	tris-EDTA buffer
u	units
USDA- APHIS	United States Department of Agriculture Animal and Plant Health Inspection Service

Abbreviations and Definitions (cont.)

µg/g fwt	microgram per gram fresh weight of tissue
U.S.	United States
USC	United States code
wt	weight

NARRATIVE SUMMARY

Food and Feed Safety and Nutritional Assessment of MON 88017 Corn

Monsanto Company has developed, through the use of recombinant DNA techniques, MON 88017 corn plants that are tolerant to the action of the Roundup® family of agricultural herbicides and are protected from damage caused by corn rootworm (CRW) larval feeding. MON 88017 produces a 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4 (CP4 EPSPS), which confers tolerance to glyphosate, the active ingredient in Roundup agricultural herbicides, and a modified *Bacillus thuringiensis (*subspecies *kumamotoensis)* Cry3Bb1 protein that selectively controls CRW species. The safety of both the CP4 EPSPS and Cry3Bb1 proteins has been assessed previously by FDA for other corn products.

Corn (*Zea mays* L.) is the largest crop grown in the United States in terms of acreage planted and net value. In 2002, its production covered 79.1 million acres that yielded nine billion bushels and had a net value of $21.2 billion. Weed control is essential is cornfields, as weeds compete with the crop for sunlight, water and nutrients. Failure to control weeds results in decreased yields and reduced crop quality. Introduction of MON 88017 will offer U.S. farmers the ability to apply Roundup agricultural herbicides over the top of corn for broad-spectrum weed control with minimal risk of crop injury. In addition the use of glyphosate in conjunction with MON 88017 will allow the grower to utilize reduced tillage techniques, which provide significant environmental benefits, such as reduced soil erosion, reduced use of fossil fuels, and improved soil quality.

Corn yields are also negatively impacted by a number of insect pests. One of the most pernicious in the U.S. cornbelt is corn rootworm. Corn rootworm larvae damage corn by feeding on the roots, reducing the ability of the plant to absorb water and nutrients from soil, and causing harvesting difficulties due to plant lodging. Corn rootworm is the most significant insect pest problem for corn production in the U.S. cornbelt from the standpoint of chemical insecticide usage, as over 16 million acres of corn were treated with organophosphate, carbamate, and pyrethroid insecticides to control corn rootworm in 1998. Corn rootworm has been described as the billion-dollar pest complex based on costs associated with the application of soil insecticides and crop losses due to pest damage. Introduction of MON 88017 will offer U.S. farmers a safe and effective alternative to the use of synthetic chemical insecticides for control of the corn rootworm pest.

Corn hybrids containing both the corn rootworm protection and glyphosate tolerance traits have been developed previously using traditional breeding techniques, i.e., two inbreds containing the individual traits were crossed to produce the stacked trait product. However, the traditional breeding process can be inefficient, requiring long development

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times. MON 88017 was therefore developed using a vector that contains both the *cp4 epsps* and *cry3Bb1* genes. This approach increases the efficiency of simultaneous introduction of both traits into new corn hybrids, thereby providing growers access to a variety of elite corn germplasms containing both traits.

MON 88017 was produced by *Agrobacterium*-mediated transformation of corn cells with plasmid vector PV-ZMIR39. This plasmid contains a gene encoding the CP4 EPSPS protein that provides tolerance to the action of Roundup agricultural herbicides and a gene encoding the Cry3Bb1 protein that has activity against corn rootworm. The *Agrobacterium tumefaciens* transformation vector is a disarmed, binary vector that contains both left and right transfer-DNA (T-DNA) border sequences to facilitate transformation. The DNA region that was integrated into the corn genome during the transformation process contains the *cp4 epsps* and *cryBb1* gene expression cassettes. Molecular analyses of MON 88017 confirmed that single copies of the *cp4 epsps* and *cry3Bb1* genes are integrated at a single locus in the corn genome with all expression elements intact and no plasmid bacterial backbone present.

Segregation analysis across ten generations confirmed the heritability and stability of the *cp4 epsps* and *cry3Bb1* coding sequences. The results of this analysis are consistent with the finding of a single active site of insertion that segregates according to the Mendelian laws of genetics.

The CP4 EPSPS protein in MON 88017 is structurally homologous to EPSPSs naturally present in food crops (e.g., soybean and corn) and in microbial food sources such as Baker's yeast that have a long history of safe consumption by humans and animals. The amino acid sequence of the CP4 EPSPS protein in MON 88017 is the same or >99% identical to the CP4 EPSPS protein produced in Roundup Ready crops such as soybean, NK603 corn, cotton and canola, which have already completed the FDA consultation process. Roundup Ready crops have been consumed directly or as processed products by humans and animals since Roundup Ready soybeans were first commercialized in 1996. The CP4 EPSPS protein is not toxic, as assessed by the history of safe use of the EPSPS class of proteins and as confirmed by the lack of any treatment-related adverse affects in mice administered the CP4 EPSPS protein by oral gavage. This lack of toxicity was expected based on the rapid degradation of the CP4 EPSPS protein and its enzymatic activity in simulated mammalian gastric and intestinal fluids. In addition, the CP4 EPSPS protein is not homologous to known protein toxins or allergens, is not glycosylated and is present at very low levels in MON 88017.

The mean levels of CP4 EPSPS protein in MON 88017 are 5.8 μg/g of dry weight in corn grain and 57 μg/g of dry weight in forage. These levels are comparable to levels found in Roundup Ready corn NK603, which has a history of safe consumption by humans and animals. Using a no observed effect level (NOEL) of 572 mg/kg body weight from the acute mouse gavage study, the margin of exposure for consumption of the CP4 EPSPS protein, based on the mean adult consumption of corn in the U.S., is calculated to be $>3.6\times10^5$. This exposure calculation makes the conservative assumption that there is no loss of the CP4 EPSPS protein during the processing of corn grain into human food. It

also assumes that 100% of the corn grain in human food is derived from MON 88017, which is highly unlikely considering the variety of commercial corn hybrids that exist in the marketplace.

Bacillus thuringiensis (B.t.) Cry proteins also have a long history of safe and widespread use. The Cry3Bb1 protein produced in MON 88017 is a member of the Cry3Bb class of proteins and shares >99% amino acid sequence identity with the wild type Cry3Bb1 protein contained in the topically applied commercial microbial product, Raven® Oil Flowable Bioinsecticide, and differs by only a single amino acid from the Cry3Bb1 variant produced in YieldGard® Rootworm corn (MON 863), which has completed the FDA consultation process. Cry3Bb1 had no effect when administered orally to mice at the highest dose tested of 1930 mg/kg. *In vitro* digestive fate studies have shown that the protein is rapidly degraded to small peptide fragments. Furthermore, the protein is not glycosylated in corn, and it lacks sequence similarity to known allergens and toxins.

The level of Cry3Bb1 protein in corn was determined in multiple plant tissues collected from replicated field trials. The mean level of Cry3Bb1 protein in corn grain is 15 µg/g dry weight and 95 µg/g dry weight in forage. Using the same conservative assumptions as described above, the margin of exposure for the Cry3Bb1 protein, based on the mean adult consumption of corn in the U.S., is calculated to be > 4.7×10^5.

MON 88017 was shown to be compositionally equivalent to conventional corn with similar genetic background as well as to other conventional corn varieties. A total of 77 significant nutrients, anti-nutrients and secondary metabolites were evaluated as part of the nutritional assessment of MON 88017 (68 in grain and nine in forage). Compositional analyses of the forage samples included proximates (protein, fat, ash, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), minerals (calcium, phosphorous), and carbohydrates by calculation. Compositional analyses of the grain samples included proximates (protein, fat, ash, and moisture), ADF, NDF, total dietary fiber (TDF), amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium, and zinc), vitamins (B_1, B_2, B_6, E, niacin, and folic acid), anti-nutrients (phytic acid and raffinose), secondary metabolites (furfural, ferulic acid, and p-coumaric acid), and carbohydrates by calculation. Sixty-two components were statistically assessed and 15 components were below the limit of quantitation. Results of the analysis showed that there were no statistically significant differences between MON 88017 and conventional corn for 232 of the 248 comparisons conducted. No statistically significant differences were found in forage. Statistically significant differences ($p<0.05$) were observed for only 16 comparisons in grain and all values fell within the 99% tolerance interval. Therefore it is unlikely that these differences are biologically meaningful. It is concluded, based on these data, that the forage and grain produced from MON 88017 are compositionally equivalent to the forage and grain produced from other commercial corn currently on the market.

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Collectively, the data summarized in this document support a conclusion that food and feed products derived from MON 88017 are as safe and nutritious as those derived from conventional corn.

PART II: SYNOPSIS OF CONSULTATION SUMMARY

SECTION 1. Name and address of the submitter

The submitter of this safety and nutritional assessment summary for corn MON 88017 is:

Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

Communications with regard to this submission should be directed to [redacted], Regulatory Affairs Manager, at the Monsanto address. He can also be contacted by telephone at [redacted].

SECTION 2. The subject of this summary and the plant species from which they were derived

The subject of this summary is corn MON 88017 derived from a Monsanto proprietary corn inbred.

SECTION 3. Distinctive designations given to the subject of this summary

The corn rootworm-protected and glyphosate-tolerant corn that is subject of this summary has been designated MON 88017. In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants," MON 88017 has been assigned the unique identifier MON-88Ø17-3.

SECTION 4. Identity and sources of the genetic material introduced into MON 88017

Agrobacterium-mediated transformation utilizing plasmid vector PV-ZMIR39 (Figure 1, Part IV) was used to generate MON 88017. This vector contains two expression cassettes: one coding for the native CP4 EPSPS protein from *Agrobacterium* sp. strain CP4 (Padgette et al., 1996), and the other coding for a variant of the wild-type Cry3Bb1 protein from *Bacillus thuringiensis* subsp. *kumamotoensis*. The genetic elements present in PV-ZMIR39 are listed in Table 1, Part IV.

The expression cassette for the coding sequence of the native CP4 EPSPS protein from *Agrobacterium sp*. strain CP4 (Padgette et al., 1996) consists of the promoter from the

rice actin gene (McElroy et al., 1990), an intron from the rice actin gene (McElroy et al., 1991), the *cp4 epsps* coding sequence fused to a chloroplast transit peptide 2 (CTP2) sequence derived from *Arabidopsis* (Klee et al., 1987) and a 3' nontranslated region of the nopaline synthase gene of *Agrobacterium tumifaciens* T-DNA that ends transcription and directs polyadenylation (Bevan et al., 1983).

The synthetic *cry3Bb1* gene expression cassette that produces the Cry3Bb1 protein consists of the enhanced 35S promoter (Odell et al., 1985), 5' untranslated leader of the wheat chlorophyll a/b/-binding protein (Lamppa, 1985), intron from the rice actin gene (McElroy et al., 1991), the synthetic *cry3Bb1* coding sequence, and the 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and provides the signal for mRNA polyadenylation (McElwain and Spiker, 1989).

The molecular analyses described in Part IV demonstrate that MON 88017 contains a single copy of introduced DNA approximately 6.8 kb in size inserted at a single locus. This insert contains one intact copy each of the *cp4 epsps* and *cry3Bb1* gene expression cassettes. There are no detectable plasmid backbone sequences and no additional elements, linked or unlinked to intact cassettes, from transformation vector PV-ZMIR39.

These results support the conclusion that only the two expected full-length proteins, CP4 EPSPS and Cry3Bb1, are encoded by the insert present in MON 88017.

SECTION 5. The intended technical effect of MON 88017

MON 88017 corn plants produce a CP4 EPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium sp.* strain CP4) that provides tolerance to the action of Roundup agricultural herbicides and a modified *Bacillus thuringiensis* (subspecies *kumamotoensis*) Cry3Bb1 protein that has activity against corn rootworm.

The CP4 EPSPS protein is structurally and functionally similar to native plant EPSPS enzymes, but has a much reduced affinity for glyphosate (Padgette et al., 1996). Typically, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby depriving plants of these essential components (Steinrucken and Amrheim, 1980; Haslam, 1993). In Roundup Ready plants, requirements for growth and development are met by continued action of the CP4 EPSPS enzyme in the presence of glyphosate.

The Cry3Bb1 protein is selectively active against corn rootworm species and protects plants from damage due to corn rootworm feeding. MON 88017 is afforded a level of protection from corn rootworm feeding damage that is comparable or superior to that offered by currently available conventional insecticides. The Cry3Bb1 protein produced in MON 88017 (Cry3Bb1.pvzmir39) is a member of the Cry3Bb class of proteins and variants that shares >99% amino acid sequence identity with the wild type Cry3Bb1 protein and differs only by a single amino acid from the Cry3Bb1 variant produced in

YieldGard® Rootworm corn (MON 863), which has completed the FDA consultation process. It is referred to as Cry3Bb1 in this submission.

SECTION 6. The applications and uses of MON 88017

Monsanto Company has developed, through the use of recombinant DNA techniques, corn plants that are tolerant to the action of the Roundup family of agricultural herbicides and protected from damage due to corn rootworm larval feeding. MON 88017 produces a CP4 EPSPS protein, which confers tolerance to glyphosate, and a modified *Bacillus thuringiensis* (subspecies *kumamotoensis*) Cry3Bb1 protein that is active against corn rootworm species. The *cp4 epsps* gene expressed in MON 88017 is identical to one of the *cp4 epsps* genes produced in Roundup Ready corn NK603, and the *cry3Bb1* gene is a synthetic variant of the *cry3Bb1* gene produced in YieldGard Rootworm corn.

One of the benefits of MON 88017 corn to growers will result from the use of Roundup herbicides to control weeds. Many benefits of Roundup Ready technology have been documented in studies of Roundup Ready soybeans (Gianessi and Carpenter, 2000). The benefits of the use of MON 88017 are likely to be similar to those of Roundup Ready corn, which was commercialized in the U.S. in 1998.

The benefits of Roundup Ready corn include:

- *Broad-spectrum weed control.* Roundup agricultural herbicide controls both broadleaf weeds and grasses, including difficult-to-control weed species (Franz et al., 1997).
- *Excellent crop safety.* When used according to label recommendations, Roundup agricultural herbicides control weeds without injury to Roundup Ready corn plants.
- *Minimal environmental impact.* Roundup agricultural herbicides have been used for more than 30 years in various applications. Glyphosate is recognized for its lack of soil persistence, low risk of ground water contamination, and lack of toxicity to birds, mammals, fish, and invertebrates (Ahrens, 1994; Franz et al., 1997; Giesy et al., 2000).
- *Flexibility in treating for weed control.* Since Roundup agricultural herbicides are applied onto the foliage of weeds after crop emergence, applications are only necessary if weed infestation reaches the threshold level for yield reductions.
- *Excellent fit with reduced-tillage systems.* Benefits of conservation tillage include improved soil quality, improved water infiltration, reduced soil erosion and sedimentation of water resources, reduced runoff of nutrients and pesticides to surface water, improved wildlife habitat, increased carbon retention in soil, reduced fuel usage, and sustainable agricultural practices (Hebblethwaite, 1995; Reicosky et al., 1995). In addition, data collected during 1999-2001 have shown that the adoption of Roundup Ready corn can lead to a significant reduction in the levels

of major pre-emergence corn herbicides in Illinois watersheds (Wauchope et al., 2001).

- *Cost effective weed control.* The costs of weed control with Roundup agricultural herbicides are competitive with the cost of alternative weed-control options, especially in view of the high weed-control efficacy of glyphosate. Both large- and small-scale farmers can benefit equally from use of this technology.
- *Use of a herbicide with low risk to humans.* Roundup agricultural herbicides pose a minimal health risk to humans (EPA, 1993; WHO, 1994).

Another major benefit from MON 88017 is protection from feeding damage by corn rootworm larvae. Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2002, corn production covered 79.1 million acres, yielding nine billion bushels of corn grain, at an average yield of 130 bu/A, and a total net value of $21.2 billion (NCGA, 2003). Corn yields are negatively impacted by a number of insect pests. One of the most pernicious in the U.S. Cornbelt is corn rootworm larvae, which damage corn by feeding on the roots, reducing the ability of the plant to absorb water and nutrients from soil, and causing harvesting difficulties due to plant lodging (Reidell, 1990; Spike and Tollefson, 1991). Corn rootworm is the most significant insect pest problem for corn production in the U.S. from the standpoint of chemical insecticide usage. Approximately 14 million acres of corn were treated in 2000 with organophosphate, carbamate and pyrethroid insecticides to control corn rootworm (Doane, 2001). Corn rootworm has been described as the billion-dollar pest complex, based on costs associated with the application of soil insecticides and crop losses due to pest damage (Metcalf, 1986).

In 2003, Monsanto commercialized YieldGard Rootworm corn (MON 863) that provides an alternative and more effective solution for the control of corn rootworm larvae based on biotechnology. The potential benefits of MON 88017 will be similar to those of MON 863 because MON 88017 contains a Cry3Bb1 protein that is equally efficacious against corn rootworm larvae. In addition, growers will have the ability to apply Roundup agricultural herbicides over the top of corn for broad-spectrum weed control. The rapid development of elite corn hybrids containing these traits is made possible by vector stack technology, i.e., the incorporation of multiple genes into a single transformation event. In traditional breeding methods, inbreds containing individual traits are crossed to produce the desired stacked hybrid. This process can be inefficient, requiring long development times for the introduction of new hybrids into the market place. Vector stack technology increases the efficiency of the introduction of multiple traits into new hybrids, thereby providing the grower access to a variety of germplasms containing these traits.
MON 88017 will offer growers many practical advantages over the use of chemical insecticides, including savings in time and money for application, labor, fuel, equipment, storage and disposal costs. In addition, MON 88017 is likely to be labeled for general use and will replace current reliance on restricted use products, providing growers and other occupational workers with greater safety, protect water bodies from run-off, and mitigate spray-drift and potential impacts on nontarget organisms (NTO), such as bird populations.

The National Agricultural Statistics Service (NASS) of the U. S. Department of Agriculture (USDA) has compiled statistics of corn insecticide usage across 15 states, comprising 68.3 million acres of corn in 1999 (USDA-NASS, 2000). These statistics indicate that chemical insecticides registered for corn rootworm control were applied on over 30% of this corn acreage in 1999. Many of these insecticides have come under increased regulatory scrutiny because of concerns about worker safety, environmental risk, and exposure to infants and children. The introduction of MON 88017 will offer U.S. farmers a safe and effective alternative to the use of synthetic chemical insecticides for control of corn rootworm pests. Such a product offers the potential to significantly reduce the amount of chemical insecticides applied in the U.S. cornbelt, benefiting the environment and leading to a reduction in actual and potential adverse effects throughout the manufacturing, distribution, and use chain.

Based on the dual traits of glyphosate tolerance and corn rootworm protection, MON 88017 is expected to provide substantial benefits to growers by limiting yield losses from corn rootworm feeding damage and weed pressure, while at the same time reducing the risk to humans and the environment as a result of anticipated reductions in the use of chemical insecticides.

SECTION 7. Applications for which MON 88017 is not suitable

Monsanto Company is aware of no food or feed uses of conventional corn that are not applicable to MON 88017.

PART III: STATUS OF SUBMISSIONS TO OTHER REGULATORY AGENCIES

SECTION 1. Status of submission to USDA-APHIS

Monsanto will be requesting a Determination of Nonregulated Status for MON 88017 from USDA-APHIS in 2004, including all progenies derived from crosses between MON 88017 and other corn. Under regulations administered by USDA-APHIS (7 CFR 340), MON 88017 is currently considered a “regulated article.” Monsanto will continue to conduct all field tests for MON 88017 in strict compliance with USDA field regulations until a Determination of Nonregulated Status is obtained for MON 88017.

SECTION 2. Status of submission to EPA

Substances that are pesticides as defined under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) [7 U.S.C. §136(u)] are subject to regulation by the U.S. Environmental Protection Agency (EPA).

An application for registration of “*Bacillus thuringiensis* Cry3Bb1 Protein and the Genetic Material (Vector ZMIR39) Necessary for its Production in Corn” has been submitted to the EPA. An application for an exemption from the requirement of a tolerance for *B.t.* Cry3Bb1 proteins, pursuant to §408(d) of the Federal Food Drug and Cosmetic Act [21 U.S.C. 346 a(d)], also was submitted to EPA (PP 7F4888). On May 11, 2001, EPA established a time-limited exemption from the requirement of a tolerance for Cry3Bb1 and the genetic material necessary for its production in all corn commodities (EPA, 2001a). On April 29, 2003 Monsanto petitioned the EPA to amend 40 CFR Part 180 by removing the time limitation for the exemption from the requirement of a tolerance for the plant-incorporated protectant *Bacillus thuringiensis* Cry3Bb1 protein and the genetic material necessary for its production in corn in or on field corn, sweet corn, and popcorn. In October 2003, a Federal Register notice was published announcing a public comment period (EPA, 2003b) to remove the time limitation.

Substances that are pesticidal as defined under the FIFRA (7U.S.C. section 136(u)), are subject to EPA’s regulatory authority. The initial registration for use of Roundup Ultra® Herbicide (EPA Reg. No. 524-475) over the top of Roundup Ready corn was granted by the EPA on March 28, 1997 (62 FR 17723-17730). Since that time a number of label amendments have been approved by the Agency and multiple glyphosate end-use products have been labeled for use in Roundup Ready corn. The most recent label for use in Roundup Ready corn, approved on June 23, 2003, for Roundup WeatherMAX®

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herbicide (EPA Reg. No 524-537), provides greater flexibility for in-crop applications. This new label allows increased over-the-top application rates and a wider window for applications with use of directed sprays up to 48-inch corn. EPA's approval of the label was based on the review of supporting glyphosate residue data and the establishment of an increased tolerance level for glyphosate in field corn forage of 6 ppm (68 FR 39460, July 2, 2003).

Pursuant to section 408(d) of the Federal Food Drug and Cosmetic Act (FFDCA), 21 U.S.C. 346 a(d), the EPA previously has reviewed and established an exemption from the requirement of a tolerance for CP4 EPSPS and the genetic material necessary for the production of this protein in or on all raw agricultural commodities (40 CFR §180.1174).

SECTION 3. Status of submissions to foreign governments

Monsanto obtained an environmental approval from the Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) for MON 88017 on April 23, 2003. Regulatory submissions for import and production approvals will be made to countries that import U.S. corn grain and have regulatory approval processes in place. These will include submissions to a number of foreign government regulatory agencies, including Japan's MAFF (for use as animal feed), the Japanese Ministry of Health, Labor and Welfare (MHLW), as well as the Canadian Food Inspection Agency (CFIA) and Health Canada. As appropriate, notifications of import will be made to importing countries that do not have a formal approval process.

PART IV: DEVELOPMENT OF MON 88017

SECTION 1. History and biology of corn

The history and biology of corn is summarized in OECD consensus document (OECD, 2003). Corn, *Zea mays* L., originated in Mexico and was grown as a food crop as early as 2700 B.C. (Salvador, 1997). It is now grown on more than 345 million acres globally (James, 2003a). The history of corn has been studied extensively, and multiple hypotheses for its origin and parentage have been advanced (Mangelsdorf, 1974). The preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988). Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2002, its production covered 79 million acres that yielded 9 billion bushels and had a net value of $21.2 billion (NCGA, 2003).

Corn is a highly productive crop, yielding an average of 130 bushels per acre in the U.S. during 2002 (NCGA, 2003). Its high yield makes it one of the most economical sources of metabolizable energy for feeds, and of starch and sugar for food and industrial products. Approximately two-thirds of the corn produced in the U.S. is fed to livestock. Therefore, indirect consumption is much greater than direct consumption for humans.

Corn, the host plant, has been a staple of the human diet for centuries. Corn grain and its processed fractions are consumed in a multitude of food and animal feed products. Corn forage is extensively consumed as an animal feed by ruminants. Corn does not contain any known allergens or produce significant quantities of toxins or anti-nutritional factors warranting analytical or toxicological tests (Watson, 1982; White and Pollak, 1995).

1.1. Scientific name and taxonomic classification of corn

Corn is a member of the tribe Maydae, which is included in the subfamily Panicoideae of the grass family Gramineae. The genera included in the tribe Maydae include *Zea* and *Tripsacum* in the Western Hemisphere and *Coix*, *Polytoca*, *Chionachne*, *Schlerachne*, and *Trilobachne* in Asia. Although the Asian genera have been implicated by some as the origin of corn, the evidence is not as extensive and convincing as for the genera located in the Western Hemisphere.

There has been some fluctuation in Latin binomial designations of the species included in *Zea* in recent years (Doebley and Iltis, 1980). The genus *Zea* includes two subgenera: *Luxuriantes* and *Zea*. Corn (*Zea mays* L.) is a separate species within the subgenus *Zea,* along with three subspecies. All of the species within the genus *Zea*, except corn, are different species of teosinte. Until recently, the teosinte species were included in the genus *Euchlaena* rather than the genus *Zea*.

The other genus included in the Maydae tribe is *Tripsacum*. *Tripsacum* includes 16 species with a basic set of 18 chromosomes (n = 18), and the various species of *Tripsacum* include multiples of 18 chromosomes, ranging from 2n = 36 to 2n = 108.

Five genera are included in the tribe Maydeae that originated in Asia. Except for *Coix*, the basic chromosome number is n = 10. Within *Coix*, n = 5 and n = 10 have been reported.

Taxonomic classification of corn and its close relatives

Family - Gramineae
- Subfamily - Panicoideae
  - Tribe - Maydae
    - Western Hemisphere:
      - I. Genus - *Zea*
        - A. Subgenus - *Luxuriantes*
          1. *Zea luxurians* (2n = 20)
          2. *Zea perennis* (2n = 40)
          3. *Zea diploperennis* (2n = 20)
        - B. Subgenus - *Zea*
          1. *Zea mays* (2n = 20)
             Subspecies
             1. *Z. mays parviglumis* (2n = 20)
             2. *Z. mays huehuetenangensis* (2n = 20)
             3. *Z. mays mexicana* (Schrad.) (2n = 20)
      - II. Genus - *Tripsacum*
        Species—

T. andersomii (2n = 64)	*T. latifolium* (2n = 36)
T. australe (2n = 36)	*T. percuvianum* (2n = 72, 90, 108)
T. bravum (2n = 36, 72)	*T. zopilotense* (2n = 36, 72)
T. cundinamarce (2n = 36)	*T. jalapense* (2n = 72)
T. dactyloides (2n = 72)	*T. lanceolatum* (2n = 72)
T. floridanum (2n = 36)	*T. fasciculatum* (2n = 36)
T. intermedium (2n = 72)	*T. maizar* (2n = 36, 72)
T. manisuroides (2n = 72)	*T. pilosum* (2n = 72)

    - Asia:
      I. Genera—

Chionachne (2n = 20)	*Schlerachne* (2n = 20)
Coix (2n = 10, 20)	*Trilobachne* (2n = 20)
Polytoca (2n = 20)	

  - Tribe—Andropogoneae
    I. Genus – *Manisuris*

1.2. Growth and reproductive characteristics of corn

Corn is genetically one of the best developed and best characterized of the higher plants. Because of the separation of male and female inflorescence, the large number of seeds produced on the female inflorescence, the ease in handling (growing and hand pollinating), the nature of the chromosomes, and the low basic chromosome number (n = 10), corn has been accessible for study at all levels of genetics.

Corn evolved as an open-pollinated (cross-fertilizing) crop species and, until the 20th century, corn cultivars were what we designate today as open-pollinated corn varieties. Because corn is essentially 100% cross-pollinated, the corn varieties were a collection of heterozygous and heterogeneous individuals (genotypes). Varieties were developed by simple mass selection by the indigenous natives prior to the arrival of Columbus. Their methods of selection were simple by present-day standards, but they were obviously effective in developing races, varieties, and strains to satisfy their food, fuel, feed, and cultural needs. Hybridization occurred between varieties as cultures moved within the Western Hemisphere, releasing genetic variability to develop other unique varieties.

The fundamental concepts for development of hybrid corn were defined by 1920. Basic studies on the genetic composition of a corn variety were conducted to determine the effects of self pollinating (or inbreeding, which is the opposite of outcrossing) within a corn variety (Shull, 1909). Because corn naturally cross- fertilizes, the genetic composition of each plant is not known. Continuous self-pollination of individuals for seven to ten generations resulted in pure lines (or inbred lines) within which every plant had similar traits. The correct interpretation of what occurred during inbreeding was based on Mendelian genetics: the heterozygous loci were eliminated by inbreeding to homozygous loci of either one of the two alleles at each locus. The fixation of alleles in pure lines caused a general reduction in vigor and productivity.

It was found upon crossing two pure lines that vigor was restored. If no selection occurred during inbreeding, the average performance (e.g., grain yield) of all possible crosses was similar to performance of the original variety in which inbreeding was initiated. Some crosses, however, were better than the original open-pollinated variety and could be reproduced from the cross of the pure-line parents of the cross. Hence, the concept of hybrid corn was determined: self to develop pure lines, cross the pure lines to produce hybrids, evaluate hybrids to determine the best hybrid, and use pure-line parents to reproduce the superior hybrid and distribute it for use by growers (Shull, 1909).

1.3. History of corn development

Corn (*Zea mays* L.), or maize, is one of the few major crop species indigenous to the Western Hemisphere. Corn is grown in nearly all areas of the world and ranks third behind rice (*Oryza sativa* L.) and wheat (*Triticum* sp.) in total production. Corn has been studied extensively and it seems the probable domestication of corn was in southern Mexico more that 7,000-10,000 years ago. The putative parents of corn have not been

recovered, but it is likely that teosinte played an important role in the genetic background of corn. The transformation from a wild, weedy species to one dependent on humans for its survival probably evolved over a long period of time by the indigenous inhabitants of the Western Hemisphere. Corn, as we known it today, cannot survive in the wild because the female inflorescence (the ear) restricts seed dispersal.

Corn originated in the highlands of Mexico 7,000 to 10,000 years ago. By the time Columbus visited the Western Hemisphere, corn was being grown by indigenous civilizations from Chile to southern Canada. Columbus noted the presence of corn on the north coast of Cuba in 1492, and introduced corn to Europe upon his return to Spain (Goodman, 1988). Within two generations after the introduction of corn to Europe, corn became distributed throughout those regions of the world where it could be cultivated. Today, corn ranks third after wheat and rice as one of the world's three leading food crops. However, unlike wheat and rice, the majority of corn produced in the U.S. is consumed by livestock. In the tropics and in the Southern Hemisphere corn is a significant component of the human diet.

The original corn-growing areas did not include the north-central area (U.S. cornbelt) of the United States. The highly productive U.S. cornbelt dent corns were derived after the colonization of North America. The European settlers accepted the local native American varieties and incorporated them with other crops to provide food, feed, and fuel for their survival. The current U.S. cornbelt dent corns evolved during the gradual mingling of those settlements that spread north and west from southeastern North America and those settlements that spread south and west from northeastern North America.

The corn types grown in the northeast are called northern flints; their origin is not clear, but races from the highlands of Guatemala have similar ear morphology (Goodman and Brown, 1988). Northern flints are largely eight-rowed with cylindrical ears, are early maturing, and are short-statured plants with tillers. The southern dent corns grown in the southeast U. S. seemed to have originated from the southeast coast of Mexico. Southern dent corns are characterized as having tall, late-maturing, non-tillered, poorly rooted plants with soft-textured white kernels on many-rowed, tapering ears. It seems the Tuxpeno race contributed to the development of southern dents. The intentional and/or unintentional crossing between the early northern flints and late southern dents led eventually to the highly productive U.S. cornbelt dent corns that are used extensively throughout the world today.

The origin of corn has been studied extensively, and hypotheses for the origin and for the parentage of corn have been advanced (Mangelsdorf, 1974). Hypotheses suggested for the origin of corn include the following: 1) cultivated corn is a descendent of pod corn; 2) corn originated by direct selection from teosinte; 3) corn, teosinte, and *Tripsacum* descended independently from a common, unknown ancestor; and 4) the tripartite theory: a) corn originated from pod corn, b) teosinte derived from a cross of corn and *Tripsacum*, and c) modern corn varieties evolved by corn intercrossing with teosinte or *Tripsacum* or both (Mangelsdorf, 1974).

It has been suggested that modern corn originated from corn grass by a single-gene mutation causing ear development. Other suggestions have included *Coix* and species of the genus *Manisuris* in the tribe *Andropogoneae* for contributing to the genome of corn. The hypotheses have been tested by the study of crosses for genome commonality, fertility, variation, and segregation of morphological plant traits, by archeological evidence, and by use of molecular genetic markers.

Evidence has been reported to support the different hypotheses, but the preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988). The teosinte genome is similar to corn, teosinte easily crosses with corn, and teosinte has several plant morphological traits similar to corn. Teosinte has a more weedy appearance and more tillers than modern corn varieties. The one major distinguishing difference between corn and teosinte is the female inflorescence, or ear. Modern corn varieties have one to three lateral branches that terminate in an ear with eight to 24 kernel rows of 50 seeds, and the ear is enclosed in modified leaves or husks. Teosinte also has lateral branches, but they terminate in two-rowed spikes of perhaps 12 fruit cases, with each fruit case having one seed enclosed by an indurated glume (Goodman, 1988).

SECTION 2. Characterization of the vector used in transformation

Plasmid vector PV-ZMIR39 (Figure 1) was used for the transformation of corn cells to produce MON 88017. It was constructed at Monsanto's research laboratories in St. Louis, Missouri, using standard molecular biology techniques. It is a disarmed, binary *Agrobacterium tumefaciens* transformation vector that contains both the left and right transfer-DNA (T-DNA) border sequences to facilitate transformation. The inserted DNA, the portion of plasmid PV-ZMIR39 that integrated into the corn genome during the transformation process, contains the *cp4 epsps* and *cryBb1* gene expression cassettes. The specific genetic elements and origins of the various components used to construct plasmid vector PV-ZMIR39 are provided in Table 1.

2.1. The *cp4 epsps* gene and CP4 EPSPS protein

The *cp4 epsps* coding sequence has the potential to provide high levels of tolerance to glyphosate when introduced into the plants (Padgette et al., 1996; OECD, 1999). Glyphosate binds to and blocks the activity of the native EPSPS, an enzyme of the aromatic amino acid biosynthetic pathway. In plants, the EPSPS enzyme is located in the chloroplast. Thus, in vector PV-ZMIR39, a chloroplast transit peptide 2 (CTP2) coding sequence was joined to the *cp4 epsps* coding sequence to target transport of the encoded protein to the chloroplast. The *cp4 epsps* coding sequence has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The targeted CTP2~CP4 EPSPS protein contains 531 amino acids and is approximately 55.8 kDa.

2.1.a. The *Arabidopsis thaliana* EPSPS transit peptide (CTP2)

In the plant gene expression cassette, the *cp4 epsps* coding sequence is joined to a chloroplast transit peptide 2 (CTP2) isolated from the *Arabidopsis thaliana epsps* gene (Klee et al., 1987). This transit peptide directs the CP4 EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid biosynthesis (Kishore and Shah, 1988). Transit peptides are typically cleaved and degraded from the full-length protein upon delivery to the plastid (Della-Cioppa et al., 1986).

2.1.b. The *cp4 epsps* regulatory sequences

As noted above, the CTP2-CP4 EPSPS coding sequence is under the control of the 5' noncoding end of the rice actin 1 sequence (ract1) containing the promoter and first intron (McElroy et al., 1990) introduced upstream of the CTP2 sequence. The CP4 EPSPS sequence is joined to the 0.3-kb nopaline synthase 3' nontranslated sequence, NOS 3', from *Agrobacterium tumefaciens* (Fraley et al., 1983), that provides the transcription termination and the mRNA polyadenylation signal.

2.2. The *cry3Bb1* gene and Cry3Bb1 protein

The Cry3Bb1 protein present in MON 88017 is a member of the Cry3Bb class of proteins that share >95% amino sequence homology (Crickmore et al., 1998). It is a variant of the wild-type Cry3Bb1 protein isolated from *Bacillus thuringiensis* (subsp. *kumamotoensis*) strain EG4691 (Donovan et al., 1992). The amino acid sequences of the Cry3Bb1 proteins present in MON 88017 and *Bacillus thuringiensis* (subsp. *kumamotoensis*) share 99.1% identity[1] because they only differ by six of 652 amino acid residues. The amino acid sequences of the Cry3Bb1 variants present in MON 88017 and YieldGard Rootworm corn (MON 863) share 99.8% identity (differ by only one of 653 amino acid residues). The Cry3Bb1 proteins in MON 863 and MON 88017 have been extensively characterized.

The synthetic *cry3Bb1* gene expression cassette that produces the Cry3Bb1 protein consists of the enhanced 35S promoter (Odell et al., 1985), the 5' untranslated leader of the wheat chlorophyll a/b/-binding protein (Lamppa et al., 1985), and the intron from the rice actin gene (McElroy et al., 1991) joined to the synthetic *cry3Bb1* coding sequence at the 5' end. Joined to the 3' end of the synthetic *cry3Bb1* coding sequence is the 3' nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and provides the signal for mRNA polyadenylation (McElwain and Spiker, 1989).

[1] The wild-type Cry3Bb1 protein consists of 652 amino acid residues (Donovan et al., 1992). In MON 88017, the Cry3Bb1 protein (653 amino acid residues) contains an additional amino acid (alanine) at position 2, because it was necessary to create an *Nco* I restriction endonuclease site for the development of the plant gene expression plasmid vector PV-ZMIR39.

2.3. T-DNA borders

Plasmid vector PV-ZMIR39 contains DNA sequences that are necessary for transfer of T-DNA into the plant cell. These sequences are termed the Right and Left Border regions. Within each of the regions is the border, a 24-26 bp sequence that generally defines the extent of the DNA that should be transferred into the plant genome. The Right Border present in PV-ZMIR39 is a 24 bp nucleotide sequence that was originally isolated from *A. tumifaciens* plasmid pTiT37 (Depicter et al., 1982). The Left Border in PV-ZMIR39 is a 25 bp nucleotide sequence from *A. tumifaciens* plasmid pTi5955, a derivative of plasmid pTiA6 (Barker et al., 1983).

2.4. Genetic elements outside the T-DNA borders

The backbone region outside of the inserted DNA, which was not integrated into the corn genome during transformation, contains two origins of replication necessary for replication and maintenance of the plasmid in bacteria, as well as a bacterial selectable marker gene, *aad*, which encodes an aminoglycoside-modifying enzyme that is resistant to the action of the antibiotics spectinomycin and streptomycin. Detailed descriptions of all elements in the bacterial backbone region are presented in Table 1.

Figure 1. Plasmid map of vector PV-ZMIR39

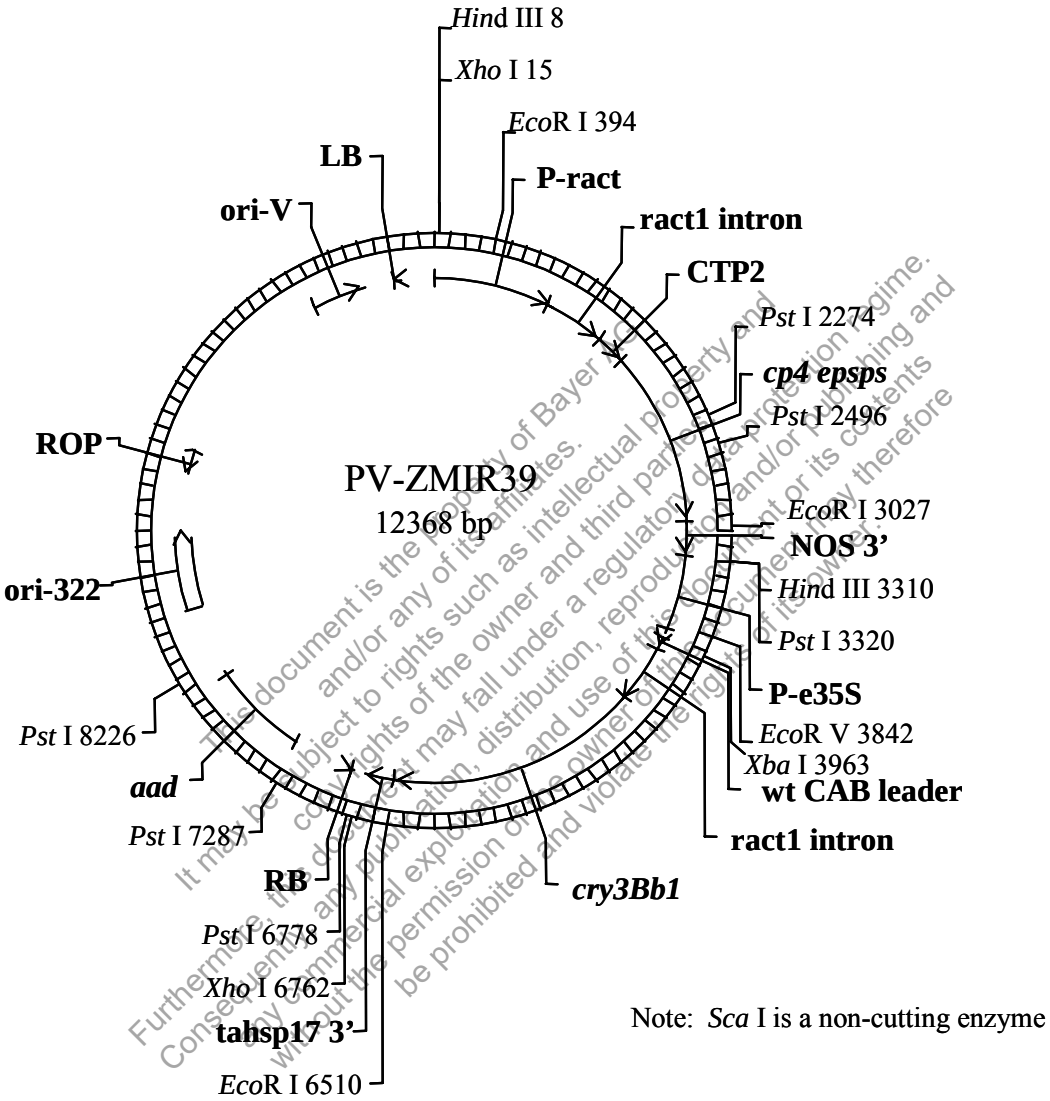


Table 1. Summary of genetic elements in vector PV-ZMIR39

Genetic elements in *cp4 epsps* expression cassette				
Genetic Element	**Localization (bp)**	**Donor**	**Function**	**Bibliographic reference**
LB (Left Border)	12067-12090	Octopine Ti plasmid, pTi15955	Left border sequence essential for transfer of T-DNA from the octopine Ti plasmid, pTi15955	Barker et al., 1983
Intervening sequence	12091-12364	*Agrobacterium*	Polylinker	Barker et al., 1983
Intervening sequence	12365-12	Synthetic	Polylinker	
P-ract1	13 - 946	Rice actin gene	Promoter	McElroy et al., 1990
ract1 intron	947 - 1407	Rice actin gene	Intron	McElroy et al., 1991
Intervening sequence	1408-1423	Synthetic	Polylinker	
CTP2	1424 -1651	*Arabidopsis thaliana*	Chloroplast transit peptide sequence	Klee et al., 1987
cp4 epsps	1652-3019	*Agrobacterium sp.* strain CP4	Coding sequence for the native CP4 EPSPS protein	Padgette et al., 1996
Intervening sequence	3020-3031	Synthetic	Polylinker	
NOS 3'	3032-3287	*Agrobacterium tumefaciens*	3' nontranslated region of the nopaline synthase (NOS) coding sequence, which terminates transcription and directs polyadenylation	Bevan et al., 1983
Intervening sequence	3288-3320	Synthetic	Polylinker	

Table 1 (cont.) Summary of genetic elements in vector PV-ZMIR39

Genetic elements in *cry3Bb1* expression cassette				
Genetic Element	**Localization (bp)**	**Donor**	**Function**	**Bibliographic reference**
P-e35S	3321-3933	Cauliflower mosaic virus	Promoter with the duplicated enhancer region	Odell et al., 1985 Kay et al., 1987
Intervening sequence	3934-3957	Synthetic	Polylinker	
wt CAB leader	3958-4028	Wheat	5' untranslated leader of the wheat chlorophyll a/b-binding protein	Lamppa et al., 1985
Intervening sequence	4029-4056	Synthetic	Polylinker	
ract1 intron	4057-4517	Rice actin gene	Intron	McElroy et al., 1991
Intervening sequence	4518-4533	Synthetic	Polylinker	
cry3Bb1	4534-6495	*Bacillus thuringiensis* subsp. *kumamotoensis*	Coding sequence for a synthetic variant of Cry3Bb1 protein	Romano, C.P., 2002
Intervening sequence	6496-6510	Synthetic	Polylinker	
tahsp17 3'	6511-6744	Wheat heat shock protein	3'nontranslated region of the coding sequence for wheat heat shock protein 17.3, which ends transcription and directs polyadenylation	McElwain and Spiker, 1989
Intervening sequence	6745-6840	*E. coli* and synthetic	Polylinker and synthetic	Depicker et al., 1982
RB (Right Border)	6841-6865	Nopaline Ti plasmid, pTiT37	Right border sequence essential for transfer of T-DNA from the nopaline Ti plasmid, pTiT37	Depicker et al, 1982

Table 1 (cont.) Summary of genetic elements in vector PV-ZMIR39

Genetic elements for expression of the plasmid in *E. coli*				
Genetic Element	**Localization (bp)**	**Donor**	**Function**	**Bibliographic reference**
Intervening sequence	6866-7350	Synthetic	Polylinker	Depicker et al, 1982 Sutcliffe, 1978 Fling et al., 1985
aad	7351-8139	Bacterial transposon Tn7	Bacterial promoter and coding sequence for an aminoglycoside-modifying enzyme, 3'(9)-O-nucleotidyltransferase from the transposon Tn7	Fling et al., 1985 GenBank accession X03043
Intervening sequence	8140-8681	Synthetic	Polylinker	Fling et al., 1985 Sutcliffe, 1978
ori-322	8682-9310	Plasmid pBR322 from *E. coli*	Origin of replication from pBR322 for maintenance of plasmid in *E. coli*	Sutcliffe, 1978
Intervening sequence	9311-9727	Plasmid pBR322 from *E. coli*	Portion of the plasmid	Sutcliffe, 1978
ROP	9728-9919	*E. coli*	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli*	Giza and Huang, 1989
Intervening sequence	9920-11182	Plasmid pBR322 from *E. coli*	Plasmid DNA	Sutcliffe, 1978
Intervening sequence	11183-11430	*E. coli*	Plasmid DNA	Stalker et al., 1981
ori-V	11431-11824	*Agrobacterium*, plasmid RK2	Origin of replication for *Agrobacterium*	Stalker et al., 1981
Intervening sequence	11825-11910	*E. coli* & Synthetic	Plasmid DNA	Stalker et al., 1981
Intervening sequence	11911-12066	*Agrobacterium*	DNA sequences	Barker et al, 1983

Figure 2. Deduced amino acid sequence of the CP4 EPSPS protein produced in MON 88017

```
  1  MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51  LKKSGMTLIG SELRPLKVMS SVSTACMLHG ASSRPATARK  SSGLSGTVRI
101  PGDKSISHRS FMFGGLASGE TRITGLLEGE DVINTGKAMQ AMGARIRKEG
151  DTWIIDGVGN GGLLAPEAPL DFGNAATGCR LTMGLVGVYD FDSTFIGDAS
201  LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP ITYRVPMASA
251  QVKSAVLLAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
301  TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR
351  TGLILTLQEM GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM
401  IDEYPILAVA AAFAEGATVM NGLEELRVKE SDRLSAVANG LKLNGVDCDE
451  GETSLVVRGR PDGKGLGNAS GAAVATHLDH RIAMSFLVMG LVSENPVTVD
501  DATMIATSFP EFMDLMAGLG AKIELSDTKA A
```


Sequence includes the CTP2 transit peptide (amino acids 1-76 underlined).

Figure 3. Deduced amino acid sequence of the Cry3Bb1 protein produced in MON 88017

```
  1  MANPNNRSEH DTIKVTPNSE LQTNHNQYPL ADNPNSTLEE LNYKEFLRMT
 51  EDSSTEVLDN STVKDAVGTG ISVVGQILGV VGVPFAGALT  SFYQSFLNTI
101  WPSDADPWKA FMAQVEVLID KKIEEYAKSK ALAELQGLQN NFEDYVNALN
151  SWKKTPLSLR SKRSQDRIRE LFSQAESHFR NSMPSFAVSK FEVLFLPTYA
201  QAANTHLLLL KDAQVFGEEW GYSSEDVAEF YRRQLKLTQQ YTDHCVNWYN
251  VGLNGLRGST YDAWVKFNRF RREMTLTVLD LIVLFPFYDI RLYSKGVKTE
301  LTRDIFTDPI  FLLTTLQKYG PTFLSIENSI RKPHLFDYLQ GIEFHTRLRP
351  GYFGKDSFNY WSGNYVETRP SIGSSKTITS PFYGDKSTEP VQKLSFDGQK
401  VYRTIANTDV AAWPNGKVYL GVTKVDFSQY DDQKNETSTQ TYDSKRNNGH
451  VSAQDSIDQL PPETTDEPLE KAYSHQLNYA ECFLMQDRRG TIPFFTWTHR
501  SVDFFNTIDA EKITQLPVVK AYALSSGASI IEGPGFTGGN LLFLKESSNS
551  IAKFKVTLNS AALLQRYRVR IRYASTTNLR LFVQNSNNDF LVIYINKTMN
601  KDDDLTYQTF DLATTNSNMG FSGDKNELII GAESFVSNEK IYIDKIEFIP
651  VQL
```

The six amino acids that differ from the wild-type Cry3Bb1 protein are indicated in underlined bold. The differences at the six positions are: 2A (insertion), H232R, S312L, N314T, E318K, Q349R.

SECTION 3. Characterization of the introduced genetic material

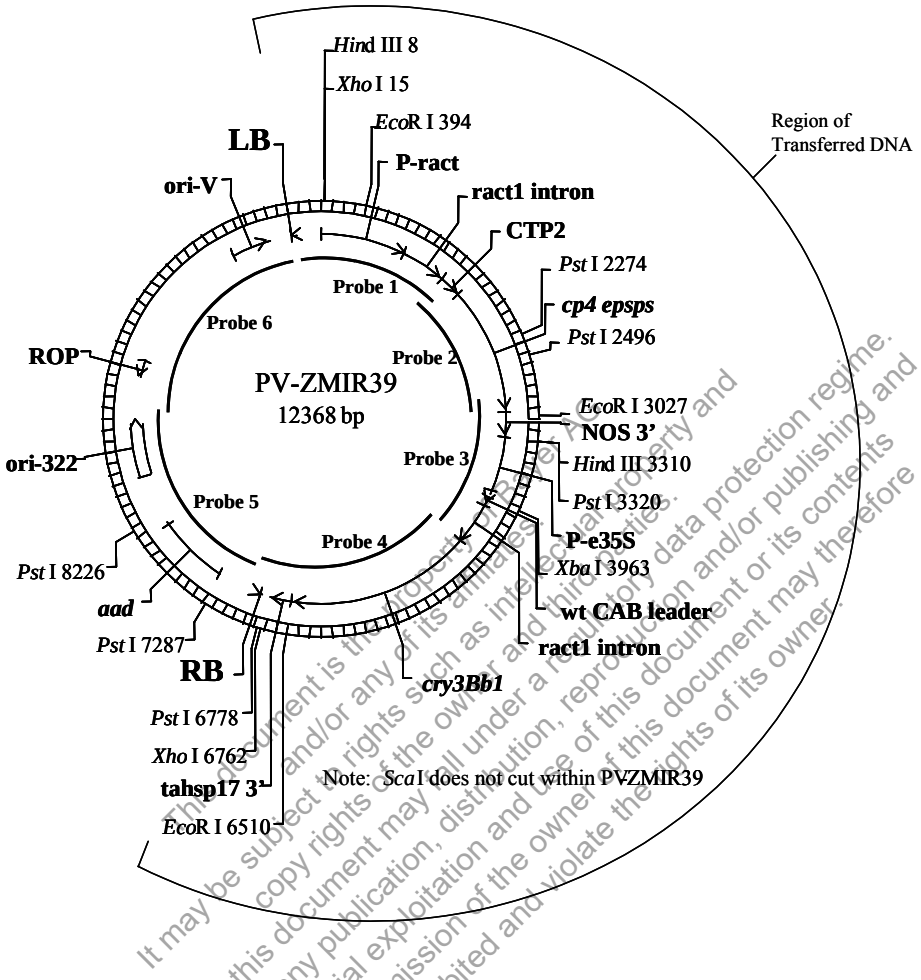
3.1 Molecular analysis – introduced DNA

Molecular analysis was performed to characterize the integrated DNA in MON 88017. Southern blot analysis was used to assess the following:

1) number of insertions of the integrated expression cassettes,
2) number of copies of the integrated expression cassettes,
3) intactness of both expression cassettes, and
4) stability of the inserted DNA during conventional breeding.

Detailed materials and methods for the molecular analysis are provided in Appendix I. A plasmid map illustrating the location of each element in PV-ZMIR39 is presented in Figure 1. Restriction enzyme sites and probes used for Southern blot analysis are presented in Figures 4a and 4b. A schematic presentation of the DNA inserted into the genome of MON 88017, including restriction enzyme sites and expected restriction fragments, is provided in Figure 5.

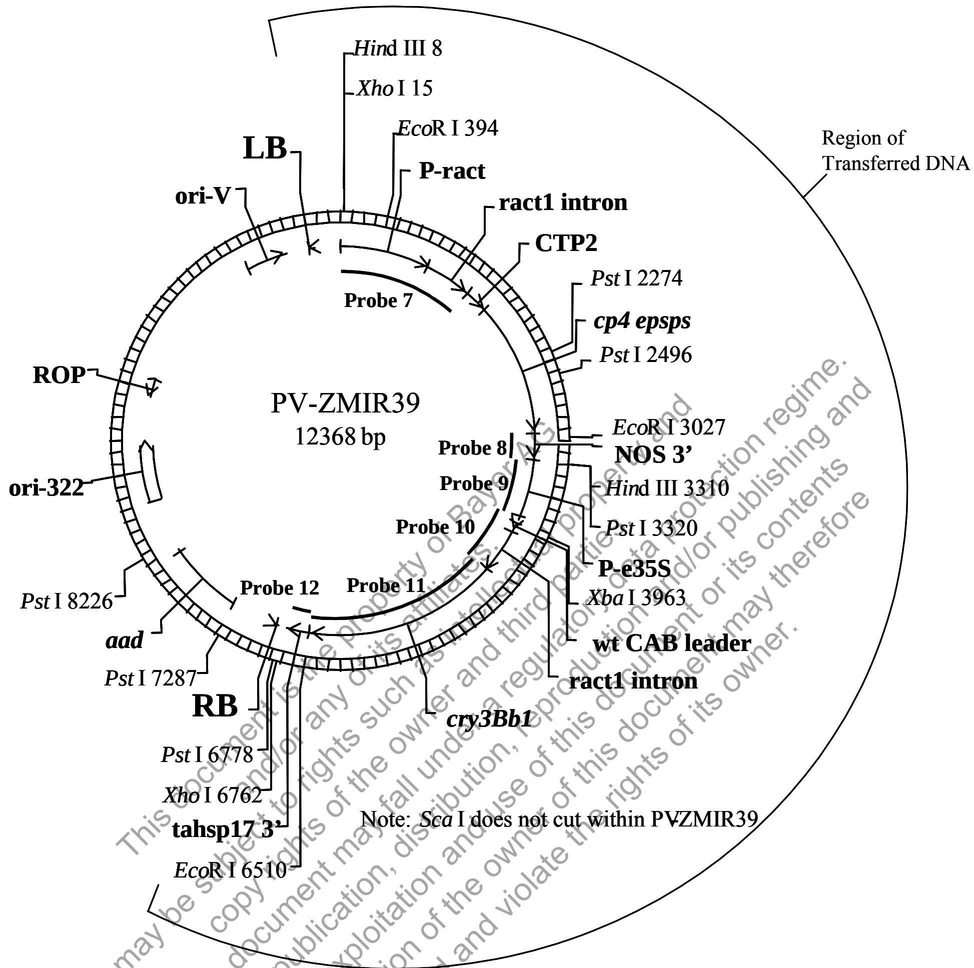
Figure 4a. Schematic representation of the probes 1- 6 generated from the vector PV-ZMIR39 for Southern blot analyses



A circular map of the plasmid vector PV-ZMIR39 used in *Agrobacterium sp.*-mediated transformation to produce MON 88017. Genetic elements are annotated in bold and restriction sites (with positions relative to the size of the plasmid vector) are shown for enzymes used in the Southern analyses. DNA probes were prepared by PCR amplification of plasmid PV-ZMIR39. The table describes probes 1-6 used in the Southern analyses.

Probe	Genetic Element(s)	Start	End	Total Length (bp)
1	Partial LB + P-ract + partial CTP2	12080	1482	1771
2	CTP2 + *cp4 epsps*	1423	3019	1597
3	Partial *cp4 epsps* + NOS 3' + P-e35S + wt CAB leader + ract1 intron	2991	4533	1543
4	*cry3Bb1* + tahsp17 3' + RB	4534	6865	2332
5	Partial backbone	6866	9492	2627
6	Partial backbone	9465	12066	2602

Figure 4b. Schematic representation of the probes 7- 12 generated from the vector PV-ZMIR39 for Southern blot analyses

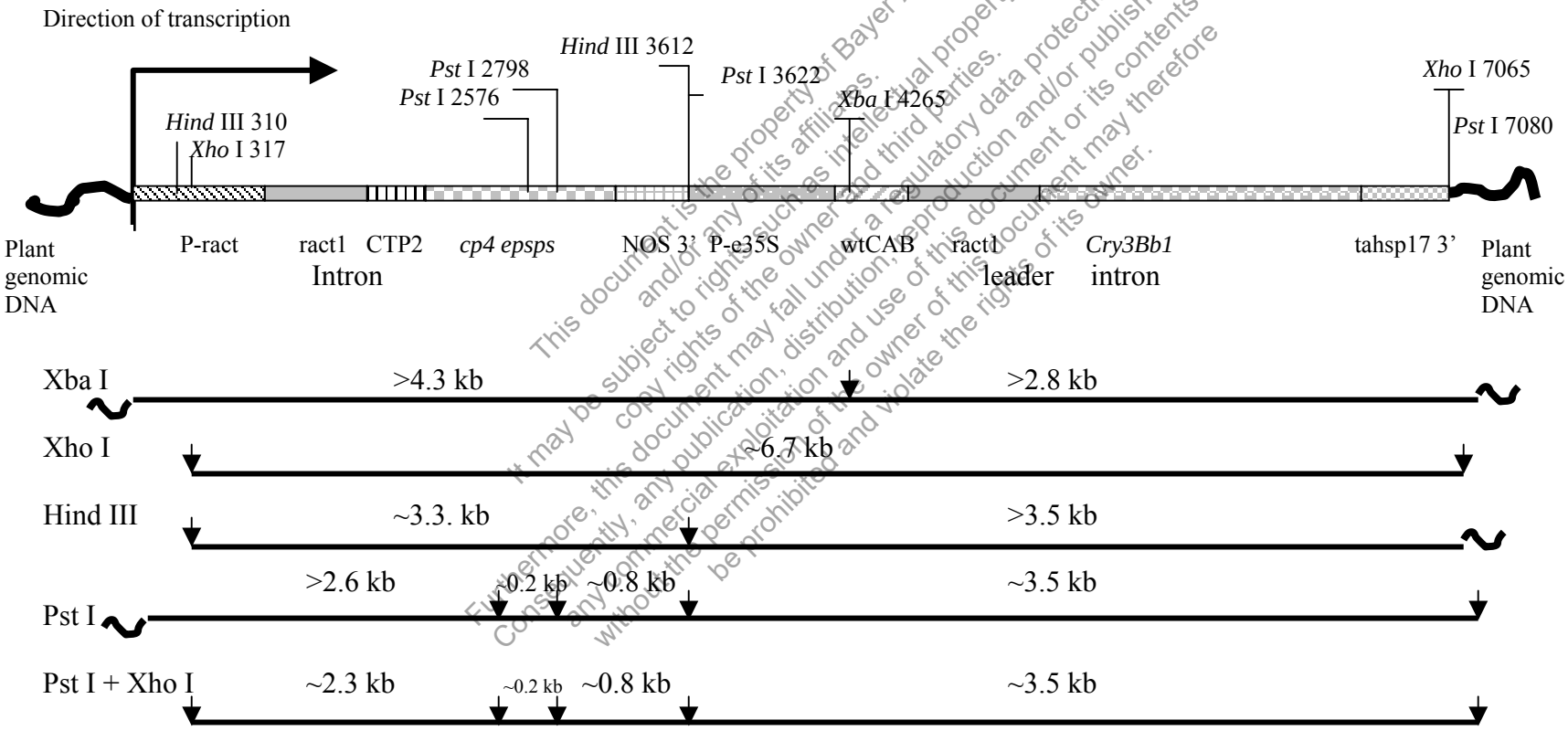


A circular map of the plasmid vector PV-ZMIR39 used in *Agrobacterium sp.*-mediated transformation to produce MON 88017. Genetic elements are annotated in bold and restriction sites (with positions relative to the size of the plasmid vector) are shown for enzymes used in the Southern analyses. DNA probes were prepared by PCR amplification of plasmid PV-ZMIR39. The table describes probes 7 – 12 used in the Southern analyses.

Probe	Genetic Element(s)	Start	End	Total Length (bp)
7	P-ract + ract1 intron	13	1407	1395
8	NOS 3'	3032	3287	256
9	P-e35S	3321	3933	613
10	wt CAB leader + ract1 intron	3958	4533	576
11	*cry3Bb1*	4534	6510	1977
12	tahsp17 3'	6511	6744	234

Figure 5. Schematic representation of the insert present in MON 88017

A linear map of the inserted DNA from transformation vector PV-ZMIR 39 is shown. Genetic elements are annotated. Positions of the restriction sites for enzymes used in the Southern blot analyses are included for reference. Arrows indicate sites of the restriction digest. Sizes of predicted restriction fragments, calculated from the size of the linear map are identified. MON 88017 contains one copy of the insert at a single integration locus (Figure 6).



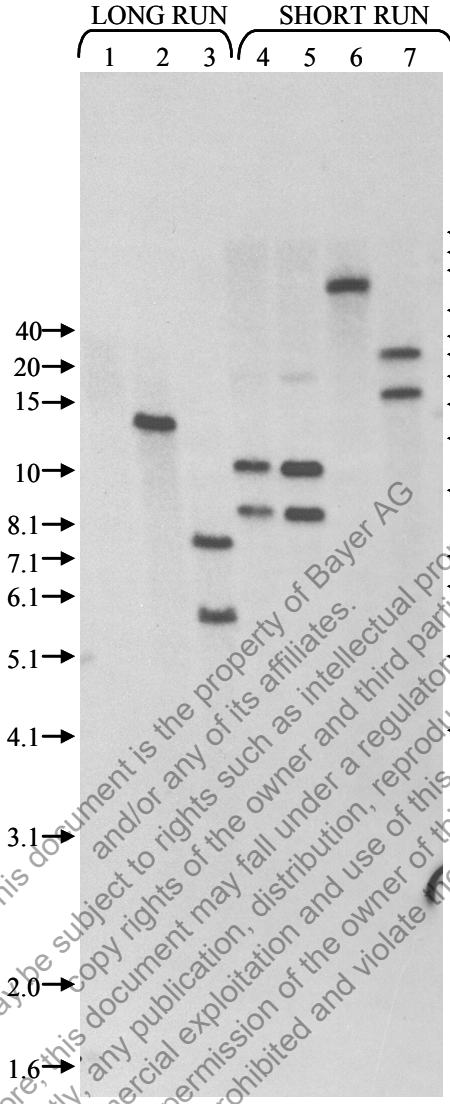
3.1.a. Insert and copy number

The insert number (the number of integration sites of the introduced DNA in the corn genome) was evaluated by digesting the MON 88017 and conventional corn genomic DNA (control in Figure 6) with the restriction enzyme *Sca* I, which does not cleave within the plasmid PV-ZMIR39. This enzyme should release a restriction fragment containing the inserted DNA and adjacent plant genomic DNA. The number of restriction fragments detected indicates the number of inserts present.

The number of copies of the introduced DNA was determined by digesting the MON 88017 genomic DNA with *Xba* I, a restriction enzyme that cuts only once within PV-ZMIR39. The blot was probed simultaneously with four radiolabeled fragments of plasmid DNA that span the entire length of the insert (Probes 1, 2, 3 and 4, Figure 4a). If one copy of the introduced DNA is present, two bands should be produced, representing two border fragments. Each of these border fragments would contain a portion of the inserted DNA and flanking corn genomic DNA. Therefore, from this analysis it was possible to determine the number of copies of the introduced DNA inserted in MON 88017. Plasmid PV-ZMIR39 DNA, previously digested with *Eco*R I, was mixed with control corn genomic DNA digested with *Sca* I, and then loaded on the gel to serve as a positive hybridization control.

The results of these analyses are shown in Figure 6. Plasmid PV-ZMIR39 DNA, previously digested with *Eco*R I, mixed with control corn genomic DNA digested with *Sca* I, produced the expected size bands of approximately 6.3 kb (faint), 3.5 kb and 2.6 kb, correlating to the sizes of PV-ZMIR39 *Eco*R I fragments (lanes 4 and 5). The ~6.3 kb band produced a weaker signal, because a smaller portion of the target DNA sequence is present on this ~6.3 kb *Eco*R I restriction fragment in comparison to the ~3.5 kb and ~2.6 kb fragments. MON 88017 genomic DNA digested with *Sca* I (lanes 2 and 6) produced one band at approximately 13 Kb. This result establishes that MON 88017 contains one insert located on an approximately 13 Kb *Sca* I restriction fragment. MON 88017 DNA digested with *Xba* I (lanes 3 and 7) produced two unique bands at approximately 7.4 kb and 5.5 kb, representing two border fragments. In combination with the insert number analysis, this result establishes that MON 88017 contains one copy of the introduced DNA at a single locus of integration.

Figure 6. Southern blot analysis of MON 88017: insert and copy number



The blot was probed simultaneously with four ^{32}P-labeled probes that span the full length of the insert (Probes 1, 2, 3, and 4, Figure 4a). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Sca* I)
2: MON 88017 (*Sca* I)
3: MON 88017 (*Xba* I)
4: Control (*Sca* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
5: Control (*Sca* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
6: MON 88017 (*Sca* I)
7: MON 88017 (*Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

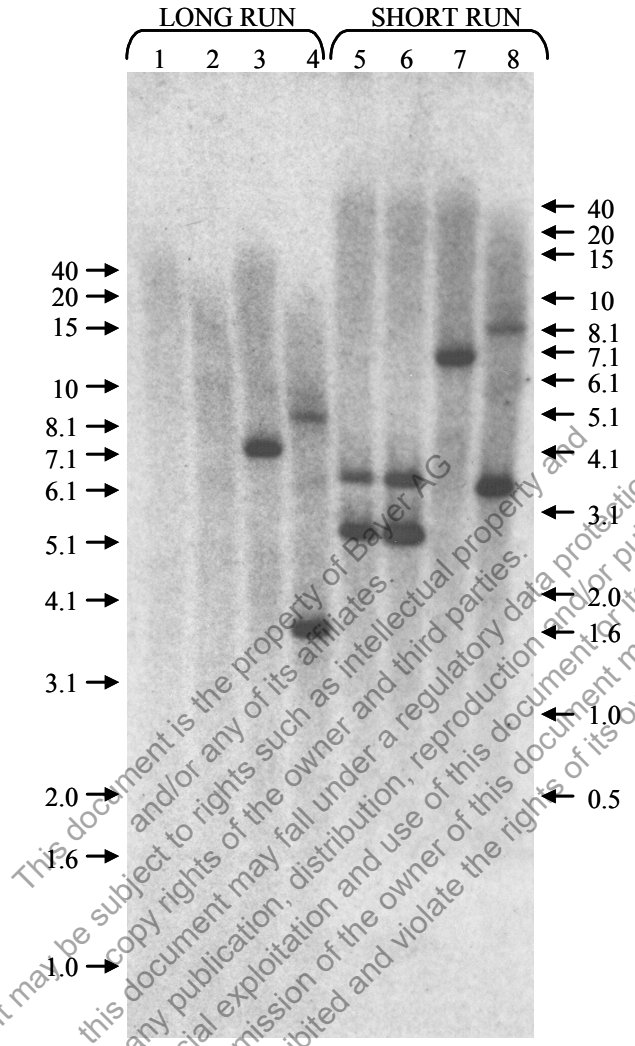
3.1.b. *cp4 epsps* cassette intactness

MON 88017 corn genomic DNA was digested individually with *Xho* I to release the inserted DNA and with *Hin*d III to release the *cp4 epsps* cassette. Control genomic DNA from conventional corn was digested with *Xho* I, or in some experiments with *Hin*d III, to serve as a negative control. Plasmid PV-ZMIR39 DNA, previously digested with *Eco*R I, was mixed with control DNA digested with *Xho* I, and the mixture was then loaded on the gel to serve as a positive hybridization control. Individual Southern blots were probed with the rice actin promoter (P-ract) + ract1 intron, the CTP2 + *cp4 epsps* coding region, or the NOS 3' polyadenylation sequence (Probes 7, 2, and 8, respectively, Figure 4b). The presence of a band representing the expected size of the full inserted DNA (*Xho* I digest, ~6.7 kb) or the *cp4 epsps* cassette (*Hin*d III digest, ~3.3 kb) indicates that the *cp4 epsps* cassette and each of its elements are intact. For reference, the expected sizes of the full insert and *cp4 epsps* cassette upon restriction enzyme digestion are shown in Figure 5. In these Southern analyses, the migration of the ~6.7 kb *Xho* I fragment containing the entire insert was slightly slower than expected. This altered migration is likely due to the difference in salt concentrations between the test DNA sample and the molecular weight marker (Sambrook, 2001).

3.1.c p-ract + ract1 intron probe

The results of this analysis are shown in Figure 7. The Southern blot containing control, plasmid and MON 88017 genomic DNA digested with *XhoI* or *HindIII* was hybridized with the P-ract + ract intron probe (Probe 7, Figure 4b). Control corn genomic DNA digested with *Xho* I or *Hin*d III (lanes 1 and 2, respectively) showed faint endogenous hybridization, indicating regions of homology between the corn genome and the probe. Plasmid PV-ZMIR39 DNA, previously digested with *Eco*R I, mixed with control genomic DNA digested with *Xho* I, produced the expected size bands at approximately 3.5 kb and 2.6 kb (lanes 5 and 6). MON 88017 DNA digested with *Xho* I (lanes 3 and 7) produced a band corresponding to the expected size of the insert (approximately 6.7 kb) in addition to the faint endogenous hybridization pattern seen in the negative control (lane 1). MON 88017 DNA digested with *Hin*d III (lanes 4 and 8) produced bands at approximately 8.5 kb and 3.3 kb in addition to the faint endogenous hybridization pattern seen in the control (lane 2). The ~3.3 kb band corresponds to the expected size of an intact *cp4 epsps* cassette. The ~8.5 kb band corresponds to a border fragment predicted by hybridization of the ract1 intron portion of the probe to the ract1 intron target sequence present in the *cry3Bb1* cassette. No unexplained bands were detected, indicating that MON 88017 does not contain any additional, detectable rice actin promoter or ract1 intron elements other than those associated with the intact *cp4 epsps* or *cry3Bb1* cassettes.

Figure 7. Southern blot analysis of MON 88017: p-ract + ract1 intron probe



The blot was probed with ^{32}P-labeled rice actin promoter and intron (Probe 7, Figure 4b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Xho* I)
2: Control (*Hin*d III)
3: MON 88017 (*Xho* I)
4: MON 88017 (*Hin*d III)
5: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
6: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
7: MON 88017 (*Xho* I)
8: MON 88017 (*Hin*d III)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

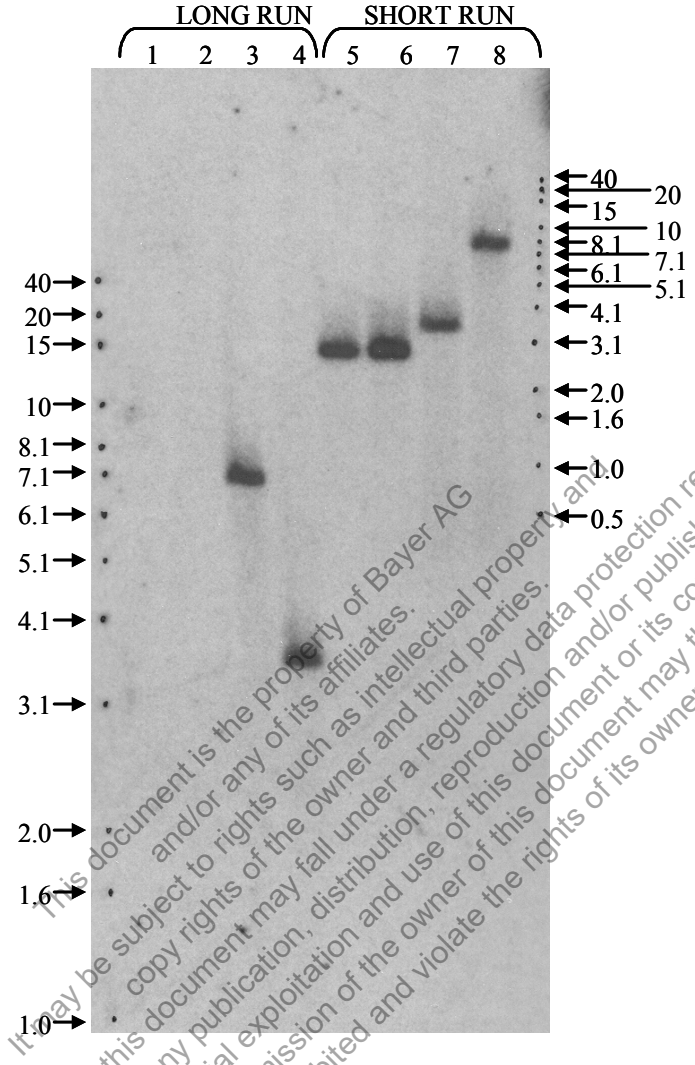
3.1.d. *ctp2 + cp4 epsps* probe

The results of this analysis are shown in Figure 8. The Southern blot containing control, plasmid and MON 88017 genomic DNA digested with *XhoI* or *HindIII* was hybridized with the *CTP2* + *cp4 epsps* probe (Probe 2, Figure 4a). Control corn genomic DNA digested with *Xho* I or *Hin*d III (lanes 1 and 2, respectively) showed no detectable hybridization bands, as expected for a negative control. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I, mixed with control DNA digested with *Xho* I, produced the expected size band at approximately 2.6 kb (lanes 5 and 6). MON 88017 genomic DNA digested with *Xho* I (lanes 3 and 8) produced a band corresponding to the expected size of the insert (approximately 6.7 kb). MON 88017 DNA digested with *Hin*d III (lanes 4 and 7) produced a band at approximately 3.3 kb, corresponding to the expected size of an intact *cp4 epsps* cassette. No unexpected bands were detected, indicating that MON 88017 does not contain any additional, detectable *CTP2* + *cp4 epsps* coding regions other than that associated with the intact *cp4 epsps* cassette.

3.1.e. NOS 3' probe

The results of this analysis are shown in Figure 9. The Southern blot containing control, plasmid and MON 88017 genomic DNA digested with *XhoI* or *HindIII* was hybridized with the NOS 3' probe (Probe 8, Figure 4b). Control corn genomic DNA digested with *Xho* I (lane 1) showed no detectable hybridization bands, as expected for a negative control. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I, mixed with control DNA digested with *Xho* I, produced the expected size band at approximately 3.5 kb (lanes 4 and 5). MON 88017 DNA digested with *Xho* I (lanes 2 and 6) produced a band corresponding to the expected size of the insert (approximately 6.7 kb). MON 88017 DNA digested with *Hind* III (lanes 3 and 7) produced a band at approximately 3.3 kb, corresponding to the expected size of an intact *cp4 epsps* cassette. No unexpected bands were detected, indicating that MON 88017 does not contain any additional, detectable NOS 3' polyadenylation elements other than that associated with the intact *cp4 epsps* cassette.

Figure 8. Southern blot analysis of MON 88017: *ctp2 + cp4 epsps* probe

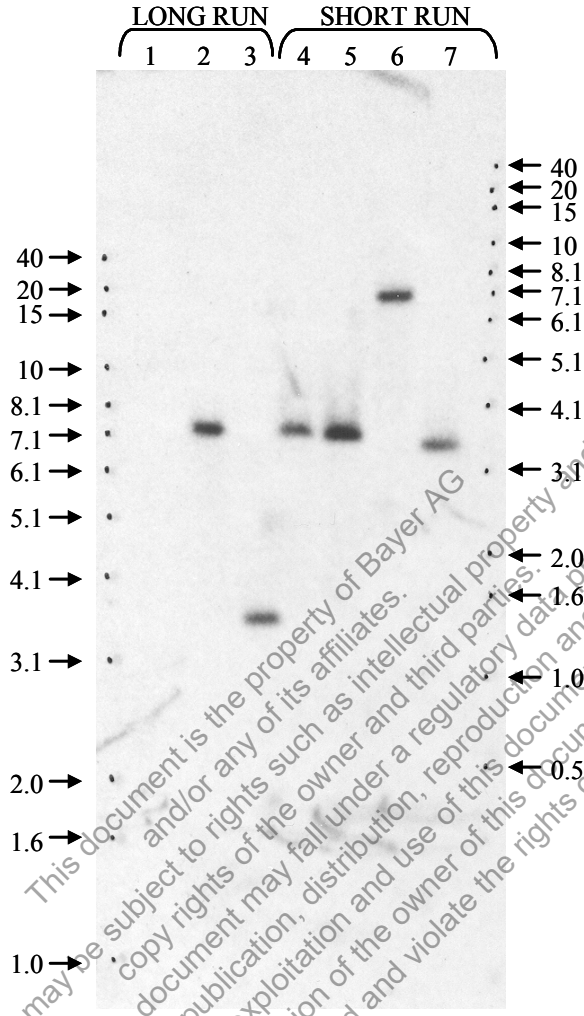


The blot was probed with ^{32}P-labeled CTP2 + *cp4 epsps* (Probe 2, Figure 4a). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Xho* I)
2: Control (*Hin*d III)
3: MON 88017 (*Xho* I)
4: MON 88017 (*Hin*d III)
5: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
6: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
7: MON 88017 (*Hin*d III)
8: MON 88017 (*Xho* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

Figure 9. Southern blot analysis of MON 88017: NOS 3' probe



The blot was probed with ^{32}P-labeled NOS 3' polyadenylation sequence (Probe 8, Figure 4b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Xho* I)
2: MON 88017 (*Xho* I)
3: MON 88017 (*Hin*d III)
4: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
5: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
6: MON 88017 (*Xho* I)
7: MON 88017 (*Hin*d III)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

3.1.f. *cry3Bb1* cassette intactness

MON 88017 genomic DNA was digested with *Xho* I to release the inserted DNA. Additionally, MON 88017 genomic DNA was digested with *Pst* I to release the *cry3Bb1* cassette. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I, was mixed with control DNA digested with *Xho* I, and the mixture was then loaded on the gel to serve as a positive hybridization control. Control genomic DNA was digested with *Xho* I to serve as a negative control. Individual Southern blots were hybridized with the enhanced 35S promoter (P-e35S), the wt CAB leader + ract1 intron, the *cry3Bb1* coding region, or the tahsp17 3' polyadenylation sequence (Probes 9, 10, 11 and 12, respectively, Figure 4b). The presence of a band representing the expected size of the full insert (*Xho* I digest, ~6.7 kb) or the *cry3Bb1* cassette (*Pst* I digest, ~3.5 kb) indicates that the *cry3Bb1* cassette and each of its elements are intact. For reference, the expected sizes of the full insert and the *cry3Bb1* cassette upon restriction enzyme digestion are shown in Figure 5. Similar to the *cp4 epsps* Southern analyses, occasionally the migration of the ~6.7 kb *Xho* I fragment containing the entire insert was slightly slower than expected. This altered migration is likely due to the difference in salt concentrations between the test DNA sample and the molecular weight marker (Sambrook, 2001).

3.1.g. p-e35S probe

The results of this analysis are shown in Figure 10. The Southern blot containing control, plasmid and MON 88017 genomic DNA digested with *XhoI* or *PstI* was hybridized with the P-e35S probe (Probe 9, Figure 4b). Control DNA digested with *Xho* I (lane 1) showed no detectable hybridization bands, as expected for the negative control. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I, mixed with control DNA digested with *Xho* I, produced the expected size band at approximately 3.5 kb (lanes 4 and 5). MON 88017 genomic DNA was digested with *Xho* I (lanes 2 and 6) produced a band corresponding to the expected size of the inserted DNA (approximately 6.7 kb). MON 88017 genomic DNA digested with *Pst* I (lanes 3 and 7) produced a band at approximately 3.5 kb, corresponding to the expected size of an intact *cry3Bb1* cassette. No unexpected bands were detected, indicating that MON 88017 does not contain any additional, detectable enhanced 35S promoter elements other than that associated with the intact *cry3Bb1* cassette.

3.1.h. wt CAB leader + ract1 intron probe

The results of this analysis are shown in Figure 11. The Southern blot containing control, plasmid and MON 88017 genomic DNA digested with *XhoI* or *PstI* was hybridized with the wtCAB leader + ract intron probe (Probe 10, Figure 4b). Control corn genomic DNA digested with *Xho* I (lane 1) showed no detectable hybridization bands, as expected for a negative control. Plasmid PV-ZMIR39 DNA, previously digested with *Eco*R I, mixed with control DNA digested with *Xho* I, produced the expected size bands at approximately 3.5 kb and 2.6 kb (lanes 4 and 5). MON 88017 genomic DNA digested with *Xho* I (lanes 2 and 7) produced a band corresponding to the expected size of the insert (approximately 6.7 kb). MON 88017 genomic DNA digested with *Pst* I (lanes 3

and 6) produced bands at approximately 3.8 kb and 3.5 kb. The ~3.5 kb band corresponds to the expected size of the intact *cry3Bb1* cassette. The ~3.8 kb band corresponds to a border fragment predicted by hybridization of the ract1 intron portion of the probe to the ract1 intron target sequence present in the *cp4 epsps* cassette. No unexpected bands were detected, indicating that MON 88017 corn does not contain any additional, detectable wt CAB leader or ract1 intron elements other than those associated with the intact *cry3Bb1* or *cp4 epsps* cassettes.

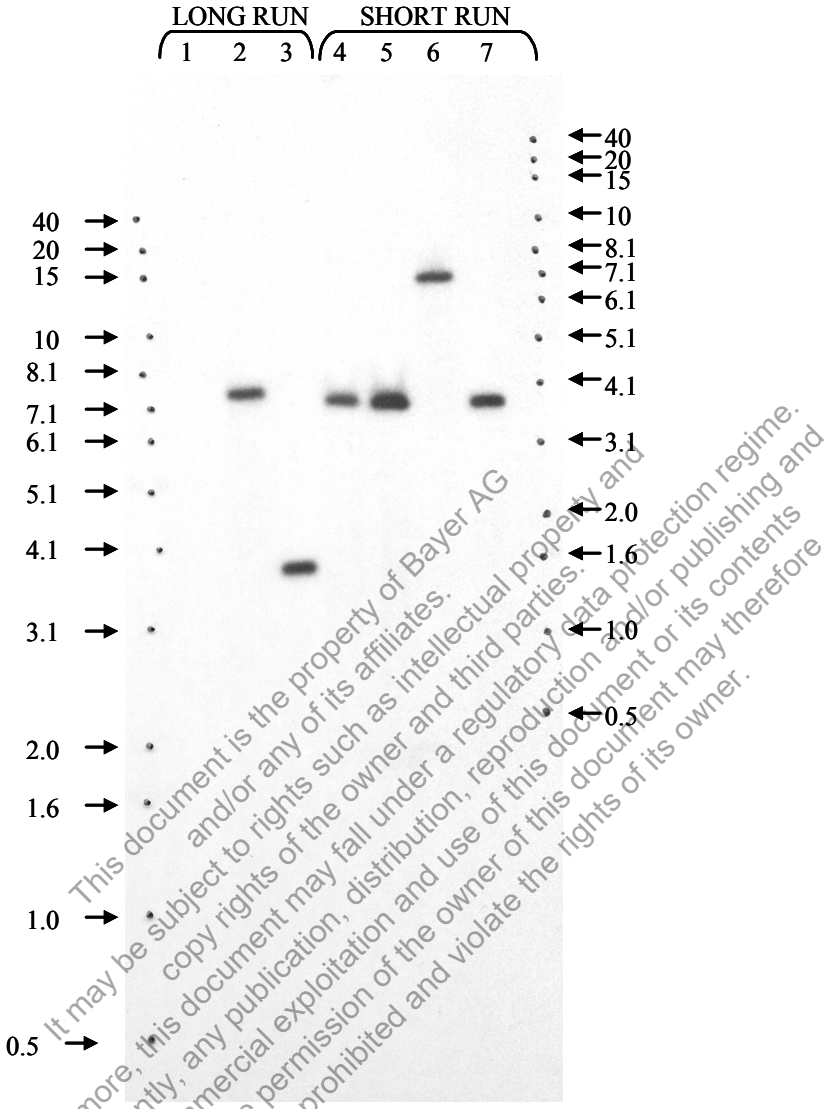
3.1.i. *cry3Bb1* probe

The results of this analysis are shown in Figure 12. The Southern blot containing control, plasmid and MON 88017 genomic DNA digested with *XhoI* or *HindIII* was hybridized with the *cry3Bb1* probe (Probe 11, Figure 4b). Control corn genomic DNA digested with *Xho* I (lane 1) showed no detectable hybridization bands, as expected for the negative control. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I, mixed with control DNA digested with *Xho* I, produced the expected size band at approximately 3.5 kb (lanes 4 and 5). MON 88017 genomic DNA digested with *Xho* I (lanes 2 and 6) produced a band corresponding to the expected size of the insert (approximately 6.7 kb). MON 88017 DNA digested with *Pst* I (lanes 3 and 7) produced a band at approximately 3.5 kb, corresponding to the expected size of an intact *cry3Bb1* cassette. No unexpected bands were detected, indicating that MON 88017 does not contain any additional, detectable *cry3Bb1* coding regions other than that associated with the intact *cry3Bb1* cassette.

3.1.j. tahsp17 3' probe

The results of this analysis are shown in Figure 13. The Southern blot containing control, plasmid and MON 88017 genomic DNA digested with *XhoI* or *PstI* was hybridized with the *tahsp17 3'* probe (Probe 12, Figure 4b). Control corn genomic DNA digested with *Xho* I (lane 1) showed no detectable hybridization bands, as expected for a negative control. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I, mixed with control DNA digested with *Xho* I, produced the expected size band at approximately 6.3 kb (lanes 4 and 5). MON 88017 genomic DNA digested with *Xho* I (lanes 2 and 6) produced a band corresponding to the expected size of the insert (approximately 6.7 kb). MON 88017 DNA digested with *Pst* I (lanes 3 and 7) produced a band at approximately 3.5 kb, corresponding to the expected size of an intact *cry3Bb1* cassette. A small area of hybridization was present in lane 3 directly above the expected hybridization signal. This spot does not span the entire lane, and does not appear in the short run of the same sample in lane 7, indicating that this signal is an artifact. No unexpected bands were detected, indicating that MON 88017 does not contain any additional, detectable tahsp17 3' polyadenylation elements other than that associated with the intact *cry3Bb1* cassette.

Figure 10. Southern blot analysis of MON 88017: p-e35S probe

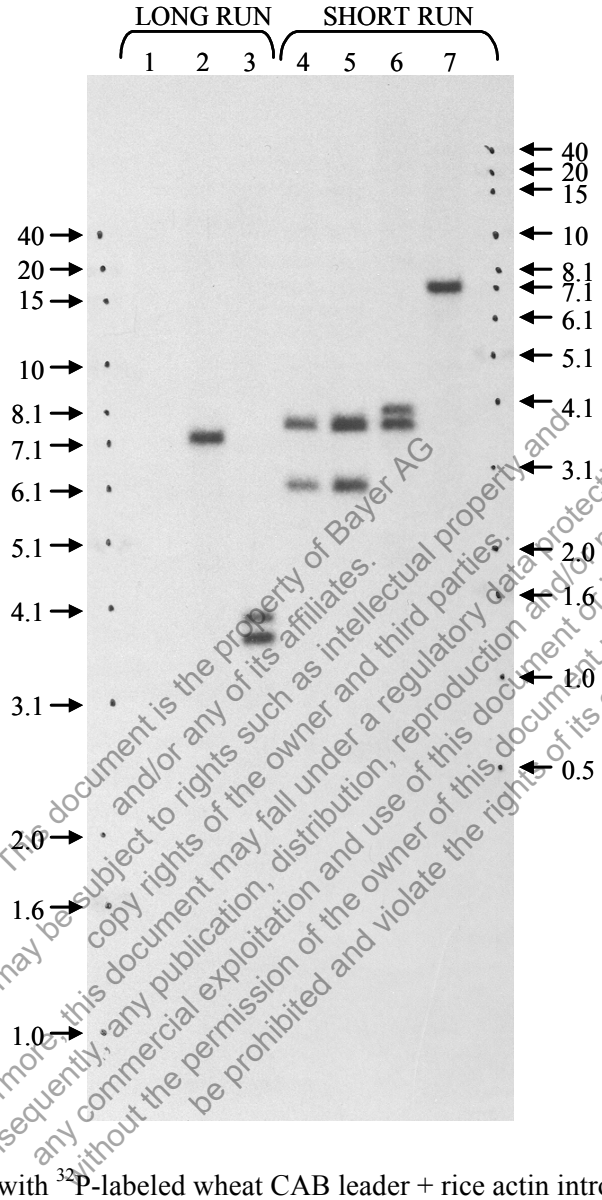


The blot was probed with ^{32}P-labeled enhanced 35S promoter (Probe 9, Figure 4b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Xho* I)
2: MON 88017 (*Xho* I)
3: MON 88017 (*Pst* I)
4: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
5: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
6: MON 88017 (*Xho* I)
7: MON 88017 (*Pst* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

Figure 11. Southern blot analysis of MON 88017: wtCAB leader + ract1 intron probe

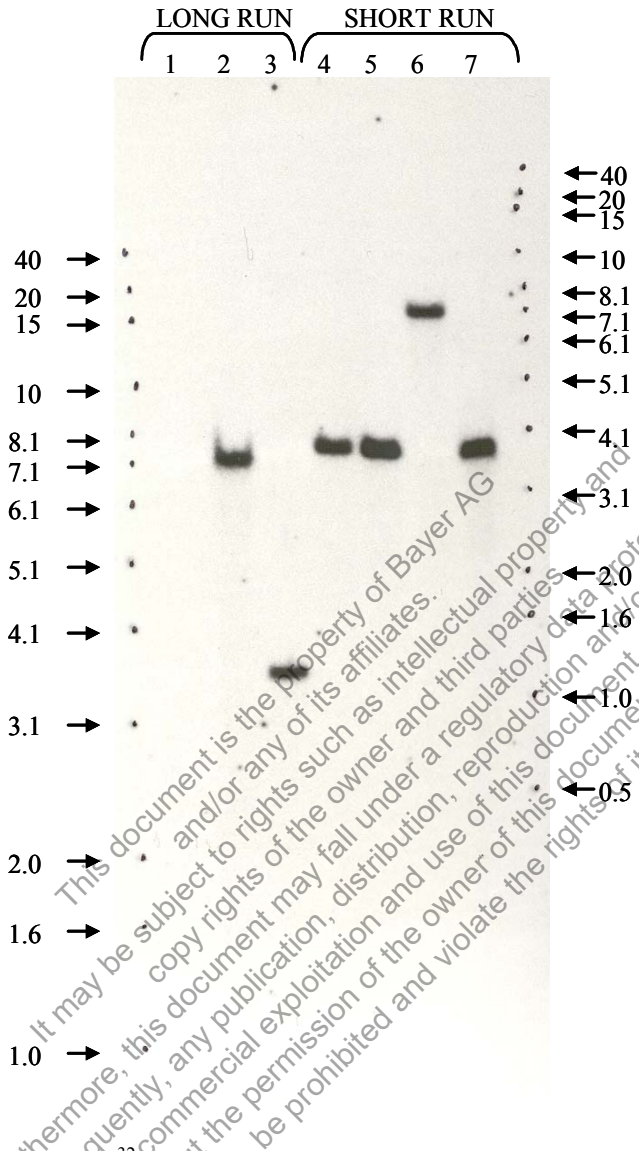


The blot was probed with ^{32}P-labeled wheat CAB leader + rice actin intron (Probe 10, Figure 4b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Xho* I)
2: MON 88017 (*Xho* I)
3: MON 88017 (*Pst* I)
4: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
5: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
6: MON 88017 (*Pst* I)
7: MON 88017 (*Xho* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

Figure 12. Southern blot analysis of MON 88017: *cry3Bb1* probe

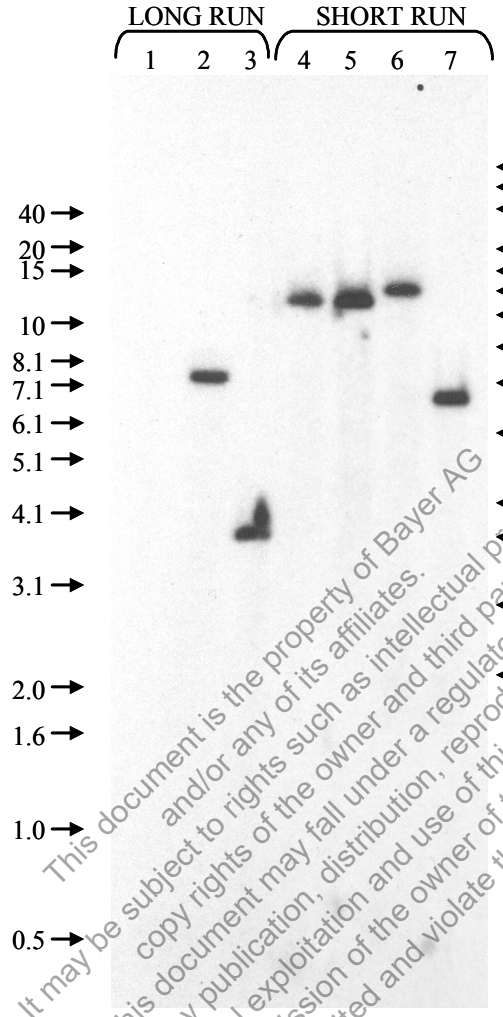


The blot was probed with ^{32}P-labeled *cry3Bb1* coding region (Probe 11, Figure 4b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Xho* I)
2: MON 88017 (*Xho* I)
3: MON 88017 (*Pst* I)
4: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
5: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
6: MON 88017 (*Xho* I)
7: MON 88017 (*Pst* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

Figure 13. Southern blot analysis of MON 88017: tahsp17 3' probe



The blot was probed with ^{32}P-labeled tahsp17 3' polyadenylation sequence (Probe 12, Figure 4b). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control (*Xho* I)
2: MON 88017 (*Xho* I)
3: MON 88017 (*Pst* I)
4: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
5: Control (*Xho* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
6: MON 88017 (*Xho* I)
7: MON 88017 (*Pst* I)

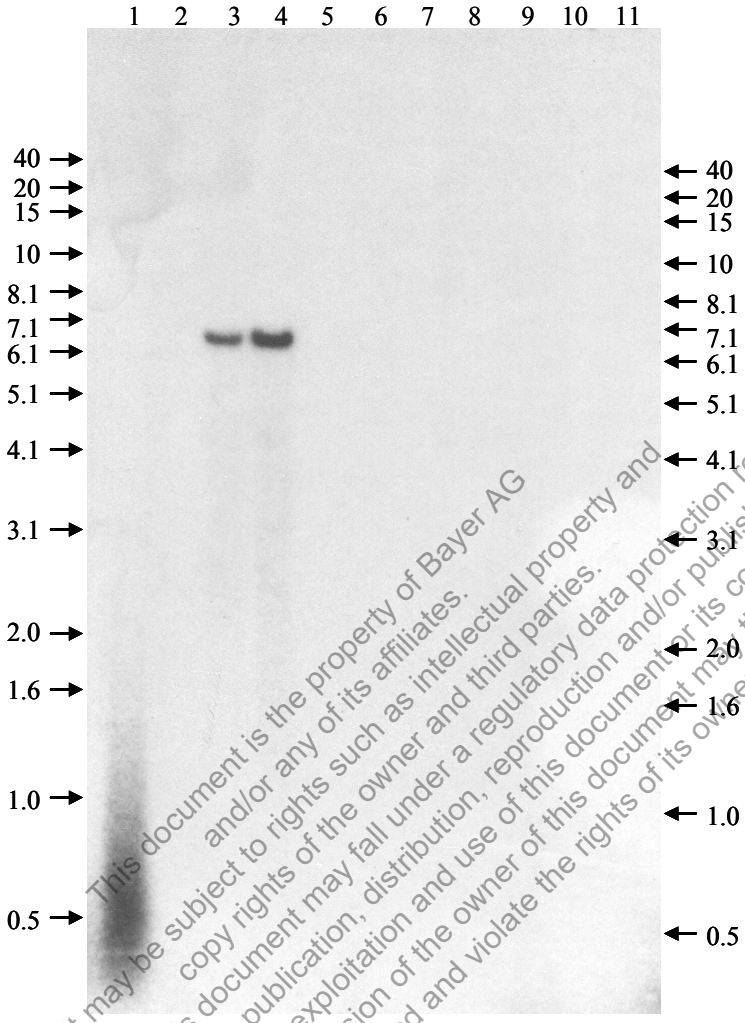
→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

3.2. Analysis for backbone fragments

For this analysis, MON 88017 and control DNA samples were digested with *Xba* I, which cuts only once within PV-ZMIR39. The blot was hybridized simultaneously with two radiolabeled probes that span the entire backbone sequence in PV-ZMIR39 (Probes 5 and 6, Figure 4a). The MON 88017 samples were obtained from the same generation used to test for stability of the insert during breeding (Figure 16). These generations are shown on the MON 88017 breeding tree (Figure 15). Because of expected cross-hybridization of the probes to the molecular weight marker bands, these lanes were trimmed from the blot prior to hybridization. Aligning these lanes to the blot after hybridization allowed for appropriate annotation of the molecular weight marker band sizes on the film.

The results of this analysis are shown in Figure 14. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I and mixed with digested control corn genomic DNA (lanes 3 and 4) produced one expected size band at approximately 6.3 kb. The control LH198 DNA (lane 1) produced a diffuse hybridization signal below 1.6 kb, which is likely the result of bacterial contamination of this source of grain. As expected, the second source of control DNA (lane 2) did not produce any detectable hybridization. Genomic DNA from plants representing six generations of MON 88017 (lanes 5 through 11) also showed no detectable hybridization bands. These results establish that the MON 88017 does not contain any detectable backbone sequences from the transformation vector PV-ZMIR39.

Figure 14. Southern blot analysis of MON 88017: backbone analysis



The blot was probed simultaneously with two ^{32}P-labeled probes that span the backbone in PV-ZMIR39 (Probes 5 and 6, Figure 4a). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control LH198 (*Xba* I)
2: Control LH59 x LH198 (*Xba* I)
3: Control LH59 x LH198 (*Xba* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
4: Control LH59 x LH198 (*Xba* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
5: MON 88017 [LH198BC0F_1 x LH59]F_2 (*Xba* I)
6: MON 88017 LH198BC0F_1 (*Xba* I)
7: MON 88017 LH198BC0F_2 (*Xba* I)
8: MON 88017 LH198BC1F_1 (*Xba* I)
9: MON 88017 LH198BC1F_3 x $LH59_{(H)}$ (*Xba* I)
10: MON 88017 LH59 x LH198BC3$F_{3(H)}$ (*Xba* I)
11: MON 88017 HC33 x LH59BC2$F_{3(H)}$ (*Xba* I)

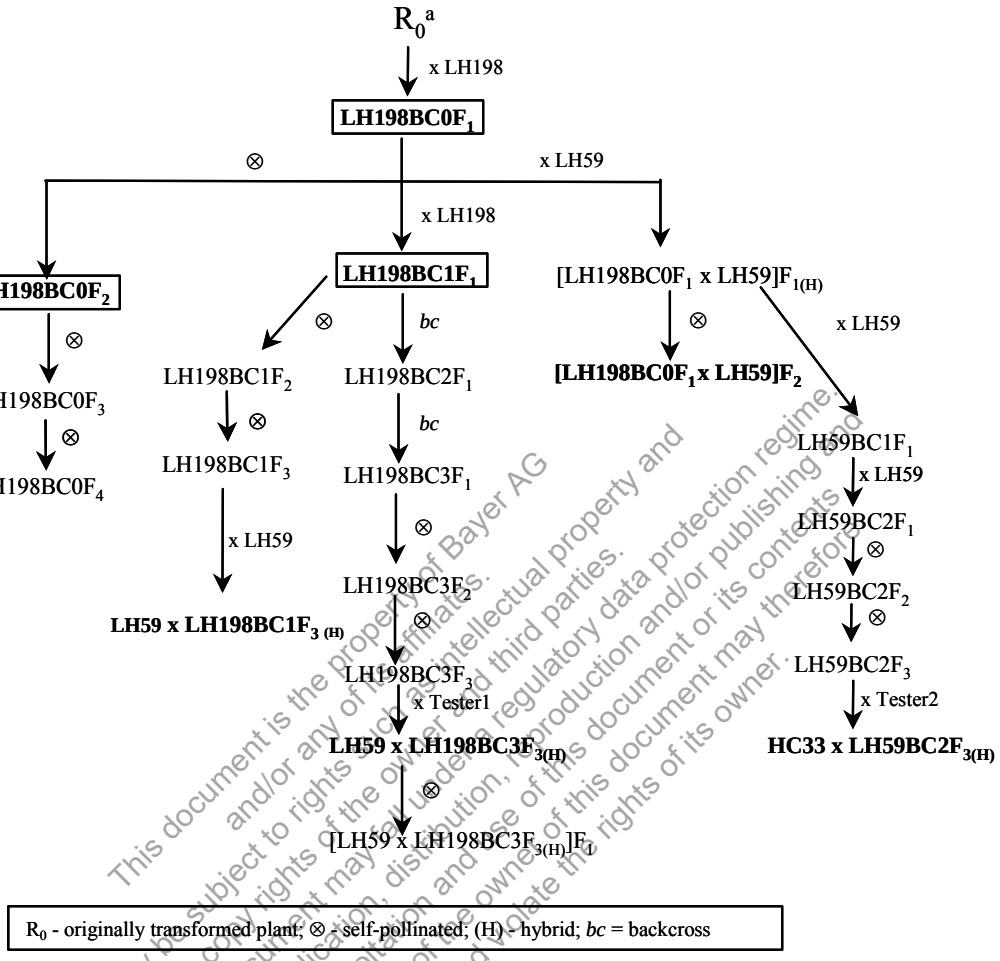
→Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

3.3. Insert stability in multiple breeding generations

In order to determine generational stability of the integrated DNA, a Southern blot analysis to determine the number of copies of integrated transgenes was performed on multiple generations from the breeding tree of MON 88017. For reference, the breeding tree of MON 88017 is presented in Figure 15. The specific generations tested are indicated in bold. The [LH198BC0F$_1$ x LH159]F$_2$ generation, which was used as a test substance in the molecular characterization analyses described in Sections 3.1 – 3.4 of this Part IV, was included as a reference in this analysis. For this analysis, MON 88017 and conventional corn genomic DNA samples were digested with *Xba* I, which digests only once within PV-ZMIR39. The blot was probed simultaneously with four radiolabeled probes that span the entire length of the insert (Probes 1, 2, 3 and 4, Figure 4a).

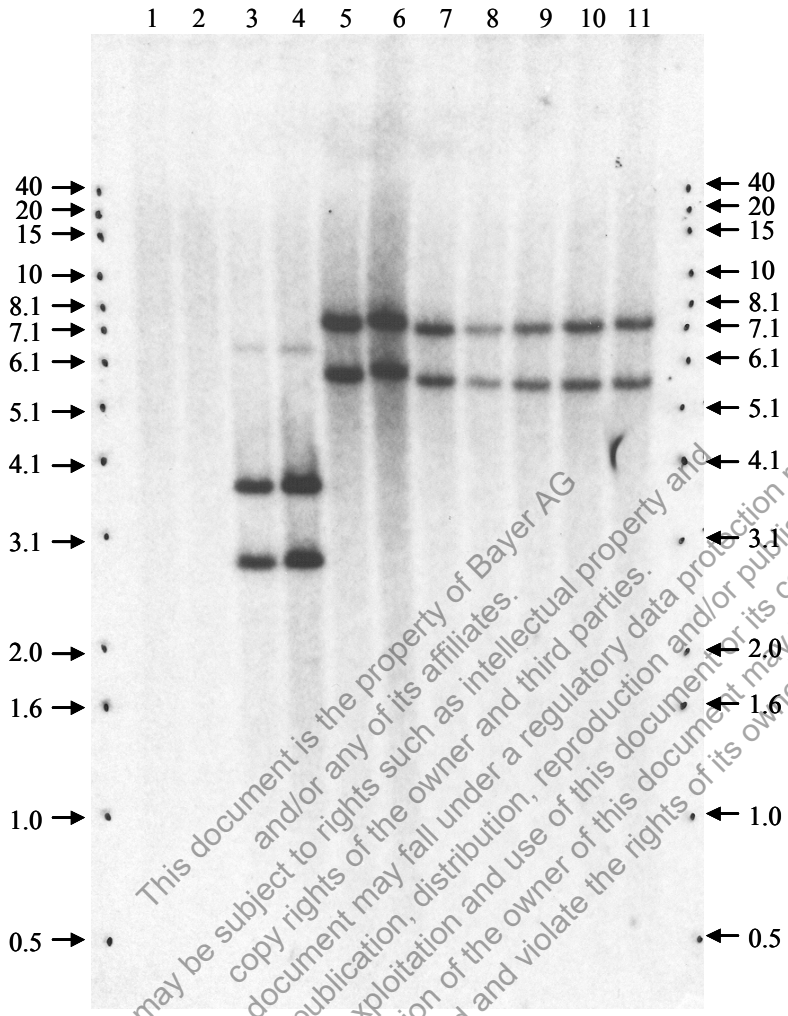
The results of the Southern blot analysis are shown in Figure 16. Plasmid PV-ZMIR39 DNA previously digested with *Eco*R I, mixed with digested control DNA, produced the expected size bands of approximately 6.3 kb, 3.5 kb and 2.6 kb, correlating to the sizes of PV-ZMIR39 *Eco*R I fragments (lanes 3 and 4). The ~6.3 kb band produced a weaker signal because a smaller portion of the target insert sequence is present on this ~6.3 kb *Eco*R I restriction fragment in comparison to the ~3.5 kb and ~2.6 kb fragments. The previously characterized generation (lane 5) produced the expected size bands of approximately 7.4 kb and 5.5 kb that had been produced in the previous copy number analysis (Figure 6). In addition, six generations of MON 88017 (lanes 6 through 11) produced the same size bands as the previously characterized generation (~7.4 kb and ~5.5 kb). These results demonstrate that the expected Southern fingerprint of the MON 88017 insert has been maintained across the branches of the breeding tree that were tested. Therefore, the stability of the integrated DNA in MON 88017 has been established over multiple generations.

Figure 15. Breeding tree of MON 88017



Data	**MON 88017 Generation**
Molecular analysis:	[LH198BC0F$_1$ x LH59]F$_2$
Insert stability (Southern. blot):	**generations bolded**
Protein stability (western blot):	**generations bolded**
2002 T/C/R trials:	LH59 x LH198BC3F$_{3(H)}$ seed; [LH59 x LH198BC3F$_{3(H)}$]F$_1$ grain
Expression/composition:	LH59 x LH198BC3F$_{3(H)}$ seed; [LH59 x LH198BC3F$_{3(H)}$]F$_1$ grain

Figure 16. Stability of integrated DNA in MON 88017



The blot was probed simultaneously with four ^{32}P-labeled probes that span the full length of the inserted DNA (Probes 1, 2, 3, and 4, Figure 4a). Each lane contains ~10 µg of digested genomic DNA isolated from grain. Lane designations are as follows:

Lane 1: Control LH198 (*Xba* I)
2: Control LH59 x LH198 (*Xba* I)
3: Control LH59 x LH198 (*Xba* I) spiked with PV-ZMIR39 (*Eco*R I) [0.5 copy]
4: Control LH59 x LH198 (*Xba* I) spiked with PV-ZMIR39 (*Eco*R I) [1.0 copy]
5: MON 88017 [LH198BC0F_1 x LH59]F_2 (*Xba* I)
6: MON 88017 LH198BC0F_1 (*Xba* I)
7: MON 88017 LH198BC0F_2 (*Xba* I)
8: MON 88017 LH198BC1F_1 (*Xba* I)
9: MON 88017 LH198BC1F_3 x $LH59_{(H)}$ (*Xba* I)
10: MON 88017 LH59 x LH198BC3$F_{3(H)}$ (*Xba* I)
11: MON 88017 HC33 x LH59BC2$F_{3(H)}$ (*Xba* I)

→ Symbol denotes size of DNA, in kilobase pairs, obtained from MW markers on ethidium stained gel.

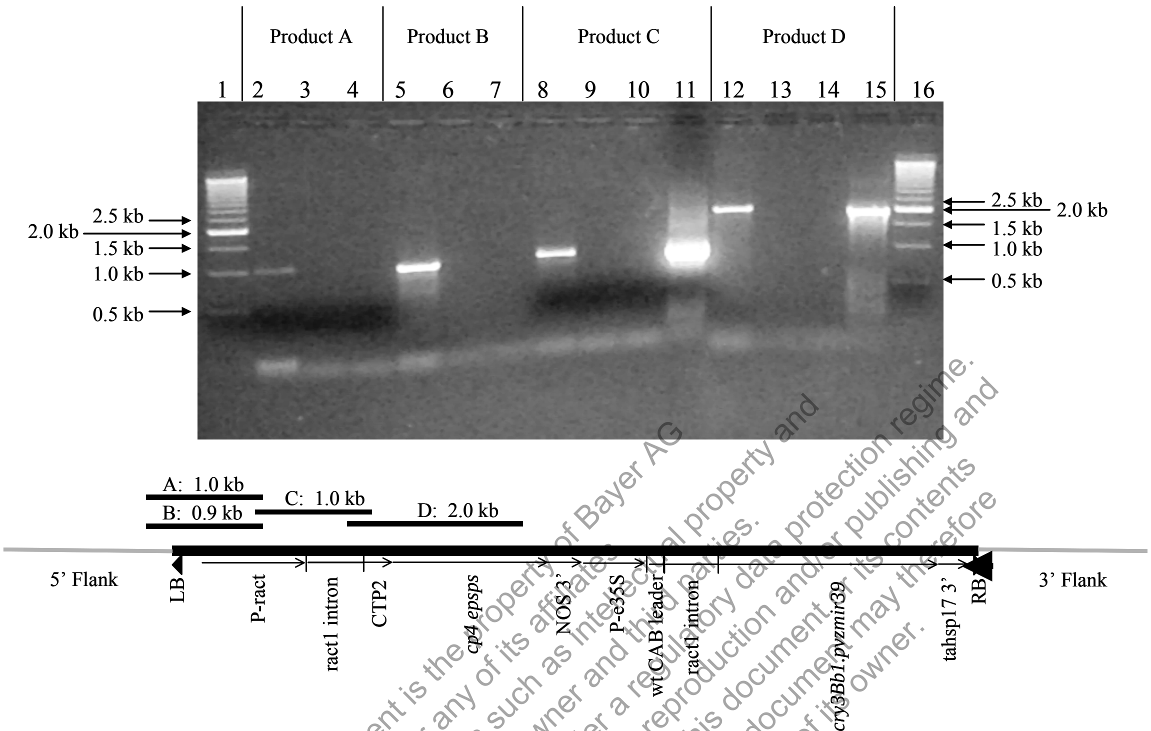
3.4. Organization of the insert in MON 88017

The organization of the elements within the insert in MON 88017 was confirmed using PCR analysis by amplifying seven overlapping regions of DNA that span the entire length of the insert. The location of the PCR products generated in relation to the insert, as well as the results of the PCR analyses, are shown in Figures 17 and 18.

The control reactions containing no template DNA (Figure 17: lanes 4, 7, 10 and 14; Figure 18: lanes 4, 8 and 11) did not generate PCR products with any of the primer sets, as expected. The control reactions containing DNA from conventional corn (Figure 17: lanes 3, 6, 9 and 13) did not generate PCR products with four out of the seven primer sets. The primer set used to generate Products E, F and G did amplify PCR products from the control DNA (Figure 18: lane 3, 7 and 10); however, none of these products were of the expected size for the primer set. The same PCR products were also generated using MON 88017 genomic DNA as a template (Figure 18: lane 2, 6 and 9). The generation of these PCR products is likely the result of nonspecific amplification of sequences in the corn genome due to one or both of the PCR primers in the primer set.

The plasmid PV-ZMIR39 was used as a positive control in the three PCR analyses (Products C-E) that amplified products containing only inserted DNA rather than the genomic DNA flanking the insert. In these three analyses, amplification from genomic DNA from MON 88017, as well as the plasmid PV-ZMIR39, generated the expected size PCR products of 1.0 kb for Product C (Figure 17: lanes 8 and 11), 2.0 kb for Product D (Figure 17: lanes 12 and 15) and 2.3 kb for Product E (Figure 18: lanes 2 and 5). In the amplification of Product E using plasmid PV-ZMIR39 as template DNA, faint, lower molecular weight products were visible that were most likely due to nonspecific amplification (Figure 18: lane 5). Amplification from MON 88017 genomic DNA also generated the expected size PCR products of 1.0 kb for Product A (Figure 17: lane 2); 0.9 kb for Product B (Figure 17: lane 5); 2.3 kb for Product F (Figure 18: lane 6); and 2.2 kb for Product G (Figure 18: lane 9). The generation of the predicted size PCR products from MON 88017 establishes that the arrangement or linkage of elements in the insert is the same as those in plasmid PV-ZMIR39 and that the elements within each gene cassette are arranged as depicted in the schematic of the insert in Figure 5.

Figure 17. Overlapping PCR analyses across the insert in MON 88017: Part I



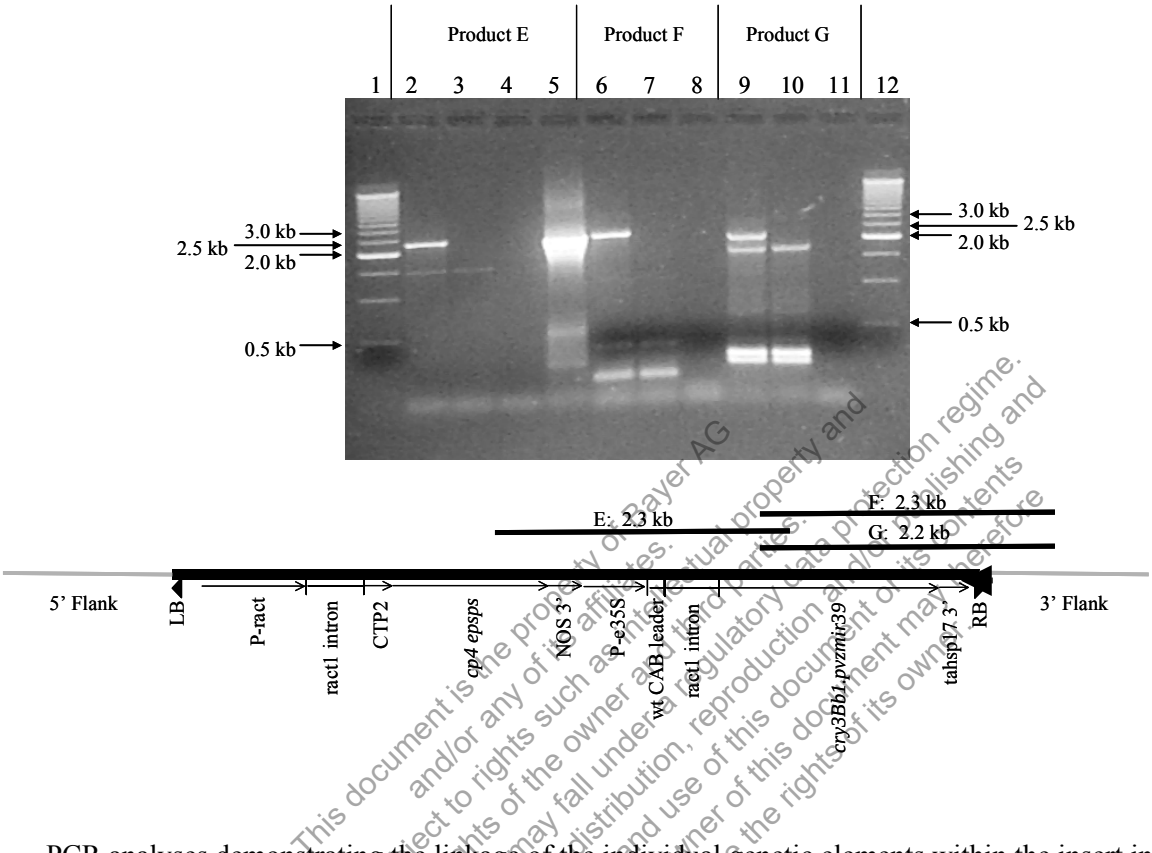
PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 88017 were performed on DNA extracted from grain of MON 88017 and conventional corn. The expected product size for each amplicon is highlighted in the illustration of the insert in MON 88017 that appears at the bottom of the figure. 20 µl of each of the PCR reactions were loaded on the gel.

Lane designation is as follows:

1: 500 bp DNA ladder (Life Technologies)
2: MON 88017 genomic DNA
3: control LH59 x LH198 DNA
4: control no DNA template
5: MON 88017 genomic DNA
6: control LH59 x LH198 DNA
7: control no DNA template
8: MON 88017 genomic DNA
9: control LH59 x LH198 genomic DNA
10: control no DNA template
11: plasmid PV-ZMIR39 DNA
12: MON 88017 genomic DNA
13: control LH59 x LH198 genomic DNA
14: control no DNA template
15: plasmid PV-ZMIR39 DNA
16: 500 bp DNA ladder (Life Technologies)

⟶ Symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

Figure 18. Overlapping PCR analyses across the insert in MON 88017: Part II



PCR analyses demonstrating the linkage of the individual genetic elements within the insert in MON 88017 were performed on plasmid PV-ZMIR39 DNA, conventional corn and MON 88017 genomic DNA extracted from grain. The expected product size for each amplicon is highlighted in the illustration of the insert in MON 88017 that appears at the bottom of the figure. 20 µl of each of the PCR products were loaded on the gel.

Lanes designation is as follows:

1: 500 bp DNA molecular weight ladder (Life Technologies)
2: MON 88017 genomic DNA
3: control LH59 x LH198 genomic DNA
4: control no DNA template
5: plasmid PV-ZMIR39 DNA
6: MON 88017 genomic DNA
7: control LH59 x LH198 genomic DNA
8: control no DNA template
9: MON 88017 genomic DNA
10: control LH59 x LH198 genomic DNA
11: control no DNA template
12: 500 bp DNA molecular weight ladder (Life Technologies)

⟶ Symbol denotes sizes obtained from MW markers on ethidium bromide stained gel.

3.5. Inheritance of corn rootworm protection and glyphosate tolerance traits in MON 88017

Chi square analysis of Mendelian inheritance data over ten generations was performed to determine the heritability and stability of the *cry3Bb1* and *cp4 epsps* genes in MON 88017. Expected and observed segregation frequencies of MON 88017 progeny that are positive for the corn rootworm (CRW)-protected and glyphosate-tolerant phenotypes are presented in Table 2. Plants were identified as positive for the CRW-protected phenotype based on the detection of the Cry3Bb1 protein by ELISA. Because the *cp4 epsps* and *cry3Bb1* genes are linked in the vector used for transformation, and are expected to have identical segregation ratios in the progeny of MON 88017, the results were, also applicable to inheritance of the glyphosate tolerant trait in MON 88017.

The breeding history of the ten generations evaluated for Mendelian inheritance (Figure 15) was as follows:

- The LH198BC0F_1 generation was derived from cross-pollinating the original transformed plant with an inbred line, LH198
- The LH198BC0F_2 generation was derived from self-pollinating individual LH198BC0F_1 plants
- The LH198BC1F_1 generation was derived from backcrossing LH198BC0F_1 plants to conventional inbred line LH198
- The LH198BC2F_1 generation was derived from backcrossing LH198BC1F_1 plants to conventional inbred line LH198
- The LH198BC3F_1 generation was derived from backcrossing LH198BC2F_1 plants to conventional inbred line LH198
- The LH198BC3F_2 generation was derived from self-pollinating individual LH198BC3F_1 plants
- The LH198BC0F_1xLH59 generation was derived from crossing LH198BC0F_1 plants to conventional inbred line LH59
- The LH59BC1F_1 generation was derived from backcrossing LH198BC0F_1xLH59 plants to conventional inbred line LH59
- The LH59BC2F_1 generation was derived from backcrossing LH59BC1F_1 plants to conventional inbred line LH59
- The LH59BC2F_2 generation was derived from self-pollinating individual LH59BC2F_1 plants

Genotype frequency was compared by means of a Chi square test (Little and Hills, 1978). The Chi square value (χ^2) was computed as follows:

$$\chi^2 = \Sigma \left[\left(|o - e| - 0.5 \right)^2 / e \right]$$

where, o = observed frequency of the genotype; e = expected frequency of the genotype; and 0.5 = Yates correction factor for analysis with one degree of freedom (df).

Results from the Mendelian heritance analysis are summarized in Table 2. A chi square value of at least 3.84 is needed to indicate statistical difference ($p \leq 0.05$). With only two exceptions, all χ^2 values were less than the critical value of 3.84, thus indicating no significant differences between observed and expected frequency for the CRW-protected /glyphosate-tolerant phenotypes in eight breeding generations of MON 88017. Although the results obtained for the LH198BC1F_1 generation were significant at $p \leq 0.05$ (Chi square = 3.84, 1 df), they were not significant at $p \leq 0.01$ (Chi square = 6.63). The results obtained for the LH198BC0F_1 x LH59 generation are attributed to gamete selection caused by Roundup agricultural herbicide application to plants of the previous (LH198BC0F_1) generation. Roundup agricultural herbicide application was necessary to obtain non-segregating seed for purposes of field evaluation. Gamete selection caused by glyphosate application has been previously observed for Roundup Ready corn NK603 (USDA petition 00-011-01p).

Table 2. Comparison of expected and observed segregation frequencies for progeny of MON 88017 plants

	Observed[a]		Expected[a]		
Generation	**+**	**-**	**+**	**-**	**χ^2**
LH198BC$_0$F$_1$	21	14	17.5	17.5	1.03[†]
LH198BC$_0$F$_2$	53	12	48.75	16.25	1.15[†]
LH198BC$_1$F$_1$	21	9	15	15	4.03[#]
LH198BC$_2$F$_1$	10	15	12.5	12.5	0.64[†]
LH198BC$_3$F$_1$	8	5	6.5	6.5	0.31[†]
LH198BC3F$_2$	21	3	18	6	1.39[†]
LH198BC$_0$F$_1$xLH59	29	0	14.5	14.5	27.03[*]
LH59 BC$_1$F$_1$	7	5	6	6	0.08[†]
LH59 BC$_2$F$_1$	8	5	6.5	6.5	0.31[†]
LH59 BC$_2$F$_2$	35	13	36	12	0.03[†]

[a] Symbol (+) denotes number of plants that are positive for the two traits; (-) denotes number of plants that are negative for the two traits.

[†] Not significant at $p \leq 0.05$ (Chi square = 3.84, 1 df).

Significant at $p \leq 0.05$ (Chi square = 3.84, 1 df); but not significant at $p \leq 0.01$ (Chi square = 6.63).

[*] Significant at $p \leq 0.05$ (Chi square = 3.84, 1 df) and $p \leq 0.01$ (Chi square = 6.63).

The results of this analysis are consistent with the finding of a single locus of insertion of the *cry3Bb1* and *cp4 epsps* genes that segregate according to Mendel's laws of genetics. The stability of the insert has been demonstrated through seven generations of cross-fertilization and three generations of self-pollination.

Results of the Southern blot analysis performed on multiple generations from the breeding tree of MON 88017 to determine insert stability were discussed in Section 3.3 and presented in Figure 16.

3.6. Summary of the molecular characterization of MON 88017

MON 88017 was produced by *Agrobacterium sp.*-mediated transformation of a corn with plasmid vector PV-ZMIR39, which contains both the *cp4 epsps* and *cry3Bb1* gene expression cassettes. MON 88017 contains one copy of the introduced DNA at a single integration locus on an approximately 13 kb *Sca* I restriction fragment. No additional elements from transformation vector PV-ZMIR39, linked or unlinked to the intact cassettes, were detected in the genome of MON 88017. PCR analyses were performed to determine the 5' and 3' insert-to-plant junctions and confirm the organization of the elements within the insert. Insert stability analysis demonstrated that the expected Southern blot fingerprint of MON 88017 insert was maintained across the branches of the breeding tree that were tested, thereby confirming the stability of the integrated DNA over multiple generations. No backbone sequences from the transformation vector PV-ZMIR39 were detected in the tested generations. These data support the conclusion that only the two expected full-length proteins, Cry3Bb1 and CP4 EPSPS, are encoded by the insert in MON 88017.

SECTION 4. Other data and information about the development of MON 88017

All relevant information regarding development of MON 88017 is described in Parts II – VII of this summary.

PART V: PRESENCE OF GENES THAT ENCODE RESISTANCE TO ANTIBIOTICS

No genes that encode resistance to an antibiotic were inserted into the genome of MON 88017. Molecular characterization data presented in Part IV Section 3 confirms the absence of the *add* gene encoding an antibiotic resistance marker.

PART VI: CHARACTERIZATION OF THE PROTEINS INTRODUCED INTO MON 88017

SECTION 1. Identity and function of the CP4 EPSPS and Cry3Bb1 proteins present in MON 88017

MON 88017 contains a 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* spp. strain CP4 (CP4 EPSPS). The *cp4 epsps* coding region encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The CP4 EPSPS protein is structurally and functionally similar to plant EPSPS enzymes, but has a much reduced affinity for glyphosate (Padgette et al., 1996). Typically, glyphosate binds to the plant EPSPS enzyme and blocks the biosynthesis of aromatic amino acids, thereby depriving plants of these essential components (Steinrucken and Amrhein, 1980; Haslam, 1993). In Roundup Ready plants, however, requirements for growth and development are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate. A safety assessment of the CP4 EPSPS protein has been described previously in the literature (Harrison et al., 1996).

The Cry3Bb1 protein expressed in MON 88017 is a member of the Cry3Bb class of proteins that share >95% amino sequence homology (Crickmore et al., 1998). It is a variant of the wild-type Cry3Bb1 protein isolated from *Bacillus thuringiensis* (subsp. *kumamotoensis*) strain EG4691 (Donovan et al., 1992) and a variant of the Cry3Bb1 protein present in commercial YieldGard Rootworm corn (MON 863). The amino acid sequence identity between the Cry3Bb1 proteins present in MON 88017, YieldGard Rootworm corn, and in *Bacillus thuringiensis* (subsp. *kumamotoensis*) ranges between 99.1-99.8%. The amino acid sequence of the Cry3Bb1 protein present in MON 88017 and *Bacillus thuringiensis* (subsp. *kumamotoensis*) shares 99.1% identity (differs by six of 652 amino acid residues). The Cry3Bb1 protein present in MON 88017 and YieldGard Rootworm corn share 99.8% amino acid identity (differs by only one of 653 amino acid residues). YieldGard Rootworm corn has been granted full regulatory clearance in the U.S. FDA consultation was completed in December 2001. In 2001 EPA established an exemption from the requirement of a tolerance for the Cry3Bb1 protein and the genetic material necessary for its production in field corn, sweet corn and popcorn (40CFR §180.1214). Non-regulated status was granted by USDA in October 2002, and EPA registration was granted in February 2003. Thus, the Cry3Bb1 protein, as expressed in MON 88017, has been well characterized and its safety for human and animal consumption has been established.

1.1. Characterization of the CP4 EPSPS protein produced in MON 88017

The CP4 EPSPS protein was purified from MON 88017 grain and from *E. coli* culture that produced the CP4 EPSPS protein. A panel of analytical tests was used to identify, characterize and compare the plant- and *E. coli*-produced CP4 EPSPS proteins: (1)

western blot analysis and densitometry, (2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry, (3) matrix- assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS), (4) N-terminal sequence analysis, (5) glycosylation analysis, and (6) CP4 EPSPS activity assay.

These data provide a detailed characterization of the CP4 EPSPS protein isolated from grain of MON 88017 and establish the equivalence of the plant-produced CP4 EPSPS protein to the *E. coli*-produced CP4 EPSPS protein. The materials and methods for the analytical tests are provided in Appendix II. The results are summarized in the following sections.

1.1.a. Western blot analysis to confirm the identity of the CP4 EPSPS protein

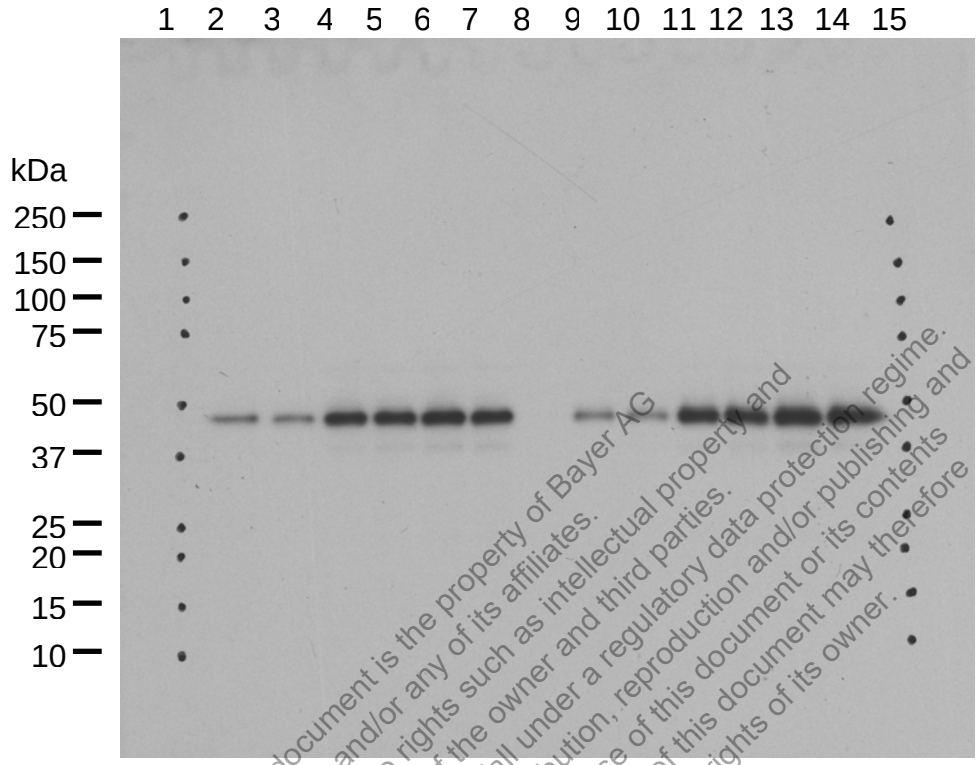
A protein band migrating at an approximate molecular weight of 45 kDa on SDS-PAGE gel was identified as the CP4 EPSPS protein by western blot analysis using a goat anti-CP4 EPSPS antibody (Figure 19, Lanes 9-14). This serum previously was shown to be specific for the CP4 EPSPS protein and therefore was used to confirm the identity of the plant-produced CP4 EPSPS protein. One immunoreactive band migrating at approximately 45 kDa was observed in both the *E. coli*- and plant-produced CP4 EPSPS samples, confirming the identity of the CP4 EPSPS protein produced in MON 88017.

1.1.b. Molecular weight and purity of the CP4 EPSPS protein

Molecular weight and purity of the plant-produced CP4 EPSPS protein were estimated using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-polyacrylamide gel (Figure 20). Out of three bands observed on the SDS-polyacrylamide gel, the 45kDa band was identified as CP4 EPSPS protein by western blot analysis. The predicted molecular weight of the CP4 EPSPS protein, based on the deduced amino acid sequence, is 47.7 kDa. The apparent molecular weight of the plant-produced CP4 EPSPS protein, estimated by comparison to molecular weight markers on the SDS-polyacrylamide gel, was 45 kDa (Figure 19) and the plant- and *E. coli*-produced proteins co-migrated on a Tris-glycine buffered 4-20% polyacrylamide gel (Figure 20). The purity value for the plant-produced CP4 EPSPS protein calculated as the average of three separate loads was 45.5%.

The molecular weight of the plant-produced CP4 EPSPS protein was further confirmed using MALDI-TOF mass spectrometry. The average mass (MH+), determined from three separate spectral acquisitions, was 47,447.25 daltons. This value compares well with the predicted mass for CP4 EPSPS, 47,730 daltons.

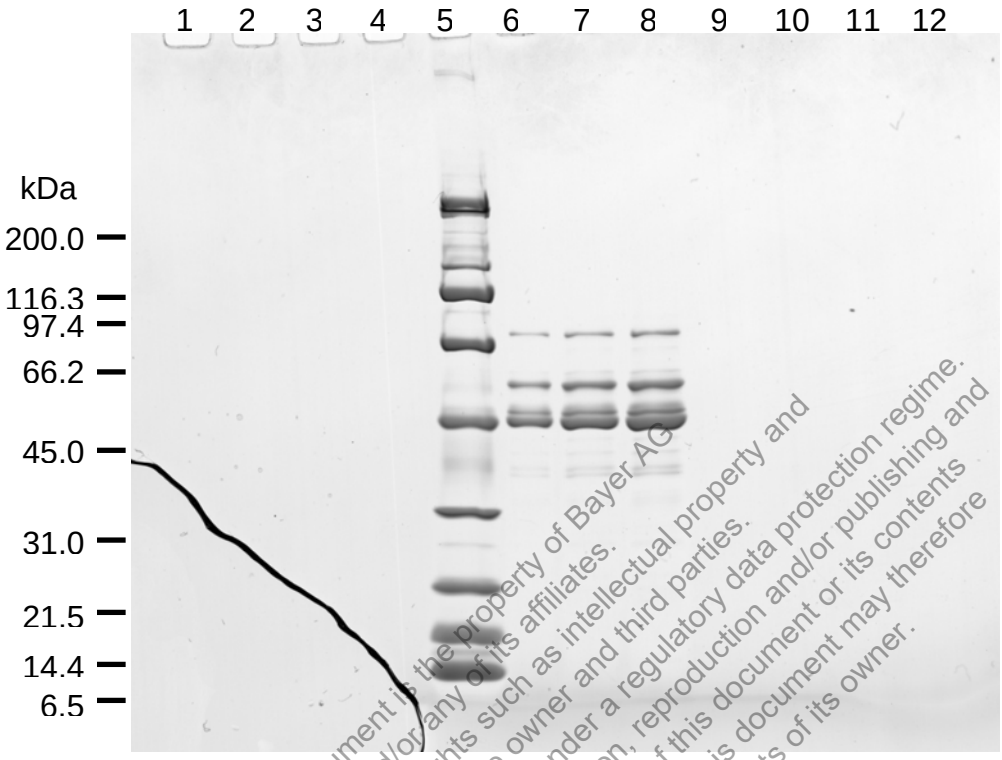
Figure 19. Western blot analysis demonstrating equivalence of CP4 EPSPS proteins produced in MON 88017 and *E. coli*



Samples of the plant- and *E. coli*-produced CP4 EPSPS proteins (1, 2, and 3 ng) were separated using SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with goat anti-CP4 EPSPS polyclonal antiserum to detect the presence of CP4 EPSPS protein. The dots (Lanes 1 and 15) represent the location of the molecular weight markers (MWM) on the membrane. A 10 sec exposure is shown.

Lane	Sample	Amount (ng)
1	Bio-Rad Precision Plus Dual Color MWM	---
2	*E. coli*-produced CP4 EPSPS	1
3	*E. coli*-produced CP4 EPSPS	1
4	*E. coli*-produced CP4 EPSPS	2
5	*E. coli*-produced CP4 EPSPS	2
6	*E. coli*-produced CP4 EPSPS	3
7	*E. coli*-produced CP4 EPSPS	3
8	loading buffer	---
9	plant-produced CP4 EPSPS	1
10	plant-produced CP4 EPSPS	1
11	plant-produced CP4 EPSPS	2
12	plant-produced CP4 EPSPS	2
13	plant-produced CP4 EPSPS	3
14	plant-produced CP4 EPSPS	3
15	Bio-Rad Precision Plus Dual Color MWM	

Figure 20. Molecular weight and purity of the plant-produced CP4 EPSPS protein demonstrated by SDS-PAGE analysis



1, 2 and 3 µg total protein of plant-produced CP4 EPSPS protein sample were separated by electrophoresis on a 4-20% gradient polyacrylamide gel under reducing and denaturing conditions. The stained gel was analyzed using densitometry to determine the purity and molecular weight of the CP4 EPSPS protein.

Lane	Sample	Amount (µg)
1	empty	---
2	empty	---
3	loading buffer	---
4	loading buffer	---
5	Bio-Rad Broad Range MWM	---
6	plant-produced CP4 EPSPS	1
7	plant-produced CP4 EPSPS	2
8	plant-produced CP4 EPSPS	3
9	loading buffer	---
10	loading buffer	---
11	empty	---
12	empty	---

1.1.c. MALDI-TOF MS tryptic map mass analysis

MALDI-TOF mass spectrometry analysis was performed on the CP4 EPSPS protein produced in MON 88017. The protein band identified as CP4 EPSPS was excised from the gel, chemically reduced, alkylated, digested with trypsin, and analyzed using MALDI-TOF MS. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with theoretical tryptic peptide fragment masses. With sufficient mass accuracy, four tryptic peptides may be sufficient to identify a protein (Jiménez et al., 1998). Peptides were considered to match when a difference in molecular weight of less than one dalton was found between the observed and theoretical fragment masses. Matches were made without consideration for potential natural amino acid modifications. For the 45kDa protein, a total of 22 observed peptide mass fragments matched the theoretical tryptic peptide mass map for the CP4 EPSPS protein.

The amino acid sequence of the plant-produced CP4 EPSPS protein was deduced from the coding region of the *cp4 epsps* gene present in MON 88017. A coverage map was generated using the identified masses from the MALDI-TOF MS tryptic mass analysis. Approximately 55.2% (251 of 455 amino acids) of the expected protein sequence was identified (Figure 21). The predicted molecular weight of the full-length CP4 EPSPS protein, based on the deduced amino acid sequence, is 47.7 kDa. Using the above criteria, the 45kDa plant-produced protein band was identified as CP4 EPSPS protein.

Figure 21. MALDI-TOF MS coverage map of the CP4 EPSPS protein sequence produced in MON 88017

```
  1   MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL
 51   LEGEDVINTG KAMQAMGARI RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101   TGCRLTMGLV GVYDFDSTFI GDASLTKRPM GRVLNPLREM GVQVKSEDGD
151   RLPVTLRGPK TPTPITYRVP MASAQVKSAV LLAGLNTPGI TTVIEPIMTR
201   DHTEKMLQGF GANLTVETDA DGVRTIRLEG RGKLTGQVID VPGDPSSTAF
251   PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED
301   VADLRVRSST LKGVTVPEDR APSMIDEYPI LAVAAAFAEG ATVMNGLEEL
351   RVKESDRLSA VANGLKLNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401   HLDHRIAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451   DTKAA
```

1.1.d. N-terminal sequence analysis

An SDS-PAGE analysis of the plant-produced CP4 EPSPS protein revealed three protein bands (migrating at approximately 45, 48, and 55 kDa, Figure 22), each representing greater than 10% of the total protein in the plant-produced CP4 EPSPS sample. The identity of these bands was evaluated using N-terminal sequence analysis (Table 3). The observed amino acid sequence for the 45 kDa protein band matched the expected N-terminal amino acid sequence of the CP4 EPSPS protein. Two sequences, both

consistent with the N-terminus of the CP4 EPSPS protein were observed in the plant-produced CP4 EPSPS protein sample. The primary sequence originates at residue 2 (leucine) and matches the expected N-terminal amino acid sequence of the CP4 EPSPS protein from residues 2 through 16 (Table 3). It is not uncommon for the initiator methionine to be removed from proteins in eukaryotic organisms by an endogenous methionine aminopeptidase (Arfin and Bradshaw, 1988). The secondary sequence starts at residue 4 (glycine) and matches the expected N-terminal amino acid sequence of the CP4 EPSPS protein from residues 4 through 18 (Table 3). The loss of several N-terminal amino acid residues may be due to protease action when plant cells are homogenized. Despite the ragged N-terminus, the sequence data confirm the identity of the 45 kDa protein in the plant-produced sample as the CP4 EPSPS protein.

The N-terminal sequence of the 48 kDa protein band was obtained and the sequence did not match that of the CP4 EPSPS protein. The sequence obtained was used to search the non-redundant public database and the PIR Protein database but no matches were identified. Only five amino acid residues were obtained from the N-terminal sequence analysis of the 55 kDa protein and therefore no positive identification could be made for this protein.

Table 3. N-terminal sequence of the CP4 EPSPS protein produced in MON 88017

MW	Observed Sequence	Identity
45 kDa	Major Sequence LHGASSRPATARKS(S)	Residues 2-16 of CP4 EPSPS
	Secondary Sequence GASSRPATARKS(S)G(L)	Residues 4-18 of CP4 EPSPS
48 kDa	GGFKVTRISEGPVKX	Not Identified
55 kDa	(G)LIDGXXXXXXXXXX	Not Identified

Sequences obtained for each protein band were compared with the known sequence for CP4 EPSPS and/or against protein sequence databases. The ( ) denotes tenuous amino acid designations and Xs refer to undesignated amino acids. Amino acid residues 1-18 (using the IUPAC-IUB amino acid codes) of the CP4 EPSPS protein are MLHGASSRPATARKSSGL.

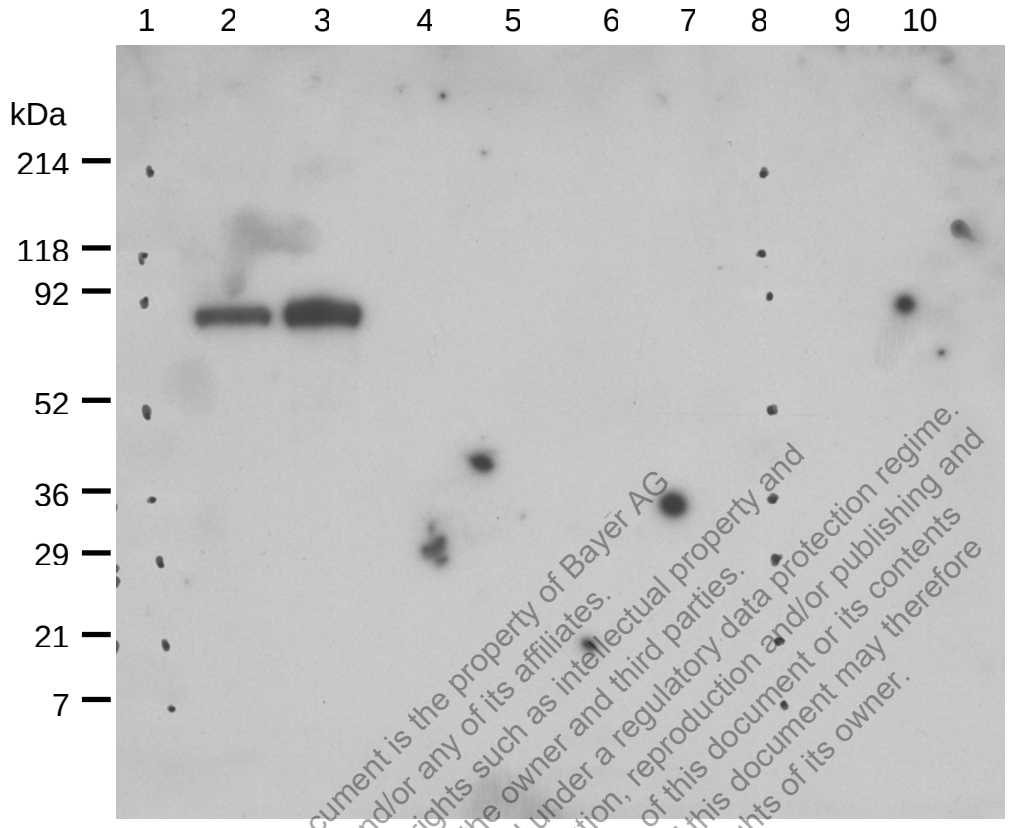
1.1.e. Functional activity

The specific activity of the plant- and *E. coli*-produced CP4 EPSPS proteins was estimated concurrently using a phosphate release assay. The specific activity of the plant-produced protein was 7.1 U/mg of CP4 EPSPS, and the specific activity of the *E. coli*-produced CP4 EPSPS protein was 6.0 U/mg. This confirmed that the functional activity of the CP4 EPSPS produced in MON 88017 is similar to that of the *E. coli*-produced CP4 EPSPS.

1.1.f. Glycosylation analysis

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex branched polysaccharide structures or simple monosaccharides. In contrast, prokaryotic organisms such as *E. coli* lack the necessary organelles and biochemical pathways required for protein glycosylation. To test if post-translational glycosylation of the plant-produced CP4 EPSPS protein occurred, this protein was analyzed for covalently bound carbohydrate moeities. The *E. coli*-produced CP4 EPSPS protein was analyzed concurrently as a negative control in this experiment. Transferrin protein was analyzed as a positive control. The positive control (transferrin) was clearly detected in a concentration-dependent manner at loadings of 0.5 and 1 µg/lane (Figure 22, Lanes 2-3). No bands were observed in the *E. coli*- or plant-produced protein samples (Lanes 4-7). Several dots present on the blot were considered to be non-specific background because of their form and location. Because no band was observed at the expected molecular weight for the plant- or *E. coli*-produced CP4 EPSPS proteins, it was concluded that the proteins were not glycosylated.

Figure 22. Glycosylation analysis of CP4 EPSPS protein produced in MON 88017



Proteins were separated by SDS-PAGE under reducing and denaturing conditions and transferred to a PVDF membrane. The dots (Lanes 1 and 8) represent the location of the molecular weight markers (MWM) on the membrane. A three minute exposure is shown.

Lane	Sample	Amount (µg)
1	Bio-Rad Prestained Broad Range MWM	---
2	transferrin	0.5
3	transferrin	1.0
4	*E. coli*-produced CP4 EPSPS	0.5
5	*E. coli*-produced CP4 EPSPS	1.0
6	plant-produced CP4 EPSPS	0.5
7	plant-produced CP4 EPSPS	1.0
8	Bio-Rad Prestained Broad Range MWM	---
9	empty	---
10	empty	

1.1.g. Conclusions for characterization of the CP4 EPSPS protein produced in MON 88017

A panel of analytical tests was used to characterize and compare the plant- and *E. coli*-produced CP4 EPSPS proteins. The equivalence of the plant-produced CP4 EPSPS protein to the *E. coli*-produced CP4 EPSPS protein was evaluated by comparing their molecular weights, immunoreactivity with anti-CP4 EPSPS antibodies, glycosylation status and functional activity.

The plant-produced CP4 EPSPS protein was identified as a 45 kDa band using western blot analysis. The N-terminal sequence of this 45 kDa protein band was consistent with the N-terminal sequence of the CP4 EPSPS protein. MALDI-TOF mass spectrometry analysis of the plant-produced CP4 EPSPS protein after trypsin digestion yielded peptide masses encompassing 55.2% of the deduced amino acid sequence (251/455 amino acids) of the plant-produced CP4 EPSPS. The purity of the plant-produced CP4 EPSPS protein was 45.5%. The approximate molecular weight of the protein band identified as the plant-produced CP4 EPSPS protein, estimated by comparison to molecular weight markers on a Colloidal Brilliant Blue G stained SDS-polyacrylamide gel, was 45 kDa. The molecular weight of the plant-produced CP4 EPSPS, as determined using the mass average (MH+) molecular weight using MALDI-TOF MS, was 47,447.25 Da. The plant-produced CP4 EPSPS protein was not glycosylated. The functional activity of the plant- and *E. coli*-produced CP4 EPSPS proteins was determined using a phosphate release assay and shown to be similar. The specific activity of the plant- and *E. coli*-produced CP4 EPSPS proteins was 7.1 U/mg and 6.0 U/mg, respectively.

These results lead to the conclusion that the plant-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS protein.

1.2. Characterization of Cry3Bb1 protein produced in MON 88017

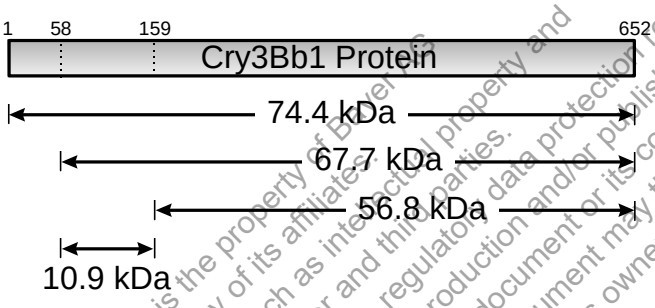
The Cry3Bb1 protein was purified from the grain of MON 88017 and from *E. coli* cultures that express the Cry3Bb1 protein. Proteins of three molecular weights were identified as the full-length Cry3Bb1 protein and protease-cleaved products of Cry3Bb1 (Figure 23). The Cry3Bb1 protein (predicted molecular weight of 74.4 kDa) is processed in corn to produce peptides with apparent molecular weights of ~66 and ~55 kDa. The ~55 kDa peptide is thought to correspond to the *B.t*. tryptic core protein described in the literature (Schnepf et al., 1998). Four immunoreactive bands migrating at approximately 77, 66, 55, and 46 kDa were observed in the sample purified from MON 88017 grain. The immunoreactive band of 46 kDa may represent a degradation product of Cry3Bb1 protein but was not considered for further analysis because it was smaller than the expected size of the tryptic core protein.

A panel of analytical tests was used to characterize the plant-produced Cry3Bb1 protein: (1) western blot analysis and densitometry, (2) SDS-PAGE and densitometry, (3)

MALDI-TOF mass spectrometry, (4) glycosylation analysis, and (5) a Cry3Bb1 bioactivity assay. The equivalence of the plant-produced Cry3Bb1 protein to the *E. coli*-produced Cry3Bb1 protein was evaluated by comparing their full-length molecular weights, immunoreactivity with anti-Cry3Bb1 antibodies, glycosylation status and functional activity.

The materials and methods used in these experiments are provided in Appendix II. Results obtained from the analyses conducted to characterize the Cry3Bb1 protein produced in MON 88017 are discussed in the following sections.

Figure 23. Polypeptides derived from the full-length Cry3Bb1 protein



The amino acid positions are shown on top and correspond to residue numbers obtained from N-terminal sequence analysis of a homologous Cry3Bb1 protein. The predicted molecular weights for each polypeptide are shown.

1.2.a. Western blot analysis to confirm the identity of the Cry3Bb1 protein

Western blot analysis was performed using a goat anti-Cry3Bb1 serum. That serum was shown to be specific for Cry3Bb1 protein and was used to confirm the identity of the plant-produced Cry3Bb1 protein. Four immunoreactive bands migrating at approximately 77, 66, 55, and 46 kDa were observed (Figure 24, lanes 9-14) in the plant-produced Cry3Bb1 sample. Full-length Cry3Bb1 protein with predicted molecular weight of 74.4 kDa is processed in corn to produce peptides with apparent molecular weight ~66 kDa and ~ 55 kDa (Figure 23). The immunoreactive band at 46 kDa may represent a degradation product of the Cry3Bb1 protein. This band was not analyzed because it was smaller than the expected size of the tryptic core determined for homologous Cry3Bb1 proteins.

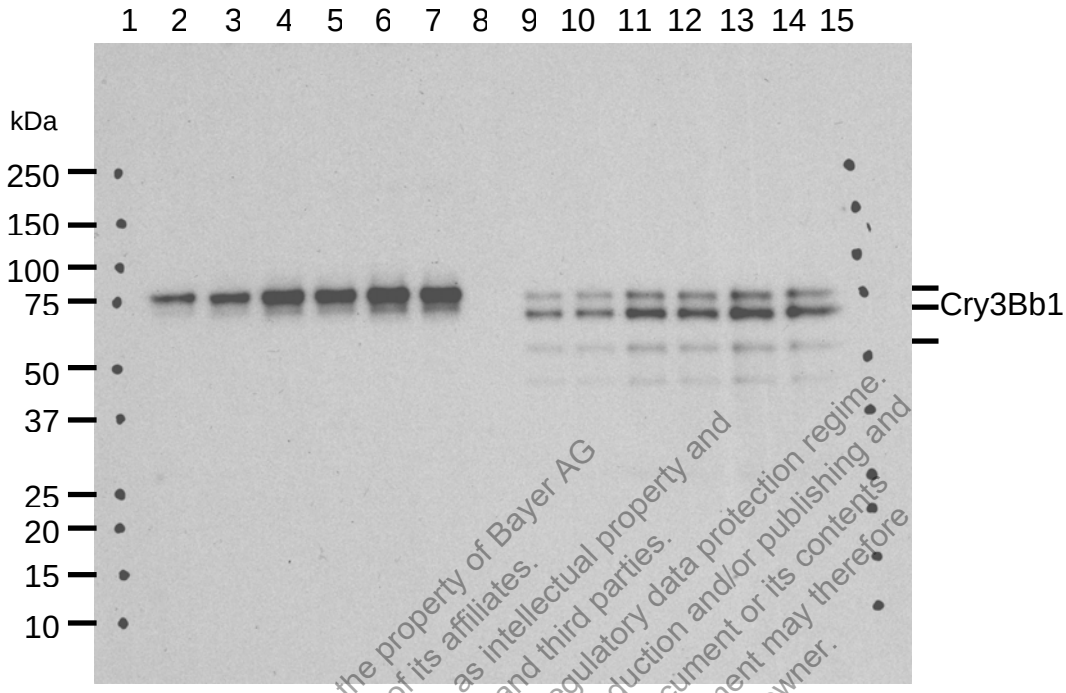
The plant- and *E. coli*-produced Cry3Bb1 proteins were loaded in duplicate at three purity-corrected protein amounts: 1, 2, and 3 ng (Figure 24). The percent difference in the immunoreactivity between the *E. coli*- and plant-produced Cry3Bb1 protein was calculated based on densitometric analysis of the bands for two different exposures (15

and 30 sec) of the western blot. The Cry3Bb1 antibody recognizes the plant-produced full-length protein and several smaller protein fragments that represent proteolytically cleaved forms of this protein. Bands greater than 55 kDa were included in the densitometric analysis of the plant-produced sample. The average difference in immunoreactivity between the plant- and *E. coli*-produced Cry3Bb1 proteins was 9%.

1.2.b. Molecular weight and purity of the Cry3Bb1 protein

The molecular weight and purity of the plant-produced Cry3Bb1 protein were determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS-polyacrylamide gel (Figure 25). The predicted molecular weight of the full-length Cry3Bb1 protein, based on the deduced amino acid sequence, is 74.6 kDa. The full-length Cry3Bb1 protein in the plant-produced sample had an estimated molecular weight of 77.2 kDa. The apparent molecular weight of the full-length *E. coli*-produced Cry3Bb1 protein, on the same gel, was estimated to be 77.7 kDa (Lane 7). The molecular weights of the additional protein fragments, previously identified as Cry3Bb1 protein (Section 1.2.a), were estimated to be 66.2 and 55.4 kDa. The purity of the plant-produced Cry3Bb1 protein was calculated as the sum of the three protein bands (77.2, 66.2, and 55.4 kDa) and the average from three separate loads (1, 2, and 3 µg) of total protein (Lanes 4-6). The average purity of the plant-produced Cry3Bb1 protein was 66.1%.

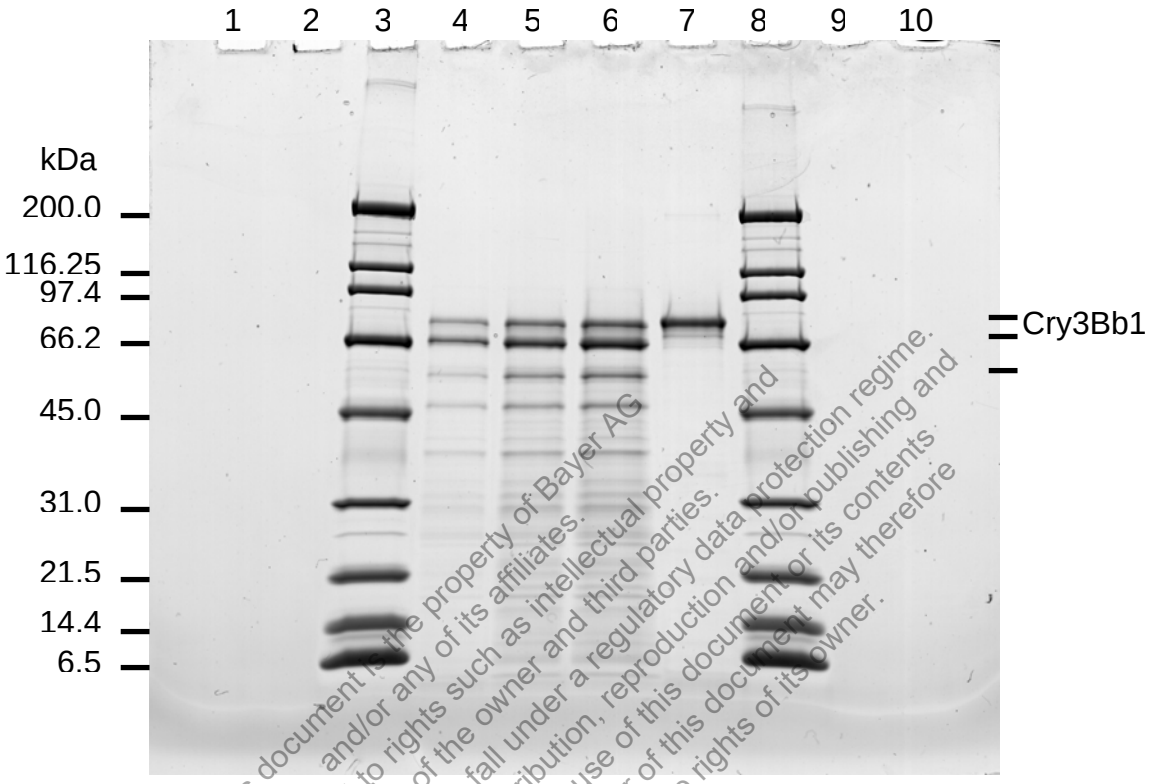
Figure 24. Western blot analysis demonstrating equivalence of plant- and *E.coli*-produced Cry3Bb1 protein



Samples of the *E. coli*- and plant- produced Cry3Bb1 proteins (1, 2, and 3 ng) were separated using SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with goat anti-Cry3Bb1 antibodies to detect the presence of Cry3Bb1 protein. The dots (Lanes 1 and 15) represent the location of the molecular weight markers (MWM) on the membrane. A 15 sec exposure is shown.

Lane	Sample	Amount (ng)
1	Bio-Rad Precision Plus Dual Color MWM	---
2	*E. coli*-produced Cry3Bb1 protein	1
3	*E. coli*-produced Cry3Bb1 protein	1
4	*E. coli*-produced Cry3Bb1 protein	2
5	*E. coli*-produced Cry3Bb1 protein	2
6	*E. coli*-produced Cry3Bb1 protein	3
7	*E. coli*-produced Cry3Bb1 protein	3
8	2× loading buffer	---
9	plant-produced Cry3Bb1 protein	1
10	plant-produced Cry3Bb1 protein	1
11	plant-produced Cry3Bb1 protein	2
12	plant-produced Cry3Bb1 protein	2
13	plant-produced Cry3Bb1 protein	3
14	plant-produced Cry3Bb1 protein	3
15	Bio-Rad Precision Plus Dual Color MWM	---

Figure 25. Molecular weight and purity of Cry3Bb1 protein demonstrated by SDS-PAGE analysis



1, 2, and 3 µg of total protein from the plant-produced Cry3Bb1 protein sample and 1 µg of total proten from the *E. coli*-produced Cry3Bb1 protein sample were separated by electrophoresis on a 4-20% gradient polyacrylamide gel under reducing and denaturing conditions. The stained gel was analyzed using densitometry to determine the purity and molecular weight of the Cry3Bb1 protein.

Lane	Sample	Amount (µg)
1	empty	---
2	empty	---
3	Bio-Rad Broad Range MWM	---
4	plant-produced Cry3Bb1 protein	1
5	plant-produced Cry3Bb1 protein	2
6	plant-produced Cry3Bb1 protein	3
7	*E. coli*-produced Cry3Bb1 protein	1
8	Bio-Rad Broad Range MWM	---
9	empty	---
10	empty	---

1.2.c. MALDI-TOF MS tryptic mass map analysis

The identity of the plant-produced Cry3Bb1 protein was also assessed using MALDI-TOF mass spectrometry. The ability to identify a protein using this method depends upon matching a sufficient number of observed to expected (theoretical) mass fragments. With sufficient mass accuracy, four tryptic peptides may be enough to identify a protein (Jiménez et al., 1998). Peptides were considered to match when a difference in molecular weight of less than one dalton was found between the observed and theoretical fragment mass. Matches were made without consideration for potential natural amino acid modifications.

The identity of each of the three protein bands (~77, ~66 and ~55 kDa) observed in western blot analysis (Figure 24) was confirmed. For the 77 kDa protein, 20 observed peptide mass fragments were matched to the theoretical tryptic peptide mass map for the full-length Cry3Bb1 protein. For the 66 kDa protein fragment, 20 observed peptide mass fragments were matched to the theoretical tryptic peptide mass map for the Cry3Bb1 protein. For the 55 kDa protein fragment, 15 observed peptide mass fragments were matched to the theoretical tryptic peptide mass map for the Cry3Bb1 protein. The identified masses were used to assemble a combined coverage map (Figure 26) for the three bands identified as Cry3Bb1 protein in the western blot analysis. In total, approximately 44% (287 of 653 amino acids) of the expected protein sequence was identified.

Figure 26. MALDI-TOF MS coverage map of the Cry3Bb1 protein sequence produced in MON 88017

```
  1  MANPNNRSEH DTIKVTPNSE LQTNHNQYPL ADNPNSTLEE LNYKEFLRMT
 51  EDSSTEVLDN STVKDAVGTG ISVVGQILGV VGVPFAGALT SFYQSFLNTI
101  WPSDADPWKA FMAQVEVLID KKIEEYAKSK ALAELQGLQN NFEDYVNALN
151  SWKKTPLSLR SKRSQDRIRE LFSQAESHFR NSMPSFAVSK FEVLFLPTYA
201  QAANTHLLLL KDAQVFGEEW GYSSEDVAEF YRRQLKLTQQ YTDHCVNWYN
251  VGLNGLRGST YDAWVKFNRF RREMTLTVLD LIVLFPFYDI RLYSKGVKTE
301  LTRDIFTDPI FLLTTLQKYG PTFLSIENSI RKPHLFDYLQ GIEFHTRLRP
351  GYFGKDSFNY WSGNYVETRP SIGSSKTITS PFYGDKSTEP VQKLSFDGQK
401  VYRTIANTDV AAWPNGKVYL GVTKVDFSQY DDQKNETSTQ TYDSKRNNGH
451  VSAQDSIDQL PPETTDEPLE KAYSHQLNYA ECFLMQDRRG TIPFFTWTHR
501  SVDFFNTIDA EKITQLPVVK AYALSSGASI IEGPGFTGGN LLFLKESSNS
551  IAKFKVTLNS AALLQRYRVR IRYASTTNLR LFVQNSNNDF LVIYINKTMN
601  KDDDLTYQTF DLATTNSNMG FSGDKNELII GAESFVSNEK IYIDKIEFIP
651  VQL
```

Shaded regions correspond to tryptic peptide masses that were identified from the 77, 66, and 55 kDa bands, analyzed separately, using MALDI-TOF MS. The gray shaded sequence corresponds to the region identified from the 77 kDa band. The black shaded sequence corresponds to additional sequence identified from the 66 kDa band. No additional amino acids were identified from the 55 kDa band that were not observed in the 77 or 66 kDa bands.

1.2.d. Functional activity of the Cry3Bb1 protein

The biological activity of *E. coli*- and plant-produced Cry3Bb1 proteins was estimated in a diet-incorporated bioassay. *E. coli*- and plant-produced Cry3Bb1 proteins were incorporated into a diet and fed to larvae of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, a susceptible insect. Biological activity was measured as an LC_{50}, which is defined as the estimated lethal concentration of protein (µg/ml diet) required to kill 50% of the CPB larvae. The LC_{50} values for CPB when fed the plant- and *E. coli*-produced Cry3Bb1 proteins were 0.414 µg Cry3Bb1 protein/mL diet and 0.554 µg Cry3Bb1 protein/mL diet, respectively. Statistical analysis demonstrated that the two LC_{50} values were not different ($p>0.05$), leading to the conclusion that the *E. coli*- and plant-produced Cry3Bb1 proteins are functionally equivalent.

1.2.e. Glycosylation analysis of the Cry3Bb1 protein

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex branched polysaccharide structures or simple monosaccharides. In contrast, prokaryotic organisms such as *E. coli* lack the necessary organelles and biochemical pathways required for protein glycosylation. To test if post-translational glycosylation of the plant-produced Cry3Bb1 protein occurred, this protein was analyzed for covalently bound carbohydrate moieties (Figure 27). The *E. coli*-produced Cry3Bb1 protein and transferrin protein were analyzed concurrently as negative and positive controls, respectively. The positive control (transferrin) was clearly detected in a concentration-dependent manner at loadings of 0.25 and 0.5 µg/lane (Lanes 2-3). A band at ~25 kDa was observed in the *E. coli*-produced protein sample (Lanes 4-5) and no band was observed in the plant-produced Cry3Bb1 sample (Lanes 6-7). The band observed in the *E. coli*-produced Cry3Bb1 sample was at a lower molecular weight than the Cry3Bb1 protein. This band likely represents a naturally biotinylated protein that co-purified with the *E. coli*-produced Cry3Bb1 protein (Choi-Rhee et al., 2003), because it would bind to the streptavidin-HRP conjugate and be detected by the ECL system. No band was observed at the expected molecular weights for the Cry3Bb1 protein; therefore, it was concluded that it was not glycosylated.

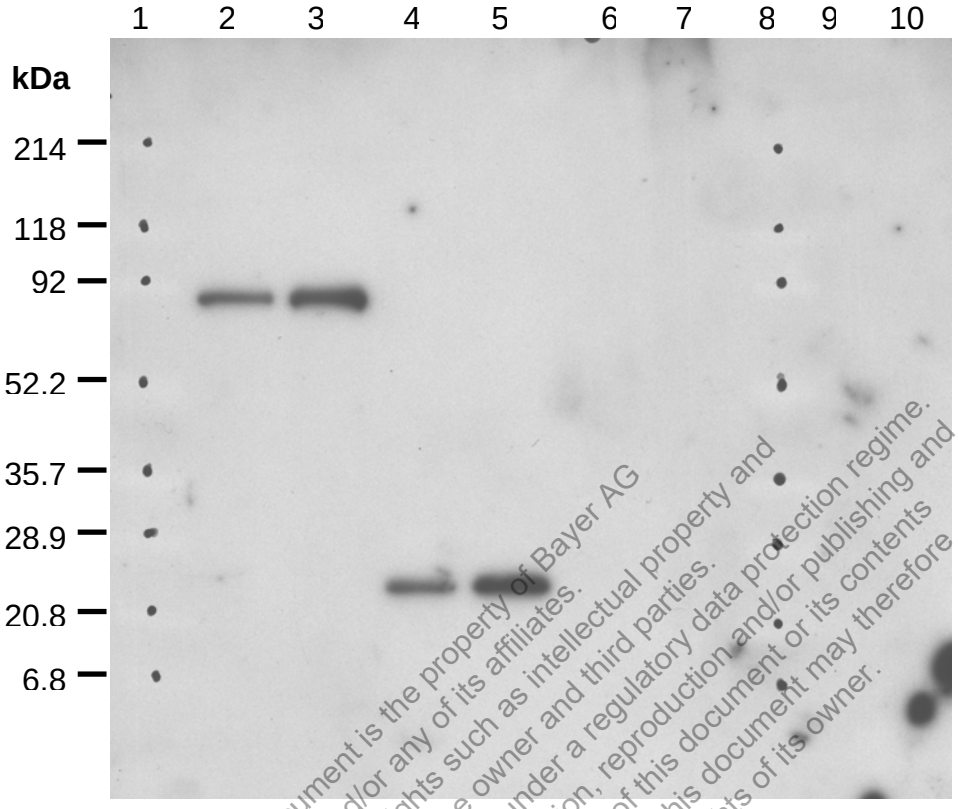
1.2.f. Conclusions of the characterization of the Cry3Bb1 protein produced in MON 88017 corn

A panel of analytical tests was used to characterize and compare the plant- and *E. coli*-produced Cry3Bb1 proteins: (1) western blot analysis and densitometry, (2) SDS-PAGE and densitometry, (3) MALDI-TOF mass spectrometry, (4) glycosylation analysis, and (5) a Cry3Bb1 activity assay. The equivalence of the plant-produced Cry3Bb1 protein to the *E. coli*-produced Cry3Bb1 protein was evaluated by comparing their full-length molecular weights, immunoreactivity with anti-Cry3Bb1 antibodies, glycosylation status, and functional activity.

Protein bands migrating at approximate molecular weights of 77, 66, and 55 kDa were identified as plant-produced Cry3Bb1 protein fragments using western blot analysis with anti-Cry3Bb1 antibody. The purity, calculated as the sum of the percent optical density of the three bands identified as Cry3Bb1 protein, was 66.1%. The approximate molecular weights of each of the three Cry3Bb1 protein bands identified in the plant-produced Cry3Bb1 protein sample, estimated by comparison to molecular weight markers on a Colloidal Brilliant Blue G stained SDS-polyacrylamide gel, were 77, 66, and 55 kDa. MALDI-TOF mass spectrometry analysis of these bands after trypsin digestion yielded peptide masses consistent with the peptide masses expected for the Cry3Bb1 protein. Together, masses identified for each protein band yielded 44% overall coverage (287 out of 653 amino acids) of the peptide sequence of the plant-produced Cry3Bb1 protein. The plant-produced Cry3Bb1 protein was not glycosylated. The functional activities of the plant- and *E. coli* - produced Cry3Bb1 proteins determined by insect bioassay were not significantly different ($p>0.05$), leading to the conclusion that the proteins are functionally equivalent.

Based on the results of these tests it was concluded that the plant- and *E. coli*-produced Cry3Bb1 proteins are physicochemically and functionally equivalent.

Figure 27. Glycosylation analysis of the Cry3Bb1 protein produced in MON 88017



Proteins were separated using SDS-PAGE under reducing and denaturing conditions and transferred to a PVDF membrane. The dots in lanes 1 and 8 represent the location of the molecular weight markers (MWM) on the membrane. A 10 minutes exposure is shown.

Lane	Sample	Amount (µg)
1	Bio-Rad Prestained Broad Range MWM	---
2	transferrin	0.25
3	transferrin	0.5
4	*E. coli*-produced Cry3Bb1 protein	0.25
5	*E. coli*-produced Cry3Bb1 protein	0.5
6	plant-produced Cry3Bb1 protein	0.25
7	plant-produced Cry3Bb1 protein	0.5
8	Bio-Rad Prestained Broad Range MWM	---
9	empty	---
10	empty	

SECTION 2. Levels of the CP4 EPSPS and Cry3Bb1 proteins in MON 88017

The levels of the CP4 EPSPS and Cry3Bb1 proteins in various tissues of MON 88017 were estimated using enzyme-linked immunosorbent assay (ELISA). The materials and methods for the ELISA analysis, as well as a description of the tissue types, are provided in Appendix III. To produce the tissues for analysis, MON 88017 and conventional corn hybrids were planted at three field locations during the 2002 growing season. The sites were located in the major corn-growing region of the United States. A randomized complete block design with three replications was used at all sites. Pollen, silk, forage, forage root, grain, stover and young leaf were collected at appropriate times of plant development. Cry3Bb1 and CP4 EPSPS protein levels in these tissues are presented in Table 4. The following tissues were collected over the season from the V2 to the R6 vegetative growth stages: overseason leaf samples (OSL 1 – 4), overseason whole plant (OSWP 1-4), and overseason root (OSR 1-4). The levels of CP4 EPSPS and Cry3Bb1 proteins in overseason tissues are presented in Tables 5 and 6. Limits of detection and quantitation are presented in Table 7.

The mean CP4 EPSPS protein levels in analyzed tissues collected across three sites ranged during the season from 150-220 µg/g dwt in leaf and 70-150 µg/g dwt in root (Table 5). The mean CP4 EPSPS protein levels across three sites were 220 µg/g dwt in young leaf, 390 µg/g dwt in pollen, 57 µg/g dwt in forage, and 5.8 µg/g dwt in grain (Table 4). CP4 EPSPS levels were not assessed in whole plant, silk and stover. In general, levels of the CP4 EPSPS protein declined during the growing season.

The mean Cry3Bb1 protein levels in analyzed tissues collected across the three sites ranged from 260-570 µg/g dwt in leaf, 220-500 µg/g dwt in the whole plant, and 100-370 µg/g dwt in root tissues harvested throughout the growing season (Table 6). The mean levels across three sites were 570 µg/g dwt in young leaf, 25 µg/g dwt in pollen, 95 µg/g dwt in forage, 380 µg/g dwt in silk, 130 µg/g dwt in forage root, 15 µg/g dwt in grain, and 88 µg/g dwt in stover (Table 4).

Table 4. Levels of the Cry3Bb1 and CP4 EPSPS proteins in tissues of MON 88017

Tissue Type[1]	Growth Stage	Cry3Bb1 Mean (SD)[2] [range][3]		CP4 EPSPS Mean (SD)[2] [range][3]	
		(µg/g dwt)	(µg/g fwt)	(µg/g dwt)	(µg/g fwt)
Young leaf	V2-V3 (14-22 DAP)	570 (170) [230-820]	76 (23) [28-110]	220 (30) [160-260]	30 (5.3) [19-36]
Pollen	R1 (62-69 DAP)	25 (4.2) [17-32]	14 (2.5) [11-20]	390 (85) [210–470]	220 (43) [130-280]
Silk	R1 (62-69 DAP)	380 (65) [300-500]	37 (5.6) [30-45]	NM	NM
Forage	R4-R6 (early dent) (97-124 DAP)	95 (19) [75-130]	27 (5.5) [22-39]	57 (7.6) [42-69]	16 (2.1) [12-19]
Forage root	R4-R6 (early dent) (97-124 DAP)	130 (29) [98-170]	21 (3.1) [17-27]	70 (20) [47-110]	11 (2.8) [6.6-15]
Grain	R6 (133–146 DAP)	15 (3.6) [10-22]	13(3.1) [8.7-19]	5.8 (0.97) [4.1-7.1]	5.1 (0.89) [3.7-6.3]
Stover	R6 (after harvest) (133-147 DAP)	88 (13) [71-110]	30 (4.4) [25-39]	NM	NM

[1] Description of the tissue types is provided in Appendix III. Limits of detection and quantitation are described in Table 7.
[2] The mean and standard deviation were calculated across sites and replicates. (n=9)
[3] Minimum and maximum values were determined for each tissue type across sites.

DAP = days after planting
n = number of samples
SD = standard deviation. The mean and SD were calculated across sites (n=9)
NM = not measured

Table 5. Levels of the CP4 EPSPS protein (μg/g dwt) in overseason tissues of MON 88017

Tissues[1,2] (n=9)		V2-V3 (14-22 DAP)	V5 (26-34 DAP)	V8 (40-45 DAP)	V11-V17 (55-62 DAP)	R4-R6 (97-124 DAP)
Leaf	Mean (SD)	220 (30)	190 (26)	170 (37)	150 (19)	NA
	Range	160-260	130-250	140-240	120-170	
Root	Mean (SD)	150 (34)	110 (29)	100 (30)	97 (18)	70 (20)
	Range	110-220	74-160	62-160	72-130	47-110

[1] Growth stages V2-V3, V5, V8 and V11-V17 correspond to overseason tissue samples -1, -2, -3 and -4, respectively (e.g., OSL-1, etc.). The root samples collected at the R4-R6 and R6 growth stages correspond to forage root and senescent root, respectively. The DAP values were estimated from information in the production report
[2]Description of tissue types is provided in Appendix III. Limits of detection and quantitation are described in Table 7.

DAP = days after planting.
NA = not applicable.
dwt = dry weight tissue
SD = standard deviation

Table 6. Levels of the Cry3Bb1 protein (µg/g dwt) in overseason tissues of MON 88017

Tissues[1] (n=9)		V2-V3 (14-22 DAP)	V5 (26-34 DAP)	V8 (40-45 DAP)	V11-V17 (55-62 DAP)	R4-R6 (97-124 DAP)	R6 (133-147 DAP)
Leaf	Mean (SD)	570 (170)	430 (58)	310 (45)	260 (44)	NA	NA
	Range	230-820	310-510	240-380	190-340		
Whole plant	Mean (SD)	500 (64)	380 (170)	310 (48)	220 (23)	NA	NA
	Range	410-590	150-600	230-380	190-250		
Root	Mean (SD)	370 (80)	250 (71)	210 (78)	180 (37)	130 (29)	100 (19)
	Range	240-510	190-420	150-410	110-230	98-170	77-140

[1] Growth stages V2-V3, V5, V8 and V11-V17 correspond to over-season tissue samples -1, -2, -3 and -4, respectively (e.g., OSL-1, etc.). The root samples collected at the R4-R6 and R6 growth stages correspond to forage root and senescent root, respectively.

DAP = days after planting
NA = not applicable
dwt = dry weight tissue
SD = standard deviation

Table 7. Limits of detection and quantitation for the ELISA of Cry3Bb1 and CP4 EPSPS proteins

Tissue[1]	Cry3Bb1		CP4 EPSPS	
	LOD (µg/g fwt)	LOQ (µg/g fwt)	LOD (µg/g fwt)	LOQ (µg/g fwt)
Leaf	0.0073	0.044	0.090	0.17
Root	0.032	0.040	0.050	0.10
Pollen	0.020	0.039	0.12	0.18
Silk	0.012	0.041	NM	NM
Forage	0.010	0.047	0.11	0.21
Grain	0.0097	0.051	0.18	0.28

[1] Description of the tissue types is provided in Appendix III. Whole plant and stover were analyzed using the forage assay. Senescent root was analyzed using the root assay.

LOD = limit of detection.
LOQ = limit of quantitation.
NM = not measured.

SECTION 3. Estimate of dietary exposure

Use of corn as food and animal feed is discussed in Part VII, Section 1. Humans and animals will be exposed to CP4 EPSPS and Cry3Bb1 proteins through dietary intake of food and feed derived from MON 88017. The quantity of corn consumed on a daily basis by humans and livestock, as well as the levels of CP4 EPSPS and Cry3Bb1 proteins in corn, are necessary to derive an estimate of daily dietary exposure (DDE). Humans consume only corn grain or processed food products derived from corn grain. Livestock consume both corn grain and forage.

The mean adult consumption of corn grain (endosperm fraction) in the United States is 0.27 g/kg body weight/day. This consumption figure is based on the CSFII 1994-1998 food consumption surveys for adults in the United States (DEEM US™ data base (acute version 7.77) – Exponent, Inc.).

Consumption of corn is much higher in livestock. The daily consumption of corn for the young pig is 37 g/kg body wt/day (assuming 60% dietary inclusion rate) and 23 g/kg body wt/day for the finishing pig (assuming 75% dietary inclusion rate). The four-week old broiler consumes 57 g/kg body wt/day when the inclusion rate of corn is 60% of the diet. The lactating dairy cow (~570 kg body wt) producing 37 kg of milk per day consumes about 4.4 kg of corn per day and 10.4 kg of corn silage per day or 7.7g/kg body wt/day and 18.2 g/kg body wt/day, respectively (Ouellet et al., 2003). Daily dietary exposure (DDE) is computed as follows:

DDE = Corn consumption (g/kg) x Cry3Bb1 or CP4 EPSPS protein concentration (µg/g)

Using upper bound estimates of human and animal daily corn consumption, it is possible to calculate the margin of exposure (MOE) for these proteins. The margin of exposure is defined as the ratio of the no observed effect level (NOEL) derived from toxicology tests to the estimate of human and animal daily dietary exposure (DDE). The MOE is computed as follows:

$$\text{MOE} = \text{NOEL (µg/kg)} \div \text{DDE (µg/kg)}$$

The exposure calculation makes the conservative assumption that there is no loss of the introduced protein during the processing of corn grain into human food. It also assumes that 100% of the corn grain ending up in human food is derived from the MON 88017, which is highly unlikely given the variety of commercial corn hybrids that exist in the market place.

3.1. Estimated dietary exposure to the CP4 EPSPS protein

The mean level of CP4 EPSPS protein in MON 88017 grain is 5.8 µg/gram (dwt) (range 4.1-7.1) and 57 µg/gram (dwt) (range 42 – 69) in forage (Table 4, Section 2, Part VI). The highest dose of CP4 EPSPS protein that was administered by gavage to mice, 572

mg/kg (572,000 µg/kg), produced no adverse effects; therefore the no observed effect level (NOEL) in the CP4 EPSPS mouse gavage study is 572,000 µg/kg.

A dairy cow consumes both corn grain and silage, and therefore the amount of CP4 EPSPS needs to be considered from both sources. CP4 EPSPS in corn silage (40% grain and 60% forage) would have a mean CP4 CPSPS concentration of (5.8 µg/g x 40% + 57 µg/g x 60%) = 36.5 µg/g. The high end of the range is calculated to be (7.1µg/g x 40% + 69 µg/g x 60%) = 44.2 µg/g CP4 EPSPS. A summary of the calculated margins of exposure for humans and livestock for CP4 EPSPS protein produced in MON 88017 is presented in Table 8.

3.2. Estimated dietary exposure to the Cry3Bb1 protein

The mean level of Cry3Bb1 protein in corn grain is 15 µg/gram (dwt) (range 10 - 22) and 95 µg/gram (dwt) (range 75 – 130) in forage. The highest dose of Cry3Bb1 protein that was administered by gavage to mice, 1930 mg/kg, produced no adverse effects; therefore, the NOEL in the Cry3Bb1 mouse gavage study is 1,930,000 µg/kg.

For dairy cow, the amount of consumed Cry3Bb1 was considered from both corn grain and silage. Cry3Bb1 in corn silage (40% grain and 60% forage) would have a mean Cry3Bb1 concentration of (15 µg/g x 40% + 95 µg/g x 60%) = 63 µg/g. The high end of the range is calculated to be (22 µg/g x 40% + 130 µg/g x 60%) = 86.8 µg/g Cry3Bb1. A summary of the calculated margins of exposure to Cry3Bb1 protein produced in MON 88017 by humans and farm animals is presented in Table 8.

3.3. Margins of exposure

Large margins of exposure were noted for CP4 EPSPS protein for humans (364,331) and livestock (806.8- 4,287), and for Cry3Bb1 for humans (470,731) and livestock (1,529 – 5,594) (Table 8). These calculated margins of exposure indicate that there is virtually no risk to human and animal health that will be associated with dietary exposure to food and feed products derived from MON 88017.

Table 8. Margins of exposure for dietary consumption of CP4 EPSPS and Cry3Bb1 proteins in MON 88017 when used as food or animal feed.

Parameter	Adult Human	Broiler Chicken	Young Pig	Finishing Pig	Dairy Cow
Daily grain consumption (g/kg body wt)	0.27	57	37	23	7.7
Daily forage consumption (g/kg body wt)	NA	NA	NA	NA	18.2
CP4 EPSPS					
DDE (mg/ kg body wt/day)	0.0016 (0.0019) [1]	0.331 (0.405)	0.215 (0.263)	0.133 (0.163)	0.709 (0.859)
MOE	364,331 (301,053)	1,730 (1,413)	2,665 (2,177)	4,287 (3,503)	806.8 (666)
Cry3Bb1					
DDE (mg/ kg body wt/day)	0.0041 (0.0059)	0.855 (1.254)	0.555 (0.814)	0.345 (0.506)	1.262 (1.749)
MOE	470,731 (327,119)	2,257 (1,539)	3,477 (2,371)	5,594 (3,814)	1,529 (1,103)

SECTION 4. Assessment of the potential for allergenicity of the CP4 EPSPS and Cry3Bb1 proteins produced in MON 88017

This assessment of the allergenic potential of a protein addresses the following questions, which identify characteristics of known allergens:

1. Is the protein from a known allergenic source?
2. Does the protein represent a relatively large portion of the total protein in MON 88017?
3. Is the protein structurally similar, based on amino acid sequence, to known allergens?
4. Is the protein resistant to digestion in simulated mammalian digestive fluid?

In the following sections, these four questions are addressed in detail for the CP4 EPSPS and Cry3Bb1 proteins produced in MON 88017. General information on the methods used in assessing the last two questions – structural similarity to known allergens and stability in simulated digestive fluids- is provided below.

[1] Numbers calculated using high end of the expression range given in parentheses.

Structural similarity to known allergens

In order to assess potential similarity to allergens, bioinformatic analyses were performed on the CP4 EPSPS and Cry3Bb1 proteins expressed in MON 88017. The comparisons were performed using the allergen (AD4) database.

Exposure to allergens in foods may cause sudden, severe, life-threatening reactions in susceptible individuals. Gliadins are suspected to cause celiac disease (gluten-sensitive enteropathy) and are also considered important immunologically active proteins. Screening the amino acid sequences of proteins introduced into plants by modern biotechnology for similarity to sequences of known allergens and gliadins is one of many assessments performed to evaluate product safety. Similarly, the amino acid sequences of introduced proteins are also screened against known toxins as well as all known proteins in publicly available genetic databases (see Section 5 of Part VI).

The FASTA algorithm can be used to evaluate the extent of sequence similarity between a query protein sequence and a database sequence. In principle, if two proteins share sufficient linear sequence similarity, they also will share three-dimensional structure and, therefore, functional homology. By definition, homologous proteins share secondary structure and common three-dimensional folds (Pearson, 2000). Because the degree of similarity between homologs varies widely, the data need to be carefully evaluated in order to maximize their value to the allergy assessment process.

The bioinformatics assessment is used to identify similarities between the query protein and known or clinically cross-reactive allergens. While related (homologous) proteins may share only 25% amino acid identity in a 200 amino acid overlap (Pearson, 2000), this is not generally sufficient to indicate IgE-mediated cross-reactivity (Aalberse et al., 2001). Indeed, allergenic cross-reactivity caused by proteins sharing conformational or linear epitopes with known allergens is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). Such high levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins (the proteins that trigger celiac disease, a non-IgE mediated allergic disorder) can be easily identified using FASTA. It is possible that proteins structurally unrelated to allergens and gliadins may still contain smaller immunologically significant epitopes. For this comparison an immunologically relevant sequence was defined as eight linearly contiguous, identical amino acids (Metcalfe et al., 1996). A second bioinformatics tool (IDENTITYSEARCH) was used to specifically identify short linear polypeptide matches to known or suspected allergens. The results of the bioinformatics analyses of CP4 EPSPS and Cry3Bb1 amino acid sequences are described below in Sections 4.1.c. and 4.2.c, respectively.

Stability in simulated digestive fluids

A factor that increases the likelihood of allergic oral sensitization to proteins is the stability of the protein to gastrointestinal digestion. Protein allergens tend to be stable to the peptic and acidic conditions of the digestive system if they are to reach and pass

through the intestinal mucosa to elicit an allergenic response (Astwood et al., 1996; Metcalfe et al., 1996). A relationship between digestibility in simulated gastric fluid (*in vitro* pepsin digestibility) and food safety has been previously demonstrated using a digestion model (Astwood et al., 1996). Simulated intestinal fluid is frequently used for *in vitro* studies to assess the digestibility of food components (Yagami et al., 2000; Okunuki et al., 2002).

Recently, the pepsin digestibility assay protocol was standardized by the International Life Science Institute (ILSI) based on results obtained from an international, multi-laboratory ring study (Thomas et al., in press). This test showed that the results of *in vitro* pepsin digestion assays are reproducible when standard protocols were followed. Using these protocols, the pepsin digestion assay was used to assess the susceptibility of the CP4 EPSPS and Cry3Bb1 proteins to pepsin digestion *in vitro.* In addition, *in vitro* susceptibility of these proteins to pancreatin was assessed for stability in simulated intestinal fluid according to methods described in the United States Pharmacopeia (1995). The stability of the CP4 EPSPS and Cry3Bb1 proteins in simulated digestive fluids is discussed in Sections 4.1.d. and 4.2.d., respectively.

4.1. Assessment of the potential for allergenicity of the CP4 EPSPS protein

4.1.a. Source of the CP4 EPSPS protein

As described in Part VI, Section 1, the *cp4 epsps* coding sequence was obtained from a naturally occurring, glyphosate-degrading bacterium and has been identified by the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are not known human or animal pathogens and are not commonly allergenic. A number of food crops have been improved by addition of a single functional gene from *Agrobacterium tumefaciens* (FAO/WHO, 1991) and there are no reports of allergies to *Agrobacterium* species (Section 5.3, Part VI). Furthermore, according to FAO/WHO (2001), there is no known population of individuals sensitized to bacterial proteins. Therefore, it can be concluded that the CP4 EPSPS protein is not from a known allergenic source.

4.1.b. Proportion of total protein – CP4 EPSPS

Data presented in Section 2 of Part VI show that the overall mean level of CP4 EPSPS protein in MON 88017 grain is 5.8 µg/g (dwt). The mean % dry weight of total protein in MON 88017 grain is 12.51% (Table 16). The percent of CP4 EPSPS protein in one gram of MON 88017 grain is calculated as follows:

$$5.8\ \mu g/g \div 125{,}100\ \mu g \times 100\% = 0.0046362\ \%$$

Therefore, the CP4 EPSPS protein represents a very small portion of total protein in MON 88017 grain.

4.1.c. Bioinformatics analyses of sequence similarity of the CP4 EPSPS protein produced in MON 88017 to allergens

Using the bioinformatics analyses described above, potential structural similarities between the CP4 EPSPS protein and proteins in the allergen database were evaluated using the FASTA sequence alignment tool. Nine identified proteins were ranked according to their degree of similarity. The highest similarity was to a small portion of the *Dermatophagoides farinae* allergen Der f 2 (Accession No. AAB30829). This alignment demonstrated 30.5% identity over an 82 amino acid (aa) window with a high *E*-score of 0.41. Moreover, the alignment contained four gaps, and the longest stretch of contiguous amino acid identities consisted of five amino acids. The length of the overlap is relatively short (18%) when compared to the full length (455 aa) of the CP4 EPSPS protein. Consequently, no structural and/or functional homology between the CP4 EPSPS protein and the Der f 2 allergen can be inferred. It is unlikely that allergenic cross-reactivity will be observed even when there is ≥ 50% identity across the entire length of the protein (Aalberse, 2000). Therefore, it is highly unlikely that cross-reactivity exists between the CP4 EPSPS protein and the Der f 2 allergen. Inspection of the remaining eight alignments did not show any significant similarities between the CP4 EPSPS protein and aligned allergens. No immunologically relevant sequences (eight contiguous amino acid identities) were detected when the CP4 EPSPS protein sequence was compared to the AD4 sequence database.

4.1.d. Stability of the CP4 EPSPS protein in simulated digestive fluids

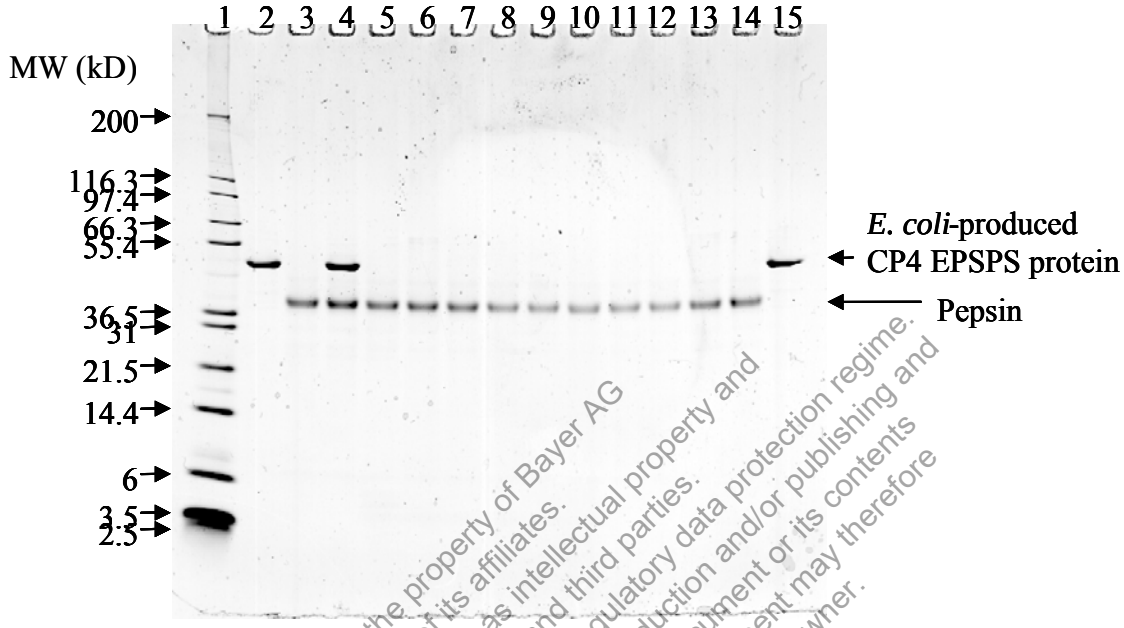
Harrison et al. (1996) demonstrated that the CP4 EPSPS protein is rapidly degraded in simulated digestive fluids. The half-life for CP4 EPSPS was less than 15 seconds in the gastric system and less than 10 minutes in the intestinal system, based on western blot analysis. Therefore, if any of the CP4 EPSPS protein were to survive in the gastric system, it would be rapidly degraded in the intestine. As a comparison, 50% of solid food has been estimated to empty from the human stomach in two hours, while 50% liquid empties in approximately 25 minutes (Sleisenger and Fordtran, 1989). Based on this information, CP4 EPSPS protein is expected to degrade rapidly in the mammalian digestive tract.

Subsequent experiments were performed to assess the *in vitro* digestibility of the CP4 EPSPS protein in simulated gastric fluid (SGF). As with the previous study (Harrison et al., 1996), the CP4 EPSPS protein used was produced in and purified from *E. coli*. Digestibility was assessed by three methods, including SDS-PAGE gel staining, western blot analysis, and EPSPS enzymatic activity assay.

The results of these experiments demonstrate that the *E. coli*-produced CP4 EPSPS protein was rapidly digested after incubation in SGF. The SDS-PAGE Colloidal Blue gel staining method demonstrated that at least 98% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 seconds (Figure 28). No protein bands due to degradation of the CP4 EPSPS protein were observed. Western blot analysis (Figure 29)

confirmed that greater than 95% of the immunoreactive *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 seconds. Similarly, it was demonstrated that the EPSPS activity was reduced to <10% within 15 seconds of incubation of the CP4 EPSPS protein in SGF (Table 9). In summary, and in complement to the earlier study, the results of the SDS PAGE, western blot, and functional assays demonstrate that the *E. coli*-produced CP4 EPSPS protein is rapidly degraded in simulated gastric fluid.

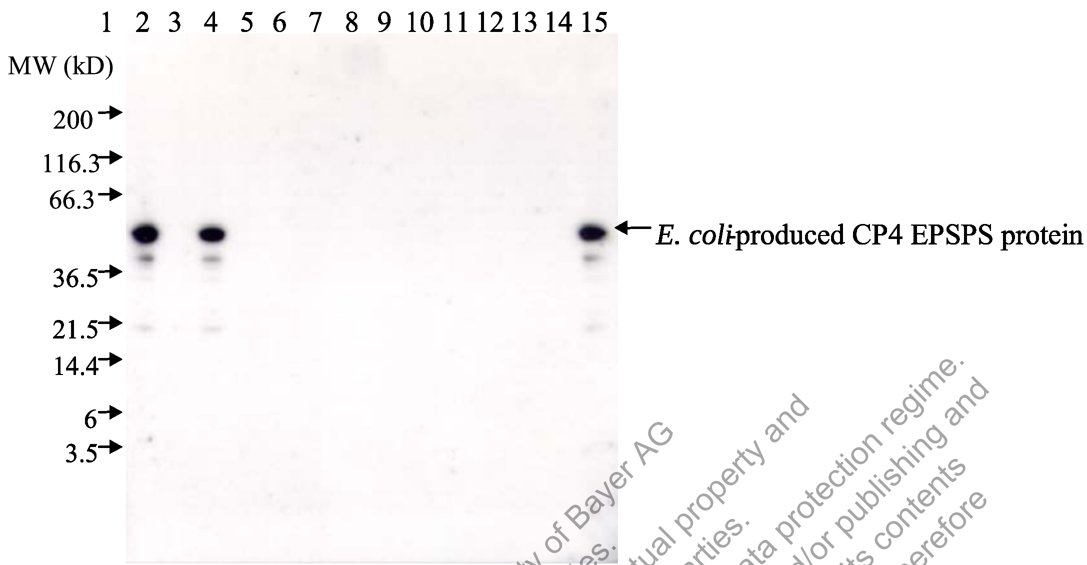
Figure 28. Digestion of CP4 EPSPS protein in simulated gastric fluid demonstrated by SDS PAGE



Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. *E. coli*-produced CP4 EPSPS protein was loaded at 500 ng per lane based on pre-digestion concentration.

Lane	Description	Incubation time
1	Molecular weight markers	
2	Experimental control without pepsin (P0)	0 s
3	Experimental control without CP4 EPSPS (N0)	0 s
4	CP4 EPSPS protein in SGF, T = 0	0 s
5	CP4 EPSPS protein in SGF, T = 1	15 s
6	CP4 EPSPS protein in SGF, T = 2	30 s
7	CP4 EPSPS protein in SGF, T = 3	1 min
8	CP4 EPSPS protein in SGF, T = 4	2 min
9	CP4 EPSPS protein in SGF, T = 5	4 min
10	CP4 EPSPS protein in SGF, T = 6	8 min
11	CP4 EPSPS protein in SGF, T = 7	15 min
12	CP4 EPSPS protein in SGF, T = 8	30 min
13	CP4 EPSPS protein in SGF, T = 9	60 min
14	Experimental control without CP4 EPSPS (N9)	60 min
15	Experimental control without pepsin (P9)	60 min

Figure 29. Digestion of CP4 EPSPS protein in simulated gastric fluid demonstrated by western blot analysis



Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. *E. coli*-produced CP4 EPSPS protein was loaded at 1 ng per lane based on 90% purity and pre-digestion concentration.

Lane	Description	Incubation Time
1	Molecular weight markers	
2	Experimental control without pepsin (P0)	0 sec
3	Experimental control without CP4 EPSPS (N0)	0 sec
4	CP4 EPSPS protein in SGF, T = 0	0 sec
5	CP4 EPSPS protein in SGF, T = 1	15 sec
6	CP4 EPSPS protein in SGF, T = 2	30 sec
7	CP4 EPSPS protein in SGF, T = 3	1 min
8	CP4 EPSPS protein in SGF, T = 4	2 min
9	CP4 EPSPS protein in SGF, T = 5	4 min
10	CP4 EPSPS protein in SGF, T = 6	8 min
11	CP4 EPSPS protein in SGF, T = 7	15 min
12	CP4 EPSPS protein in SGF, T = 8	30 min
13	CP4 EPSPS protein in SGF, T = 9	60 min
14	Experimental control without CP4 EPSPS (N9)	60 min
15	Experimental control without pepsin (P9)	60 min

Table 9. Specific activity of *E. coli*-produced CP4 EPSPS protein after digestion in simulated gastric fluid

Sample	Specific Activity (Units/mg protein)
Experimental control without pepsin incubated for 0 seconds	4.92
Experimental control without pepsin incubated for 60 minutes	2.10
E. coli-produced CP4 EPSPS protein in SGF incubated for 0 seconds	5.63
E. coli-produced CP4 EPSPS protein in SGF incubated for 15 seconds	0.27
E. coli-produced CP4 EPSPS protein in SGF incubated for 30 seconds	0.15
E. coli-produced CP4 EPSPS protein in SGF incubated for 60 seconds	0.15
Experimental control without CP4 EPSPS incubated for 0 seconds	0.02
Experimental control without CP4 EPSPS incubated for 60 minutes	0.05
Buffer Blank	0.01

4.2. Assessment of the potential for allergenicity of the Cry3Bb1 protein

4.2.a. Source of the Cry3Bb1 protein

The Cry3Bb1 protein is a variant of the wild-type Cry3Bb1 protein isolated from *Bacillus thuringiensis* (subsp. *kumamotoensis*) strain EG4691 (Donovan et al., 1992). *Bacillus thuringiensis* subspecies *kumamotoensis* is a spore-forming, gram-positive bacterium that is found naturally in soil. *B.t.* strains have been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). There are no known reports of allergies to *Bacillus thuringiensis* species.

4.2.b. Proportion of total protein – Cry3Bb1

The mean level of Cry3Bb1 protein in corn grain is 15 µg/g (dwt). The mean % dry weight of total protein in MON 88017 grain is 12.51% (Table 16). The percent of Cry3Bb1 protein in one gram of MON 88017 grain is calculated as follows:

$$15\ \mu g/g \div 125100\ \mu g \times 100\% = 0.01199\ \%$$

Therefore, the Cry3Bb1 protein represents a very small portion of total protein in MON 88017 grain.

4.2.c. Bioinformatics analysis of sequence similarity of the Cry3Bb1 protein produced in MON 88017 to allergens

Using the methods described above, potential structural similarities shared between the Cry3Bb1 protein and proteins in the allergen database were evaluated using the FASTA sequence alignment tool. Six identified proteins were ranked according to their degree of similarity. None of these proteins had an *E*-score of less than $1x10^{-5}$. The best similarity observed was to the *Anisakis simplex* muscle tropomyosin allergen, Ani s 3 (Accession No. Q9NAS5). In this alignment, the overlap of 120 aa contained four gaps, showed only 27.5% identity, and had an *E*-score of 1.1. The length of the overlap was relatively short when compared to the full-length (653 aa) Cry3Bb1 protein. The longest stretch of contiguous amino acid identities consisted of three amino acids. Consequently, no structural and/or functional homology between the Cry3Bb1 protein and the Ani s 3 allergen can be inferred. As allergenic cross-reactivity is unlikely to be observed even when there is ≥ 50% identity across the entire length of the protein (Aalberse, 2000), it is highly unlikely that cross-reactivity exists between the Cry3Bb1 protein and the Ani s 3 allergen. Inspection of the remaining five alignments did not show any significant similarities between the Cry3Bb1 protein and aligned allergens.

No immunologically relevant sequences (eight contiguous amino acid identities) were detected when the Cry3Bb1 protein sequence was compared to the AD4 sequence database.

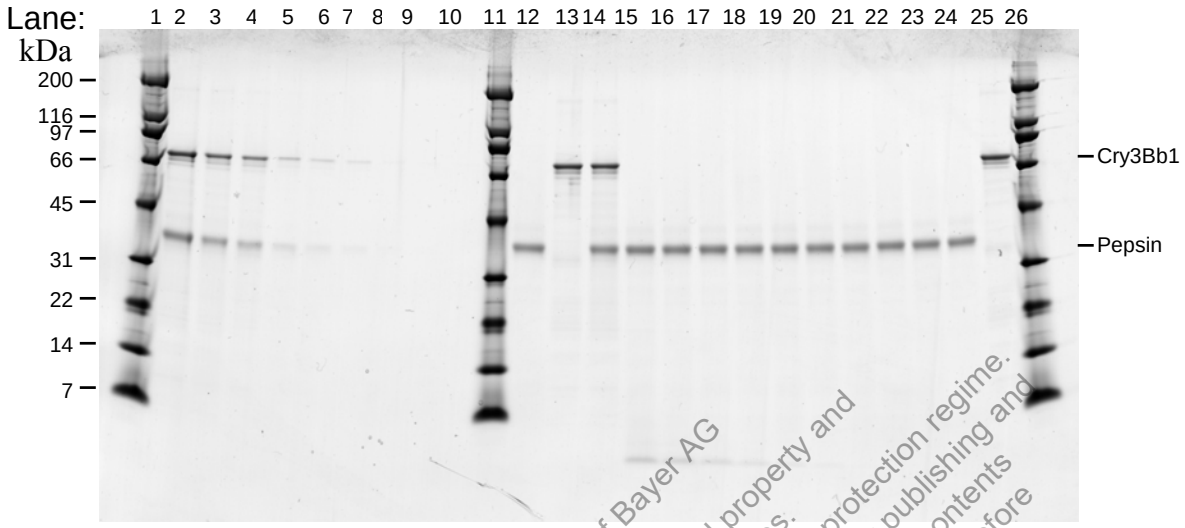
4.2.d. Stability of the Cry3Bb1 protein in simulated digestive fluids

Using the ILSI pepsin digestion protocol described above (Section 4, Thomas et al., in press)) *in vitro* digestibility of the Cry3Bb1 protein was assessed in simulated gastric (SGF) and simulated intestinal (SIF) fluids. SGF contains the proteolytic enzyme pepsin. Digestibility in SGF was assessed using Colloidal Brilliant Blue G stained SDS polyacrylamide gels and western blot analysis. SIF contains a mixture of proteolytic enzymes (including trypsin) known as pancreatin. Digestibility in SIF was assessed using western blot analysis.

The Cry3Bb1 protein was rapidly digested when incubated in SGF. At least 98% of the full-length (~75 kDa) Cry3Bb1 protein was digested within 15 seconds in SGF when analyzed using Colloidal Brilliant Blue G stained SDS polyacrylamide gels (Figure 30). A proteolytic fragment (migrating at the dye front) was observed at several of the initial time points, but was absent after four minutes of incubation. At least 99.8% of the full-length Cry3Bb1 protein was digested within 15 seconds in SGF and no proteolytic fragments were observed for samples evaluated by western blot analysis (Figure 31).

The full-length Cry3Bb1 protein was also rapidly digested when incubated in SIF. At least 99.5% of the full-length Cry3Bb1 protein was digested within 1 minute in SIF when evaluated by western blot analysis (Figure 32). As expected, protease-resistant fragments were observed throughout the 24-hour time course. Proteolytic (trypsin) conversion of the full-length protoxin to an active toxin has been described for all members of the Cry1, Cry2, and Cry3 families (EPA, 2001b, 2002, 2003a). These results suggest that the Cry3Bb1 protein will be readily digestible in the mammalian digestive tract.

Figure 30. Digestion of the Cry3Bb1 protein in simulated gastric fluid demonstrated by SDS-PAGE analysis

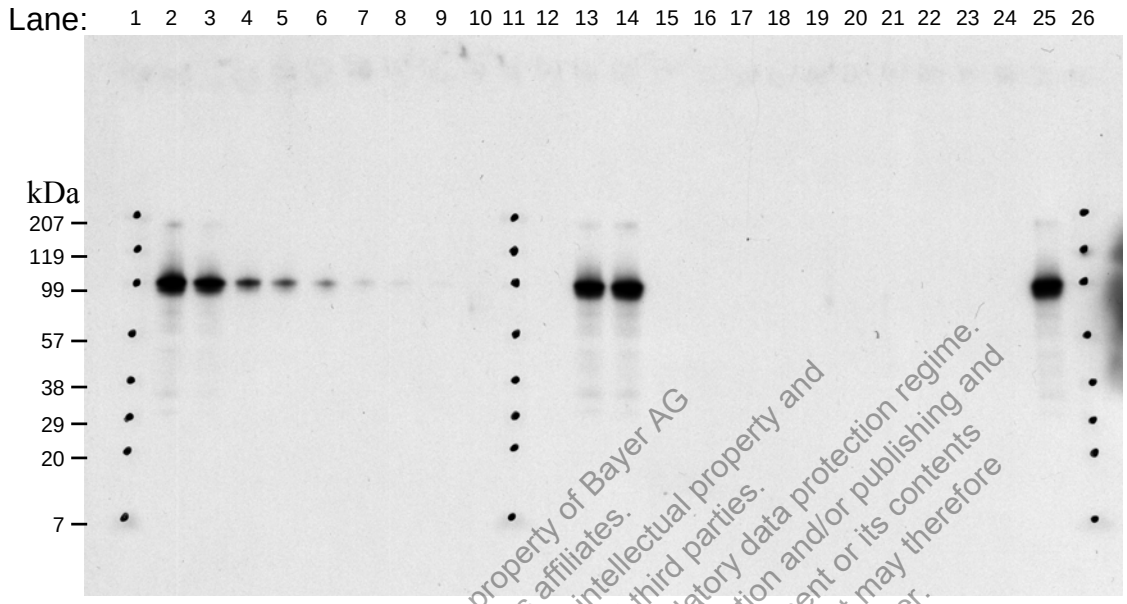


Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient Tris-tricine buffered gel and visualized by staining with Colloidal Brilliant Blue G stain. Cry3Bb1 protein was loaded at 500 ng per lane based on pre-digestion concentration and purity values.

Lane	Description	Incubation Time	Lane	Description	Incubation Time
1	MWM*	---	14	SGF-2 T0	0 sec
2	500 ng SGF-2 T0	---	15	SGF-2 T1	15 sec
3	200 ng SGF-2 T0	---	16	SGF-2 T2	30 sec
4	100 ng SGF-2 T0	---	17	SGF-2 T3	1 min
5	50 ng SGF-2 T0	---	18	SGF-2 T4	2 min
6	25 ng SGF-2 T0	---	19	SGF-2 T5	4 min
7	10 ng SGF-2 T0	---	20	SGF-2 T6	8 min
8	5 ng SGF-2 T0	---	21	SGF-2 T7	15 min
9	2.5 ng SGF-2 T0	---	22	SGF-2 T8	30 min
10	1 ng SGF-2 T0	---	23	SGF-2 T9	60 min
11	MWM*	---	24	SGF-2 N60	60 min
12	SGF-2 N0	0 sec	25	SGF-2 P60	60 min
13	SGF-2 P0	0 sec	26	MWM*	---

*MWM (molecular weight markers) = Bio-Rad SDS-PAGE Standards, Broad Range

Figure 31. Digestion of the Cry3Bb1 protein in simulated gastric fluid demonstrated by western blot analysis

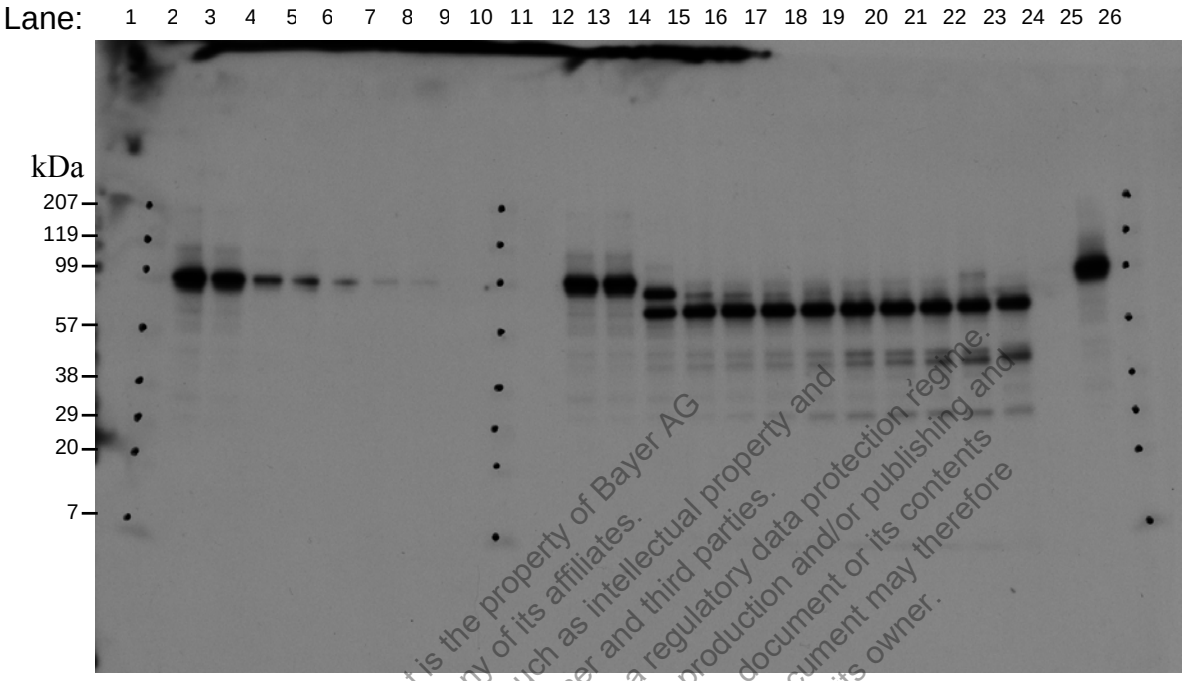


Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a Tris-tricine buffered gel. Proteins were transferred to PVDF for western blot analysis. Immunoreactivity was detected by ECL; the above image is a 5 sec exposure.

Lane	Description	Incubation Time	Lane	Description	Incubation Time
1	MWM*	---	14	SGF-2 T0	0 sec
2	10 ng SGF-2 T0	---	15	SGF-2 T1	15 sec
3	5 ng SGF-2 T0	---	16	SGF-2 T2	30 sec
4	1 ng SGF-2 T0	---	17	SGF-2 T3	1 min
5	0.5 ng SGF-2 T0	---	18	SGF-2 T4	2 in
6	0.2 ng SGF-2 T0	---	19	SGF-2 T5	4 min
7	0.1 ng SGF-2 T0	---	20	SGF-2 T6	8 min
8	0.05 ng SGF-2 T0	---	21	SGF-2 T7	15 min
9	0.02 ng SGF-2 T0	---	22	SGF-2 T8	30 min
10	0.01 ng SGF-2 T0	---	23	SGF-2 T9	60 min
11	MWM*	---	24	SGF-2 N60	60 min
12	SGF-2 N0	0 sec	25	SGF-2 P60	60 min
13	SGF-2 P0	0 sec	26	MWM*	---

*MWM (molecular weight markers) = Bio-Rad Prestained SDS-PAGE Standards, Broad Range

Figure 32. Digestion of Cry3Bb1 protein in simulated intestinal fluid demonstrated by western blot analysis



Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a Tris-tricine buffered gel. Proteins were transferred to PVDF for western blot analysis. Immunoreactivity was detected by ECL; the above image is a 10 second exposure. The dots (Lanes 1, 10, and 26) represent the location of the molecular weight markers on the membrane.

Lane	Description	Incubation Time	Lane	Description	Incubation Time
1	MWM*	---	14	SIF T1	1 min
2	10 ng SIF T0	---	15	SIF T2	5 min
3	5 ng SIF T0	---	16	SIF T3	15 min
4	1 ng SIF T0	---	17	SIF T4	30 min
5	0.5 ng SIF T0	---	18	SIF T5	1 hr
6	0.2 ng SIF T0	---	19	SIF T6	2 hr
7	0.1 ng SIF T0	---	20	SIF T7	4 hr
8	0.05 ng SIF T0	---	21	SIF T8	6 hr
9	0.01 ng SIF T0	---	22	SIF T9	12 hr
10	MWM*	---	23	SIF T10	24 hr
11	SIF N0	0 min	24	SIF N24	24 hr
12	SIF P02	0 min	25	SIF P24	24 hr
13	SIF T0	0 min	26	MWM*	---

*MWM (molecular weight markers) = Bio-Rad Prestained SDS-PAGE Standards, Broad Range

4.3. Discussion and conclusions

The data and information provided in this section addressed the questions important to an assessment of allergenic potential of a protein. There are no reports of allergies to either of the donor organisms – *Agrobacterium* or *Bacillus thuringiensis* species. Thus, CP4 EPSPS and Cry3Bb1 proteins are not from known, commonly allergenic sources. The CP4 EPSPS and Cry3Bb1 proteins represent no more than 0.00144% and 0.01199 % of the total protein in the grain of MON 88017, respectively. Therefore, the CP4 EPSPS and Cry3Bb1 proteins would constitute a very small portion of the total protein present in feed and food derived from MON 88017. Bioinformatics analyses demonstrated that the CP4 EPSPS and Cry3Bb1 proteins do not share structurally or immunologically relevant amino acid sequence similarities with known allergens. Thus, it is highly unlikely that the CP4 EPSPS or Cry3Bb1 proteins contain immunologically cross-reactive allergenic epitopes. Digestive fate experiments conducted with *E. coli*-produced CP4 EPSPS and Cry3Bb1 demonstrated that both proteins are rapidly digested in simulated digestive fluids, a characteristic shared among proteins with a history of safe consumption. Thus, it is concluded that MON 88017 does not pose a significant allergenic risk.

SECTION 5. Safety assessment of the CP4 EPSPS and Cry3Bb1 proteins

The previous section described an assessment of the potential for allergenicity of the CP4 EPSPS and Cry3Bb1 proteins in MON 88017 based on the sources of the proteins, their proportion of the total protein in MON 88017, a comparison of their sequence to known allergenic proteins, and their stability in simulated digestive fluids. In this section, safety assessments of the CP4 EPSPS and Cry3Bb1 proteins are provided, including an evaluation of the structural similarity of the CP4 EPSPS and Cry3Bb1 to known proteins and toxins, acute oral toxicity by mouse gavage, the safety of the donor organisms (*Agrobacterium* and *Bacillus thuringiensis,* respectively), the similarity of CP4 EPSPS to EPSPSs derived from food sources with a long history of safe consumption, and the history of safe use of *B.t.* proteins are discussed.

5.1. Safety assessment of the CP4 EPSPS protein

5.1.a. Structural similarity of CP4 EPSPS to known proteins and toxins

Potential structural similarities shared between the CP4 EPSPS protein and proteins in the ALLPEPTIDES database were evaluated using the FASTA sequence alignment tool. Although the FASTA program directly compares amino acid sequences (i.e., primary protein structure), the alignment data may be used to infer higher order structural similarities (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire length are often homologous. Homologous proteins share secondary structure and common three-dimensional folds. Identified proteins were ranked according to their degree of similarity. The most significant

alignment was to the CP4 EPSPS protein found in genetically enhanced *Glycine max* (Accession No. AY125353), demonstrating 100.0% identity over a 455 aa overlap window with an *E*-score of 1.4 e-165. This result was expected as the CP4 EPSPS proteins in Roundup Ready soybean and MON 88017 are identical. All the remaining alignments with significant *E*-scores (i.e., < 1 e-5) were to other members of the EPSPS protein family and, therefore, do not present a risk of adverse biological activity toward humans and animals.

Potential structural similarities shared between the CP4 EPSPS protein and proteins in the toxin database were also evaluated using the FASTA sequence alignment tool. Identified proteins were ranked according to their degree of similarity. The most significant alignment was to the *Bacillus cereus* sphingomyelinase c precursor protein (Accession No. P11889), demonstrating only 28.2% identity over a 131 aa overlap window with an – *E*-score of 0.26. These data demonstrate that the CP4 EPSPS protein is highly unlikely to share any structural homology to any known toxin proteins.

Results of the FASTA sequence alignments demonstrated a lack of structurally relevant similarity between the CP4 EPSPS protein and any known toxic or pharmacologically active proteins relevant to human or animal health.

5.1.b. Acute oral toxicity study with the CP4 EPSPS protein

An oral acute toxicity study was conducted with *E. coli*-produced CP4 EPSPS protein (Harrison et al., 1996). The *E. coli*-produced CP4 EPSPS protein has been shown to be equivalent to the plant-produced CP4 EPSPS present in MON 88017 (Section 1, Part VI). Acute administration was considered appropriate to assess the potential toxicity of CP4 EPSPS protein because toxic proteins generally act via acute mechanisms (Sjobad et al., 1992; Pariza and Foster, 1983). The no observed effect level (NOEL) for oral toxicity of CP4 EPSPS in mice was 572 mg/kg, the highest dose tested (Harrison et al., 1996). There were no significant differences in body weight, cumulative body weight, or food consumption between the vehicle or bovine serum albumin protein control groups and the CP4 EPSPS-treated groups.

5.1.c. Safety of the donor organism – *Agrobacterium sp.* strain CP4

Agrobacterium sp. strain CP4 was chosen as the donor organism because this bacterium exhibited tolerance to glyphosate by producing a naturally occurring glyphosate-tolerant EPSPS (Padgette et al., 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are not known human or animal pathogens and are not commonly allergenic (FAO/WHO, 1991). Furthermore, according to FAO/WHO (2001), there is no known population of individuals sensitized to bacterial proteins.

Agrobacterium sp. strain CP4 has been reviewed previously as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA

regarding Roundup Ready soybean (*Glycine max*) (1994), canola (*Brassica napus*) (1995), cotton (*Gossypium hirsutum*) (1995), NK603 corn (*Zea mays*) (2000), and sugarbeet line 77 (*Beta vulgaris*) (1998).

5.1.d. Similarity of CP4 EPSPS to EPSPSs derived from food sources with a long history of safe consumption

The CP4 EPSPS protein present in MON 88017 is substantially similar to EPSPSs consumed in a variety of food and feed sources. As shown in Table 10, the CP4 EPSPS protein is homologous to EPSPSs naturally present in food crops (e.g., soybean and corn) and in microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*), which have a history of safe human consumption (Padgette et al., 1996; Harrison et al., 1996). The similarity of the CP4 EPSPS protein to EPSPSs in a variety of foods and extensive human and animal consumption of the family of EPSPS proteins support the lack of health concerns.

The ubiquitous presence of homologous EPSPS enzymes in plant material used as food and feed establishes that EPSPS proteins and their enzyme activity pose no hazards for humans and animals. Furthermore, the EPA has established an exemption from the requirement of a tolerance for residues of CP4 EPSPS and the genetic material necessary for its production in all plants (40 CFR §180.1174).

Table 10. Comparison of the deduced amino acid sequence of native CP4 EPSPS to that of other EPSPSs

	Soybean (food/ feed)	Corn (food/ feed)	*E. coli* (present in human gut)	*S. cerevisiae* (Baker's yeast)
CP4 EPSPS				
% sequence identity	26	24	26	30
% sequence similarity	51	49	52	54

5.1.e. Presence of CP4 EPSPS protein in commercial food and feed crops

Herbicide-tolerant crops, primarily those with the Roundup Ready trait, were planted on 49.7 million hectares globally in 2003 (James, 2003b). Roundup Ready soybean is a significant example of the growth of these crops since its introduction in 1996. In the first year of introduction, Roundup Ready soybean was planted on 400,000 hectares in the U.S. (1% of the U.S. soybean area) and has grown to 55% of the 76 million hectares of soybean planted worldwide in 2003. Globally in 2003, Roundup Ready soybean containing the CP4 EPSPS protein was produced on approximately 41.4 million hectares, including almost 13 million hectares in Argentina and half a million hectares in Canada.

The amino acid sequence of the CP4 EPSPS protein produced in MON 88017 is identical to, or shares greater than 99% sequence identity with, the amino acid sequence of the CP4 EPSPS protein produced in a number of other Roundup Ready crops that have already completed the FDA consultation process and are produced commercially, including soybean, NK603 corn, cotton, and canola. As described above, humans and animals have consumed these crops and/or their processed products, since 1996 (James, 2003b). This demonstrates a history of safe consumption of the CP4 EPSPS proteins present in Roundup Ready crops.

5.2. Safety assessment of the Cry3Bb1 protein

5.2.a. Structural similarity of the Cry3Bb1 to known proteins and toxins

Using the same procedures as described above, potential structural similarities between the Cry3Bb1 protein and other proteins in the ALLPEPTIDES database were evaluated using the FASTA sequence alignment tool. Identified proteins were ranked according to their degree of similarity. The best similarity observed was to the *Bacillus thuringiensis* pesticidal crystal protein Cry3Bb (Accession No. Q06117), an isoform of the Cry3Bb1 protein, demonstrating an overlap of 651 aa, with 99.2% identity and an *E*-score of zero, which was not unexpected. The remaining alignments with significant *E*-scores were also Cry protein homologues. The Cry3Bb1 protein produced in MON 88017 is a member of the Cry3Bb class of protein variants that shares >99% amino acid sequence identity with the wild type Cry3Bb1 protein contained in the topically applied commercial microbial product *Raven* Oil Flowable Bioinsecticide.

Potential structural similarities shared between the Cry3Bb1 protein and proteins in the TOXIN5 database were also evaluated using the FASTA sequence alignment tool. Identified proteins were ranked according to their degree of similarity. The greatest similarity observed was to the *Bacillus thuringiensis* pesticidal crystal protein Cry3Bb (Accession No. Q06117), an isoform of the Cry3Bb1 protein. In this alignment, the overlap of 651 aa contained no gaps, showed 99.2% identity, and had an *E*-score of zero. This alignment is not surprising, because the Cry3Bb protein is listed within GenBank as an insecticidal toxin and as such was included in the TOXIN5 database during its construction.

Results of the FASTA sequence alignments demonstrated the lack of structurally relevant similarity between the Cry3Bb1 protein and any known toxic or pharmacologically active proteins relevant to human or animal health. These data demonstrate the lack of structurally relevant similarities between Cry3Bb1 and toxins or other pharmacologically active proteins that may adversely impact human or animal health.

5.2.b. Acute oral toxicity study with Cry3Bb1 protein

An acute oral toxicity assessment was conducted to evaluate potential adverse clinical signs or detrimental effects on mice from exposure to *E. coli*-produced Cry3Bb1 protein.

The results of this study were consistent with the study conducted for the Cry3Bb1 protein variant produced in YieldGard Rootworm, which completed the consultation process with FDA in 2002.

The Cry3Bb1 protein produced in *E. coli* was shown to be identical to the plant-produced Cry3Bb1 as expressed in MON 88017 (Part VI). Two groups of ten male and female CD1 mice received an acute high dose of the Cry3Bb1 protein by gavage. The target dose of 2,442 mg/kg was based on the maximum attainable Cry3Bb1 concentration of the dosing solution (estimated at 37 mg/mL) and a total dose volume of 66.6 mL/kg body weight. The limited solubility of theCry3Bb1 protein precluded its administration as a single dose. Therefore, dosing was subdivided into two doses of 33.3 mL/kg body weight (66.6 mL/kg total) to achieve the target dose of 2,442 mg/kg. On the day of dosing (Day 0) the two individual doses of 33.3 mg/mL body weight were separated by approximately four hours. A separate group of ten male and ten female protein control animals received bovine serum albumin (BSA) at a dose of 1900 mg/kg. Analysis of the dosing solutions revealed that the concentration of Cry3Bb1 protein was 79% of target and, therefore, the achieved dose was 1930mg/kg.

No significant differences were observed in food consumption during the study. No gross pathological findings related to consumption of Cry3Bb1 protein were observed at necropsy. Under the conditions of this test, no toxicity was observed in any of the groups. Therefore, the acute oral LD_{50} of *E. coli*-produced Cry3Bb1 protein in mice is greater than 1930 mg/kg body weight. This dose was determined to be the NOEL.

For comparison, the results of rodent acute oral toxicity tests conducted with other Cry3 proteins, including the Cry3Bb1 variant produced in YieldGard Rootworm corn (MON 863), are summarized in Table 11. In each acute test the highest achievable dose level failed to produce evidence of treatment-related adverse effects and was, therefore, considerate to be a no observable effect level (NOEL).

Table 11. Acute oral NOELs for various *B.t.* and plant-produced Cry3 proteins

Cry3 Protein	NOEL (mg/kg) [a]
Cry3Bb1 – MON 88017	≥ 1930
Cry3Bb1 – YieldGard Rootworm corn	≥ 3200
Cry3Bb1 – Wild type present in Raven Oil Flowable Bioinsecticide [b]	≥ 30
Cry3Aa4 - NewLeaf® potato	≥ 5000

a - NOEL in rodent acute gavage study. In all instances, the highest dose tested was the NOEL.
b - Raven contains a mixture of Cry3Bb1, Cry3Aa4 and CrylAc proteins. In the batch tested, Cry3 proteins constituted 40% (w/w) active ingredient; Cry3Bbl protein represents 66-75% of the Cry3 proteins present in Raven. The highest dose tested was 10^8 CFU/rat, which is approximately a 100 mg/kg body weight dose of total active ingredients.

5.2.c. Safety of the donor organism: *Bacillus thuringiensis*

The safety of *B.t.* as a donor organism has been previously reviewed in Monsanto consultations with FDA for genetically modified cotton, corn and potato products. *Bacillus thuringiensis* subspecies *kumamotoensis* is a spore-forming, gram-positive bacterium that is found naturally in soil. *B.t.* strains have been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity (EPA, 1988). Many strains of *B.t.* have been shown to produce protein crystals or inclusion bodies that are selectively toxic to certain orders and species of insect pests. The current nomenclature, based on amino acid identity, allows closely related proteins to be ranked together. Each protein is assigned a unique name incorporating four hierarchical ranks consisting (in order) of an Arabic numeral, upper case letter, lower case letter, and an Arabic numeral (e.g., Cry3Bb1 protein). Thus, proteins with identity of

a) <45% differ in a primary rank (e.g., Cry2, Cry3, etc.),
b) >45% but <78% differ in secondary rank (e.g., Cry3A, Cry3B),
c) >78% but <95% differ in tertiary rank (e.g., Cry3Ba, Cry3Bb), and
d) >95% differ in quaternary rank (e.g., Cry3Bb1, Cry3Bb2) and are considered allelic variants.

In general, the primary rank of the *B.t.* proteins denotes its specific insecticidal activity; for example, Cry1, Cry2, Cry3, and Cry4 proteins are toxic to Lepidopteran, Lepidopteran/Dipteran, Coleopteran, and Dipteran pests, respectively (Bravo, 1997; Höfte and Whitely, 1989).

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An exemption from the requirement of a tolerance for the first microbial *B.t.* product was granted in 1960 by the FDA after an extensive toxicity and infectivity evaluation program. The testing program consisted of acute, subchronic, and chronic studies, which resembled the testing required for conventional chemical pesticides. Registration was granted by the USDA later that same year. In 1971, EPA assumed responsibility for all pesticide tolerance exemptions. Since then, a variety of naturally occurring and genetically modified microbial *B.t.* products have been registered and included under this tolerance exemption. EPA has established separate tolerance exemptions by amendment for various Cry proteins (e.g., Cry1Ab, Cry1Ac, Cry2Ab2 and Cry3Aa) expressed in genetically-modified food crops (EPA 1995a, 1995b, 1995c, 1996, 1997 and 2001). The conclusion of reasonable certainty of no harm and the resultant tolerance exemptions for this wide array of *B.t.* mixtures and Cry proteins in food or feed were based on the lack of adverse effects to mammals in numerous toxicological studies. This conclusion is supported by a history of safe use in agriculture for over 40 years (McClintock et al., 1995). There are no adverse effects known to have occurred in humans during this prolonged period of use (EPA, 1998).

5.2.d. Human and animal safety of *B.t.* Cry proteins

FDA's statement of policy (FDA, 1992) and the proposed rule on Plant-Pesticides Subject to the Federal Insecticide, Fungicide and Rodenticide Act (*Federal Register* November 23, 1994) stipulate that EPA will exercise regulatory oversight of plant-expressed pesticidal proteins and the markers used in the selection of transformed plants. On May 11, 2001, a time-limited exemption from the requirement for a tolerance was established by EPA for the Cry3Bb1 protein and the genetic material necessary for its production in corn (EPA, 2001a[1]). As part of the decision to grant this tolerance exemption, EPA stated: "The lack of mammalian toxicity at high levels of exposure to the Cry3Bb1 ... proteins demonstrate the safety of the product at levels well above maximum exposure levels anticipated in the crop." The EPA further stated: "There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry3Bb1 ... proteins and the genetic material necessary for their production. This includes all anticipated dietary exposures and other exposures for which there is reliable information." Furthermore, no receptors for Cry proteins have been identified on intestinal cells of mammals (Noteborn, 1994; Sacchi et al., 1986; Van Mellaert et al., 1988).

5.3. Conclusions

Numerous factors have been considered in the safety assessment of the CP4 EPSPS and Cry3Bb1 proteins that are expressed in MON 88017. An assessment of safety of the CP4 EPSPS leads to the following conclusions, which are consistent with the conclusions reached for the CP4 EPSPS protein produced in a number of Roundup Ready crops:

[1] An application to amend the current time-limited tolerance exemption to a permanent tolerance is currently under review by the EPA. A notice of filing published in October 2003 (EPA 2003b).

a) The donor organism, *Agrobacterium sp*. strain CP4, is not known for human or animal pathogenicity and is not commonly allergenic. Additionally, *Agrobacterium sp*. strain CP4 and the CP4 EPSPS protein it produces have been reviewed previously as a part of the safety assessment for other Roundup Ready crops.
b) A history of the safe use of CP4 EPSPS protein has been demonstrated, based on the similarity of the CP4 EPSPS protein in MON 88017 to EPSPSs naturally present in food crops (e.g., soybean and corn) and in microbial food sources such as Baker's yeast (*Saccharomyces cerevisiae*), and to the CP4 EPSPS protein produced in a number of other Roundup Ready crops that have already completed the FDA consultation process, including soybean, NK603 corn, cotton and canola.
c) The CP4 EPSPS protein purified from *E. coli* was found to be physicochemically and functionally equivalent to the protein produced in MON 88017.
d) Large margins of exposure for CP4 EPSPS protein indicate that there is virtually no risk to human and animal health associated with dietary exposure to food and feed products derived from MON 88017.
e) Digestibility studies demonstrate that the CP4 EPSPS protein is rapidly degraded in simulated digestive fluids and would be unlikely to elicit potential toxic effects.
f) No biologically relevant structural similarities were observed between the CP4 EPSPS protein and pharmacologically active proteins that are known to cause adverse health effects in humans or animals.
g) Results from the acute oral toxicity study demonstrate that the CP4 EPSPS protein is not toxic and does not cause any adverse effects.

Similarly, analysis of the Cry3Bb1 protein leads to the following conclusions, which are consistent with the conclusions reached for the Cry3Bb1 protein produced in YieldGard Rootworm corn:

a) The donor organism, *Bacillus thuringiensis,* has been used commercially in the U.S. since 1958 to produce microbial-derived products with insecticidal activity. The extremely low mammalian toxicity of *B.t.*-based insecticide products has been demonstrated in numerous safety studies, and there are no confirmed cases of allergic reactions to Cry proteins in applicators of microbial-derived *B.t.* products during 40 years of use.
b) A history of safe use of Cry3Bb1 protein has been demonstrated, based on similarity to the Cry3Bb1 protein produced in YieldGard Rootworm corn (MON 863) and to the wild type Cry3Bb1 protein present in Raven bioinsecticide. The safety and nutritional assessment of the YieldGard Rootworm corn has been previously reviewed by FDA. The conclusion that "corn derived from these new varieties is not materially different in composition, safety and other relevant parameters from corn currently on the market and that genetically modified corn does not raise issues that would require pre-market review or approval by FDA" was reached on December 31, 2001.
c) The Cry3Bb1 protein purified from *E. coli* was found to be physicochemically and functionally equivalent to the protein produced in MON 88017.

d) Large margins of exposure for the Cry3Bb1 protein indicate that there is virtually no risk to human and animal health associated with dietary exposure to food and feed products derived from MON 88017.
e) Digestibility studies demonstrate that the Cry3Bb1 protein is rapidly degraded in simulated digestive fluids and would be unlikely to elicit potential toxic effects.
f) No biologically relevant structural similarities were observed between the Cry3Bb1 protein and pharmacologically active proteins that are known to cause adverse health effects in humans or animals.
g) Results from the acute oral toxicity study demonstrate that the Cry3Bb1 protein is not toxic and does not cause any adverse effects.

Using the guidance provided by the FDA, a conclusion of "no concern" is reached for the donor organisms and the CP4 EPSPS and Cry3Bb1 proteins. One of the decision trees from the FDA Food Policy (FDA, 1992) is reproduced in Figure 33 and identifies the considerations to be used in evaluating the safety of the donor organism. The information provided in this section and summarized above leads to a finding of "no concerns" for the source of the donor organism. The pathway leading to no concerns for MON 88017 is highlighted with bold arrows. Considerations used in evaluating the safety of the proteins introduced from the donor are identified in Figure 34, another decision tree reproduced from the FDA Food Policy (FDA, 1992). As with the donor, the information provided in this section and summarized above leads to a finding of "no concerns" for the CP4 EPSPS and Cry3Bb1 proteins in MON 88017. The pathway leading to no concerns for MON 88017 is highlighted with bold arrows. It is therefore concluded that the data and information provided in Section 5, and supported by other data and information in this Part VI, demonstrate that the CP4 EPSPS and Cry3Bb1 proteins in MON 88017 are safe for human and animal consumption.

Figure 33. Safety assessment of new varieties: the donor

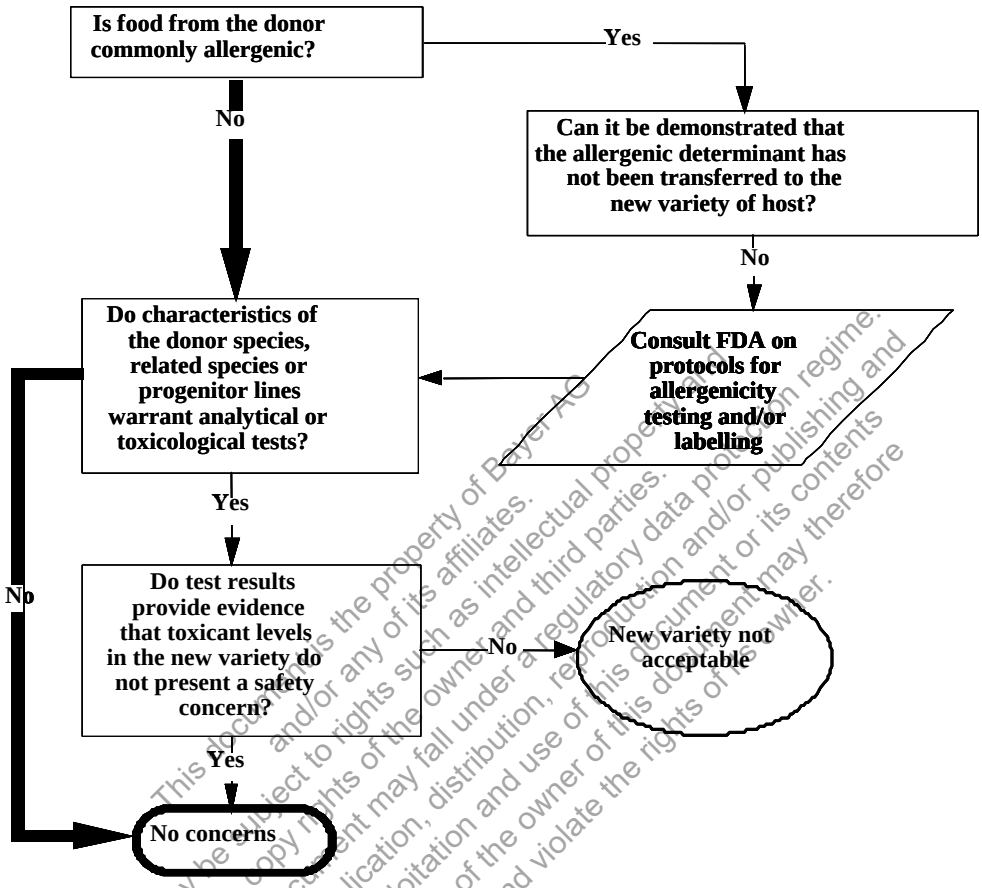
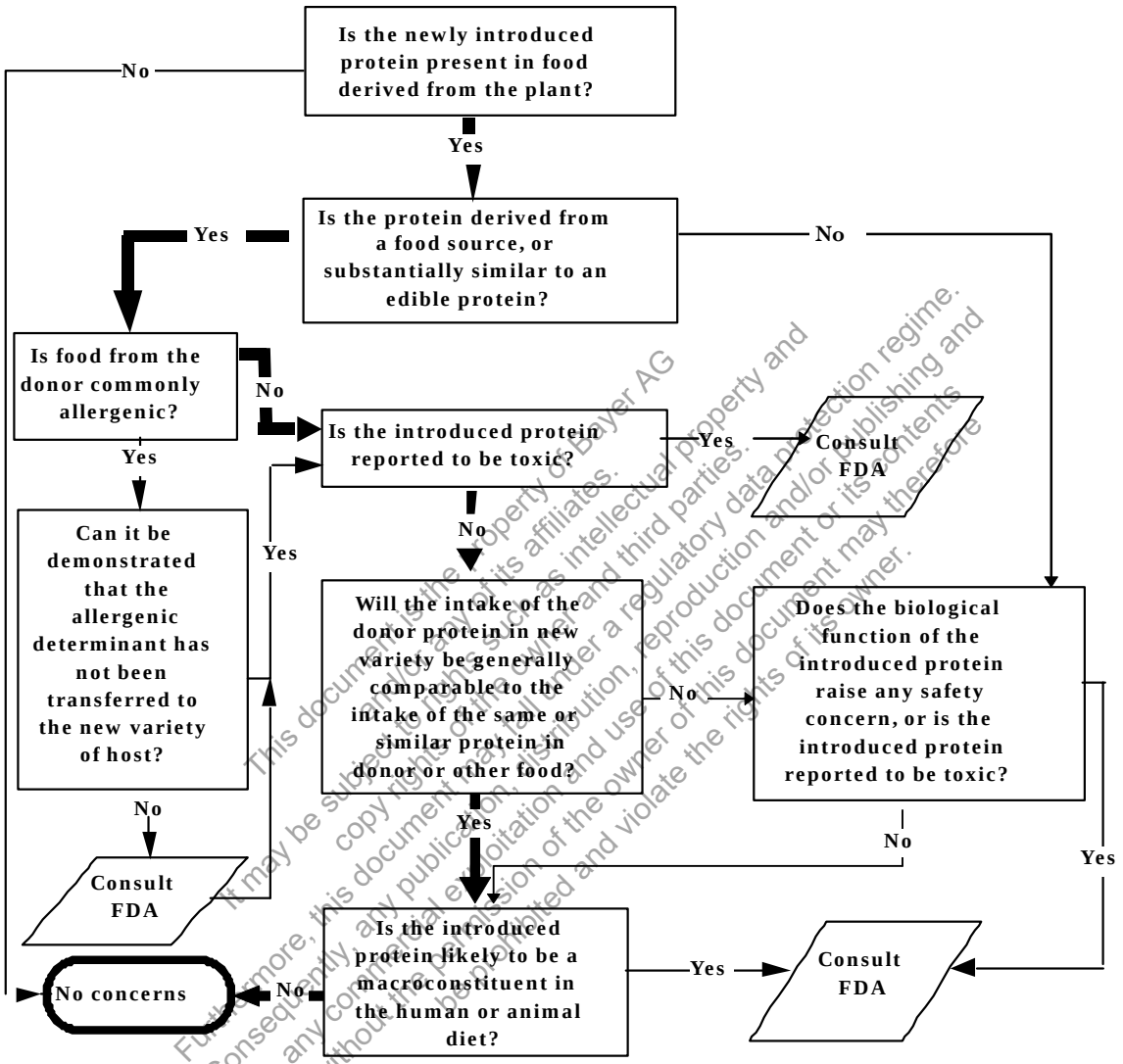


Figure 34. Safety assessment of new varieties: proteins introduced from donor.



PART VII. FOOD/FEED SAFETY AND NUTRITIONAL ASSESSMENT OF MON 88017 CORN

SECTION 1. Corn as the comparable food and feed

Corn, *Zea mays* L., the host plant, has been a staple of the human diet for centuries. Corn grain and its processed fractions are consumed in a multitude of food and animal feed products. Corn forage is extensively consumed as an animal feed by ruminants. Corn does not contain any known allergens or produce significant quantities of toxins or anti-nutritional factors warranting analytical or toxicological tests (Watson, 1982; White and Pollak, 1995).

Corn is a highly productive crop, yielding an average of 130 bushels per acre in the U.S. during 2002 (NCGA, 2003). Its high yield makes it one of the most economical sources of metabolizable energy for feeds, and of starch and sugar for food and industrial products. Approximately two-thirds of the corn produced in the U.S. is fed to livestock. Therefore, indirect consumption is much greater than direct consumption for humans.

SECTION 2. Historical uses of corn

Corn (*Zea mays* L.) originated in Mexico and was grown as a food crop as early as 2700 B.C. (Salvador, 1997). It is now grown on more than 296 million acres globally. The history of corn has been studied extensively and multiple hypotheses for its origin and parentage have been advanced (Mangelsdorf, 1974). The preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988). Corn is the largest crop grown in the U.S. in terms of acreage planted and net value. In 2002, its production covered 79.1 million acres that yielded 9 billion bushels and had a net value of $21.2 billion (NCGA, 2003).

2.1. History and utilization of corn

The history and development of corn has been discussed previously in Part IV, Section 1.3. Corn has been a staple of the human diet for centuries. Corn grain and its processed fractions are consumed in a multitude of food and animal feed products. Corn forage is extensively consumed as an animal feed by ruminants.

2.2. Corn as a food source

In spite of its great value as a source of energy, little whole kernel corn is consumed by humans when compared to corn-based food ingredients (Hodge, 1982; Watson, 1988). The low price and ready availability of processed corn products has resulted in the development of large volume food and industrial uses. Corn is an excellent raw material for the manufacture of starch (Anderson and Watson, 1982). Nearly one quarter of corn

starch produced is sold as starch products, whereas three quarters of the starch is converted to a variety of sweetener and fermentation products including high fructose corn syrup and ethanol (Watson, 1988; NCGA, 2003; Anderson and Watson, 1982; White and Pollack, 1995). Additionally, corn oil is commercially processed from the germ and accounts for approximately 9% of domestic vegetable oil production (Orthoefer and Sinram, 1987). Each of these materials is a component of many foods including bakery and dairy goods, beverages, confections, and meat products.

2.3. Corn as a feed source

Animal feeding represents the largest use of corn in the U.S. with approximately two-thirds of annual production being fed to cattle, chicken and swine (Hodge, 1982; Perry, 1988; Watson, 1988). Approximately 100 million metric tons of grain is fed to livestock directly as grain. Another 1.5 to 2 million metric tons of wet and dry milling by-products (primarily corn gluten meal and feed) are fed directly or in formulated feeds (Perry, 1988).

SECTION 3. Comparison of the composition and characteristics of MON 88017 to conventional corn

3.1. Levels of significant nutrients, anti-nutrients, and secondary metabolites

The composition of forage and grain produced by MON 88017 was evaluated and compared to a conventional control corn with similar genetic background, as well as other commercially available corn hybrids. MON 88017 and the conventional control corn were grown at three replicated field sites in major corn-growing areas of the U.S. (Iowa, Illinois and Nebraska) during the 2002 field season. Four commercially available corn hybrids were grown also at each of the same field sites to provide a total of 12 different reference substances. At each field site, the test, control and reference seed was planted in a randomized complete block design with three replicates per block. All the plants were grown under normal agronomic field conditions for their respective geographic regions. All test plots received an application of Roundup UltraMAX herbicide according to label directions. Bulk forage samples were harvested at the late dough/early dent stage, and bulk grain samples were harvested when the grain was mature. The materials and methods for the compositional analyses are described in the Appendix IV.

Compositional analyses of the forage samples included proximates (protein, fat, ash, and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), minerals (calcium, phosphorous), and carbohydrates by calculation. Compositional analyses of the grain samples included proximates (protein, fat, ash, and moisture), ADF, NDF, total dietary fiber (TDF), amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium, and zinc), vitamins (B_1, B_2, B_6, E, niacin, and folic acid), anti-nutrients (phytic acid and raffinose), secondary

metabolites (furfural, ferulic acid, and p-coumaric acid), and carbohydrates by calculation. In all, 77 different analytical components (nine in forage and 68 in grain) were analyzed. The following 15 compositional analytes with >50% of observations below the limit of quantitation (LOQ) of the assay were excluded from statistical analysis: sodium, 2-furaldehyde, 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma linolenic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, and 20:4 arachidonic acid. Therefore, 62 components were statistically assessed (nine in forage and 53 in grain) for the compositional equivalence of MON 88017.

Statistical analyses of the compositional data were conducted using a mixed model analysis of variance method. Four sets of analyses were made based on data from each of the three replicated field sites plus data from a combination of all three field sites. Statistical evaluation of the composition data involved comparison of the forage and grain from the test substance to the control. Statistically significant differences were determined at the 5% level of significance ($p<0.05$). There were 248 comparisons (four sets of analyses x 62 components assessed) conducted on the test material. Using the data for each component obtained from the 12 different commercial hybrids, a 99% tolerance interval was calculated to contain, with 95% confidence, 99% of the values contained in the population of commercial corn hybrids. For those comparisons in which the test was statistically different from the control, the test range was compared to the 99% tolerance interval in order to determine if the test range was within the interval and therefore considered to be part of the population of the commercial corn.

Results of the forage and grain sample analysis showed that there were no statistically significant differences ($p<0.05$) between MON 88017 and the conventional control for 232 of the 248 comparisons conducted (Tables 12-19). There were no statistically significant differences found in forage (Table 12). For grain, statistically significant differences were observed for the content of: 16:0 palmitic acid, 18:1 oleic acid, 18:3 linolenic acid, 20:0 arachidic acid, copper, methionine, moisture, niacin, and serine (one comparison each); 18:2 linoleic acid (three comparisons); and vitamin B_1 (four comparisons) (Table 19). Five percent, or approximately 12 (0.05×248) comparisons, were expected to be statistically significant based upon chance alone. Except for vitamin B_1, none of the statistically significant differences were in all four analyses (each individual site and the combination of all sites). The vitamin B_1 values in grain were statistically lower in MON 88017 compared to conventional corn for all four analyses, but they were similar to literature and historical values of vitamin B_1 in corn grain. All test values were also within the 99% tolerance interval for the 16 comparisons observed to be statistically different between MON 88017 and the conventional control. Therefore it is unlikely that these differences are biologically meaningful. The summary of the results is presented in Tables 12 through 19. Literature values and historic ranges for components of corn forage and grain are presented in Table 20. In addition, Tables 1-21 in Appendix IV provide the results of the analysis for each independent site. Supplemental summary information presented in Appendix IV provides calculations of fatty acid composition as percent of dry weight (Table 22), percent of total fat (Table 23),

and amino acid content calculated as percent of dry weight (Table 24) and percent of total protein (Table 25).

Based on these data, it is concluded that the forage and grain produced from MON 88017 are compositionally equivalent to the forage and grain produced from corn currently on the market.

Table 12. Comparison of proximates, fiber, and mineral content in forage from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ash (% dwt)	3.99 ± 0.24 (3.30 - 5.53)	4.04 ± 0.24 (3.59 - 4.67)	-0.051 ± 0.28 (-1.37 – 1.55)	-0.74,0.64	0.861	(2.62 - 6.78) [0.72,7.42]
Carbohydrates (% dwt)	86.19 ± 0.62 (83.54 - 87.88)	86.48 ± 0.62 (84.43 - 87.71)	-0.29 ± 0.40 (-2.58 – 1.73)	-1.11,0.54	0.478	(81.86 - 89.90) [78.70,93.43]
Fat, total (% dwt)	1.61 ± 0.29 (0.80 - 3.13)	1.65 ± 0.29 (0.83 - 2.97)	-0.039 ± 0.25 (-1.47 – 1.99)	-0.56,0.48	0.878	(0.69 - 2.92) [0.80,2.95]
Moisture (% fw)	70.86 ± 0.66 (68.50 - 72.70)	70.66 ± 0.66 (69.10 - 72.70)	0.20 ± 0.39 (-1.40 – 1.90)	-0.61,1.01	0.615	(65.20 - 78.60) [59.37,80.83]
Protein (% dwt)	8.20 ± 0.31 (7.44 - 8.97)	7.82 ± 0.31 (6.79 - 8.54)	0.38 ± 0.25 (-0.99 – 1.65)	-0.13,0.88	0.137	(6.31 - 9.96) [4.17,11.81]
ADF (% dwt)	26.54 ± 1.25 (24.29 - 29.97)	25.45 ± 1.25 (23.34 - 28.13)	1.10 ± 1.76 (-2.58 – 4.08)	-2.97,5.16	0.549	(19.16 - 35.55) [13.95,38.96]
NDF (% dwt)	37.34 ± 1.22 (33.44 - 45.05)	38.33 ± 1.22 (35.86 - 41.18)	-0.99 ± 1.42 (-4.63 – 6.97)	-3.90,1.91	0.490	(30.27 - 57.93) [23.80,54.73]
Calcium (% dwt)	0.22 ± 0.014 (0.19 - 0.26)	0.23 ± 0.014 (0.18 - 0.31)	-0.0092 ± 0.014 (-0.054 – 0.024)	-0.044,0.026	0.542	(0.13 - 0.32) [0.11,0.32]
Phosphorus (% dwt)	0.25 ± 0.011 (0.21 - 0.30)	0.25 ± 0.011 (0.20 - 0.30)	0.0017 ± 0.013 (-0.060 - 0.079)	-0.029,0.032	0.899	(0.16 - 0.31) [0.095,0.38]

[a]dwt=dry weight; ADF=acid detergent fiber; NDF=neutral detergent fiber; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval

[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 13. Comparison of the amino acid content in grain from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Alanine (% total AA)	7.55 ± 0.084 (7.29 - 7.70)	7.55 ± 0.084 (7.34 - 7.79)	-0.0026 ± 0.039 (-0.19 - 0.18)	-0.097,0.092	0.949	(7.24 - 8.16) [6.66,8.49]
Arginine (% total AA)	4.42 ± 0.11 (4.10 - 4.74)	4.29 ± 0.11 (4.01 - 4.63)	0.13 ± 0.060 (-0.12 - 0.36)	-0.013,0.28	0.066	(3.72 - 5.08) [3.34,5.67]
Aspartic acid (% total AA)	6.22 ± 0.050 (6.09 - 6.34)	6.25 ± 0.050 (6.04 - 6.45)	-0.032 ± 0.067 (-0.34 - 0.18)	-0.20,0.13	0.648	(6.18 - 6.81) [5.77,7.16]
Cystine (% total AA)	2.14 ± 0.054 (1.93 - 2.26)	2.15 ± 0.054 (1.93 - 2.30)	-0.013 ± 0.042 (-0.20 - 0.17)	-0.098,0.073	0.766	(1.82 - 2.58) [1.46,2.89]
Glutamic acid (% total AA)	20.40 ± 0.18 (19.80 - 20.87)	20.44 ± 0.18 (19.91 - 20.84)	-0.036 ± 0.086 (-0.52 - 0.48)	-0.25,0.17	0.686	(19.46 - 21.57) [18.01,22.15]
Glycine (% total AA)	3.45 ± 0.063 (3.32 - 3.62)	3.45 ± 0.063 (3.18 - 3.61)	0.0061 ± 0.031 (-0.081 - 0.19)	-0.058,0.070	0.844	(3.29 - 4.03) [2.81,4.54]
Histidine (% total AA)	2.99 ± 0.049 (2.90 - 3.10)	2.95 ± 0.049 (2.83 - 3.14)	0.032 ± 0.022 (-0.056 - 0.10)	-0.023,0.087	0.200	(2.50 - 3.12) [2.16,3.60]
Isoleucine (% total AA)	3.59 ± 0.037 (3.43 - 3.71)	3.57 ± 0.037 (3.45 - 3.76)	0.025 ± 0.044 (-0.15 - 0.25)	-0.065,0.11	0.577	(3.39 - 3.79) [3.30,3.84]
Leucine (% total AA)	13.28 ± 0.20 (12.69 - 13.62)	13.31 ± 0.20 (12.76 - 14.11)	-0.037 ± 0.098 (-0.69 - 0.56)	-0.28,0.20	0.717	(12.11 - 14.35) [10.72,15.18]

[a]AA=amino acids; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
[c]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 13 (cont). Comparison of the amino acid content in grain from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Lysine (% total AA)	2.69 ± 0.058 (2.42 - 2.87)	2.66 ± 0.058 (2.49 - 2.82)	0.024 ± 0.047 (-0.072 - 0.11)	-0.074,0.12	0.614	(2.44 - 3.27) [2.06,3.73]
Methionine (% total AA)	1.98 ± 0.059 (1.85 - 2.05)	2.01 ± 0.059 (1.83 - 2.20)	-0.030 ± 0.043 (-0.15 - 0.12)	-0.14,0.076	0.515	(1.70 - 2.47) [1.37,2.60]
Phenylalanine (% total AA)	5.18 ± 0.059 (4.97 - 5.31)	5.14 ± 0.059 (5.01 - 5.32)	0.035 ± 0.055 (-0.13 - 0.25)	-0.10,0.17	0.545	(4.82 - 5.39) [4.57,5.71]
Proline (% total AA)	9.39 ± 0.094 (9.02 - 9.69)	9.34 ± 0.094 (8.85 - 9.80)	0.046 ± 0.11 (-0.61 - 0.71)	-0.18,0.27	0.676	(8.35 - 9.72) [7.60,10.37]
Serine (% total AA)	4.83 ± 0.049 (4.65 - 5.04)	4.91 ± 0.049 (4.63 - 5.13)	-0.081 ± 0.068 (-0.47 - 0.42)	-0.22,0.059	0.244	(4.81 - 5.23) [4.60,5.43]
Threonine (% total AA)	3.22 ± 0.040 (3.10 - 3.38)	3.25 ± 0.040 (3.06 - 3.37)	-0.026 ± 0.045 (-0.25 - 0.24)	-0.12,0.067	0.572	(2.96 - 3.55) [2.89,3.84]
Tryptophan (% total AA)	0.54 ± 0.027 (0.48 - 0.60)	0.55 ± 0.027 (0.41 - 0.68)	-0.0090 ± 0.018 (-0.17 - 0.096)	-0.046,0.028	0.627	(0.44 - 0.83) [0.36,0.77]
Tyrosine (% total AA)	3.35 ± 0.16 (2.35 - 3.66)	3.43 ± 0.16 (2.58 - 3.66)	-0.079 ± 0.23 (-1.18 - 0.98)	-0.61,0.46	0.743	(2.26 - 3.80) [2.62,4.26]
Valine (% total AA)	4.79 ± 0.039 (4.60 - 4.92)	4.74 ± 0.039 (4.60 - 4.94)	0.043 ± 0.052 (-0.25 - 0.26)	-0.064,0.15	0.414	(4.44 - 5.04) [4.22,5.27]

[a]AA=amino acids; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval

[b] With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 14. Comparison of the fatty acid content in grain from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
16:0 palmitic (% total FA)	10.24 ± 0.43 (10.07 - 10.52)	11.27 ± 0.43 (10.14 - 14.57)	-1.03 ± 0.60 (-4.35 - 0.36)	-2.42,0.37	0.128	(9.29 - 17.81) [6.51,16.50]
16:1 pamitoleic (% total FA)	0.18 ± 0.010 (0.16 - 0.21)	0.18 ± 0.010 (0.16 - 0.22)	-0.0030 ± 0.0064 (-0.029 - 0.025)	-0.019,0.013	0.655	(0.054 - 0.21) [0.0017,0.28]
18:0 stearic (% total FA)	2.01 ± 0.073 (1.80 - 2.19)	2.07 ± 0.073 (1.76 - 2.23)	-0.052 ± 0.046 (-0.28 - 0.25)	-0.15,0.042	0.266	(1.68 - 2.30) [1.41,2.53]
18:1 oleic (% total FA)	22.74 ± 0.23 (22.20 - 23.53)	22.87 ± 0.23 (21.43 - 23.51)	-0.13 ± 0.24 (-0.94 - 1.13)	-0.71,0.46	0.613	(19.79 - 34.46) [9.25,44.14]
18:2 linoleic (% total FA)	62.85 ± 0.39 (61.86 - 63.72)	61.52 ± 0.39 (59.10 - 63.18)	1.34 ± 0.53 (-0.64 - 4.19)	0.093,2.58	0.038	(51.64 - 64.12) [41.22,74.09]
18:3 linolenic (% total FA)	1.21 ± 0.062 (1.15 - 1.26)	1.32 ± 0.062 (1.19 - 1.77)	-0.11 ± 0.077 (-0.53 - 0.043)	-0.30,0.079	0.205	(0.84 - 1.91) [0.42,1.95]
20:0 arachidic (% total FA)	0.37 ± 0.010 (0.35 - 0.39)	0.38 ± 0.010 (0.35 - 0.41)	-0.0085 ± 0.0032 (-0.028 - 0.0088)	-0.015,-0.0019	0.012	(0.36 - 0.45) [0.31,0.49]
20:1 eicosenoic (% total FA)	0.24 ± 0.0056 (0.23 - 0.26)	0.25 ± 0.0056 (0.24 - 0.26)	-0.0034 ± 0.0034 (-0.019 - 0.019)	-0.010,0.0036	0.323	(0.24 - 0.36) [0.18,0.40]
22:0 behenic (% total FA)	0.15 ± 0.0027 (0.14 - 0.16)	0.15 ± 0.0027 (0.14 - 0.17)	-0.0062 ± 0.0038 (-0.018 - 0.014)	-0.014,0.0016	0.116	(0.074 - 0.24) [0.071,0.25]

[a]FA=fatty acids; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 15. Comparison of the mineral content in grain from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Calcium (% dwt)	0.0054 ± 0.00035 (0.0047 - 0.0060)	0.0058 ± 0.00035 (0.0049 - 0.0069)	-0.00040 ± 0.00025 (-0.0013 - -0.00006)	-0.0010,0.00021	0.159	(0.0032 - 0.0060) [0.0017,0.0062]
Copper (mg/kg dwt)	1.73 ± 0.086 (1.48 - 2.05)	1.99 ± 0.086 (1.64 - 2.63)	-0.26 ± 0.12 (-0.95 - 0.41)	-0.54,0.016	0.061	(1.01 - 2.34) [0.17,3.00]
Iron (mg/kg dwt)	21.51 ± 0.59 (20.07 - 22.92)	21.84 ± 0.59 (20.31 - 23.93)	-0.33 ± 0.62 (-2.16 - 2.12)	-1.60,0.93	0.595	(16.42 - 26.03) [12.60,31.26]
Magnesium (% dwt)	0.14 ± 0.0034 (0.13 - 0.15)	0.14 ± 0.0034 (0.13 - 0.16)	-0.0022 ± 0.0044 (-0.024 - 0.018)	-0.011,0.0069	0.618	(0.10 - 0.14) [0.088,0.16]
Manganese (mg/kg dwt)	9.72 ± 0.38 (9.01 - 10.76)	9.37 ± 0.38 (7.55 - 10.44)	0.35 ± 0.38 (-0.39 - 1.56)	-0.57,1.27	0.384	(4.96 - 9.81) [2.45,10.60]
Phosphorus (% dwt)	0.39 ± 0.010 (0.37 - 0.41)	0.39 ± 0.010 (0.36 - 0.43)	-0.0042 ± 0.013 (-0.052 - 0.042)	-0.032,0.023	0.754	(0.28 - 0.41) [0.24,0.44]
Potassium (% dwt)	0.41 ± 0.012 (0.39 - 0.44)	0.42 ± 0.012 (0.38 - 0.47)	-0.0063 ± 0.012 (-0.052 - 0.037)	-0.030,0.018	0.592	(0.29 - 0.43) [0.27,0.48]
Zinc (mg/kg dwt)	24.53 ± 0.98 (22.31 - 27.27)	24.92 ± 0.98 (22.02 - 27.18)	-0.39 ± 0.62 (-3.87 - 1.90)	-1.67,0.89	0.534	(17.15 - 26.18) [13.42,31.37]

[a]dwt=dry weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 16. Comparison of the proximates and fiber content in grain from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ash (% dwt)	1.54 ± 0.077 (1.31 - 1.68)	1.59 ± 0.077 (1.23 - 1.97)	-0.049 ± 0.087 (-0.45 - 0.43)	-0.23,0.13	0.573	(1.04 - 1.86) [0.94,1.73]
Carbohydrates (% dwt)	82.32 ± 0.40 (81.61 - 83.39)	82.33 ± 0.40 (80.67 - 83.62)	-0.019 ± 0.25 (-1.39 - 0.94)	-0.62,0.58	0.940	(81.46 - 86.68) [79.39,89.67]
Fat, total (% dwt)	3.64 ± 0.13 (3.44 - 3.96)	3.79 ± 0.13 (3.53 - 4.36)	-0.16 ± 0.080 (-0.63 - 0.15)	-0.35,0.041	0.100	(2.38 - 4.43) [0.74,6.01]
Moisture (% fw)	11.10 ± 0.99 (9.03 - 13.20)	11.60 ± 0.99 (9.73 - 14.20)	-0.49 ± 0.35 (-1.10 - -0.10)	-1.36,0.37	0.212	(9.15 - 14.90) [4.67,17.56]
Protein (% dwt)	12.51 ± 0.35 (11.63 - 13.00)	12.28 ± 0.35 (11.22 - 13.82)	0.23 ± 0.24 (-0.82 - 1.37)	-0.36,0.82	0.379	(9.26 - 13.37) [6.20,15.35]
ADF (% dwt)	3.77 ± 0.16 (3.31 - 4.40)	3.54 ± 0.16 (2.97 - 4.69)	0.23 ± 0.18 (-0.62 - 1.16)	-0.13,0.59	0.203	(2.39 - 4.89) [1.89,5.23]
NDF (% dwt)	12.44 ± 0.62 (10.99 - 13.58)	11.87 ± 0.62 (10.38 - 14.29)	0.57 ± 0.50 (-1.21 - 2.64)	-0.66,1.79	0.299	(8.41 - 16.54) [3.51,21.65]
TDF (% dwt)	16.24 ± 0.71 (13.57 - 18.64)	15.40 ± 0.71 (13.18 - 17.84)	0.84 ± 0.96 (-2.39 - 4.19)	-1.51,3.20	0.414	(11.80 - 23.04) [5.72,27.10]

[a]ADF=acid detergent fiber; NDF=neutral detergent fiber; TDF=total dietary fiber; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval

[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 17. Comparison of the vitamin content in grain from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Folic acid (mg/kg dwt)	0.48 ± 0.021 (0.38 - 0.60)	0.48 ± 0.021 (0.42 - 0.59)	0.0012 ± 0.030 (-0.074 - 0.11)	-0.072,0.075	0.969	(0.28 - 0.61) [0.12,0.77]
Niacin (mg/kg dwt)	20.94 ± 1.20 (17.04 - 24.14)	21.75 ± 1.20 (19.08 - 23.92)	-0.81 ± 0.42 (-2.04 - 0.23)	-1.67,0.050	0.063	(14.11 - 27.77) [3.19,34.49]
Vitamin B_1 (mg/kg dwt)	2.47 ± 0.14 (2.30 - 2.69)	3.24 ± 0.14 (2.99 - 3.60)	-0.77 ± 0.12 (-1.02 - -0.35)	-1.06,-0.48	<0.001	(2.69 - 3.73) [1.96,4.38]
Vitamin B_2 (mg/kg dwt)	1.10 ± 0.041 (0.98 - 1.22)	1.13 ± 0.041 (0.99 - 1.33)	-0.025 ± 0.037 (-0.17 - 0.14)	-0.12,0.066	0.524	(0.88 - 1.32) [0.67,1.51]
Vitamin B_6 (mg/kg dwt)	7.16 ± 0.22 (6.57 - 8.06)	7.10 ± 0.22 (5.65 - 8.54)	0.063 ± 0.28 (-1.27 - 2.40)	-0.59,0.72	0.828	(4.93 - 7.24) [4.29,7.84]
Vitamin E (mg/kg dwt)	14.15 ± 1.70 (6.08 - 16.93)	14.07 ± 1.70 (1.74 - 17.77)	0.070 ± 1.46 (-11.15 - 14.39)	-2.93,3.07	0.962	(8.09 - 21.97) [0,29.69]

[a]dwt=dry weight; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval

[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 18. Comparison of the secondary metabolites and anti-nutrients content in grain from MON 88017 and conventional corn for combined field sites

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% CI (Lower, upper)	p-Value	
Ferulic acid (µg/g dwt)	2175.34 ± 46.31 (1986.75 - 2275.48)	2121.05 ± 46.31 (1927.55 - 2339.71)	54.29 ± 49.66 (-200.92 - 347.92)	-47,14,155.72	0.283	(1717.17 - 2687.57) [1415.19,3173.90]
p-Coumaric acid (µg/g dwt)	169.26 ± 7.26 (148.45 - 215.25)	154.83 ± 7.26 (141.41 - 173.24)	14.43 ± 9.88 (-14.72 - 72.55)	-9.75,38.61	0.194	(152.30 - 319.15) [43.13,384.34]
Phytic acid (% dwt)	0.95 ± 0.043 (0.83 - 1.05)	0.89 ± 0.043 (0.72 - 1.03)	0.058 ± 0.056 (-0.15 - 0.24)	-0.058,0.17	0.309	(0.45 - 1.00) [0.28,1.12]
Raffinose (% dwt)	0.17 ± 0.013 (0.14 - 0.20)	0.17 ± 0.013 (0.14 - 0.23)	0.00080 ± 0.0081 (-0.035 - 0.036)	-0.019,0.021	0.924	(0.073 - 0.22) [0,0.32]

[a]dwt=dry weight; S.E.= standard error of the mean; C.I.= confidence interval; T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 19. Summary of statistically significant differences in composition of MON 88017 and conventional corn

Tissue/Site/ Component (Units)[a]	Mean MON 88017	Mean Control	Mean Diff. (% of Control Value)	Signif. (p-value	MON 88017 (Range)	99% Tolerance Interval[b]
Grain						
IA						
16:0 palmitic (% total FA)	10.16	12.94	-21.50	0.029	(10.11-10.23)	[6.51, 16.50]
18:2 linoleic (% total FA)	63.25	60.41	4.70	0.017	(62.73-63.72)	[41.22, 74.09]
18:3 linolenic (% total FA)	1.25	1.57	-20.26	0.036	(1.24-1.26)	[0.42, 1.95]
Methionine (% total AA)	2.02	2.16	-6.39	<0.001	(1.96-2.05)	[1.37, 2.60]
Moisture (% fw)	9.38	9.93	-5.54	0.034	(9.03-9.70)	[4.67, 17.56]
Vitamin B_1 (mg/kg dwt)	2.54	3.07	-17.37	<0.001	(2.42-2.65)	[1.96, 4.38]
IL						
18:1 oleic (% total FA)	22.53	23.29	-3.26	<0.001	(22.50-22.56)	[9.25, 44.14]
18:2 linoleic (% total FA)	63.11	62.15	1.55	0.003	(62.84-63.29)	[41.22, 74.09]
Niacin (mg/kg dwt)	21.10	22.52	-6.30	0.014	(20.39-21.52)	[3.19, 34.49]
Vitamin B_1 (mg/kg dwt)	2.30	3.10	-25.63	<0.001	(2.30-2.30)	[1.96, 4.38]
NE						
Copper (mg/kg dwt)	1.57	2.21	-28.80	0.023	(1.48-1.68)	[0.17, 3.00]
Serine (% total AA)	4.80	4.97	-3.37	0.042	(4.80-4.81)	[4.60, 5.43]
Vitamin B_1 (mg/kg dwt)	2.58	3.56	-27.53	<0.001	(2.47-2.69)	[1.96, 4.38]

[a]dwt=dry weight; fw=fresh weight; AA=amino acids; FA=fatty acids;
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 19 (cont). Summary of statistically significant differences between MON 88017 and conventional corn

Tissue/Site/ Component (Units)[a]	Mean MON 88017	Mean Control	Mean Diff. (% of Control Value)	Signif. (p-value)	MON 88017 (Range)	99% Tolerance Interval[b]
Grain						
Combination of all sites						
18:2 linoleic (% total FA)	62.85	61.52	2.17	0.038	(61.86-63.72)	[41.22, 74.09]
20:0 arachidic (% total FA)	0.37	0.38	-2.24	0.012	(0.35-0.39)	[0.31, 0.49]
Vitamin B_1 (mg/kg dwt)	2.47	3.24	-23.72	<0.001	(2.30-2.69)	[1.96, 4.38]

[a]dwt=dry weight; fw=fresh weight; AA=amino acids; FA=fatty acids;
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 20. Literature and historical ranges of components of corn forage and grain

Tissue/Component[1]	Literature Range[2]	Historical Range[3]
Forage		
Proximates (% dwt)		
Ash	2.43-9.64[a]; 2-6.6[b]	2.03-8.23
Carbohydrates	83.2-91.6[b]; 76.5-87.3[a]	80.6-90.8
Fat, total	0.35-3.62[b]; 1.42-4.57[a]	0.61-4.02
Moisture (% fw)	56.5-80.4[a];55.3-75.3[b]	42-78.8
Protein	4.98-11.56[a]	3.86-11.0
Fiber (% dwt)		
Acid detergent fiber (ADF)	18.3-41.0[b]; 17.5-38.3[a]	17.6-36.7
Neutral detergent fiber (NDF)	26.4-54.5[b]; 27.9-54.8[a]	29.6-55.2
Minerals (% dwt)		
Calcium	0.0969-0.3184[b]	0.0866-0.2754
Phosphorous	0.1367-0.2914[b]	0.1602-0.2914
Grain		
Proximates (% dwt)		
Ash	1.1-3.9[d]; 0.89-6.28[b]	0.81-3.09
Carbohydrates	77.4-87.2[b]; 82.2-88.1[a]	79.8-89.6
Fat, total	3.1-5.7[d]; 2.48-4.81[b]	1.74-4.83
Moisture (% fw)	7-23[d]; 8.18-26.2[b]	6.07-24.7
Protein	6-12[d]; 9.7-16.1[c]	6.15-14.8
Fiber (% dwt)		
Acid detergent fiber (ADF)	3.3-4.3[d]; 2.46-11.34[a,b]	2.3-9.33
Neutral detergent fiber (NDF)	8.3-11.9[d]; 7.58-15.91[b]	6.88-18.1
Total dietary fiber (TDF)	10.99-11.41[h]	-

[1]fw=fresh weight; dw=dry weight; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine

[2]Literature range references: [a]Ridley et al., 2002. [b]Sidhu et al., 2000. [c]Jugenheimer, 1976. [d]Watson, 1987. [e]Watson, 1982. [f]Classen et al., 1990. [g]Dowd and Vega, 1996. [h]Choi et al., 1999.

[3] Historical range is from control samples analyzed in previous Monsanto Company studies.

Conversions: % dw x 10^4 = µg/g dw; mg/g dw x 10^3 = mg/kg dw; mg/100g dw x 10 = mg/kg dw

Table 20 (cont). Literature and historical ranges of components of corn forage and grain

Tissue/Component[1]	Literature Range[2]	Historical Range[3]
<u>Grain</u>		
Minerals		
Calcium (% dwt)	0.01-0.1[d]	0.0024-0.0089
Copper (mg/kg dwt)	0.9-10[d]	0.98-3.43
Iron (mg/kg dwt)	1-100[d]	10.4-30.7
Magnesium (% dwt)	0.09-1[d]	0.082-0.16
Manganese (mg/kg dwt)	0.7-54[d]	3.2-9.89
Phosphorous (% dwt)	0.26-0.75[d]	0.24-0.46
Potassium (% dwt)	0.32-0.72[d]	0.29-0.53
Zinc (mg/kg dwt)	12-30[d]	14.1-37.2
Amino Acids	**(% total protein)**	**(% total amino acid)**
Alanine	6.4-9.9[e]	7.06-8.19
Arginine	2.9-5.9[e]	3.49-5.48
Aspartic acid	5.8-7.2[e]	5.97-7.36
Cystine	1.2-1.6[e]	1.61-2.94
Glutamic acid	12.4-19.6[e]	17.3-20.4
Glycine	2.6-4.7[e]	3.22-4.91
Histidine	2.0-2.8[e]	2.46-3.35
Isoleucine	2.6-4.0[e]	2.95-4.08
Leucine	7.8-15.2[e]	11.2-14.6
Lysine	2.0-3.8[e]	2.35-4.18
Methionine	1.0-2.1[e]	1.61-2.89
Phenylalanine	2.9-5.7[e]	4.6-5.76
Proline	6.6-10.3[e]	8.03-9.9
Serine	4.2-5.5[e]	3.45-5.63
Threonine	2.9-3.9[e]	2.87-4.01
Tryptophan	0.5-1.2[e]	0.39-1.04
Tyrosine	2.9-4.7[e]	1.93-4.32
Valine	2.1-5.2[e]	3.94-5.46

[1]fw=fresh weight; dw=dry weight; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine
[2]Literature range references: [a]Ridley et al., 2002. [b]Sidhu et al., 2000. [c]Jugenheimer, 1976. [d]Watson, 1987. [e]Watson, 1982. [f]Classen et al., 1990. [g]Dowd and Vega, 1996. [h]Choi et al., 1999.
[3] Historical range is from control samples analyzed in previous Monsanto Company studies.

Conversions: % dw x 10^4 = µg/g dw; mg/g dw x 10^3 = mg/kg dw; mg/100g dw x 10 = mg/kg dw

Table 20 (cont). Literature and historical ranges of components of corn forage and grain

Tissue/Component[1]	Literature Range[2]	Historical Range[3]
Grain		
Fatty Acids	**(% total fat)**	**(% total fatty acid)**
16:0 Palmitic	7-19[e]	8.41-12.5
16:1 Palmitoleic	1[e]	0.05-0.18
18:0 Stearic	1-3[e]	1.33-2.61
18:1 Oleic	20-46[e]	20.1-37.7
18:2 Linoleic	35-70[e]	48.0-66.1
18:3 Linolenic	0.8-2[e]	0.74-1.45
20:0 Arachidic	0.1-2[e]	0.31-0.56
20:1 Eicosenoic	-	0.15-0.44
22:0 Behenic	-	0.075-0.3
Vitamins	**(mg/kg dwt)**	
Folic acid	0.3[d]	0.33-0.75 µg/g dwt
Niacin	9.3-70[d]	-
Vitamin B1	3-8.6[e]	0.2-0.33 mg/100g dwt
Vitamin B2	0.25-5.6[e]	0.83-1.74 µg/g dwt
Vitamin B6	5.3[d]; 9.6[e]	-
Vitamin E	3-12.1[e]; 17-47[d]	0.005-0.037 mg/g dwt

[1]fw=fresh weight; dw=dry weight; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine

[2]Literature range references: [a]Ridley et al., 2002. [b]Sidhu et al., 2000. [c]Jugenheimer, 1976. [d]Watson, 1987. [e]Watson, 1982. [f]Classen et al., 1990. [g]Dowd and Vega, 1996. [h]Choi et al., 1999.

[3] Historical range is from control samples analyzed in previous Monsanto Company studies.

Conversions: % dw x 10^4 = µg/g dw; mg/g dw x 10^3 = mg/kg dw; mg/100g dw x 10 = mg/kg dw

3.2. Levels of naturally occurring toxicants and anti-nutrients

According to OECD, "in considering the anti-nutrients and natural toxins in maize, only phytic acid is significant to the animal feed" (OECD, 2002). Levels of anti-nutrients (phytic acid and raffinose) and secondary metabolites (ferulic acid and p-coumaric acid) were measured in grain of MON 88017 and compared to conventional corn (Table 18). No statistically significant differences were observed. Therefore, it is concluded that the levels of anti-nutrients and secondary metabolites in MON 88017 are comparable to those found in conventional corn.

3.3. Any intended changes to the composition of food and feed

There have been no intended changes to the composition of food and feed derived from MON 88017 compared to conventional corn. Introduction of the *cp4 epsps* and *cry3Bb1* coding sequences and the expressed CP4 EPSPS and Cry3Bb1 proteins did not alter the compositional profile of MON 88017 compared to conventional corn. No changes other than intended traits that confer tolerance to Roundup agricultural herbicides and corn rootworm protection have been observed.

SECTION 4. Other information relevant to the safety and nutritional assessment of MON 88017

The long history of safe consumption of corn as food and feed, combined with the demonstration of the compositional equivalence of MON 88017 to conventional corn, is considered sufficient to support the safety and nutritional assessment of MON 88017.

SECTION 5. Substantial equivalence of MON 88017 to conventional corn

The data and information presented in Part VII, Section 3 establish that MON 88017 corn and the food and feed derived from it are compositionally equivalent to conventional corn and the comparable foods/feeds derived from it. Further, the compositional data support the conclusions derived from the agronomic/phenotypic studies presented to USDA-APHIS where no biologically meaningful changes were associated with MON 88017, including insect and disease susceptibility, growth, reproduction, and seed dormancy, and insect and disease susceptibility.

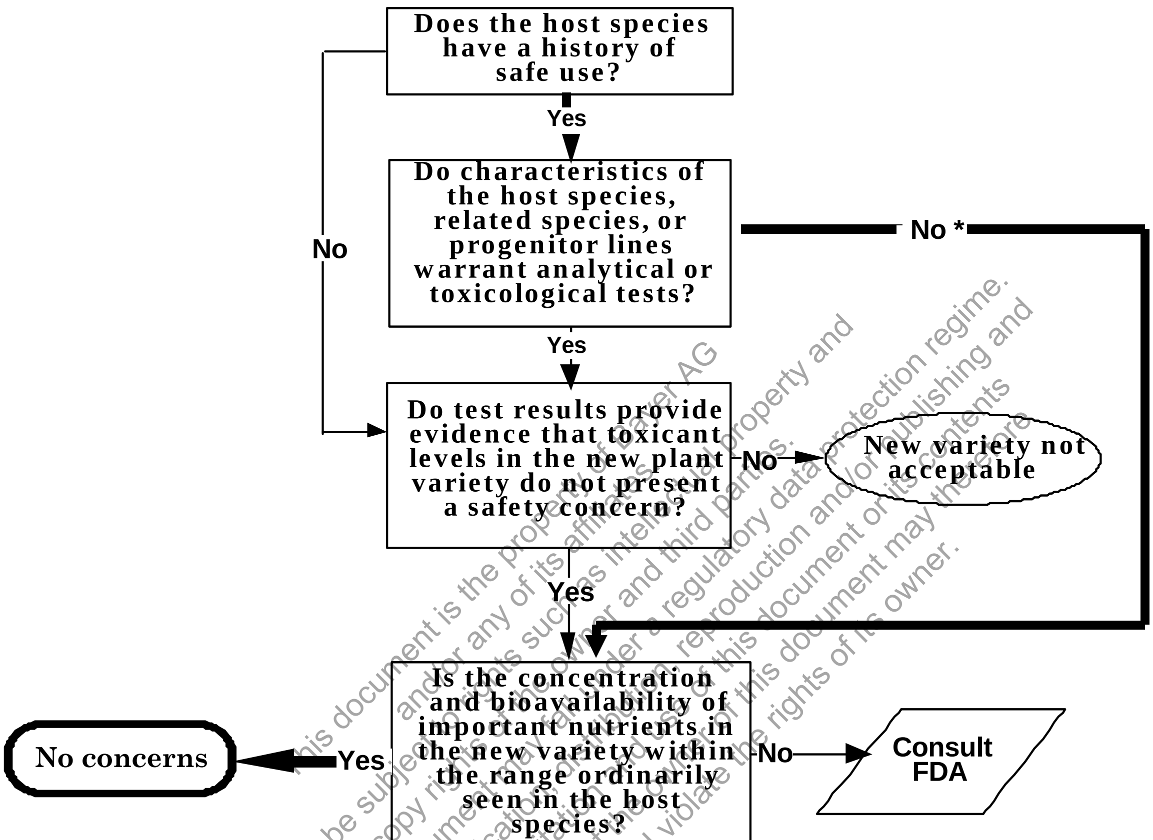
A detailed nutritional assessment of forage and grain from MON 88017 statistically compared the levels of 62 components (nine in forage and 53 in grain) to the conventional control and reference corn. Results of the forage and grain analysis showed that there were no statistically significant differences ($p<0.05$) between MON 88017 and the conventional control for 232 of the 248 comparisons conducted. There were no statistically significant differences found in forage. For grain, statistically significant differences were observed for 16 comparisons between MON 88017 and conventional

corn. However, all of the test values were within the 99% tolerance interval. Therefore it is unlikely that these differences are biologically meaningful. It is concluded, based on these data, that the forage and grain produced from MON 88017 are compositionally equivalent to the forage and grain produced from conventional corn currently on the market.

Conclusions

Collectively, these data and a history of safe use of the host organism, corn, as a common source of human food and animal feed, support a conclusion of "no concerns" for every criterion specified in the flowcharts outlined in the FDA's Food Policy document (Figure 35). Corn MON 88017 is not materially different in composition, safety or agronomic characteristics from conventional corn other than its tolerance to Roundup agricultural herbicides and protection from corn rootworm feeding damage, the intended traits. Sales and consumption of corn grain and processed products derived from MON 88017 would be fully consistent with the FDA's Food Policy, the Federal Food, Drug and Cosmetic Act, and current practices for the development and introduction of new corn varieties.

Figure 35. Safety assessment of new varieties: the host plant



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APPENDIX I

Materials and Methods for Molecular Analysis of MON 88017

Molecular analysis was performed using genomic DNA isolated from MON 88017 in order to characterize the integrated DNA. MON 88017 genomic DNA was analyzed by Southern blot analysis for the number of integration sites, the number of copies of the integrated DNA at each locus, the integrity of the inserted gene cassettes, the presence or absence of plasmid backbone sequence and to determine the stability of the introduced DNA across multiple generations. Additionally, PCR amplification and DNA sequencing were used to confirm the 5’ and 3’ insert-to-plant junctions, confirm the organization of the elements within the insert, and determine the complete DNA sequence of the integrated DNA.

A. Test Substance

The test substance was MON 88017. Genomic DNA was isolated from grain of MON 88017 generation [LH198BC0F$_1$ x LH59] F$_2$ harvested in production plan 00-01-39-05 generated from seed lot TRI-0005-10343-I. Additional test substances from the MON 88017 breeding tree (Figure 15) were used in the insert stability analysis. For this analysis, DNA was isolated from the following seed lots: GLP-0203-12151-S, GLP-0203-12152-S, GLP-0203-12153-S, GLP-0203-12154-S, GLP-0203-12155-S, and GLP-0203-12156-S.

B. Control Substance

The control substance was a conventional corn with similar genetic background as the test substance. DNA was isolated from grain harvested in production plan 00-01-39-05 generated from seed lot TPC-0005-10351-I. An additional control substance, the conventional corn LH198, was used in the insert stability analysis. For this analysis, DNA was isolated from seed lot GLP-0203-12165-S.

C. Reference Substances

The primary reference substance was plasmid PV-ZMIR39 that was used in the transformation process to produce MON 88017. For Southern blot analyses of corn genomic DNA, digested plasmid PV-ZMIR39 (approximately 0.5 and 1 genome copy equivalents) was mixed with digested DNA from the control substance and separated by electrophoresis on agarose gels. Additional reference standards included the 1 kb DNA Extension Ladder and 500 bp DNA Ladder from Life Technologies, which were used for size estimations on Southern blots and PCR agarose gels, respectively.

D. Characterization of Test, Control and Reference Substances

Event-specific PCR assays were used to confirm the identity of the test and control substances prior to use in the study. The stability of the DNA isolated from the test and control substances was determined in each Southern analysis by observation of the digested DNA sample on an ethidium bromide stained agarose gel. The identity of the test and control substances used in the insert stability analysis was confirmed by the molecular fingerprints generated from the Southern blot analyses.

E. Genomic DNA Isolation for Southern Blot Analyses

Genomic DNA from the test and control substances was extracted from corn grain by first processing the grain to a fine powder. Approximately 6 g of the processed grain were transferred to a 50 ml conical tube, then ~16 ml of CTAB extraction buffer [1.5% (w/v) CTAB, 75 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 1.05 M NaCl, and 0.75% (w/v) PVP (MW 40,000)] and 8 µl of RNase (10 mg/ml, Roche) were added to the processed grain. The samples were incubated at 65°C for 30-60 minutes with intermittent mixing and then allowed to cool to room temperature. Approximately 15 ml of chloroform:isoamyl alcohol (24:1 (v/v)) were added to the samples. The suspension was mixed for 5 minutes and the two phases separated by centrifugation at ~16,000 x *g* for 5 minutes at room temperature. The aqueous (upper) layer was transferred to a clean 50 ml conical tube. Approximately 1/10 volume (~1.5 ml) of 10% CTAB buffer [10% (w/v) CTAB and 0.7 M NaCl] and an equal volume of chloroform:isoamyl alcohol [24:1 (v/v)] were added to the aqueous phase, which was then mixed for 5 minutes. The samples were centrifuged at ~16,000 x *g* for 5 minutes to separate the phases. The aqueous (upper) layer was removed, mixed with an equal volume (~15 ml) of CTAB precipitation buffer [1% (w/v) CTAB, 50 mM Tris pH 8.0, and 10 mM EDTA pH 8.0] and allowed to stand at room temperature for 1-2 hours. The samples were centrifuged at ~10,000 x *g* for 10 minutes at room temperature to pellet the DNA. The supernatant was discarded, and the pellet was dissolved in approximately 2 ml of high salt TE buffer (10mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, and 1 M NaCl). Gentle swirling at 37°C was performed to aid in dissolution of the pellet. If necessary, samples were centrifuged at ~23,000 x *g* at room temperature for 2 minutes to pellet and remove debris. Approximately 1/10 volume (~150 µl) of 3 M NaOAc (pH 5.2) and 2 volumes (~4 ml relative to the supernatant) of chilled 100% ethanol were added to precipitate the DNA. The precipitated DNA was spooled into a microcentrifuge tube containing 70% ethanol. The DNA was pelleted in a microcentrifuge at maximum speed (~14,000 rpm) for ~5 minutes, vacuum-dried, and re-dissolved in TE buffer (pH 8.0). The DNA was then stored in a 4°C refrigerator.

F. Quantitation of Genomic DNA

Quantity of the DNA in the samples was determined by using a Hoefer DyNA Quant 200 Fluorometer with Roche molecular size marker IX as a DNA calibration standard.

G. Restriction Enzyme Digestion of Genomic DNA

Approximately 20 µg of genomic DNA from the test substance and 10 µg of genomic DNA from the control substance were used for restriction enzyme digestions. For the insert stability analysis, approximately 10 µg of genomic DNA from the test substance were used. Overnight digests were performed at 37°C in a total volume of ~500 µl using 100 units of the appropriate restriction enzyme. After digestion, the samples were precipitated by adding 1/10 volume (50 µl) of 3 M NaOAc (pH 5.2) and 2 volumes (1 ml relative to the original digest volume) of 100% ethanol, followed by incubation in a -20°C freezer for at least 30 minutes. The digested DNA was pelleted at maximum speed in a microcentrifuge, washed with 70% ethanol, dried, and re-dissolved in TE buffer.

H. DNA Probe Preparation for Southern Blot Analyses

DNA probe templates outlined in Figure 4a and 4b were prepared by PCR amplification of plasmid PV-ZMIR39. Approximately 25 ng of each probe template (except the NOS 3’ and tahsp17 3’ polyadenylation sequences) were labeled with ^{32}P-dCTP (6000 Ci/mmol) by a random priming method (RadPrime DNA Labeling System, Life Technologies). The NOS 3’ and tahsp17 3’ polyadenylation sequences were labeled by PCR using 25 ng of DNA probe template in the following manner: sense and antisense primers specific to the template (0.25 µM each); 1.5 mM $MgCl_2$; 3 µM each of dATP, dGTP and dTTP; ~100 µCi of ^{32}P-dCTP (6000 Ci/mmol); and 2.5 Units of *Taq* DNA polymerase in a final volume of 20 µl. The cycling conditions were as follows: 1 cycle at 94^{o}C for 3 minutes; 2 cycles at 94^{o}C for 45 seconds, 52^{o}C for 30 seconds, 72^{o}C for 2 minutes; and 1 cycle at 72^{o}C for 10 minutes. All radiolabeled probes were purified using a Sephadex G-50 column (Roche).

I. Southern Blot Analyses of Genomic DNA

Southern blot analyses were performed based on the method described by Southern (Southern, 1975). The samples of DNA digested with restriction enzymes were separated, based on size, using 0.8% agarose gel electrophoresis. A ‘long run’ and ‘short run’ were performed during this gel electrophoresis. The ~20 µg samples of digested test substance DNA were divided in half for loading ~10 µg on the long run and ~10 µg on the short run. The long run enabled greater separation of higher molecular weight DNAs while the short run allowed smaller molecular weight DNAs to be retained on the gel. The long run samples were loaded onto the gel and typically separated by electrophoresis for ~3-5 hours at 60-70 volts. The short run samples were then loaded in adjacent lanes on the same gel and separated by electrophoresis for ~14-17.5 additional hours at 30-35 volts. In some cases, the long run samples were loaded onto the gel and separated by electrophoresis for 15-17 hours at 30-35 volts. The short run samples in these cases were then loaded in adjacent lanes on the same gel and separated by electrophoresis for an additional 3.5-4.5 hours at 75-85 volts. For the insert stability analysis, a long run and short run were not performed. In this case, all samples were loaded onto the gel and separated by electrophoresis for 14.5 hours at 35 V and then for 3 hours at 60 V. After electrophoresis, the gels were stained in 1.5 µg/ml ethidium bromide for 10-15 minutes

and photographed. After photographing, the gels were placed in a depurination solution (0.125 N HCl) for 10-15 minutes followed by a denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30-40 minutes and then a neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) for 30-40 minutes. The gels were then placed in a 20X SSC buffer for 5-15 minutes. The DNA from the agarose gels was transferred to Hybond-N nylon membranes (Amersham) using a Turboblotter™ (Schleicher & Schuell). The DNA was allowed to transfer overnight (using 20X SSC as the transfer buffer) and covalently cross-linked to the membrane with a UV Stratalinker® 1800 (Stratagene) using the auto-crosslink setting. The blots were prehybridized for 0.5-7 hours at 60-65°C in an aqueous solution of 250 mM $Na_2HPO_4 \bullet 7H_2O$, 7% SDS, and 0.1 mg/ml tRNA. Hybridization with the radiolabeled probe was performed in fresh prehybridization solution for 15-28 hours at 60°C (NOS 3' and tahsp17 3' polyadenylation sequence probes) or 65°C (all other probes). Membranes were washed in an aqueous solution of 0.1% SDS and 0.1X SSC for four 15-20 minute periods at the hybridization temperature using fresh solution for each of the four washes. Multiple exposures of the blots were then generated using Kodak Biomax™ MS-2 film in conjunction with one Kodak Biomax MS intensifying screen at approximately -80°C.

J. PCR Analyses

Overlapping PCR products were generated that span the insert in MON 88017 (Products A-G, Figures 17 and 18). These products were sequenced to verify the sequence of the insert in MON 88017 and to verify the sequence of the genomic DNA flanking the 5' and 3' ends of the insert. The PCR analyses were conducted using 50 ng of genomic DNA or 0.5 ng of plasmid PV-ZMIR39 positive control DNA template in a 50 µl reaction volume containing a final concentration of 1.5 mM $MgCl_2$, 0.4 µM of each primer, 200 µM each dNTP, and 2.5 units of *Taq* DNA polymerase. The specific polymerase used to amplify Products A, B, and D-G was REDTaq™ DNA Polymerase (Sigma). HotStarTaq™ DNA Polymerase (Qiagen) was used to amplify Product C. The amplification of Products A, B, and D-G was performed under the following cycling conditions: 94°C for 3 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes; 1 cycle at 72°C for 10 minutes. The amplification of Product C was performed under the following cycling conditions: 94°C for 15 minutes; 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes; 1 cycle at 72°C for 10 minutes. The PCR products were separated on 1.2 % agarose gels and visualized by ethidium bromide staining. Following electrophoresis, PCR products generated from MON 88017 were excised from the gel and purified using the QIAquick® Gel Extraction Kit (Qiagen). The purified products were sequenced with the initial PCR primers, as well as primers designed internal to the amplified sequences. All sequencing was performed using dye-terminator chemistry.

™ Turboblotter is a trademark of Schleicher & Schuell, Keene, New Hampshire
® Stratalinker is a registered trademark of Stratagene Cloning Systems, La Jolla, California
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APPENDIX II

Materials and Methods used for Characterization of Introduced Proteins in MON 88017

1. Materials and methods used to characterize the CP4 EPSPS protein

1.1. Plant-Produced CP4 EPSPS Protein

The CP4 EPSPS protein was isolated from MON 88017 grain (produced under production plan 02-01-50-01). The identity of the MON 88017 corn grain sample was confirmed using event-specific PCR analysis. A verification of identity of the grain is archived under lot 60-100023. The CP4 EPSPS protein was stored in a –80 °C freezer in a buffer solution [50 mM Tris-HCl, pH 7.5, 50 mM KCl, and 2 mM DTT] at a total protein concentration of 0.5 mg/mL. The data for the isolation of the CP4 EPSPS protein from MON 88017 grain is archived under lot 60-100023.

1.2. *E. coli*-Produced CP4 EPSPS Protein

The *E. coli*-produced CP4 EPSPS protein (APS lot 20-100015) was used as a reference for the functional activity assays, glycosylation analysis, and western blot analysis. The CP4 EPSPS protein was stored in a –80 °C freezer in a buffer solution [50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl, and 25% (v/v) glycerol] at a total protein concentration of 3.8 mg/mL.

1.3. Assay Controls

Protein molecular weight standards were used to calibrate SDS-polyacrylamide gels and verify protein transfer to PVDF membranes. A peptide mixture was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis. NIST BSA was used for MALDI-TOF intact mass analysis. Transferrin was used as the positive control in glycosylation analysis. An analytical reference protein (10 pmole β-lactoglobulin) was analyzed before and after the test proteins to verify that the N-terminal sequencer met acceptable performance criteria for repetitive yield and sequence identity. Phenylthiohydantoin amino acid standards were used during N-terminal sequencing analysis. The following standards and controls were used during amino acid analysis; NIST BSA, NIST AA Standards, and norvaline standard.

1.4. Protein Purification

The CP4 EPSPS protein was purified from extracts of ground MON 88017 grain using a combination of anion exchange chromatography, ammonium sulfate fractionation, hydrophobic interaction chromatography, and affinity chromatography.

Defatted corn grain was extracted with a 50 mM Tris-HCl buffer, pH 8.0 [1:10 (w/v) grain to buffer ratio]. The extract was clarified by centrifugation and the supernatant was concentrated, dialyzed into 50 mM sodium carbonate/bicarbonate, 1 mM EDTA buffer, and applied to a Q-sepharose Fast Flow anion exchange column. The column was washed with 50 mM sodium carbonate/bicarbonate, 1 mM EDTA buffer and the eluate was collected. The eluate was clarified by centrifugation and the proteins were precipitated with ammonium sulfate [70% (w/v)]. The ammonium sulfate pellet was re-suspended in 50 mM Tris-HCl, pH 7.5, 1.25 M ammonium sulfate buffer and an aliquot was applied to a Source 15PHE hydrophobic interaction column. Proteins bound to the column were eluted with a linear ammonium sulfate gradient (1.25 M to 0 M). Fractions were pooled based on immunoreactivity with anti-CP4 EPSPS antibodies on lateral flow strips, concentrated, and the buffer was exchanged to 25 mM Bis-Tris Propane, pH 6.5, containing protease inhibitors. The sample was applied to a Source 15 Q anion exchange column. The column was washed with 25 mM Bis-Tris propane, 1 mM DTT, 1 mM benzamidine, 0.5 mM PMSF, 10% (v/v) glycerol, pH 6.5 buffer. Proteins bound to the column were eluted with a linear sodium chloride gradient (0 M to 0.5 M NaCl). Fractions containing CP4 EPSPS protein were pooled based on immunoreactivity with anti-CP4 EPSPS antibodies on lateral flow strips, concentrated, and the buffer was exchanged to 50 mM MES, pH 5.85, 2 mM DTT. The sample was applied to a cellulose phosphate column for affinity chromatography. The column was washed with 50 mM MES, pH 5.85, 2 mM DTT. CP4 EPSPS protein was eluted from the column with 0.5 mM PEP, 0.5 mM S-3-P, 50 mM MES pH 5.85, 2 mM DTT buffer. Fractions were pooled based on immunoreactivity with CP4 EPSPS antibodies on lateral flow strips and SDS-PAGE analysis. The protein was concentrated and the buffer was exchanged to 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT.

1.5. Total Protein Concentration Determination

The total protein concentration of the plant-produced CP4 EPSPS protein sample was determined using amino acid analysis. Aliquots of the sample were subjected to vapor phase acid hydrolysis followed by amino acid analysis on a Hitachi L-8800 amino acid analysis system. Amino acids were detected using post-column ninhydrin derivatization. Each protein sample was analyzed in triplicate. Total protein concentration was calculated as an average of the triplicate analysis.

1.6. Western Blot Analysis

Prior to western blot analysis, proteins were subjected to SDS-PAGE and transferred to a PVDF membrane. Aliquots of the plant- and *E. coli*-produced CP4 EPSPS proteins were diluted and mixed with loading buffer to a final concentration of 1× loading buffer [62.5 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 2.5% (v/v) 2-mercaptoethanol, 0.005% (w/v) bromophenol blue]. Samples were heated at 99-100 °C for 5 min prior to loading onto a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Bio-Rad (Hercules, California) pre-stained broad range molecular weight markers (10 µL/lane) were included on the gel to verify transfer of proteins to the PVDF membrane. Electrophoresis was performed at a constant voltage of 160 V for 80 min.

Following electrophoresis, samples were transferred to a 0.45 µm PVDF membrane for 90 min at a constant current of 300 mA in transfer buffer [12 mM Tris, 96 mM glycine, 20% (v/v) methanol, pH 8.3].

For western blot analysis, the following steps were performed. The membrane was blocked by incubation in 5% (w/v) NFDM in 1× PBST for 18-20 h. Antibodies (goat anti-CP4 EPSPS serum, lot 6844572) were diluted (1:2000) in 1% (w/v) NFDM in PBST and incubated with the membrane for 1 h. Excess serum was removed by washing with PBST four times for 10 min each. The membrane was then incubated with a 1:10,000 dilution of anti-goat IgG, HRP conjugated antibody in 1% (w/v) NFDM in PBST for 1 h. Excess antibody was removed by washing with PBST four times for 10 min each. All incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Pharmacia, Uppsala, Sweden) and the membrane was exposed (1 min, 30, 15, 10, 5, and 1 sec) to Hyperfilm ECL high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

Image analysis of immunoreactive bands on films was conducted using a Bio-Rad model GS-710 calibrated imaging densitometer equipped with Quantity One software version 4.3.0. The level of signal for the principal bands corresponding to the CP4 EPSPS protein detected in each lane was measured as a band contour quantity (average band OD × band area in mm^2).

The percent difference in immunoreactivity between the plant- and *E. coli*-produced proteins was calculated. The proteins were considered to have equivalent immunoreactivity if they differed by ≤ 10%.

1.7. N-terminal Sequence Analysis

Prior to N-terminal sequence analysis, the plant-produced CP4 EPSPS protein sample was separated using SDS-PAGE and transferred to a PVDF membrane. An aliquot of plant-produced CP4 EPSPS protein was diluted and mixed with loading buffer to a final concentration of 1× loading buffer. The sample was heated to ~102 °C for 4 minutes prior to loading onto a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Bio-Rad pre-stained broad range molecular weight markers were included on the gel to verify transfer of the proteins to the membrane. Electrophoresis was performed at a constant voltage of 125 V for 100 min. Following electrophoresis, the gel was soaked for 60 minutes in CAPS transfer buffer and the proteins were transferred to a PVDF membrane (0.2 µm pore size) for 90 minutes at a constant current of 300 mA. Protein bands were stained with Coomassie Brilliant Blue G stain (Bio-Rad) and then the blot was de-stained in a solution of 10% (v/v) acetic acid, 40% (v/v) methanol. Protein bands corresponding to CP4 EPSPS protein (~ 45 kDa) and two other protein bands found at ~48 kDa and ~55 kDa were excised from the membrane and sequenced.

N-terminal sequence analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise

HT Protein Sequencing System (Foster City, CA) with 140C Microgradient system and 785 Programmable Absorbance Detector and Procise Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas[99] software (version 3.59a). A PTH-amino acid standard mixture was used to calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin) was analyzed before and after the test substance protein bands to verify that the sequencer met acceptable performance criteria for repetitive yield and sequence identity.

1.8. MALDI-TOF MS Tryptic Mass Map Analysis

1.8.a. In-gel Trypsin Digestion and Sample Preparation

Prior to the generation of tryptic fragments for MALDI-TOF mass spectroscopy, aliquots of the plant-produced CP4 EPSPS protein in loading buffer were separated by electrophoresis on a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Prior to loading, the plant-produced CP4 EPSPS protein samples were heated at ~102 °C for 4 min. Bio-Rad broad range molecular weight markers were included on the gel. Electrophoresis was performed at a constant voltage of 125 V for 100 min. Following electrophoresis, the gel was stained for ~1 h with Coomassie Brilliant Blue G stain and destained for ~3 h in a solution containing 25% (v/v) methanol.

The band that migrated to ~45 kDa was excised, de-stained, reduced, alkylated, and subjected to an in-gel trypsin digest (Williams et al., 1997). Each gel fragment was destained by incubation in 100 μL of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in a microfuge tube. Following destaining, gel fragments were incubated in 100 μL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. Proteins were reduced in 100 μL of 10 mM dithiothreitol solution for two hours at 37 °C. Proteins were then alkylated by the addition of 100 μL of buffer containing 100 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. Gel fragments were incubated in 100 μL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. Acetonitrile (100 μL) was added to each microfuge tube and the reactions were incubated at room temperature for an additional 30 min. This procedure was repeated two additional times to remove the reducing and alkylating agents from the gel fragments. The gel bands were dried in a SpeedVac concentrator, rehydrated, and the protein digested in the gel overnight at 37 °C with 50 μL 25 mM ammonium bicarbonate solution containing 33 μg/mL trypsin. Digested peptides were extracted for one hour each at room temperature with 50 μL 70% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). The supernatant from each extraction sample was combined and dried in a SpeedVac concentrator. The extraction, pooling, and drying were repeated two additional times, and the final dried material was stored overnight in a 4 °C refrigerator followed by reconstitution in 5 μL of 0.1% (v/v) TFA.

A portion of the digested sample was desalted (Bagshaw et al., 2000) using Millipore (Bedford, MA) ZipTip$_{C18}$ pipette tips. Prior to desalting, the tips were wetted with methanol and equilibrated with 0.1% (v/v) TFA. A sample (4.7 μL) was applied to a ZipTip and eluted with 5 μL of Wash 1 [0.1% (v/v) TFA], 5 μL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], 5 μL of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA], and finally with 5 μL of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

1.8.b. MALDI-TOF Instrumentation and Mass Analysis

Mass spectral analysis was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF instrument with the supplied Data Explorer software (version 4.0). Calibration of the mass spectrometer was performed using an external peptide mixture from a Sequazyme Peptide Mass Standards kit (Applied Biosystems). Samples (0.3 μL) from each desalting step, as well as a sample of the solution prior to desalting, were co-crystallized with 0.75 μL of α-cyano-4-hydroxy cinnamic acid on the analysis plate. All samples were analyzed from 500 to 5000 Daltons in reflector mode using 150 laser shots per mass spectrum at a laser intensity setting of 3100 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected protein sequence. The amino acid sequence of the plant-produced CP4 EPSPS protein was deduced from the coding region of the *cp4 epsps* gene present in MON 88017. Masses were calculated for each theoretical peptide and the raw mass data was compared to the calculated theoretical masses. Experimental masses (MH+) were assigned to peaks when three (or more) isotopically resolved ion peaks were observed in the raw mass data. Peaks were not assessed if there were less than three isotopically resolved peaks in the spectra, when peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 daltons from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data.

1.8.c. Molecular Weight Determination using MALDI-TOF MS

Prior to analysis, the plant-produced CP4 EPSPS protein and NIST BSA reference protein were desalted using drop dialysis (Görisch, 1988). Briefly, 4μL of protein was placed on a Millipore 25 mm microdialysis disk (type VSWP, 0.025 μm pore size) and dialyzed for 60-120 minutes against HPLC-grade water. A portion of each protein sample (0.3 and 0.5 μL) was spotted on an analysis plate, mixed with 0.75 μL sinapinic acid solution, and air-dried. Mass spectral analysis of the plant-produced CP4 EPSPS protein was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation Matrix Assisted Laser Desorption and Ionization (MALDI) Time of Flight (TOF) instrument with the supplied Data Explorer software (version 4.0). Mass calibration of the instrument was performed using the desalted BSA reference protein. The mass of the plant-produced CP4 EPSPS protein was reported as an average of three separate mass spectral acquisitions. For comparison, the mass of the CP4 EPSPS protein

was calculated from the expected amino acid sequence of the protein using the Protean module of DNAstar software (version 5.01).

1.9. Purity and Molecular Weight Determination using SDS-PAGE

An aliquot of the plant-produced CP4 EPSPS protein was diluted and mixed with loading buffer to a final concentration of 1× loading buffer. The sample was heated at ~102 °C for 4 min prior to loading onto a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Three amounts (1, 2, and 3 μg) of total protein were loaded into three separate wells. Bio-Rad broad range molecular weight markers (1 μg each protein/lane) were included on the gel to estimate the molecular weight of the test substance. Electrophoresis was performed at a constant voltage of 125 V for 100 min. Proteins were fixed in the gel for 60 min in a solution containing 40% (v/v) methanol and 7% (v/v) glacial acetic acid, then stained for 2 h with Colloidal Brilliant Blue G stain (Sigma, St. Louis, MO). The gel was destained for 45 sec in a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, followed by 2 h in a solution containing 25% (v/v) methanol.

Analysis of the gel was performed using a Bio-Rad Laboratories GS-710 densitometer with the supplied Quantity One software, version 4.3.0. Molecular weight values of the marker proteins were supplied by the manufacturer and used to estimate the molecular weight of each observed band in the plant-produced CP4 EPSPS protein. All visible bands within each sample lane were quantified. Purity was estimated as the percent optical density of the ~45 kDa band relative to all bands detected in the lane. Molecular weight and purity were reported as an average of all three lanes containing the plant-produced CP4 EPSPS protein. The plant- and *E. coli*-produced proteins were considered to have equivalent molecular weights if the difference in the molecular weights was ≤ 5%.

1.10. Phosphate Release Assays

Prior to analysis, the plant-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS reference standard were diluted in a buffer solution containing 50 mM HEPES, pH 7.0. Enzymatic activity assays for both the plant- and the *E. coli*-produced CP4 EPSPS proteins were conducted in duplicate or triplicate and each replicate assay was subsequently analyzed in duplicate spectrophotometrically. This end-point type colorimetric assay measures the release of inorganic phosphate from one of the substrates, phosphoenolpyruvate (PEP), which is released by the action of the EPSPS enzyme in the presence of shikimate-3-phosphate (S-3-P). Reaction mixtures contained the EPSPS enzyme with 2 mM S-3-P and were initiated with 1 mM PEP (final concentration). The final reagent concentrations in the assay were 50 mM HEPES (pH 7.0), 0.1 mM ammonium molybdate and 5 mM potassium fluoride. Reactions were incubated for two minutes at 25 °C to allow for product formation. The reactions were quenched with malachite green reagent and fixed two minutes later with 33% (w/v) sodium citrate. The EPSPS-catalyzed release of inorganic phosphate from PEP was determined at a wavelength of 660 nm using a PowerWave X_i microplate reader, relative to a standard curve of inorganic phosphate treated with the malachite green reagent and

33% (w/v) sodium citrate. For EPSPS, one unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmole of inorganic phosphate from PEP per minute at 25 °C. Calculations of the specific activities were performed using Microsoft Excel 2000 version 9.0.4402 SR-1. Specific activity values were calculated based on the assay concentration of the CP4 EPSPS protein. The plant and *E. coli*-produced proteins were considered to have equivalent functional activity if their specific activities differed by ≤ two-fold.

1.11. Glycosylation Analysis

Glycosylation analysis was performed to determine whether the plant-produced CP4 EPSPS protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the test substance, the *E. coli*-produced CP4 EPSPS reference standard (a negative control), and transferrin (a positive control) were each diluted and mixed with loading buffer to a final concentration of 1× loading buffer. The samples were heated at 99-101 °C for 4 min, and two amounts (0.5 and 1 μg) of each sample were loaded, along with Bio-Rad prestained broad range molecular weight markers (10 μL/lane), on a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Electrophoresis was performed at a constant voltage of 170 V for 70 min. After electrophoresis proteins were electrotransferred to a 0.45 μm PVDF membrane for 90 min at a constant current of 300 mA.

Carbohydrate detection was performed directly on the PVDF membrane using the ECL glycoprotein detection system (Amersham Pharmacia). The PVDF membrane was gently shaken for 10 min in PBS and transferred to a solution of 100 mM sodium acetate containing 10 mM sodium metaperiodate. The membrane was incubated in the dark for 20 min in the oxidation reagent. The oxidation solution was removed from the membrane by two brief rinses with PBS followed by three sequential 10 min washes in PBS. The membrane was transferred to a solution of 100 mM sodium acetate containing 25 nM biotin hydrazide and incubated for 60 min. The biotin hydrazide solution was removed by rinsing the membrane twice in PBS followed by washing the membrane in PBS three times for 10 min each. The membrane was blocked overnight in a 4 °C refrigerator in the blocking agent supplied with the kit. The blocking solution was removed by rinsing the membrane twice in PBS followed by washing the membrane in PBS three times for 10 min each. The membrane was incubated with streptavidin-HRP conjugate (diluted 1:6000) in PBS for 30 min to detect carbohydrate moieties bound to biotin. Excess streptavidin-HRP was removed by rinsing and washing as previously described. Bands were visualized using the ECL detection system and the membrane was exposed (30 sec, 3 min, and 10 min) to Hyperfilm ECL high performance chemiluminescence film. Films were developed using a Konica SRX-101A automated film processor.

2.0. Materials and methods used to characterize Cry3Bb1 protein

2.1. Plant-Produced Cry3Bb1 Protein

The plant-produced Cry3Bb1 protein was isolated from MON 88017 grain. The identity of the MON 88017 corn grain sample was confirmed using event-specific PCR. A copy of the verification of identity certificate for the grain is archived under lot 60-100024. The Cry3Bb1 protein was stored in a –80 °C freezer in a buffer solution containing 100 mM Tris-HCl, pH 8.0. The data for the isolation of the Cry3Bb1 protein from MON 88017 grain is archived under lot 60-100024.

2.2. *E. coli*-Produced Cry3Bb1 Protein

The characterized *E. coli*-produced Cry3Bb1 protein (lot 30-100002) was used as a reference standard to establish equivalence in select analyses. The Cry3Bb1 protein was stored in a –80 °C freezer in a buffer solution [50 mM sodium carbonate-bicarbonate, pH 10.1, 1 mM EDTA] at a total protein concentration of 1.2 mg/mL by amino acid analysis.

2.3. Assay Controls

Bovine serum albumin (BSA) protein was used to estimate the total protein concentration in the Bio-Rad protein assays. Protein molecular weight markers were used to calibrate SDS-polyacrylamide gels and verify protein transfer to PVDF membranes. A peptide mixture was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis. Transferrin was used as a positive control in glycosylation analysis.

2.4. Protein Purification

The Cry3Bb1 protein was purified from extracts of ground defatted MON 88017 corn grain using a combination of anion exchange chromatography and affinity chromatography.

Cry3Bb1 protein was extracted from three batches (1-1.5 kg each) of defatted MON 88017 grain with either 50 mM Tris-HCl, pH 8.0 buffer or 50 mM sodium carbonate/bicarbonate, 1 mM EDTA, pH 10 buffer (buffer A) [1:10 (w/v) grain to buffer ratio]. The extracts were clarified by centrifugation and the supernatant was concentrated 3-4 fold. The samples not extracted in buffer A were combined and dialyzed into buffer A. Samples were loaded onto a Q-sepharose Fast Flow anion exchange column that was pre-equilibrated with buffer A. The column was washed with 25% buffer B (50 mM sodium carbonate/bicarbonate, 0.5 M NaCl, 1 mM EDTA, pH 10 (buffer B). The Cry3Bb1 protein was eluted with 45% buffer B. The protein was concentrated and dialyzed into 50 mM Tris-HCl, pH 8.0. The dialyzed sample was loaded onto protein G agarose columns coupled with monoclonal Cry3Bb1 antibodies for affinity chromatography. The sample was recirculated over the antibody column. Cry3Bb1 protein was eluted from the column with 100 mM triethylamine, pH 11.2. Fractions were

collected into 100 mM Tris-HCl, pH 8.0, to neutralize the solution. The fractions were concentrated and buffer exchanged into 100 mM Tris-HCl, pH 8.0. Fractions containing proteins similar in molecular weight to the Cry3Bb1 protein were pooled based on visual examination of Colloidal Brilliant Blue G stained SDS polyacrylamide gels.

2.5. Total Protein Concentration Determination

The total protein concentration of the plant-produced Cry3Bb1 protein was estimated using a Bio-Rad (Hercules, CA) protein concentration assay. BSA protein (concentrations ranging from 0.05 to 0.6 mg/mL) was used to create a standard curve. The Cry3Bb1 protein concentration was estimated by comparison to the linear standard curve. Data was collected using a Bio-Tek Instruments, Inc. Powerwave Xi microplate scanning spectrophotometer (Winooski, VT) employing the KC4 software version 2.6, revision 3. Readings were taken at a wavelength of 595 nm.

2.6. Western Blot Analysis

Prior to western blot analysis, proteins were separated using SDS-PAGE and transferred to a PVDF membrane. Aliquots of plant- and *E. coli*-produced Cry3Bb1 proteins were diluted and mixed with loading buffer, to a final concentration of 1× loading buffer [62.5 mM Tris, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 2.5% (v/v) 2-mercaptoethanol, 0.005% (w/v) bromophenol blue]. Samples were heated at 100 °C for 5 min prior to loading onto a Tris-glycine buffered 4-20% polyacrylamide gel. Bio-Rad (Hercules, CA) prestained broad range molecular weight markers (1 µg/band) or Precision Plus Dual Color molecular weight markers (1 µg/band) were included on the gel to verify transfer of proteins to the PVDF membrane. Electrophoresis was performed at a constant voltage of 160 V for 80 min. Following electrophoresis, samples were transferred to a 0.45 µm PVDF membrane for 90 min at a constant current of 300 mA in transfer buffer [12 mM Tris, 96 mM glycine, 20% (v/v) methanol, pH 8.3].

For western blot analysis, the following steps were performed. The membrane was blocked by incubation in 5% (w/v) NFDM in 1× PBST for 16 h and 30 min. Characterized antibodies (goat anti-Cry3Bb1 serum, lot 6844582) were diluted (1:5000) in 1% (w/v) NFDM in PBST and incubated with the membrane for 1 h. Excess serum was removed by washing with PBST four times for 5-10 min each. The membrane was then incubated with a 1:10,000 dilution of anti-goat IgG, HRP conjugated antibody in 1% (w/v) NFDM in PBST for 1 h. Excess antibody was removed by washing with PBST four times for 10 min each. All incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Pharmacia, Uppsala, Sweden) and the membrane was exposed (1 min, 30, 15, 10, 5, and 1 sec) to Hyperfilm ECL high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

Image analysis of immunoreactive bands on films was conducted using a Bio-Rad model GS-710 calibrated imaging densitometer equipped with Quantity One software version

4.3.0. The level of signal from the principal bands corresponding to the Cry3Bb1 protein detected in each lane was measured as a band contour quantity (average band OD × band area in mm^2). The percent difference in immunoreactivity between the plant- and *E. coli*-produced Cry3Bb1 proteins was calculated using Microsoft Excel 2000. The proteins were considered to have equivalent immunoreactivity if the average percent difference was ≤ 10%.

2.7. MALDI-TOF MS Tryptic Mass Map Analysis

2.7.a. In-gel Trypsin Digestion and Sample Preparation

Prior to the generation of tryptic fragments for MALDI-TOF mass spectroscopy, aliquots of the plant-produced Cry3Bb1 protein in loading buffer were separated by electrophoresis on a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Prior to loading, the plant-produced Cry3Bb1 protein samples were heated at ~104 °C for 4 min. Bio-Rad broad range molecular weight markers were included on the gel. Electrophoresis was performed at a constant voltage of 150 V for 85 min. Following electrophoresis, the gel was stained for 4 h and 20 min with Coomassie stain (Bio-Rad) and destained overnight with a solution containing 25% (v/v) methanol.

Bands that corresponded to the sizes expected for full-length Cry3Bb1 protein (~77 kDa) and its major fragments (~66 and ~55 kDa) were excised, destained, reduced, alkylated, and subjected to an in-gel trypsin digest (Williams et al., 1997). Briefly, each gel fragment was destained by incubation in 100 µL of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in a microfuge tube. Following destaining, gel fragments were incubated in 100 µL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. Proteins were reduced in 100 µL of 10 mM dithiothreitol solution for two hours at 37 °C. Proteins were then alkylated by the addition of 100 µL of buffer containing 100 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. Gel fragments were incubated in 100 µL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. Acetonitrile (100 µL) was added to each microfuge tube and the reactions were incubated at room temperature for an additional 30 min. This procedure was repeated two additional times to remove the reducing and alkylating agents from the gel fragments. The gel bands were dried in a SpeedVac concentrator, rehydrated, and digested in the gel with 50 µL 25 mM ammonium bicarbonate solution containing 33 µg/mL trypsin. The digestion was continued overnight at 37 °C. Trypsin-digested proteins were extracted for one hour each at room temperature with 50 µL 70% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). The supernatant from each extraction sample was combined and dried in a SpeedVac concentrator. The extraction, pooling, and drying were repeated two additional times, and the final dried material was stored in a 4 °C refrigerator until reconstituted in 5 µL of 0.1% (v/v) TFA.

A portion of the digested sample was desalted (Bagshaw et al., 2000) using Millipore (Bedford, MA) $ZipTip_{C18}$ pipette tips. Prior to desalting, the tips were wetted with methanol and equilibrated with 0.1% (v/v) TFA. A sample (4.7 µL) was applied to a

ZipTip and eluted with 5 μL of Wash 1 [0.1% (v/v) TFA], 5 μL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], 5 μL of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA], and finally with 5 μL of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

2.7.b. MALDI-TOF Instrumentation and Mass Analysis

Mass spectral analysis was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF instrument (Foster City, CA) with the supplied Data Explorer software (version 4.0). Calibration of the mass spectrometer was performed using an external peptide mixture from a Sequazyme Peptide Mass Standards kit (Applied Biosystems). Samples (0.3 μL) from each desalting step, as well as a sample of the solution prior to desalting, were co-crystallized with 0.75 μL of α-cyano-4-hydroxy cinnamic acid on the analysis plate. All samples were analyzed from 600 to 5000 daltons (mass standards were analyzed from 500-5000 daltons) in reflector mode using 150 laser shots per mass spectrum at a laser intensity setting of 3100, 3150, or 3300 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected protein sequence. The sequence of the Cry3Bb1 protein used was identical to the amino acid sequence deduced from the coding region of the full-length *cry3Bb1* gene present in MON 88017. Masses were calculated for each theoretical peptide and the raw mass data was compared to the calculated theoretical masses. Experimental masses (MH+) were assigned to peaks when three (or more) isotopically resolved ion peaks were observed in the raw mass data. Peaks were not assessed if there were less than three isotopically resolved peaks in the spectra, when peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 daltons from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data.

2.8. Purity and Molecular Weight Determination using SDS-PAGE

An aliquot of the plant-produced Cry3Bb1 protein was diluted and mixed with loading buffer to a final concentration of 1x loading buffer. The sample was heated at ~105 °C for 4 min prior to loading onto a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Three amounts (1, 2, and 3 μg) of total protein were loaded into three separate wells. A sample of the *E. coli*-produced protein (1 μg) was included on the gel for molecular weight comparison. Bio-Rad broad range molecular weight markers (1μg/band) were included on the gel to estimate the molecular weight of the test substance. Electrophoresis was performed at a constant voltage of 150 V for 75 min. Proteins were fixed in the gel for 60 min in a solution containing 40% (v/v) methanol and 7% (v/v) glacial acetic acid, then stained for 2 h with Colloidal Brilliant Blue G stain (Sigma, St. Louis, MO). The gel was destained for 30 sec in a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, followed by 2 h in a solution containing 25% (v/v) methanol.

Analysis of the gel was performed using a Bio-Rad Laboratories GS-710 densitometer with the supplied Quantity One software, version 4.3.0. Molecular weight values of the marker proteins were supplied by the manufacturer and used to estimate the molecular weight of each observed band in the plant-produced Cry3Bb1 protein. All visible bands within each lane were quantified. Purity was estimated as the sum of the percent optical density of the three bands identified as Cry3Bb1 protein, relative to all bands detected in the lane. Molecular weight and purity were reported as an average of all three lanes containing the plant-produced Cry3Bb1 protein. Calculations were performed using Microsoft Excel 2000. The plant and *E. coli*-produced proteins were considered to have equivalent molecular weights if their molecular weights differed by ≤ 5%.

2.9. Insect Bioassays

In order to assess functional activity of the Cry3Bb1 protein produced in MON 88017, aliquots of the plant- and *E. coli*-produced Cry3Bb1 proteins were used to estimate the bioactivity (measured as an LC_{50} value) of the Cry3Bb1 proteins incorporated into diets fed to Colorado potato beetle (CPB) larvae, a susceptible insect. The plant- and *E. coli*-produced proteins were considered to have equivalent functional activity if the difference in LC_{50} values was ≤ four-fold.

2.9.a. Samples

The test samples included the *E. coli*- and plant-produced Cry3Bb1 proteins, lot numbers 30-100002 and 60-100024, respectively, suspended in buffer. The *E. coli*-produced protein was suspended in a 100 mM Tris-HCl buffer, pH 8.0, and the plant produced Cry3Bb1 was suspended in a 50 mM sodium carbonate buffer, pH 10, with 1mM EDTA. Samples of each of the buffer types were received for use as control treatments. The test samples and buffers were stored in a -80° C freezer and a 5° C refrigerator, respectively.

2.9.b. Insects

Colorado potato beetle eggs were obtained from the New Jersey Department of Agriculture, West Trenton, NJ. Insect eggs were incubated at temperatures ranging from 15°C to 27°C depending on desired hatch times.

2.9.c. Bioassays

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, was used to measure activity of the plant- and *E. coli*-produced Cry3Bb1 protein samples. The bioassay was replicated two times on different days, each using a separate batch of insects. Each bioassay replicate consisted of a series of dilutions from each Cry3Bb1 sample yielding a dose series ranging from 0-3 µg Cry3Bb1 protein/ml diet plus a buffer control. Each bioassay also included a purified water control. The Cry3Bb1 protein doses were prepared by diluting the protein with purified water and incorporating the dilution into an agar-based Colorado potato beetle diet (Bio-

Serv, Frenchtown, NJ). This dose series in diet was expected to elicit a response range from CPB larvae that would allow for an estimate of the LC_{50} of the Cry3Bb1 protein. The diet mixture was then dispensed in 0.5 ml aliquots into a 128 well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets, approximately 16 insects per treatment, using a soft, fine, natural hair paintbrush. The infested wells were covered by a ventilated adhesive cover (#BIO-CV-16, CD International) and the insects were allowed to feed for a period of approximately seven days in an environmental chamber programmed at 27°C, ambient relative humidity and a lighting regime of 14:10 light:dark. The number of survivors was recorded after the incubation period. Control treatments weights were recorded for quality control purposes only and were not used in the analysis.

2.9.d. Statistical analysis

The bioassay data analyzed consisted of the number of surviving CPB larvae for each dose. Statistical analysis was performed with Release 8.2 of the SAS statistical program running under Windows 2000 Professional (SAS Institute Inc., 1999-2001). A Probit analysis was performed to test the effect of the *E. coli*- and plant-produced Cry3Bb1 proteins on the observed mortality and to estimate the dose level required for 50% mortality (LC_{50}) for each replicate with the following model:

$$p_k = \mathrm{C} + (1 - \mathrm{C})\mathrm{F}(Dose_k + e_k)$$

p_k: Observed proportion of mortality under a given dose;
C: Natural (threshold) mortality rate;
F: Logistic cumulative distribution function;
$Dose_k$: Dose effect in the scale of base 10 logarithm; and
e_k: Random residual effect.

An integrated Probit analysis was also performed to test the differences between two protein sources with the following model:

$$p_{ijk} = \mathrm{C} + (1 - \mathrm{C})\mathrm{F}(Treat_i + Rep_j + Dose_{ijk} + e_{ijk})$$

p_{ijk}: Observed proportion of mortality;
C: Natural (threshold) mortality rate;
F: Logistic cumulative distribution function;
$Treat_i$: Treatment effect;
Rep_j: Effect of the replicate;
$Dose_{ijk}$: Dose effect in the scale of base 10 logarithm; and
e_{ijk}: Random residual effect.

The Fisher's Exact test was used to test for significant mortality in water and buffer control samples. Significance of tests was determined at the 0.05 level.

2.10. Glycosylation Analysis

Glycosylation analysis was performed to determine whether the plant-produced Cry3Bb1 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the test substance, the *E. coli*-produced Cry3Bb1 reference standard (a negative control), and transferrin (a positive control) were each diluted and mixed with loading buffer to a final concentration of 1× loading buffer. The samples were heated at ~98 °C for 4 min and two amounts (0.25 and 0.5 µg) of each sample were loaded, along with Bio-Rad prestained broad range molecular weight markers, on a Tris-glycine buffered 4-20% gradient polyacrylamide gel. Electrophoresis was performed at a constant voltage of 160 V for 75 min. After electrophoresis, the gel was soaked in transfer buffer for 10 min and proteins were transferred to a 0.45 µm PVDF membrane for 90 min at a constant current of 300 mA.

Carbohydrate detection was performed directly on the PVDF membrane using the ECL glycoprotein detection system (Amersham Pharmacia). The PVDF membrane was gently shaken overnight at 4 °C in PBS and transferred to a solution of 100 mM sodium acetate containing 10 mM sodium metaperiodate. The membrane was incubated in the dark for 20 min in the oxidation reagent. The oxidation solution was removed from the membrane by two brief rinses with PBS followed by three sequential 10 min washes in PBS. The membrane was transferred to a solution of 100 mM sodium acetate containing 25 nM biotin hydrazide and incubated for 60 min. The biotin hydrazide solution was removed by rinsing the membrane twice in PBS followed by washing the membrane in PBS three times for 10 min each. The membrane was blocked for 60 min in the blocking agent supplied with the kit. The blocking solution was removed by rinsing and washing the membrane as described above. The membrane was incubated with streptavidin-HRP conjugate (diluted 1:6000) in PBS for 30 min to detect carbohydrate moieties bound to biotin. Excess of streptavidin-HRP was removed by rinsing and washing the membrane as described above. Bands were visualized using the ECL detection system and the membrane was exposed (30 sec, 3 min, and 10 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX-101A automated film processor.

APPENDIX III

Materials and Methods used for Analysis of Expression of the Introduced Proteins

1. Test, control, and reference substances

1.1. Test substance

The test substance for this study was MON 88017. Corn plants were grown from seed lot # GLP-0203-12154-S and tissue samples analyzed in this study were produced under Production Plan # 02-01-50-01.

1.2. Control substance

The control substance for this study was the conventional corn hybrid H1200902 with genetic background similar to MON 80017. Corn plants were grown from seed lot # GLP-0203-12164-S and tissue samples from the control plants were produced under Production Plan # 02-01-50-01.

1.3. Characterization of test and control substances.

The identity of the test and control substances was confirmed by verifying the chain-of-custody documentation prior to analysis. To further confirm the identity of the test and control substances, event-specific polymerase chain reaction (PCR) analyses were conducted on seed and grain samples. The identity of grain samples harvested from the field was verified by PCR.

1.4. Reference substances.

The *E. coli*-produced Cry3Bb1 protein standard (lot # 30-100002) was used as the reference substance for the analysis of Cry3Bb1 protein levels. The purity-corrected total protein concentration of the purified standard was 1.2 mg/ml by amino acid composition analysis. The purity was 98% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis.

The *E. coli*-produced CP4 EPSPS protein standard (lot # 20-100015) was used as the reference substance for analysis of CP4 EPSPS protein levels. The purity-corrected total protein concentration of the purified standard was 3.7 mg/ml by amino acid composition analysis. The purity was 97% as determined by SDS-PAGE and densitometric analysis. Certificates of analysis for both the Cry3Bb1 and CP4 EPSPS reference substances were archived with the study data.

2.0. Generation of plant samples

2.1. Summary of field design

A field production plan # 02-01-50-01 was initiated during the 2002 growing season to generate test and control substances. The field sites were located within the major corn-growing region of the U.S. (Benton County, IA; Stark County, IL; and York County, NE) and provided a variety of environmental conditions. At each site, three replicated plots of MON 88017 and the control H1200902 were planted using a randomized complete block field design. Overseason leaf (OSL), overseason whole plant (OSWP), overseason root (OSR), pollen, silk, forage, forage root, grain, stover, and senescent root tissues were collected from each replicated plot at all field sites. Throughout the field production, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. Upon collection, all tissue samples were placed in uniquely labeled bags or containers. All tissue samples, except grain, were stored and shipped on dry ice. Grain samples were stored and shipped at ambient temperature.

2.2. Description of the collected tissues

Grain

All hand-pollinated ears in each test and control plot were collected at the R6 growth stage (physiological maturity). The ears were shucked and dried to moisture content of 10-15%. All cobs were shelled prior to shipping.

Forage

Forage is defined as the entire plant (leaves, ears, tassels, and stalk) with the root ball removed. Two whole, self-pollinated plants at the early dent growth stage (R4 - R6) were collected from each test and control plot. The forage tissue samples were pooled from each plot during collection.

Pollen

Approximately 5 g of pollen was collected non-systematically from each test and control replicated plot at each site at the R1 plant growth stage. Any debris in the pollen sample was removed using a fine mesh sieve.

Silk

Silks were collected non-systematically from five primary ears of plants from each test and control plot at each site. The samples were collected at the time of hand pollination (the R1 plant growth stage). The silk tissue samples were pooled from each plot during collection.

Stover

Stover is defined as the above ground portion of the plant remaining after grain harvest. Two stover samples were collected from each test and control plot, after the harvest of self-pollinated ears. The stover samples were pooled from each plot during collection.

Overseason Leaf

The youngest immature whorl leaf (2 - 4 inches) samples were collected from 15 plants from each of the test and control plots. The first OSL samples (OSL-1) were collected at the V2 - V3 growth stage; OSL-2 samples were collected at the V5 stage; OSL-3 samples were collected at the V8 stage; and OSL-4 samples were collected at the V11 - V17 stage. The leaves corresponding to each growth stage were pooled from each plot during collection.

Overseason Whole Plant

Two whole plants were collected from each of the test and control plots. An OSWP sample consists of shoot tissue (above-ground portion of the plant including leaves, tassels, ears, etc.). The two whole plant samples were pooled from each plot during collection. At each field site, the OSWP tissue samples were collected to coincide with the collection of OSL-1 to OSL-4 and OSR-1 to OSR-4 samples.

Overseason Root

The OSR samples were the below ground root mass that had been cut from the corresponding OSWP sample. The two root samples were pooled from each plot during collection. At each field site, the OSR tissue samples were collected to coincide with the collection of OSL-1 to OSL-4 and OSWP-1 to OSWP-4 samples.

Forage Root

Forage root is defined as the root ball removed from the plant sampled for forage. Forage root tissue samples were collected at the early dent growth stage (R4 - R6) from each test and control plot. Collection of the forage root tissue was similar to that used for the overseason root tissue. The forage root tissue samples were pooled from two plants from each plot during collection.

Senescent Root

Senescent root is the below ground root ball removed from plants sampled for stover. Two senescent root samples were collected from each test and control plot and corresponded to the plants that had been collected for stover. Collection of the senescent root tissue was similar to that used for the overseason root tissue. The senescent root tissue samples were pooled from each plot during collection.

2.3. Tissue processing and protein extraction methods

2.3.a. Processing

All tissue samples produced at the field sites were shipped to Monsanto for processing. During the processing step, dry ice was combined with the samples (except pollen) and then vertical cutters or mixers were used to thoroughly grind and mix the tissues. All processed tissue samples were stored in a –80°C freezer during the study.

2.3.b. Extraction

The Cry3Bb1 and CP4 EPSPS proteins were extracted from corn tissues according to the validation parameters provided in Section 2.5. of this Appendix III. All processed tissues were kept on dry ice during extract preparation. All tissues were extracted using a Harbil mixer and insoluble material was removed from the extracts by using a Serum Filter System (Fisher Scientific, Pittsburgh, PA). The extracts were aliquoted and stored in a –80°C freezer until ELISA analyses. Extraction efficiency for each tissue type was determined by successive extraction of three replicates, where the last extraction employed a harsh buffer (e.g., 1X Laemmli buffer).

Tissue Type	Tissue-to-Buffer Ratio	Extraction Buffer
Forage	1:100	1X PBST with 0.1% (w/v) BSA
Leaf	1:100	1X PBST with 0.1% (w/v) BSA
Pollen	1:100	1X PBST with 0.1% (w/v) BSA
Root	1:50	1X PBST with 0.1% (w/v) BSA
Grain	1:100	1X TBA with 10 mM DCA

2.4. ELISA reagents and methods

2.4.a. CP4 EPSPS antibodies

Mouse monoclonal antibody clone 39B6 (IgG2a isotype, kappa light chain; lot # 6199732) specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography.

The concentration of the purified IgG was determined to be 3.2 mg/ml by spectrophotometric methods. Production of the 39B6 monoclonal antibody was performed by TSD Bioservices, Inc. (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 0.02 M $Na_2HPO_4 \cdot 7H_2O$, 0.15 M NaCl, and 15 ppm ProClin 300 (Sigma, St. Louis, MO). The detection reagent was goat anti-CP4 EPSPS antibody (produced by Sigma, St. Louis, MO) conjugated to HRP.

2.4.b. CP4 EPSPS ELISA method

The CP4 EPSPS ELISA was performed using an automated robotic workstation. Mouse anti-CP4 EPSPS antibody was diluted in coating buffer containing 0.15 M NaCl to a final concentration of 1.0 µg/ml and immobilized onto 96-well microtiter plates, followed by incubation in a 4°C refrigerator for ≥8 h. Plates were washed in 1X PBST, CP4 EPSPS protein standard or sample extract was then added (100 µl/well), and plates were incubated at 37°C. Forage sample extracts were diluted with a 1X PBST/0.1% BSA solution and grain samples were diluted with a solution containing 0.1 M tris, 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, 0.01 M $MgCl_2$, 0.05% (v/v) Tween-20 at pH 7.8, and 0.2% (w/v) L-ascorbic acid (TBA). Plates were washed as before followed by the addition of 100 µl per well of anti-CP4 EPSPS peroxidase conjugate and incubated at 37°C. Plates were developed by adding 100 µl per well of TMB. The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantitation of CP4 EPSPS protein levels was accomplished by interpolation from a CP4 EPSPS protein standard curve that ranged in concentration from 0.456 - 14.6 ng/ml.

2.4.c. Cry3Bb1 antibodies

Goat polyclonal antibody (lot # 7107417) specific for the Cry3Bb1 protein was purified using Protein-G Agarose affinity chromatography. The concentration of the purified IgG was determined to be 5.16 mg/ml by spectrophotometric methods. The purified antibody was stored in a phosphate buffered saline (PBS) buffer (pH 7.4) containing 0.001 M KH_2PO_4, 0.01 M $Na_2HPO_4 \cdot 7H_2O$, 0.137 M NaCl, and 0.0027 M KCl, with 0.02% NaN_3 (w/v) added as a preservative.

The purified antibody (lot # 7107417) was coupled with biotin (Sigma, St. Louis, MO) according to the manufacturer's instructions and assigned lot # 7107462. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP).

2.4.d. Cry3Bb1 ELISA method

The Cry3Bb1 ELISA was performed using an automated robotic workstation. Goat anti-Cry3Bb1 antibody was diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and immobilized onto 96-well microtiter plates at a concentration of 5.0 µg/ml, followed by incubation in a 4°C refrigerator for ≥12 h. Plates were washed with 1X PBS with 0.05% (v/v) Tween-20 (1X PBST), blocked with the addition of 150 µl per well of 1X PBS with 0.25% (w/v) casein buffer and incubated at 37°C. Plates were washed as before followed by the addition of 100 µl per well of the Cry3Bb1 protein standard or sample in 1X PBST with 0.1% (w/v) BSA and incubated at 37°C. The procedure was completed after separate incubations with the addition of 100 µl per well of biotinylated goat anti-Cry3Bb1 antibody and NeutrAvidin-HRP. Plates were developed by adding 100 µl per well of HRP substrate, 3,3',5,5'- tetramethylbenzidine (TMB, Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100

µl per well of 6 M H_3PO_4. Quantitation of Cry3Bb1 protein levels was accomplished by interpolation from a Cry3Bb1 protein standard curve that ranged in concentration from 0.35 - 11.2 ng/ml.

2.4.e. Sensitivity of the ELISA

Limit of detection

The limit of detection (LOD) was calculated as the mean value plus three SD using the data generated with control sample (conventional corn) extracts for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fwt" using the respective dilution factor, tissue-to-buffer ratio, and a tissue-specific method bias correction factor. Limits of detection for CP4 and Cry3Bb1 ELISA are summarized in the Table 7 of Section 2, Part VI.

Limit of quantitation

The limit of quantitation (LOQ) was calculated based on the lowest standard concentration. The "ng/ml" value was converted to "µg/g fwt" using the respective dilution factor, tissue-to-buffer ratio, and a tissue-specific method bias correction factor. Limits of quantitation for CP4 and Cry3Bb1 ELISA are summarized in the Table 7 of Section 2, Part VI.

APPENDIX IV

Materials and Methods used for Analysis of MON 88017 Composition

The composition of grain and forage of MON 88107 was analyzed and compared to conventional corn. For this analysis, MON 88017 and conventional corn were grown at replicated field trials at three locations: NE, IA and IL. Analysis of grain included proximates (protein, fat, ash and moisture), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), amino acids, fatty acids, minerals (calcium, copper, iron, magnesium, manganese, potassium, phosphorus, sodium and zink), vitamins (B_1, B2, B6, E, niacin, and folic acid), anti-nutrients (phytic acid and raffinose), secondary metabolites (furfural, ferulic, and p-coumaric acid), and carbohydrates by calculation. Analysis of forage included proximates, ADF, NDF, minerals (calcium and phosphorus), and carbohydrates by calculation.

The results of compositional analysis are discussed in the Section 3, Part VII and the summary of the analysis for combined sites, summary of statistical differences and the literature and historical ranges are presented in Tables 12 – 20 of Part VII. Additional results of forage analysis by site are presented in Tables 1 – 3, and results of the grain analysis by site are presented in Tables 4 – 21 of this Appendix IV.

1.0. Test, control and reference substances

MON 88017 (test), conventional corn with a similar genetic background (control), and commercial corn hybrids (references) were planted and grown in replicated field trials across the U.S. in 2002.

1.1. Test substance

The test substance was MON 88017 grown from starting corn seed lot GLP-0203-12154-S. Forage and grain samples harvested from MON 88017 were analyzed.

1.2. Control substance

The control substance was LH198xLH59, a conventional corn hybrid grown from starting seed lot GLP-0203-12164-S. Forage and grain samples were harvested from this corn hybrid.

1.3. Reference substances

The following commercial corn forage and grain samples were used as the reference substances:

Vendor/Hybrid ID	Starting Seed Lot No.	Field Site
Asgrow/ RX708	REF-0203-12304-S	IA
Dekalb/ DK579	REF-0203-12305-S	IA
Garst/ 8464 IT	REF-0203-12306-S	IA
Garst/ 8590 IT	REF-0203-12307-S	IA
Asgrow/ RX690	REF-0203-12308-S	IL
Dekalb/ DKC60-15	REF-0203-12309-S	IL
Dekalb/ DKC61-24	REF-0203-12310-S	IL
Northrup King/ N59-Q9	REF-0203-12311-S	IL
Mycogen/ 7474	REF-0203-12316-S	NE
Mycogen/ 6431	REF-0203-12317-S	NE
Northrup King/ N60-N2	REF-0203-12318-S	NE
Northrup King/ N67-H6	REF-0203-12319-S	NE

2.0. Test, control and reference substance characterization

The identity of the forage and grain samples from each test, control, and reference substance was verified by confirming the chain-of-custody documentation supplied with the forage and grain collected from the plots. The grain from the test, control, and reference substances was further characterized by event-specific PCR analysis.

3.0. Field trial description

Corn seed was planted in the spring of 2002 at three field sites (IA, IL, NE) in the U.S. At each field site, seed from the test, control, and reference starting material was planted in a randomized complete block design with three replicates per block. All the plants were grown under normal agronomic field conditions for their respective geographic regions. All test plots received an application of Roundup UltraMAX herbicide according to the label instructions. Bulk forage samples were harvested at the late dough/early dent stage and bulk grain samples were harvested when the grain was mature. Forage and grain samples were harvested from all plots and shipped on dry ice (forage) or at ambient (grain) temperature. A sub-sample specific for compositional analysis was obtained from each bulk forage and grain sample harvested. The samples were ground and stored in a –20°C freezer located at Monsanto Company (St. Louis, MO) until shipped to Covance Laboratories Inc. (Madison, WI) on dry ice for analysis. The labels on the samples listed the composition protocol number, a unique sample number, tissue type, and storage conditions.

4.0. Analytical methods

A total of 107 ground forage and grain samples were shipped for analysis to Covance Laboratories Inc. for analyses. The samples upon receipt were stored in a –20 °C freezer until their use. Forage samples were analyzed for proximates (protein, fat, ash, and moisture), ADF, NDF, and minerals (calcium, phosphorous). Grain samples were analyzed for proximates (protein, fat, ash, and moisture), ADF, NDF, TDF, amino acids, fatty acids, minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium, and zinc), vitamins (B_1, B_2, B_6, E, niacin, and folic acid), anti-nutrients (phytic acid and raffinose), and secondary metabolites (furfural, ferulic acid, and p-coumaric acid). Each analysis was based on published analytical methods that were approved in advance by the Study Director. Carbohydrate values in ground forage and grain were determined by calculation. The methods used for compositional analyses are summarized below.

4.1. 2-Furaldehyde (FURF)

The method used was based on a literature method (Albala-Hurtado et al., 1997). The grain sample was extracted with 4% trichloroacetic acid in MilliQ water, centrifuged, and filtered. The level of 2-furaldehyde (furfural) in the extract was determined by RP-HPLC with UV quantitation. The reference standard was ACROS 2-furaldehyde, 99%, lot number A013867501. The quantitation limit of this method was calculated to be approximately 0.5 ppm using the following equation:

(conc. of lowest standard) x (vol) x (dil) / (sample weight) = quantitation limit (ppm)

4.2. Acid detergent fiber (ADF)

The method used was based on an USDA Agriculture Handbook No. 379 (1970) method. The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. An acetone wash was used to remove the fats and pigments. The lignocellulose fraction was collected on the frit and determined gravimetrically. The limit of quantitation of this method was 0.1% fw.

4.3. Amino acid composition (TAAP)

The method used was based on AOAC International (2000) method 982.30 that estimates the levels of 18 amino acids in the sample: alanine, arginine, aspartic acid (including asparagine), cystine (including cysteine), glutamic acid (including glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis using sodium hydroxide. Sulfur-containing amino acids required an oxidation using performic acid prior to hydrolysis with hydrochloric acid. Analysis of the remaining amino acids was accomplished through direct hydrolysis with hydrochloric acid. The individual amino acids were quantitated using an automated amino acid analyzer. The limit of quantitation

of this method was 0.1 mg/g fw. The reference standards were Pierce K18, lot number DJ58806; Sigma-Aldrich L-Tryptophan, 100%, lot number 88HO4391; Sigma-Aldrich L-Cysteic acid monohydrate, 100%, lot number 111K2608; Sigma-Aldrich L-Methionine sulfone, 100%, lot number 12H3349.

4.4. Ash (ASHM)

The method used was based on AOAC International (2000) method 923.03. The sample was placed in an electric furnace at 550 °C and ignited to drive off volatile organic compounds. The nonvolatile matter remaining was quantitated gravimetrically and the percent ash was determined by calculation. The limit of quantitation of this method was 0.1% fw.

4.5. Carbohydrates (CHO)

The method used was based on an USDA Agriculture Handbook No. 74 (1973) method. The limit of quantitation of this method was 1.0% fw. Carbohydrate values were calculated by difference using the fresh weight-derived data and the following equation:

$$\% \text{ carbohydrates} = 100\% - (\% \text{ protein} + \% \text{ fat} + \% \text{ ash} + \% \text{ moisture})$$

4.6. Fat by acid hydrolysis (FAAH)

The method used was based on AOAC International (2000) methods 922.06 and 954.02. The forage sample was hydrolyzed with hydrochloric acid at an elevated temperature. The fat was extracted using diethyl ether followed by hexane. Extracts were washed with a dilute alkali solution and filtered through a sodium sulfate column. The extract was evaporated, dried, and weighed. The limit of quantitation of this method was 0.1% fw.

4.7. Fat by soxhlet extraction (FSOX)

The method used was based on AOAC International (2000) method 960.39. The grain sample was weighed into a cellulose thimble containing sand or sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was evaporated, dried, and weighed. This method was used for grain sample analysis. The limit of quantitation of this method was 0.1% fw.

4.8. Fatty acids (FAPM)

The method used was based on AOCS (1997) method Ce 1-62 that estimates the levels of 22 fatty acids in the sample: 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 16:0 palmitic acid, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic, 18:3 gamma linolenic acid, 20:0 arachidic acid, 20:1 eicosenoic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, and 22:0 behenic acid. Lipid in grain samples

was extracted and saponified with 0.5 N sodium hydroxide in methanol. The saponification mixture was methylated with 14% (weight/volume) boron trifluoride:methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation. The limit of quantitation of this method was 0.003% fw. The reference standards were Nu Chek Prep GLC reference standard Hazelton no. 1, used as 100%, lot number O2-M; Nu Chek Prep GLC reference standard Hazelton no. 2, used as 100%, lot number JA10-H; Nu Chek Prep GLC reference standard Hazelton no. 3, used as 100%, lot number M18-L; Nu Chek Prep GLC reference standard Hazelton no. 4, used as 100%, lot number O2-M; and Nu Chek Prep methyl gamma linolenate, used as 100%, lot number U-63M-A26-L.

4.9. Folic acid (FOAN)

The method used was based on AOAC International (2000) methods 960.46 and 992.05 and Methods of Analysis for Infant Formulas (1973), Section C-2. The sample was hydrolyzed in potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis, the sample was treated with a chicken-pancreas enzyme and incubated approximately 18 hours to liberate the bound folic acid. The amount of folic acid was turbidimetrically determined by comparing the growth response of the bacteria *Lactobacillus casei* in the sample versus the growth response in folic acid standard. The limit of quantitation of this method was 0.06 µg/g fw. The reference standard was USP, folic acid, 100%, lot number P.

4.10. Minerals/ICP emission spectrometry (ICPS)

The method used was based on AOAC International (2000) methods 984.27 and 985.01 and a literature method (Dahlquist and Knoll, 1978). Samples were dried, precharred, and ashed overnight at 500° ± 50°C. Ashed samples were treated with hydrochloric acid, dried, and dissolved in 5% (v/v) hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, using inductively coupled plasma, with the emission of the standard solutions. The limits of quantitation of this method and Spex CertiPrep reference standards are listed in the table below.

Mineral	Lot Numbers	Concentration (mg/L)	Limit of Quantitation (ppm)
Calcium	O8-67CA	10,000	20.0
Copper	9-38CU	10,000	0.500
Iron	9-02FE	1,000	2.00
Magnesium	S8-87MG	10,000	20.0
Manganese	9-16MN	10,000	0.300
Phosphorus	P8-77P	10,000	20.0
Potassium	Q8-108K	10,000	100
Sodium	P7-176NA	10,000	100
Zinc	9-34ZN	1,000	0.400

4.11. Moisture (M100).

The method used was based on AOAC International (2000) methods 926.08 and 925.09. Samples were dried in a vacuum oven at 100°C to a constant weight. The moisture loss was determined and converted to percent moisture. The limit of quantitation of this method was 0.1% fw.

4.12. Neutral detergent fiber, enzyme method (NDFE)

The method used was based on AACC (1998) method 32.20 and a USDA Agriculture Handbook No. 379 (1970) method. Samples were placed in a fritted vessel and washed with a neutral boiling detergent solution to dissolve the protein, carbohydrate, enzyme, and ash. Fats and pigments were removed using an acetone wash. The hemicellulose, cellulose, and lignin fractions were collected on a frit and determined gravimetrically. The limit of quantitation of this method was 0.1% fw.

4.13. Niacin (NIAP)

The method used was based on AOAC International (2000) method 944.13. The sample was hydrolyzed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was turbidimetrically determined by comparing the growth response of the bacteria *Lactobacillus plantarun* in the samples versus the growth response in niacin standard. The limit of quantitation of this method was 0.3 µg/g fw. The reference standard was USP, niacin, 100% lot number H-1.

4.14. p-Coumaric and ferulic acids (ACID)

The method was based on a literature method (Hagerman and Nicholson, 1982). The samples were extracted with methanol using ultrasonication, and the extracts then hydrolyzed using 4 N sodium hydroxide, buffered using acetic acid/sodium hydroxide,

acidified with 3 N hydrochloric acid, and filtered. The levels of p-coumaric and ferulic acids in the extracts were determined by RP-HPLC with UV quantitation. The reference standards were ACROS p-Hydroxycinnamic acid (p-coumaric acid), 98.8%, lot number A014307101 and ACROS 4-Hydroxy-3-methoxycinnamic acid (ferulic acid), 100%, lot number A11176601. The limit of quantitation for both analytes was calculated to be approximately 50.0 ppm using the following equation:

(conc. of lowest standard) x (vol) x (dil) / (sample weight) = quantitation limit (ppm)

4.15. Phytic acid (PHYT)

The method used was based on two literature methods (Lehrfeld 1989, 1994). Samples were extracted using 0.5 M HCl with ultrasonication. Purification and concentration was performed using a silica-based anion exchange (SAX) column. Sample analysis was conducted using a macroporous polymer HPLC column [PRP-1, 5µm (150 x 4.1 mm)] connected to a refractive index detector. The limit of quantitation of this method was approximately 0.133% fw. The reference standard was Aldrich phytic acid, dodecasodium salt hydrate, 94%, lot number 14403DO.

4.16. Protein (PGEN)

The method used was based on AOAC International (2000) methods 955.04 and 979.09 and two literature methods (Bradstreet, 1965; Kalthoff and Sandell, 1948). Protein and other nitrogenous compounds in the sample were reduced to ammonia by digestion of the sample with sulfuric acid containing a mercury catalyst mixture. The acid digest was made alkaline, and the ammonia was distilled and titrated with a standard acid. The percent nitrogen was determined and converted to percent protein by multiplication with 6.25. The limit of quantitation of this method was 0.1% fw.

4.17. Pyridoxine/vitamin B_6 (B6A)

The method used was based on AOAC International (2000) method 961.15. The sample was hydrolyzed with dilute sulfuric acid. The amount of pyridoxine was turbidimetrically determined by comparing the growth response of the yeast *Saccharromyces carlsbergenesis* in the sample with the growth response in pyridoxine standard. The limit of quantitation of this method was 0.07 µg/g fw. The reference standard was USP pyridoxine, 100%.

4.18. Raffinose (SUGT)

This method was based on two literature methods (Mason and Slover, 1971; Brobst, 1972). The grain samples were extracted with deionized water and the extracts treated with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucoside as an internal standard. The resulting oximes were converted to silyl derivatives by treatment with hexamethyldisilane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector. The reference standard was

Sigma, D(+)-raffinose, 99%, lot number 091K12741. The quantitation limit of this method was calculated to be 0.0358% fw using the following equation:

(conc. of lowest standard) x (vol) x (dil) / (sample size) = quantitation limit (%)

4.19. Riboflavin/vitamin B_2 (B2FV)

The method used was based on AOAC International (2000) method 940.33. The sample was hydrolyzed with dilute HCl. The amount of riboflavin was determined by comparing the growth response of the bacteria, *Lactobacillus casei*, in the sample hydrolysate with the bacterial growth response in varying amounts of riboflavin standard. The bacterial growth response was measured turbidimetrically. The limit of quantitation of this method was 0.2 µg/g fw. The reference standard was USP riboflavin, 100%.

4.20. Thiamin/vitamin B_1 (BIDE).

The method used was based on AOAC International (2000) methods 942.23, 953.17, and 957.17. The sample was autoclaved under weak acid conditions to extract the thiamin. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamin. The solution was purified on an ion-exchange column. An aliquot was taken and reacted with potassium ferricyanide to convert thiamin to thiochrome. The thiochrome was extracted into isobutyl alcohol and read on a fluorometer against a known standard. The limit of quantitation of this method was 0.01mg/100g fw. The reference standard was USP, thiamin, 100%.

4.21. Total dietary fiber (TDF)

The method used was based on AOAC International (2000) method 985.29. Duplicate samples were gelatinized with alpha-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The samples were filtered and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the sample was calculated using the protein and ash values. The limit of quantitation of this method was approximately 1.0% fw.

4.22. Vitamin E (EFD2)

The method used was based on three literature methods (Cort et al., 1983; McMurray et al., 1980; Speek et al., 1985). Samples were saponified to break down fat and release vitamin E. The saponified mixture was extracted with ethyl ether and quantitated directly by HPLC on a silica column. The limit of quantitation of this method was approximately 0.003 mg/g fw. The reference standard was USP alpha tocopherol, 100%.

5.0. Control of bias

The test, control, and reference substances from each respective plot within the field sites were produced under similar agronomic conditions. To control and/or minimize bias, the samples were analyzed in the order specified by a computer-generated randomized sample list. The Study Director generated the randomized sample list and forwarded it to Covance Laboratories Inc. prior to analysis.

6.0. Statistical analysis

6.1. Data processing

The data were statistically analyzed by Certus International, Inc. The following formulas were used for re-expression of the data for statistical analysis:

Component	From (X)	To	Formula
Proximates (excluding moisture), Fiber, Phytic Acid, Raffinose	% FW	% DW	X/d*
Calcium, Magnesium, Phosphorus, Potassium, Sodium	ppm FW	% DW	(X/d) X 10^{-4}
Copper, Iron, Manganese, Zinc	ppm FW	mg/kg DW	X/d
Ferulic Acid, Furfural	ppm FW	µg/g DW	X/d
Niacin, Folic Acid, Vitamin B_2, Vitamin B_6	µg/g FW	mg/kg DW	X/d
Vitamin B_1	mg/100g FW	mg/kg DW	10 (X/d)
Vitamin E	mg/g FW	mg/kg DW	10^3 (X/d)
Amino Acids (AA)	mg/g FW	% Total AA	(100)$X_j/\Sigma X_j$, for each AA j
Fatty Acids (FA)	g/100g FW	% Total FA	(100)Xj/Σ Xj, for each FA j
* d is the fraction of the sample that is dry matter.			

The following 15 compositional analytes with >50% of observations below the LOQ of the assay were excluded from statistical analysis: sodium, 2-furaldehyde, 8:0 caprylic acid, 10:0 capric acid, 12:0 lauric acid, 14:0 myristic acid, 14:1 myristoleic acid, 15:0 pentadecanoic acid, 15:1 pentadecenoic acid, 17:0 heptadecanoic acid, 17:1 heptadecenoic acid, 18:3 gamma linolenic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, and 20:4 arachidonic acid.

There were observations below the LOQ observed for 16:1 palmitoleic acid (five values), 22:0 behenic acid (one value) and vitamin E (one value). To include a complete data set for these analytes in the statistical analysis, a value equal to 50% of the LOQ was assigned for these seven data points. The data was also assessed for potential outliers using a studentized PRESS residuals calculation and no outliers were identified in this evaluation.

6.2. Statistical methodology

At the field sites, the test, control, and reference substances were grown in single plots randomly assigned within each of three replication blocks. The compositional components for the test and control substances were statistically analyzed using a mixed model analysis of variance. The three replicated sites were analyzed separately and combined. Individual replicated site analyses used the model:

$$Y_{ij} = U + T_i + B_j + e_{ij} ,$$

where Y_{ij} = unique individual observation, U = overall mean, T_i = hybrid effect, B_j = random block effect, and e_{ij} = residual error.

Combined site analyses used the model:

$$Y_{ijk} = U + T_i + L_j + B(L)_{jk} + LT_{ij} + e_{ijk} ,$$

where Y_{ijk} = unique individual observation, U = overall mean, T_i = hybrid effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by hybrid interaction effect, and e_{ijk} = residual error. For each compositional component, the forage and grain from the test substance was compared to the control.

A range of observed values from the reference substances were determined for each analytical component. Additionally, the reference substances data were used to develop population tolerance intervals. A tolerance interval is an interval that one can claim, with a specified degree of confidence, contains at least a specified proportion, p, of an entire sampled population for the parameter measured. For each compositional component, 99% tolerance intervals were calculated that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of conventional hybrids. Each tolerance interval estimate was based upon one observation per reference. Individual hybrids with multiple observations were summarized across sites to obtain a single estimate for inclusion in tolerance interval calculations. Because negative quantities are not possible, calculated negative lower tolerance bounds were set to zero.

SAS® software was used to generate all summary statistics and perform all analyses. Report tables present p-values from SAS® as either <0.001 or the actual value truncated to three decimal places.

Table 1. Comparison of the proximates, fiber, and mineral content in forage collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ash (% dw)	3.62 ± 0.24 (3.30 - 3.90)	4.06 ± 0.24 (3.74 - 4.67)	-0.44 ± 0.35 (-1.37 - 0.16)	-1.28,0.41	0.254	(2.62 - 6.78) [0.72,7.42]
Carbohydrates (% dw)	87.27 ± 0.48 (86.19 - 87.88)	87.30 ± 0.48 (86.78 - 87.71)	-0.029 ± 0.57 (-1.22 - 1.10)	-1.42,1.36	0.960	(81.86 - 89.90) [78.70,93.43]
Fat, total (% dw)	1.35 ± 0.24 (1.02 - 1.97)	1.16 ± 0.24 (0.83 - 1.63)	0.19 ± 0.31 (-0.0062 - 0.34)	-0.57,0.95	0.560	(0.69 - 2.92) [0.80,2.95]
Moisture (% fw)	69.70 ± 0.51 (68.50 - 70.70)	69.67 ± 0.51 (69.40 - 69.80)	0.033 ± 0.65 (-1.30 - 0.90)	-1.56,1.62	0.960	(65.20 - 78.60) [59.37,80.83]
Protein (% dw)	7.75 ± 0.39 (7.44 - 7.94)	7.48 ± 0.39 (6.79 - 8.43)	0.27 ± 0.55 (-0.99 - 1.10)	-1.07,1.62	0.633	(6.31 - 9.96) [4.17,11.81]
ADF (% dw)	26.05 ± 0.80 (24.32 - 28.23)	25.92 ± 0.80 (24.17 - 26.90)	0.14 ± 1.01 (-2.58 - 1.54)	-2.33,2.60	0.897	(19.16 - 35.55) [13.95,38.96]
NDF (% dw)	39.48 ± 2.39 (36.51 - 45.05)	40.11 ± 2.39 (38.08 - 41.18)	-0.63 ± 3.38 (-4.55 - 6.97)	-8.89,7.64	0.858	(30.27 - 57.93) [23.80,54.73]
Calcium (% dw)	0.24 ± 0.021 (0.22 - 0.26)	0.27 ± 0.021 (0.20 - 0.31)	-0.027 ± 0.019 (-0.049 - 0.011)	-0.073,0.018	0.190	(0.13 - 0.32) [0.11,0.32]
Phosphorus (% dw)	0.23 ± 0.011 (0.21 - 0.25)	0.26 ± 0.011 (0.25 - 0.27)	-0.034 ± 0.015 (-0.060 - -0.018)	-0.071,0.0030	0.065	(0.16 - 0.31) [0.095,0.38]

[a]dw=dry weight; fw=fresh weight; ADF=acid detergent fiber; NDF=neutral detergent fiber, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval

[c]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 2. Comparison of the of proximates, fiber, and mineral content in forage collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ash (% dw)	4.40 ± 0.43 (3.60 - 5.53)	3.86 ± 0.43 (3.59 - 4.01)	0.54 ± 0.48 (0.016 - 1.55)	-0.63,1.71	0.305	(2.62 - 6.78) [0.72,7.42]
Carbohydrates (% dw)	86.05 ± 0.62 (84.13 - 87.21)	86.56 ± 0.62 (85.94 - 87.06)	-0.51 ± 0.73 (-1.80 - 0.15)	-2.29,1.27	0.510	(81.86 - 89.90) [78.70,93.43]
Fat, total (% dw)	1.40 ± 0.24 (0.80 - 1.75)	1.61 ± 0.24 (1.32 - 1.87)	-0.21 ± 0.34 (-0.83 - 0.31)	-1.05,0.63	0.558	(0.69 - 2.92) [0.80,2.95]
Moisture (% fw)	70.77 ± 0.63 (70.20 - 71.80)	70.00 ± 0.63 (69.10 - 71.00)	0.77 ± 0.65 (-0.70 - 1.90)	-0.81,2.35	0.280	(65.20 - 78.60) [59.37,80.83]
Protein (% dw)	8.16 ± 0.20 (7.52 - 8.58)	7.97 ± 0.20 (7.72 - 8.21)	0.19 ± 0.29 (-0.48 - 0.66)	-0.52,0.89	0.541	(6.31 - 9.96) [4.17,11.81]
ADF (% dw)	25.53 ± 0.75 (24.81 - 26.17)	24.10 ± 0.75 (23.34 - 24.72)	1.43 ± 0.97 (0.88 - 1.95)	-0.93,3.80	0.188	(19.16 - 35.55) [13.95,38.96]
NDF (% dw)	37.20 ± 1.22 (33.89 - 39.06)	36.76 ± 1.22 (35.86 - 37.22)	0.44 ± 1.68 (-3.32 - 3.20)	-3.67,4.55	0.803	(30.27 - 57.93) [23.80,54.73]
Calcium (% dw)	0.22 ± 0.015 (0.20 - 0.25)	0.23 ± 0.015 (0.21 - 0.25)	-0.0045 ± 0.022 (-0.054 - 0.024)	-0.058,0.048	0.841	(0.13 - 0.32) [0.11,0.32]
Phosphorus (% dw)	0.26 ± 0.016 (0.23 - 0.28)	0.23 ± 0.016 (0.20 - 0.25)	0.034 ± 0.021 (-0.018 - 0.079)	-0.018,0.086	0.163	(0.16 - 0.31) [0.095,0.38]

[a]dw=dry weight; fw=fresh weight; ADF=acid detergent fiber; NDF=neutral detergent fiber, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval

[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 3. Comparison of the proximates, fiber, and mineral content in forage collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
Ash (% dw)	3.96 ± 0.20 (3.59 - 4.36)	4.21 ± 0.20 (4.06 - 4.32)	-0.25 ± 0.19 (-0.73 - 0.10)	-0.71,0.20	0.224	(2.62 - 6.78) [0.72,7.42]
Carbohydrates (% dw)	85.25 ± 0.69 (83.54 - 86.16)	85.58 ± 0.69 (84.43 - 86.18)	-0.32 ± 0.91 (-2.58 - 1.73)	-2.56,1.91	0.735	(81.86 - 89.90) [78.70,93.43]
Fat, total (% dw)	2.10 ± 0.46 (1.49 - 3.13)	2.19 ± 0.46 (1.14 - 2.97)	-0.095 ± 0.65 (-1.47 - 1.99)	-1.68,1.49	0.888	(0.69 - 2.92) [0.80,2.95]
Moisture (% fw)	72.10 ± 0.47 (70.90 - 72.70)	72.30 ± 0.47 (71.90 - 72.70)	-0.20 ± 0.66 (-1.40 - 0.80)	-1.81,1.41	0.771	(65.20 - 78.60) [59.37,80.83]
Protein (% dw)	8.69 ± 0.35 (8.42 - 8.97)	8.02 ± 0.35 (7.03 - 8.54)	0.67 ± 0.50 (-0.12 - 1.65)	-0.55,1.89	0.226	(6.31 - 9.96) [4.17,11.81]
ADF (% dw)	28.05 ± 1.32 (24.29 - 29.97)	26.32 ± 1.32 (24.95 - 28.13)	1.73 ± 1.83 (-0.66 - 4.08)	-2.75,6.20	0.381	(19.16 - 35.55) [13.95,38.96]
NDF (% dw)	35.35 ± 1.40 (33.44 - 37.73)	38.14 ± 1.40 (37.36 - 39.50)	-2.79 ± 1.98 (-4.63 - 0.37)	-7.63,2.05	0.207	(30.27 - 57.93) [23.80,54.73]
Calcium (% dw)	0.21 ± 0.012 (0.19 - 0.22)	0.20 ± 0.012 (0.18 - 0.24)	0.0044 ± 0.012 (-0.024 - 0.023)	-0.025,0.034	0.727	(0.13 - 0.32) [0.11,0.32]
Phosphorus (% dw)	0.27 ± 0.019 (0.23 - 0.30)	0.26 ± 0.019 (0.24 - 0.30)	0.0055 ± 0.014 (-0.0066 - 0.021)	-0.028,0.039	0.705	(0.16 - 0.31) [0.095,0.38]

[a]dw=dry weight; fw=fresh weight; ADF=acid detergent fiber; NDF=neutral detergent fiber, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4. Comparison of the amino acid content in grain collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
Alanine (% total AA)	7.37 ± 0.031 (7.29 – 7.46)	7.36 ± 0.031 (7.34 - 7.40)	0.0039 ± 0.039 (-0.055 - 0.12)	-0.091,0.099	0.923	(7.24 - 8.16) [6.66,8.49]
Arginine (% total AA)	4.66 ± 0.065 (4.57 – 4.74)	4.50 ± 0.065 (4.40 - 4.63)	0.15 ± 0.093 (-0.060 - 0.34)	-0.075,0.38	0.153	(3.72 - 5.08) [3.34,5.67]
Aspartic acid (% total AA)	6.23 ± 0.058 (6.09 – 6.32)	6.18 ± 0.058 (6.04 - 6.28)	0.047 ± 0.065 (0.0070 - 0.087)	-0.11,0.21	0.497	(6.18 - 6.81) [5.77,7.16]
Cystine (% total AA)	2.16 ± 0.037 (2.10 – 2.26)	2.26 ± 0.037 (2.20 - 2.30)	-0.11 ± 0.053 (-0.19 - -0.028)	-0.24,0.022	0.089	(1.82 - 2.58) [1.46,2.89]
Glutamic acid (% total AA)	20.08 ± 0.079 (19.80 - 20.24)	20.02 ± 0.079 (19.91 - 20.10)	0.060 ± 0.11 (-0.26 - 0.34)	-0.20,0.32	0.599	(19.46 - 21.57) [18.01,22.15]
Glycine (% total AA)	3.55 ± 0.032 (3.47 – 3.62)	3.56 ± 0.032 (3.50 - 3.61)	-0.0069 ± 0.042 (-0.058 - 0.064)	-0.11,0.096	0.875	(3.29 - 4.03) [2.81,4.54]
Histidine (% total AA)	3.07 ± 0.032 (3.02 – 3.10)	3.08 ± 0.032 (2.99 - 3.14)	-0.010 ± 0.038 (-0.034 - 0.026)	-0.10,0.082	0.796	(2.50 - 3.12) [2.16,3.60]
Isoleucine (% total AA)	3.52 ± 0.046 (3.43 – 3.57)	3.53 ± 0.046 (3.51 - 3.54)	-0.0057 ± 0.062 (-0.099 - 0.048)	-0.16,0.15	0.930	(3.39 - 3.79) [3.30,3.84]
Leucine (% total AA)	12.93 ± 0.085 (12.69 - 13.11)	12.91 ± 0.085 (12.76 - 13.04)	0.016 ± 0.094 (-0.25 - 0.23)	-0.21,0.25	0.867	(12.11 - 14.35) [10.72,15.18]

[a]AA=amino acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 4 (cont.) Comparison of the amino acid content in grain collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Lysine (% total AA)	2.77 ± 0.059 (2.67 - 2.87)	2.72 ± 0.059 (2.57 - 2.82)	0.053 ± 0.082 (-0.045 - 0.11)	-0.15,0.25	0.541	(2.44 - 3.27) [2.06,3.73]
Methionine (% total AA)	2.02 ± 0.029 (1.96 - 2.05)	2.16 ± 0.029 (2.11 - 2.20)	-0.14 ± 0.015 (-0.15 - -0.12)	-0.17,-0.10	<0.001	(1.70 - 2.47) [1.37,2.60]
Phenylalanine (% total AA)	5.10 ± 0.067 (4.97 - 5.18)	5.08 ± 0.067 (5.01 - 5.22)	0.019 ± 0.080 (-0.037 - 0.13)	-0.18,0.21	0.818	(4.82 - 5.39) [4.57,5.71]
Proline (% total AA)	9.50 ± 0.15 (9.32 - 9.69)	9.63 ± 0.15 (9.54 - 9.80)	-0.13 ± 0.21 (-0.22 - -0.050)	-0.65,0.39	0.572	(8.35 - 9.72) [7.60,10.37]
Serine (% total AA)	4.87 ± 0.093 (4.65 - 5.03)	4.85 ± 0.093 (4.64 - 4.96)	0.024 ± 0.10 (-0.0024 - 0.063)	-0.22,0.27	0.815	(4.81 - 5.23) [4.60,5.43]
Threonine (% total AA)	3.29 ± 0.056 (3.15 - 3.38)	3.26 ± 0.056 (3.15 - 3.33)	0.035 ± 0.070 (0.0034 - 0.084)	-0.14,0.21	0.637	(2.96 - 3.55) [2.89,3.84]
Tryptophan (% total AA)	0.56 ± 0.017 (0.53 - 0.58)	0.59 ± 0.017 (0.58 - 0.60)	-0.032 ± 0.024 (-0.053 - -0.013)	-0.090,0.026	0.221	(0.44 - 0.83) [0.36,0.77]
Tyrosine (% total AA)	3.59 ± 0.20 (3.48 - 3.66)	3.57 ± 0.20 (3.52 - 3.66)	0.026 ± 0.28 (-0.052 - 0.14)	-0.66,0.71	0.929	(2.26 - 3.80) [2.62,4.26]
Valine (% total AA)	4.73 ± 0.066 (4.60 - 4.82)	4.74 ± 0.066 (4.67 - 4.77)	-0.0077 ± 0.088 (-0.069 - 0.045)	-0.22,0.21	0.933	(4.44 - 5.04) [4.22,5.27]

[a]AA=amino acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 5. Comparison of the amino acid content in grain collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Alanine (% total AA)	7.65 ± 0.059 (7.56 - 7.70)	7.59 ± 0.059 (7.52 - 7.65)	0.057 ± 0.079 (-0.087 - 0.18)	-0.14,0.25	0.498	(7.24 - 8.16) [6.66,8.49]
Arginine (% total AA)	4.24 ± 0.066 (4.10 - 4.35)	4.23 ± 0.066 (4.20 - 4.27)	0.014 ± 0.060 (-0.12 – 0.15)	-0.13,0.16	0.827	(3.72 - 5.08) [3.34,5.67]
Aspartic acid (% total AA)	6.16 ± 0.071 (6.11 - 6.24)	6.29 ± 0.071 (6.06 - 6.45)	-0.13 ± 0.10 (-0.34 – 0.18)	-0.37,0.12	0.253	(6.18 - 6.81) [5.77,7.16]
Cystine (% total AA)	2.21 ± 0.045 (2.18 - 2.24)	2.17 ± 0.045 (2.12 - 2.19)	0.042 ± 0.057 (-0.0079 - 0.11)	-0.097,0.18	0.488	(1.82 - 2.58) [1.46,2.89]
Glutamic acid (% total AA)	20.66 ± 0.14 (20.43 - 20.87)	20.59 ± 0.14 (20.39 - 20.84)	0.069 ± 0.19 (-0.40 – 0.48)	-0.39,0.53	0.728	(19.46 - 21.57) [18.01,22.15]
Glycine (% total AA)	3.40 ± 0.042 (3.32 - 3.46)	3.44 ± 0.042 (3.31 - 3.54)	-0.035 ± 0.055 (-0.081 - 0.0027)	-0.17,0.10	0.554	(3.29 - 4.03) [2.81,4.54]
Histidine (% total AA)	2.95 ± 0.024 (2.93 - 2.99)	2.89 ± 0.024 (2.87 - 2.92)	0.063 ± 0.034 (0.046 - 0.073)	-0.019,0.14	0.110	(2.50 - 3.12) [2.16,3.60]
Isoleucine (% total AA)	3.64 ± 0.064 (3.53 - 3.71)	3.54 ± 0.064 (3.45 - 3.67)	0.11 ± 0.090 (-0.15 - 0.25)	-0.11,0.33	0.284	(3.39 - 3.79) [3.30,3.84]
Leucine (% total AA)	13.54 ± 0.13 (13.46 - 13.62)	13.35 ± 0.13 (13.06 - 13.69)	0.18 ± 0.16 (-0.16 - 0.56)	-0.20,0.57	0.285	(12.11 - 14.35) [10.72,15.18]

[a]AA=amino acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 5 (cont.) Comparison of the amino acid content in grain collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Lysine (% total AA)	2.64 ± 0.070 (2.42 - 2.78)	2.62 ± 0.070 (2.49 - 2.71)	0.019 ± 0.099 (-0.072 - 0.070)	-0.22,0.26	0.854	(2.44 - 3.27) [2.06,3.73]
Methionine (% total AA)	2.01 ± 0.018 (1.99 - 2.03)	1.99 ± 0.018 (1.96 - 2.02)	0.017 ± 0.026 (-0.0043 - 0.046)	-0.047,0.081	0.534	(1.70 - 2.47) [1.37,2.60]
Phenylalanine (% total AA)	5.27 ± 0.071 (5.19 - 5.31)	5.16 ± 0.071 (5.05 - 5.32)	0.11 ± 0.10 (-0.13 - 0.25)	-0.14,0.35	0.330	(4.82 - 5.39) [4.57,5.71]
Proline (% total AA)	9.34 ± 0.15 (9.02 - 9.56)	9.17 ± 0.15 (8.85 - 9.63)	0.17 ± 0.22 (-0.61 - 0.71)	-0.36,0.70	0.470	(8.35 - 9.72) [7.60,10.37]
Serine (% total AA)	4.81 ± 0.11 (4.66 - 5.04)	4.91 ± 0.11 (4.63 - 5.13)	-0.10 ± 0.16 (-0.47 - 0.42)	-0.48,0.28	0.544	(4.81 - 5.23) [4.60,5.43]
Threonine (% total AA)	3.19 ± 0.080 (3.10 - 3.30)	3.26 ± 0.080 (3.06 - 3.37)	-0.071 ± 0.11 (-0.25 - 0.24)	-0.35,0.21	0.554	(2.96 - 3.55) [2.89,3.84]
Tryptophan (% total AA)	0.56 ± 0.033 (0.51 - 0.60)	0.58 ± 0.033 (0.53 - 0.68)	-0.023 ± 0.046 (-0.17 - 0.062)	-0.14,0.089	0.628	(0.44 - 0.83) [0.36,0.77]
Tyrosine (% total AA)	2.90 ± 0.24 (2.35 - 3.41)	3.44 ± 0.24 (3.29 - 3.53)	-0.53 ± 0.34 (-1.18 - 0.12)	-1.36,0.29	0.162	(2.26 - 3.80) [2.62,4.26]
Valine (% total AA)	4.82 ± 0.084 (4.69 - 4.92)	4.77 ± 0.084 (4.60 - 4.94)	0.047 ± 0.11 (-0.25 - 0.26)	-0.23,0.32	0.691	(4.44 - 5.04) [4.22,5.27]

[a]AA=amino acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 6. Comparison of the amino acid content in grain collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Alanine (% total AA)	7.63 ± 0.038 (7.59 - 7.69)	7.70 ± 0.038 (7.59 - 7.79)	-0.068 ± 0.054 (-0.19 - 0.095)	-0.20,0.063	0.248	(7.24 - 8.16) [6.66,8.49]
Arginine (% total AA)	4.37 ± 0.069 (4.28 - 4.49)	4.13 ± 0.069 (4.01 - 4.25)	0.24 ± 0.098 (0.085 - 0.36)	-0.0022,0.48	0.051	(3.72 - 5.08) [3.34,5.67]
Aspartic acid (% total AA)	6.27 ± 0.059 (6.18 - 6.34)	6.29 ± 0.059 (6.11 - 6.40)	-0.017 ± 0.068 (-0.10 - 0.064)	-0.18,0.15	0.811	(6.18 - 6.81) [5.77,7.16]
Cystine (% total AA)	2.06 ± 0.071 (1.93 - 2.20)	2.03 ± 0.071 (1.93 - 2.14)	0.027 ± 0.10 (-0.20 - 0.17)	-0.22,0.27	0.795	(1.82 - 2.58) [1.46,2.89]
Glutamic acid (% total AA)	20.47 ± 0.085 (20.33 - 20.62)	20.71 ± 0.085 (20.47 - 20.84)	-0.24 ± 0.12 (-0.52 - 0.15)	-0.53,0.058	0.096	(19.46 - 21.57) [18.01,22.15]
Glycine (% total AA)	3.41 ± 0.052 (3.37 - 3.45)	3.35 ± 0.052 (3.18 - 3.44)	0.060 ± 0.063 (-0.037 - 0.19)	-0.094,0.21	0.378	(3.29 - 4.03) [2.81,4.54]
Histidine (% total AA)	2.93 ± 0.029 (2.90 - 2.95)	2.88 ± 0.029 (2.83 - 2.96)	0.044 ± 0.041 (-0.056 - 0.10)	-0.056,0.14	0.322	(2.50 - 3.12) [2.16,3.60]
Isoleucine (% total AA)	3.61 ± 0.056 (3.56 - 3.64)	3.63 ± 0.056 (3.45 - 3.76)	-0.027 ± 0.079 (-0.12 - 0.16)	-0.22,0.17	0.748	(3.39 - 3.79) [3.30,3.84]
Leucine (% total AA)	13.36 ± 0.14 (13.21 - 13.46)	13.67 ± 0.14 (13.26 - 14.11)	-0.31 ± 0.20 (-0.69 - 0.20)	-0.79,0.17	0.165	(12.11 - 14.35) [10.72,15.18]

[a]AA=amino acids
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 6 (cont.) Comparison of the amino acid content in grain collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Lysine (% total AA)	2.65 ± 0.061 (2.60 - 2.70)	2.65 ± 0.061 (2.50 - 2.74)	0.00084 ± 0.070 (-0.057 - 0.095)	-0.17,0.17	0.990	(2.44 - 3.27) [2.06,3.73]
Methionine (% total AA)	1.91 ± 0.042 (1.85 - 1.95)	1.88 ± 0.042 (1.83 - 1.94)	0.031 ± 0.059 (-0.085 - 0.12)	-0.11,0.18	0.614	(1.70 - 2.47) [1.37,2.60]
Phenylalanine (% total AA)	5.16 ± 0.046 (5.13 - 5.22)	5.18 ± 0.046 (5.14 - 5.20)	-0.020 ± 0.066 (-0.077 - 0.075)	-0.18,0.14	0.776	(4.82 - 5.39) [4.57,5.71]
Proline (% total AA)	9.33 ± 0.094 (9.14 - 9.64)	9.23 ± 0.094 (9.12 - 9.41)	0.098 ± 0.13 (-0.20 - 0.51)	-0.23,0.42	0.490	(8.35 - 9.72) [7.60,10.37]
Serine (% total AA)	4.80 ± 0.046 (4.80 - 4.81)	4.97 ± 0.046 (4.82 - 5.09)	-0.17 ± 0.065 (-0.29 - -0.026)	-0.33,-0.0084	0.042	(4.81 - 5.23) [4.60,5.43]
Threonine (% total AA)	3.19 ± 0.027 (3.14 - 3.24)	3.23 ± 0.027 (3.15 - 3.29)	-0.042 ± 0.038 (-0.12 - 0.0037)	-0.14,0.052	0.317	(2.96 - 3.55) [2.89,3.84]
Tryptophan (% total AA)	0.50 ± 0.018 (0.48 - 0.51)	0.47 ± 0.018 (0.41 - 0.51)	0.029 ± 0.025 (-0.012 - 0.096)	-0.032,0.090	0.292	(0.44 - 0.83) [0.36,0.77]
Tyrosine (% total AA)	3.54 ± 0.25 (3.50 - 3.58)	3.27 ± 0.25 (2.58 - 3.62)	0.27 ± 0.35 (-0.12 - 0.98)	-0.59,1.14	0.470	(2.26 - 3.80) [2.62,4.26]
Valine (% total AA)	4.81 ± 0.060 (4.77 - 4.83)	4.72 ± 0.060 (4.65 - 4.85)	0.090 ± 0.084 (-0.077 - 0.19)	-0.12,0.30	0.326	(4.44 - 5.04) [4.22,5.27]

[a]AA=amino acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 7. Comparison of the fatty acid content in grain collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
16:0 palmitic (% total FA)	10.16 ± 0.71 (10.11 - 10.23)	12.94 ± 0.71 (10.14 - 14.57)	-2.78 ± 0.98 (-4.35 - -0.033)	-5.18,-0.39	0.029	(9.29 - 17.81) [6.51,16.50]
16:1 pamitoleic (% total FA)	0.20 ± 0.0049 (0.19 - 0.21)	0.21 ± 0.0049 (0.20 - 0.22)	-0.0084 ± 0.0069 (-0.021 - -0.0021)	-0.025,0.0084	0.265	(0.054 - 0.21) [0.0017,0.28]
18:0 stearic (% total FA)	1.84 ± 0.066 (1.80 - 1.88)	2.01 ± 0.066 (1.76 - 2.15)	-0.17 ± 0.088 (-0.28 - 0.041)	-0.38,0.047	0.104	(1.68 - 2.30) [1.41,2.53]
18:1 oleic (% total FA)	22.56 ± 0.27 (22.20 - 23.07)	22.11 ± 0.27 (21.43 - 22.98)	0.45 ± 0.38 (-0.78 - 1.13)	-0.47,1.37	0.278	(19.79 - 34.46) [9.25,44.14]
18:2 linoleic (% total FA)	63.25 ± 0.66 (62.73 - 63.72)	60.41 ± 0.66 (59.10 - 62.96)	2.84 ± 0.87 (0.75 - 4.19)	0.72,4.96	0.017	(51.64 - 64.12) [41.22,74.09]
18:3 linolenic (% total FA)	1.25 ± 0.087 (1.24 - 1.26)	1.57 ± 0.087 (1.22 - 1.77)	-0.32 ± 0.12 (-0.53 - 0.016)	-0.61,-0.029	0.036	(0.84 - 1.91) [0.42,1.95]
20:0 arachidic (% total FA)	0.35 ± 0.0029 (0.35 - 0.36)	0.36 ± 0.0029 (0.35 - 0.36)	-0.0037 ± 0.0039 (-0.012 - 0.0037)	-0.013,0.0059	0.384	(0.36 - 0.45) [0.31,0.49]
20:1 eicosenoic (% total FA)	0.24 ± 0.0015 (0.24 - 0.24)	0.24 ± 0.0015 (0.24 - 0.24)	0.00044 ± 0.0019 (-0.0013 - 0.0031)	-0.0043,0.0052	0.827	(0.24 - 0.36) [0.18,0.40]
22:0 behenic (% total FA)	0.15 ± 0.0061 (0.14 - 0.15)	0.15 ± 0.0061 (0.15 - 0.16)	-0.0090 ± 0.0087 (-0.017 - 0.0059)	-0.030,0.012	0.341	(0.074 - 0.24) [0.071,0.25]

[a]FA=fatty acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 8. Comparison of the fatty acid content in grain collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
16:0 palmitic (% total FA)	10.18 ± 0.083 (10.07 - 10.36)	10.34 ± 0.083 (10.18 - 10.42)	-0.16 ± 0.12 (-0.35 - 0.19)	-0.45,0.13	0.223	(9.29 - 17.81) [6.51,16.50]
16:1 pamitoleic (% total FA)	0.18 ± 0.0084 (0.16 - 0.19)	0.17 ± 0.0084 (0.16 - 0.19)	0.0069 ± 0.012 (-0.029 - 0.025)	-0.022,0.036	0.581	(0.054 - 0.21) [0.0017,0.28]
18:0 stearic (% total FA)	2.04 ± 0.032 (2.00 - 2.08)	2.06 ± 0.032 (1.99 - 2.12)	-0.026 ± 0.045 (-0.089 - 0.094)	-0.14,0.085	0.589	(1.68 - 2.30) [1.41,2.53]
18:1 oleic (% total FA)	22.53 ± 0.084 (22.50 - 22.56)	23.29 ± 0.084 (23.08 - 23.51)	-0.76 ± 0.12 (-0.94 - -0.58)	-1.05,-0.47	<0.001	(19.79 - 34.46) [9.25,44.14]
18:2 linoleic (% total FA)	63.11 ± 0.15 (62.84 - 63.29)	62.15 ± 0.15 (62.01 - 62.25)	0.96 ± 0.21 (0.66 - 1.19)	0.44,1.48	0.003	(51.64 - 64.12) [41.22,74.09]
18:3 linolenic (% total FA)	1.20 ± 0.011 (1.19 - 1.23)	1.21 ± 0.011 (1.19 - 1.22)	-0.0053 ± 0.016 (-0.033 - 0.036)	-0.045,0.034	0.753	(0.84 - 1.91) [0.42,1.95]
20:0 arachidic (% total FA)	0.37 ± 0.0042 (0.37 - 0.38)	0.39 ± 0.0042 (0.38 - 0.39)	-0.011 ± 0.0059 (-0.021 - 0.0019)	-0.026,0.0030	0.100	(0.36 - 0.45) [0.31,0.49]
20:1 eicosenoic (% total FA)	0.24 ± 0.0045 (0.23 - 0.24)	0.25 ± 0.0045 (0.24 - 0.26)	-0.0077 ± 0.0064 (-0.019 - 0.0024)	-0.023,0.0080	0.274	(0.24 - 0.36) [0.18,0.40]
22:0 behenic (% total FA)	0.15 ± 0.0046 (0.14 - 0.16)	0.15 ± 0.0046 (0.14 - 0.15)	0.0012 ± 0.0066 (-0.014 - 0.014)	-0.015,0.017	0.856	(0.074 - 0.24) [0.071,0.25]

[a]FA=fatty acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 9. Comparison of the fatty acid content in grain collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
16:0 palmitic (% total FA)	10.38 ± 0.14 (10.10 - 10.52)	10.51 ± 0.14 (10.16 - 10.85)	-0.14 ± 0.20 (-0.44 - 0.36)	-0.62,0.35	0.520	(9.29 - 17.81) [6.51,16.50]
16:1 pamitoleic (% total FA)	0.17 ± 0.0039 (0.16 - 0.17)	0.17 ± 0.0039 (0.16 - 0.18)	-0.0074 ± 0.0055 (-0.013 - 0.00074)	-0.021,0.0060	0.224	(0.054 - 0.21) [0.0017,0.28]
18:0 stearic (% total FA)	2.16 ± 0.065 (2.12 - 2.19)	2.13 ± 0.065 (1.93 - 2.23)	0.036 ± 0.092 (-0.11 - 0.25)	-0.19,0.26	0.711	(1.68 - 2.30) [1.41,2.53]
18:1 oleic (% total FA)	23.12 ± 0.22 (22.56 - 23.53)	23.19 ± 0.22 (22.59 - 23.50)	-0.068 ± 0.22 (-0.22 - 0.048)	-0.61,0.48	0.768	(19.79 - 34.46) [9.25,44.14]
18:2 linoleic (% total FA)	62.19 ± 0.36 (61.86 - 62.54)	61.99 ± 0.36 (61.21 - 63.18)	0.20 ± 0.49 (-0.64 - 0.65)	-1.00,1.41	0.692	(51.64 - 64.12) [41.22,74.09]
18:3 linolenic (% total FA)	1.19 ± 0.019 (1.15 - 1.23)	1.20 ± 0.019 (1.19 - 1.21)	-0.0044 ± 0.026 (-0.039 - 0.043)	-0.069,0.060	0.871	(0.84 - 1.91) [0.42,1.95]
20:0 arachidic (% total FA)	0.39 ± 0.0046 (0.38 - 0.39)	0.40 ± 0.0046 (0.38 - 0.41)	-0.010 ± 0.0065 (-0.028 - 0.0088)	-0.026,0.0054	0.158	(0.36 - 0.45) [0.31,0.49]
20:1 eicosenoic (% total FA)	0.25 ± 0.0060 (0.24 - 0.26)	0.25 ± 0.0060 (0.24 - 0.26)	-0.0031 ± 0.0085 (-0.019 - 0.019)	-0.024,0.018	0.731	(0.24 - 0.36) [0.18,0.40]
22:0 behenic (% total FA)	0.15 ± 0.0042 (0.14 - 0.15)	0.16 ± 0.0042 (0.14 - 0.17)	-0.011 ± 0.0060 (-0.018 - 0.0034)	-0.025,0.0039	0.122	(0.074 - 0.24) [0.071,0.25]

[a]FA=fatty acid, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 10. Comparison of the mineral content in grain collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Calcium (% dw)	0.0047 ± 0.00017 (0.0047 - 0.0047)	0.0050 ± 0.00017 (0.0049 - 0.0051)	-0.00030 ± 0.00024 (-0.00045 - -0.00020)	-0.00090,0.00030	0.267	(0.0032 - 0.0060) [0.0017,0.0062]
Copper (mg/kg dw)	1.74 ± 0.10 (1.71 - 1.78)	1.95 ± 0.10 (1.76 - 2.27)	-0.22 ± 0.099 (-0.49 - -0.052)	-0.46,0.025	0.070	(1.01 - 2.34) [0.17,3.00]
Iron (mg/kg dw)	22.18 ± 0.81 (21.77 - 22.92)	22.31 ± 0.81 (20.80 - 23.93)	-0.13 ± 1.15 (-2.16 - 2.12)	-2.95,2.68	0.912	(16.42 - 26.03) [12.60,31.26]
Magnesium (% dw)	0.14 ± 0.0068 (0.13 - 0.15)	0.14 ± 0.0068 (0.13 - 0.16)	-0.0056 ± 0.0096 (-0.024 - 0.018)	-0.029,0.018	0.579	(0.10 - 0.14) [0.088,0.16]
Manganese (mg/kg dw)	9.05 ± 0.40 (9.01 - 9.11)	8.67 ± 0.40 (7.55 - 9.41)	0.38 ± 0.52 (-0.37 - 1.56)	-0.90,1.66	0.491	(4.96 - 9.81) [2.45,10.60]
Phosphorus (% dw)	0.39 ± 0.018 (0.37 - 0.40)	0.40 ± 0.018 (0.36 - 0.43)	-0.010 ± 0.024 (-0.052 - 0.042)	-0.070,0.049	0.682	(0.28 - 0.41) [0.24,0.44]
Potassium (% dw)	0.42 ± 0.017 (0.41 - 0.44)	0.44 ± 0.017 (0.40 - 0.47)	-0.015 ± 0.024 (-0.052 - 0.037)	-0.074,0.044	0.552	(0.29 - 0.43) [0.27,0.48]
Zinc (mg/kg dw)	24.17 ± 0.90 (23.92 - 24.50)	24.05 ± 0.90 (22.02 - 25.81)	0.11 ± 1.08 (-1.74 - 1.90)	-2.53,2.76	0.919	(17.15 - 26.18) [13.42,31.37]

[a]dw=dry weight; mg/kg=ppm (parts per million), S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 11. Comparison of the mineral content in grain collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
Calcium (% dw)	0.0058 ± 0.00015 (0.0056 - 0.0060)	0.0060 ± 0.00015 (0.0058 - 0.0061)	-0.00015 ± 0.00022 (-0.00024 - -0.00006)	-0.00068,0.00038	0.510	(0.0032 - 0.0060) [0.0017,0.0062]
Copper (mg/kg dw)	1.89 ± 0.076 (1.68 - 2.05)	1.82 ± 0.076 (1.64 - 1.91)	0.069 ± 0.11 (-0.23 - 0.41)	-0.19,0.33	0.541	(1.01 - 2.34) [0.17,3.00]
Iron (mg/kg dw)	21.91 ± 0.54 (21.40 - 22.78)	22.06 ± 0.54 (21.45 - 22.54)	-0.15 ± 0.76 (-0.64 - 0.24)	-2.01,1.72	0.854	(16.42 - 26.03) [12.60,31.26]
Magnesium (% dw)	0.14 ± 0.0024 (0.13 - 0.14)	0.14 ± 0.0024 (0.14 - 0.14)	-0.00076 ± 0.0034 (-0.0057 - 0.0028)	-0.0091,0.0076	0.830	(0.10 - 0.14) [0.088,0.16]
Manganese (mg/kg dw)	10.20 ± 0.27 (9.85 - 10.46)	9.81 ± 0.27 (9.46 - 10.29)	0.40 ± 0.36 (0.0092 - 1.00)	-0.49,1.29	0.317	(4.96 - 9.81) [2.45,10.60]
Phosphorus (% dw)	0.38 ± 0.0067 (0.37 - 0.40)	0.38 ± 0.0067 (0.38 - 0.38)	0.0010 ± 0.0095 (-0.011 - 0.016)	-0.022,0.024	0.918	(0.28 - 0.41) [0.24,0.44]
Potassium (% dw)	0.40 ± 0.0061 (0.39 - 0.42)	0.41 ± 0.0061 (0.41 - 0.42)	-0.0073 ± 0.0087 (-0.015 - 0.0084)	-0.029,0.014	0.433	(0.29 - 0.43) [0.27,0.48]
Zinc (mg/kg dw)	26.40 ± 0.55 (25.43 - 27.27)	26.66 ± 0.55 (25.99 - 27.18)	-0.26 ± 0.64 (-0.68 - 0.45)	-1.84,1.31	0.697	(17.15 - 26.18) [13.42,31.37]

[a]dw=dry weight; mg/kg=ppm (parts per million), S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 12. Comparison of the mineral content in grain collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Calcium (% dw)	0.0056 ± 0.00043 (0.0054 - 0.0057)	0.0063 ± 0.00043 (0.0056 - 0.0069)	-0.00076 ± 0.00038 (-0.0013 - -0.00029)	-0.0017,0.00017	0.093	(0.0032 - 0.0060) [0.0017,0.0062]
Copper (mg/kg dw)	1.57 ± 0.15 (1.48 - 1.68)	2.21 ± 0.15 (1.83 - 2.63)	-0.64 ± 0.21 (-0.95 - -0.35)	-1.15,-0.12	0.023	(1.01 - 2.34) [0.17,3.00]
Iron (mg/kg dw)	20.44 ± 0.92 (20.07 - 20.74)	21.16 ± 0.92 (20.31 - 22.02)	-0.72 ± 1.30 (-1.51 - 0.43)	-3.89,2.45	0.598	(16.42 - 26.03) [12.60,31.26]
Magnesium (% dw)	0.13 ± 0.0071 (0.13 - 0.14)	0.13 ± 0.0071 (0.13 - 0.14)	-0.00030 ± 0.0090 (-0.0027 - 0.0032)	-0.022,0.022	0.974	(0.10 - 0.14) [0.088,0.16]
Manganese (mg/kg dw)	9.90 ± 0.84 (9.36 - 10.76)	9.63 ± 0.84 (8.69 - 10.44)	0.28 ± 0.90 (-0.39 - 0.90)	-1.92,2.47	0.768	(4.96 - 9.81) [2.45,10.60]
Phosphorus (% dw)	0.40 ± 0.026 (0.39 - 0.41)	0.40 ± 0.026 (0.38 - 0.42)	-0.0032 ± 0.031 (-0.013 - 0.0097)	-0.080,0.073	0.923	(0.28 - 0.41) [0.24,0.44]
Potassium (% dw)	0.41 ± 0.020 (0.40 - 0.42)	0.41 ± 0.020 (0.38 - 0.42)	0.0036 ± 0.023 (-0.019 - 0.023)	-0.052,0.059	0.880	(0.29 - 0.43) [0.27,0.48]
Zinc (mg/kg dw)	23.02 ± 0.99 (22.31 - 23.77)	24.05 ± 0.99 (22.33 - 26.85)	-1.03 ± 1.40 (-3.87 - 1.43)	-4.45,2.40	0.491	(17.15 - 26.18) [13.42,31.37]

[a]dw=dry weight; mg/kg=ppm (parts per million), S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 13. Comparison of the proximates and fiber content in grain collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
Ash (% dw)	1.55 ± 0.078 (1.37 - 1.66)	1.67 ± 0.078 (1.51 - 1.82)	-0.12 ± 0.11 (-0.45 - 0.15)	-0.39,0.15	0.313	(1.04 - 1.86) [0.94,1.73]
Carbohydrates (% dw)	82.72 ± 0.27 (82.23 - 83.39)	83.23 ± 0.27 (82.75 - 83.62)	-0.50 ± 0.37 (-1.39 - 0.074)	-1.40,0.39	0.216	(81.46 - 86.68) [79.39,89.67]
Fat, total (% dw)	3.53 ± 0.064 (3.44 - 3.59)	3.58 ± 0.064 (3.53 - 3.61)	-0.044 ± 0.090 (-0.16 - 0.062)	-0.26,0.18	0.645	(2.38 - 4.43) [0.74,6.01]
Moisture (% fw)	9.38 ± 0.18 (9.03 - 9.70)	9.93 ± 0.18 (9.73 - 10.10)	-0.55 ± 0.20 (-0.70 - -0.40)	-1.05,-0.054	0.034	(9.15 - 14.90) [4.67,17.56]
Protein (% dw)	12.21 ± 0.24 (11.63 - 12.58)	11.51 ± 0.24 (11.22 - 11.96)	0.70 ± 0.30 (0.28 - 1.37)	-0.042,1.45	0.060	(9.26 - 13.37) [6.20,15.35]
ADF (% dw)	3.99 ± 0.31 (3.50 - 4.40)	3.78 ± 0.31 (3.24 - 4.69)	0.21 ± 0.37 (-0.62 - 1.16)	-0.70,1.12	0.594	(2.39 - 4.89) [1.89,5.23]
NDF (% dw)	13.24 ± 0.39 (13.07 - 13.58)	13.80 ± 0.39 (12.99 - 14.29)	-0.56 ± 0.56 (-1.21 - 0.58)	-1.93,0.80	0.353	(8.41 - 16.54) [3.51,21.65]
TDF (% dw)	16.22 ± 0.61 (15.45 - 16.71)	17.39 ± 0.61 (16.88 - 17.84)	-1.17 ± 0.87 (-1.43 - -0.96)	-3.30,0.95	0.225	(11.80 - 23.04) [5.72,27.10]

[a]ADF=acid detergent fiber; dw=dry weight; fw=fresh weight; NDF=neutral detergent fiber; TDF=total dietary fiber, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 14. Comparison of the proximates and fiber content in grain collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
Ash (% dw)	1.49 ± 0.10 (1.31 - 1.66)	1.38 ± 0.10 (1.23 - 1.59)	0.11 ± 0.14 (-0.27 - 0.43)	-0.24,0.47	0.458	(1.04 - 1.86) [0.94,1.73]
Carbohydrates (% dw)	82.27 ± 0.38 (82.16 - 82.39)	82.39 ± 0.38 (82.00 - 83.12)	-0.12 ± 0.45 (-0.96 - 0.34)	-1.22,0.98	0.800	(81.46 - 86.68) [79.39,89.67]
Fat, total (% dw)	3.82 ± 0.10 (3.72 - 3.96)	4.11 ± 0.10 (3.99 - 4.36)	-0.29 ± 0.14 (-0.63 - -0.027)	-0.63,0.057	0.087	(2.38 - 4.43) [0.74,6.01]
Moisture (% fw)	13.13 ± 0.30 (13.10 - 13.20)	13.87 ± 0.30 (13.50 - 14.20)	-0.73 ± 0.38 (-1.10 - -0.40)	-1.65,0.19	0.099	(9.15 - 14.90) [4.67,17.56]
Protein (% dw)	12.39 ± 0.34 (12.31 - 12.54)	12.11 ± 0.34 (11.56 - 12.43)	0.28 ± 0.38 (-0.10 - 0.98)	-0.65,1.21	0.489	(9.26 - 13.37) [6.20,15.35]
ADF (% dw)	3.67 ± 0.22 (3.31 - 4.34)	3.30 ± 0.22 (2.97 - 3.84)	0.37 ± 0.27 (0.27 - 0.50)	-0.29,1.03	0.219	(2.39 - 4.89) [1.89,5.23]
NDF (% dw)	12.59 ± 0.61 (11.74 - 13.58)	10.70 ± 0.61 (10.39 - 10.94)	1.89 ± 0.87 (0.97 - 2.64)	-0.23,4.01	0.072	(8.41 - 16.54) [3.51,21.65]
TDF (% dw)	17.11 ± 0.93 (15.44 - 18.64)	14.01 ± 0.93 (13.18 - 14.45)	3.10 ± 1.31 (1.04 - 4.19)	-0.10,6.31	0.055	(11.80 - 23.04) [5.72,27.10]

[a]ADF=acid detergent fiber; dw=dry weight; fw=fresh weight; NDF=neutral detergent fiber; TDF=total dietary fiber, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 15. Comparison of the proximates and fiber content in grain collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control) Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	Commercial (Range) [99% T.I.][b]
Ash (% dw)	1.57 ± 0.15 (1.37 - 1.68)	1.72 ± 0.15 (1.54 - 1.97)	-0.14 ± 0.21 (-0.28 - 0.032)	-0.66,0.38	0.528	(1.04 - 1.86) [0.94,1.73]
Carbohydrates (% dw)	81.95 ± 0.26 (81.61 - 82.29)	81.39 ± 0.26 (80.67 - 82.04)	0.57 ± 0.34 (-0.092 - 0.94)	-0.26,1.39	0.143	(81.46 - 86.68) [79.39,89.67]
Fat, total (% dw)	3.55 ± 0.084 (3.44 - 3.69)	3.69 ± 0.084 (3.54 - 3.89)	-0.14 ± 0.12 (-0.45 - 0.15)	-0.42,0.15	0.295	(2.38 - 4.43) [0.74,6.01]
Moisture (% fw)	10.80 ± 0.060 (10.80 - 10.80)	11.00 ± 0.060 (10.90 - 11.10)	-0.20 ± 0.085 (-0.30 - -0.10)	-0.41,0.0079	0.056	(9.15 - 14.90) [4.67,17.56]
Protein (% dw)	12.93 ± 0.23 (12.89 - 13.00)	13.22 ± 0.23 (12.68 - 13.82)	-0.29 ± 0.27 (-0.82 - 0.21)	-0.96,0.38	0.329	(9.26 - 13.37) [6.20,15.35]
ADF (% dw)	3.66 ± 0.22 (3.58 - 3.80)	3.55 ± 0.22 (3.00 - 4.07)	0.11 ± 0.31 (-0.50 - 0.60)	-0.66,0.88	0.743	(2.39 - 4.89) [1.89,5.23]
NDF (% dw)	11.48 ± 0.38 (10.99 - 12.33)	11.11 ± 0.38 (10.38 - 11.59)	0.38 ± 0.34 (-0.22 - 0.75)	-0.46,1.22	0.314	(8.41 - 16.54) [3.51,21.65]
TDF (% dw)	15.40 ± 0.89 (13.57 - 17.38)	14.79 ± 0.89 (13.36 - 15.96)	0.60 ± 1.21 (-2.39 - 2.30)	-2.35,3.55	0.635	(11.80 - 23.04) [5.72,27.10]

[a]ADF=acid detergent fiber; dw=dry weight; fw=fresh weight; NDF=neutral detergent fiber; TDF=total dietary fiber, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 16. Comparison of the vitamin content in grain collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Folic acid (mg/kg dw)	0.50 ± 0.026 (0.42 - 0.55)	0.45 ± 0.026 (0.42 - 0.47)	0.050 ± 0.032 (-0.032 - 0.11)	-0.029,0.13	0.170	(0.28 - 0.61) [0.12,0.77]
Niacin (mg/kg dw)	22.92 ± 0.59 (21.74 - 24.14)	23.13 ± 0.59 (22.43 - 23.92)	-0.21 ± 0.64 (-0.69 - 0.23)	-1.77,1.35	0.749	(14.11 - 27.77) [3.19,34.49]
Vitamin B_1 (mg/kg dw)	2.54 ± 0.066 (2.42 - 2.65)	3.07 ± 0.066 (2.99 - 3.23)	-0.53 ± 0.084 (-0.68 - -0.35)	-0.74,-0.33	<0.001	(2.69 - 3.73) [1.96,4.38]
Vitamin B_2 (mg/kg dw)	1.01 ± 0.021 (0.98 - 1.04)	1.02 ± 0.021 (0.99 - 1.07)	-0.0081 ± 0.029 (-0.057 - 0.042)	-0.079,0.063	0.790	(0.88 - 1.32) [0.67,1.51]
Vitamin B_6 (mg/kg dw)	7.01 ± 0.34 (6.57 - 7.28)	7.56 ± 0.34 (6.79 - 8.54)	-0.55 ± 0.36 (-1.27 - -0.18)	-1.43,0.32	0.171	(4.93 - 7.24) [4.29,7.84]
Vitamin E (mg/kg dw)	14.79 ± 0.45 (14.62 - 15.01)	13.95 ± 0.45 (13.55 - 14.46)	0.83 ± 0.59 (0.27 - 1.46)	-0.61,2.28	0.207	(8.09 - 21.97) [0,29.69]

[a]dw=dry weight; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 17. Comparison of the vitamin content in grain collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Folic acid (mg/kg dw)	0.43 ± 0.026 (0.38 - 0.50)	0.45 ± 0.026 (0.43 - 0.47)	-0.020 ± 0.037 (-0.074 - 0.064)	-0.11,0.070	0.603	(0.28 - 0.61) [0.12,0.77]
Niacin (mg/kg dw)	21.10 ± 0.30 (20.39 - 21.52)	22.52 ± 0.30 (22.20 - 23.08)	-1.42 ± 0.42 (-1.91 - -0.68)	-2.44,-0.39	0.014	(14.11 - 27.77) [3.19,34.49]
Vitamin B_1 (mg/kg dw)	2.30 ± 0.061 (2.30 - 2.30)	3.10 ± 0.061 (3.03 - 3.14)	-0.79 ± 0.086 (-0.83 - -0.73)	-1.00,-0.58	<0.001	(2.69 - 3.73) [1.96,4.38]
Vitamin B_2 (mg/kg dw)	1.15 ± 0.066 (1.12 - 1.22)	1.13 ± 0.066 (1.08 - 1.17)	0.025 ± 0.076 (-0.045 - 0.14)	-0.16,0.21	0.753	(0.88 - 1.32) [0.67,1.51]
Vitamin B_6 (mg/kg dw)	7.49 ± 0.44 (6.88 - 8.06)	6.78 ± 0.44 (5.65 - 7.46)	0.72 ± 0.58 (-0.58 - 2.40)	-0.70,2.13	0.261	(4.93 - 7.24) [4.29,7.84]
Vitamin E (mg/kg dw)	11.47 ± 3.47 (6.08 - 16.13)	11.57 ± 3.47 (1.74 - 17.23)	-0.10 ± 4.91 (-11.15 - 14.39)	-12.72,12.52	0.984	(8.09 - 21.97) [0,29.69]

[a]dw=dry weight; Vitamin B_1 =Thiamine; Vitamin B_2 =Riboflavin; Vitamin B_6 =Pyridoxine, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval

[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 18. Comparison of the vitamin content in grain collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Folic acid (mg/kg dw)	0.51 ± 0.041 (0.44 - 0.60)	0.54 ± 0.041 (0.48 - 0.59)	-0.027 ± 0.058 (-0.054 - 0.015)	-0.17,0.12	0.662	(0.28 - 0.61) [0.12,0.77]
Niacin (mg/kg dw)	18.80 ± 0.76 (17.04 - 19.73)	19.59 ± 0.76 (19.08 - 19.89)	-0.79 ± 1.02 (-2.04 - -0.16)	-3.28,1.70	0.465	(14.11 - 27.77) [3.19,34.49]
Vitamin B_1 (mg/kg dw)	2.58 ± 0.073 (2.47 - 2.69)	3.56 ± 0.073 (3.49 - 3.60)	-0.98 ± 0.10 (-1.02 - -0.90)	-1.23,-0.73	<0.001	(2.69 - 3.73) [1.96,4.38]
Vitamin B_2 (mg/kg dw)	1.14 ± 0.061 (1.10 - 1.18)	1.24 ± 0.061 (1.16 - 1.33)	-0.092 ± 0.073 (-0.17 - 0.021)	-0.27,0.088	0.256	(0.88 - 1.32) [0.67,1.51]
Vitamin B_6 (mg/kg dw)	6.99 ± 0.23 (6.67 - 7.20)	6.97 ± 0.23 (6.47 - 7.63)	0.026 ± 0.22 (-0.52 - 0.40)	-0.52,0.57	0.912	(4.93 - 7.24) [4.29,7.84]
Vitamin E (mg/kg dw)	16.18 ± 0.74 (15.70 - 16.93)	16.71 ± 0.74 (15.15 - 17.77)	-0.52 ± 1.04 (-2.08 - 1.78)	-3.08,2.03	0.633	(8.09 - 21.97) [0,29.69]

[a]dw=dry weight; S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
Vitamin B_1 =Thiamine
Vitamin B_2 =Riboflavin
Vitamin B_6 =Pyridoxine

[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 19. Comparison of the secondary metabolites and anti-nutrients content in grain collected at the IA field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ferulic acid (µg/g dw)	2092.58 ± 83.12 (1986.75 - 2275.48)	2024.32 ± 83.12 (1927.55 - 2187.67)	68.26 ± 117.54 (-200.92 - 347.92)	-219.36,355.88	0.582	(1717.17 - 2687.57) [1415.19,3173.90]
p-Coumaric acid (µg/g dw)	162.93 ± 6.63 (153.93 - 168.19)	164.32 ± 6.63 (156.20 - 173.24)	-1.39 ± 9.37 (-9.58 - 11.99)	-24.32,21.55	0.887	(152.30 - 319.15) [43.13,384.34]
Phytic acid (% dw)	0.94 ± 0.093 (0.85 - 1.03)	0.87 ± 0.093 (0.76 - 1.01)	0.069 ± 0.12 (0.014 - 0.097)	-0.22,0.35	0.575	(0.45 - 1.00) [0.28,1.12]
Raffinose (% dw)	0.15 ± 0.0049 (0.14 - 0.16)	0.14 ± 0.0049 (0.14 - 0.15)	0.0079 ± 0.0070 (-0.0018 - 0.022)	-0.0091,0.025	0.298	(0.073 - 0.22) [0,0.32]

[a]dw=dry weight, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 20. Comparison of the secondary metabolites and anti-nutrients content in grain collected at the IL field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ferulic acid (µg/g dw)	2198.75 ± 52.20 (2142.86 - 2266.97)	2140.23 ± 52.20 (2046.24 - 2241.58)	58.52 ± 73.81 (-98.72 - 220.73)	-122.10,239.14	0.458	(1717.17 - 2687.57) [1415.19,3173.90]
p-Coumaric acid (µg/g dw)	169.60 ± 10.01 (148.45 - 202.53)	155.98 ± 10.01 (149.13 - 163.17)	13.63 ± 14.16 (-14.72 - 53.40)	-21.02,48.27	0.373	(152.30 - 319.15) [43.13,384.34]
Phytic acid (% dw)	0.92 ± 0.039 (0.83 - 1.00)	0.99 ± 0.039 (0.95 - 1.03)	-0.066 ± 0.055 (-0.15 - -0.016)	-0.20,0.068	0.272	(0.45 - 1.00) [0.28,1.12]
Raffinose (% dw)	0.18 ± 0.0093 (0.17 - 0.19)	0.17 ± 0.0093 (0.16 - 0.18)	0.015 ± 0.013 (-0.013 - 0.036)	-0.017,0.047	0.305	(0.073 - 0.22) [0,0.32]

[a]dw=dry weight, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

Table 21. Comparison of the secondary metabolites and anti-nutrients content in grain collected at the NE field site from MON 88017 and conventional corn

Component (Units)[a]	MON 88017 Mean ± S.E. (Range)	Control Mean ± S.E. (Range)	Difference (MON 88017 Minus Control)			Commercial (Range) [99% T.I.][b]
			Mean ± S.E. (Range)	95% C.I. (Lower, upper)	p-Value	
Ferulic acid (µg/g dw)	2234.68 ± 53.04 (2186.10 - 2264.57)	2198.59 ± 53.04 (2098.77 - 2339.71)	36.09 ± 75.01 (-75.13 - 154.60)	-147.46,219.64	0.647	(1717.17 - 2687.57) [1415.19,3173.90]
p-Coumaric acid (µg/g dw)	175.26 ± 10.40 (153.59 - 215.25)	144.20 ± 10.40 (141.41 - 148.48)	31.06 ± 14.69 (8.47 - 72.55)	-4.88,67.01	0.078	(152.30 - 319.15) [43.13,384.34]
Phytic acid (% dw)	0.98 ± 0.071 (0.91 - 1.05)	0.81 ± 0.071 (0.72 - 0.89)	0.17 ± 0.10 (0.087 - 0.24)	-0.072,0.42	0.135	(0.45 - 1.00) [0.28,1.12]
Raffinose (% dw)	0.18 ± 0.010 (0.16 - 0.20)	0.20 ± 0.010 (0.18 - 0.23)	-0.020 ± 0.014 (-0.035 - -0.012)	-0.055,0.015	0.207	(0.073 - 0.22) [0,0.32]

[a]dw=dry weight, S.E. = standard error, C.I.= confidence interval, T.I.= tolerance interval
[b]With 95% confidence, interval contains 99% of the values expressed in the population of commercial lines. Negative limits were set to zero.

SUPPLEMENTAL SUMMARY

Fatty and amino acid composition of MON 88017 and conventional corn calculated as percent of dry weight, percent of total fat, and percent of total protein

Data Description

A SAS data set containing MON 88017 and conventional control corn forage and grain compositional analysis data was analyzed. Test and control materials were grown in single plots, randomly assigned within each of three replication blocks, at sites IA, IL and NE.

The calculation of fatty acid and amino acid composition in MON 88017 was done using following formulas:

Component	From (*X*)	To	Formula
Fatty Acid (FA)	% FW	% DW	X/d
		% Total Fat	X/(ff)
Amino Acid (AA)	mg/g FW	% Total Protein	X/(10*fp)
		% DW	X/(10*d)

d is the fraction of the sample that is dry matter.
ff is the total fat fraction of fresh weight obtained by proximate analysis; ff = % total fat / 100.
fp is the protein fraction of fresh weight obtained by proximate analysis; fp = % protein /100.

Statistical Approach

Individual site and combined site means and ranges were calculated for each test and control fatty acid compositional analysis component using SAS® software. A range of observed commercial reference hybrid values was also determined for each fatty acid compositional analysis component.

Results

For each fatty acid compositional analysis component, means and ranges are presented for each of the test and control hybrids of interest. Tables 22-25 presents means and ranges for each site individually and combined with the overall observed commercial reference range.
The calculations are presented in the Tables as follows:

Table 22 - fatty acid content calculated as percent of dry weight
Table 23 – fatty acids content calculated as percent of total fat
Table 24 – amino acid content calculated as percent of dry weight
Table 25 – amino acid content calculated as percent of total protein

Table 22. Fatty acid content in grain from MON88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Fatty Acid % DW				
16:0 Palmitic	All Sites	0.35	0.39	(0.22 - 0.50)
		(0.32 - 0.38)	(0.32 - 0.48)	
	Site IA	0.32	0.41	
		(0.32 - 0.33)	(0.32 - 0.48)	
	Site IL	0.37	0.40	
		(0.36 - 0.38)	(0.39 - 0.42)	
	Site NE	0.34	0.35	
		(0.33 - 0.35)	(0.33 - 0.38)	
16:1 Palmitoleic	All Sites	0.0061	0.0064	(0.0017 - 0.0068)
		(0.0053 - 0.0071)	(0.0052 - 0.0080)	
	Site IA	0.0064	0.0067	
		(0.0060 - 0.0071)	(0.0061 - 0.0071)	
	Site IL	0.0064	0.0067	
		(0.0056 - 0.0070)	(0.0060 - 0.0080)	
	Site NE	0.0056	0.0058	
		(0.0053 - 0.0057)	(0.0052 - 0.0063)	
18:0 Stearic	All Sites	0.068	0.072	(0.043 - 0.079)
		(0.058 - 0.077)	(0.055 - 0.083)	
	Site IA	0.059	0.064	
		(0.058 - 0.061)	(0.055 - 0.071)	
	Site IL	0.074	0.080	
		(0.072 - 0.077)	(0.078 - 0.083)	
	Site NE	0.072	0.071	
		(0.069 - 0.074)	(0.063 - 0.079)	
18:1 Oleic	All Sites	0.77	0.80	(0.46 - 1.27)
		(0.69 - 0.86)	(0.69 - 0.98)	
	Site IA	0.72	0.71	
		(0.69 - 0.76)	(0.69 - 0.72)	
	Site IL	0.82	0.91	
		(0.78 - 0.86)	(0.87 - 0.98)	
	Site NE	0.77	0.78	
		(0.73 - 0.82)	(0.74 - 0.83)	

Table 22 (cont.) Fatty acid content in grain from MON 88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Fatty Acid % DW				
18:2 Linoleic	All Sites	2.12	2.14	(1.32 - 2.48)
		(1.94 - 2.41)	(1.87 - 2.59)	
	Site IA	2.02	1.93	
		(1.95 - 2.06)	(1.87 - 1.96)	
	Site IL	2.28	2.42	
		(2.17 - 2.41)	(2.33 - 2.59)	
	Site NE	2.07	2.07	
		(1.94 - 2.17)	(1.98 - 2.17)	
18:3 Linolenic	All Sites	0.041	0.046	(0.024 - 0.058)
		(0.037 - 0.045)	(0.038 - 0.059)	
	Site IA	0.040	0.050	
		(0.038 - 0.041)	(0.038 - 0.059)	
	Site IL	0.043	0.047	
		(0.042 - 0.045)	(0.045 - 0.050)	
	Site NE	0.040	0.040	
		(0.037 - 0.041)	(0.038 - 0.043)	
20:0 Arachidic	All Sites	0.013	0.013	(0.0084 - 0.016)
		(0.011 - 0.014)	(0.011 - 0.016)	
	Site IA	0.011	0.011	
		(0.011 - 0.012)	(0.011 - 0.012)	
	Site IL	0.014	0.015	
		(0.013 - 0.014)	(0.015 - 0.016)	
	Site NE	0.013	0.013	
		(0.012 - 0.013)	(0.013 - 0.014)	
20:1 Eicosenoic	All Sites	0.0082	0.0085	(0.0055 - 0.013)
		(0.0074 - 0.0089)	(0.0074 - 0.010)	
	Site IA	0.0076	0.0075	
		(0.0074 - 0.0077)	(0.0074 - 0.0078)	
	Site IL	0.0086	0.0095	
		(0.0083 - 0.0089)	(0.0091 - 0.010)	
	Site NE	0.0084	0.0085	
		(0.0080 - 0.0086)	(0.0078 - 0.0093)	

Table 22 (cont.) Fatty acid content in grain of MON 88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Fatty Acid % DW				
22:0 Behenic	All Sites	0.0050	0.0053	(0.0017 - 0.0063)
		(0.0044 - 0.0059)	(0.0045 - 0.0064)	
	Site IA	0.0046	0.0049	
		(0.0044 - 0.0049)	(0.0045 - 0.0053)	
	Site IL	0.0055	0.0058	
		(0.0049 - 0.0059)	(0.0054 - 0.0064)	
	Site NE	0.0049	0.0053	
		(0.0047 - 0.0050)	(0.0047 - 0.0059)	

Table 23. Fatty acid content in MON88017 and control LH198xLH59 corn calculated as percent of total fat

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Fatty Acid % Total Fat				
16:0 Palmitic	All Sites	9.50	10.30	(8.61 - 16.44)
		(8.83 - 10.00)	(8.77 - 13.35)	
	Site IA	9.18	11.59	
		(8.83 - 9.42)	(8.77 - 13.35)	
	Site IL	9.63	9.78	
		(9.57 - 9.71)	(9.73 - 9.82)	
	Site NE	9.70	9.52	
		(9.51 - 10.00)	(9.29 - 9.88)	
16:1 Palmitoleic	All Sites	0.17	0.17	(0.050 - 0.20)
		(0.15 - 0.20)	(0.14 - 0.20)	
	Site IA	0.18	0.19	
		(0.17 - 0.20)	(0.17 - 0.20)	
	Site IL	0.17	0.16	
		(0.15 - 0.18)	(0.15 - 0.18)	
	Site NE	0.16	0.16	
		(0.15 - 0.16)	(0.14 - 0.17)	
18:0 Stearic	All Sites	1.87	1.89	(1.45 - 2.09)
		(1.62 - 2.10)	(1.52 - 2.03)	
	Site IA	1.66	1.80	
		(1.62 - 1.69)	(1.52 - 1.97)	
	Site IL	1.93	1.95	
		(1.90 - 1.95)	(1.90 - 2.00)	
	Site NE	2.02	1.93	
		(1.97 - 2.10)	(1.78 - 2.03)	
18:1 Oleic	Combined Sites	21.11	20.92	(18.26 - 31.94)
		(19.35 - 23.30)	(19.63 - 22.48)	
	Site IA	20.39	19.72	
		(19.35 - 21.11)	(19.63 - 19.88)	
	Site IL	21.32	22.05	
		(20.90 - 21.66)	(21.71 - 22.48)	
	Site NE	21.63	21.00	
		(20.40 - 23.30)	(20.71 - 21.42)	

Table 23 (cont.) Fatty acid content in MON88017 and control LH198xLH59 corn calculated as percent of total fat

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Fatty Acid % Total Fat				
18:2 Linoleic	All Sites	58.34	56.29	(46.22 - 59.73)
		(54.63 - 61.59)	(53.00 - 59.47)	
	Site IA	57.15	53.87	
		(54.63 - 59.42)	(53.00 - 54.46)	
	Site IL	59.71	58.83	
		(58.20 - 60.76)	(58.48 - 59.47)	
	Site NE	58.16	56.17	
		(56.35 - 61.59)	(54.32 - 58.41)	
18:3 Linolenic	All Sites	1.13	1.21	(0.77 - 1.76)
		(1.07 - 1.16)	(1.05 - 1.62)	
	Site IA	1.13	1.40	
		(1.07 - 1.16)	(1.06 - 1.62)	
	Site IL	1.14	1.14	
		(1.13 - 1.14)	(1.14 - 1.15)	
	Site NE	1.11	1.08	
		(1.09 - 1.14)	(1.05 - 1.10)	
20:0 Arachidic	All Sites	0.35	0.35	(0.31 - 0.42)
		(0.31 - 0.37)	(0.30 - 0.37)	
	Site IA	0.32	0.32	
		(0.31 - 0.33)	(0.30 - 0.33)	
	Site IL	0.35	0.37	
		(0.35 - 0.36)	(0.36 - 0.37)	
	Site NE	0.36	0.36	
		(0.36 - 0.37)	(0.36 - 0.37)	
20:1 Eicosenoic	All Sites	0.22	0.22	(0.21 - 0.33)
		(0.21 - 0.24)	(0.21 - 0.24)	
	Site IA	0.21	0.21	
		(0.21 - 0.22)	(0.21 - 0.22)	
	Site IL	0.22	0.23	
		(0.22 - 0.23)	(0.23 - 0.24)	
	Site NE	0.24	0.23	
		(0.23 - 0.24)	(0.22 - 0.24)	

Table 23 (cont.) Fatty acid content in MON88017 and control LH198xLH59 corn calculated as percent of total fat

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Fatty Acid % Total Fat				
22:0 Behenic	All Sites	0.14	0.14	(0.068 - 0.22)
		(0.12 - 0.15)	(0.13 - 0.15)	
	Site IA	0.13	0.14	
		(0.12 - 0.14)	(0.13 - 0.15)	
	Site IL	0.14	0.14	
		(0.13 - 0.15)	(0.13 - 0.15)	
	Site NE	0.14	0.14	
		(0.13 - 0.14)	(0.13 - 0.15)	

Table 24. Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % DW				
Alanine	Combined Sites	0.93	0.93	(0.67 - 1.06)
		(0.84 - 1.00)	(0.81 - 1.11)	
	Site IA	0.89	0.83	
		(0.84 - 0.92)	(0.81 - 0.84)	
	Site IL	0.91	0.91	
		(0.90 - 0.93)	(0.88 - 0.93)	
	Site NE	0.99	1.03	
		(0.98 - 1.00)	(0.96 - 1.11)	
Arginine	All Sites	0.54	0.52	(0.42 - 0.55)
		(0.50 - 0.58)	(0.49 - 0.58)	
	Site IA	0.56	0.51	
		(0.55 - 0.57)	(0.49 - 0.53)	
	Site IL	0.51	0.51	
		(0.50 - 0.52)	(0.49 - 0.52)	
	Site NE	0.57	0.55	
		(0.56 - 0.58)	(0.54 - 0.58)	
Aspartic Acid	All Sites	0.77	0.77	(0.60 - 0.82)
		(0.73 - 0.82)	(0.68 - 0.88)	
	Site IA	0.75	0.70	
		(0.73 - 0.77)	(0.68 - 0.72)	
	Site IL	0.74	0.75	
		(0.73 - 0.75)	(0.74 - 0.78)	
	Site NE	0.81	0.84	
		(0.80 - 0.82)	(0.81 - 0.88)	
Cystine	All Sites	0.26	0.26	(0.20 - 0.29)
		(0.25 - 0.28)	(0.25 - 0.28)	
	Site IA	0.26	0.26	
		(0.26 - 0.26)	(0.25 - 0.26)	
	Site IL	0.26	0.26	
		(0.26 - 0.27)	(0.25 - 0.27)	
	Site NE	0.27	0.27	
		(0.25 - 0.28)	(0.27 - 0.28)	

Table 24 (cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % DW				
Glutamic Acid	All Sites	2.51	2.50	(1.78 - 2.81)
		(2.28 - 2.68)	(2.22 - 3.00)	
	Site IA	2.42	2.26	
		(2.28 - 2.48)	(2.22 - 2.28)	
	Site IL	2.47	2.47	
		(2.43 - 2.52)	(2.38 - 2.52)	
	Site NE	2.66	2.78	
		(2.62 - 2.68)	(2.58 - 3.00)	
Glycine	All Sites	0.43	0.42	(0.35 - 0.43)
		(0.40 - 0.45)	(0.39 - 0.46)	
	Site IA	0.43	0.40	
		(0.42 - 0.44)	(0.39 - 0.41)	
	Site IL	0.41	0.41	
		(0.40 - 0.41)	(0.41 - 0.42)	
	Site NE	0.44	0.45	
		(0.44 - 0.45)	(0.43 - 0.46)	
Histidine	All Sites	0.37	0.36	(0.26 - 0.36)
		(0.35 - 0.38)	(0.34 - 0.41)	
	Site IA	0.37	0.35	
		(0.36 - 0.38)	(0.34 - 0.36)	
	Site IL	0.35	0.35	
		(0.35 - 0.36)	(0.34 - 0.35)	
	Site NE	0.38	0.39	
		(0.37 - 0.38)	(0.36 - 0.41)	
Isoleucine	All Sites	0.44	0.44	(0.32 - 0.48)
		(0.40 - 0.47)	(0.39 - 0.53)	
	Site IA	0.42	0.40	
		(0.40 - 0.44)	(0.39 - 0.40)	
	Site IL	0.44	0.42	
		(0.43 - 0.44)	(0.40 - 0.45)	
	Site NE	0.47	0.49	
		(0.46 - 0.47)	(0.44 - 0.53)	

Table 24 (cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % DW				
Leucine	All Sites	1.64	1.63	(1.10 - 1.87)
		(1.46 - 1.75)	(1.43 - 2.03)	
	Site IA	1.56	1.46	
		(1.46 - 1.61)	(1.43 - 1.48)	
	Site IL	1.62	1.60	
		(1.60 - 1.64)	(1.51 - 1.68)	
	Site NE	1.73	1.84	
		(1.70 - 1.75)	(1.67 - 2.03)	
Lysine	All Sites	0.33	0.33	(0.27 - 0.36)
		(0.29 - 0.35)	(0.29 - 0.36)	
	Site IA	0.33	0.31	
		(0.33 - 0.34)	(0.29 - 0.32)	
	Site IL	0.32	0.31	
		(0.29 - 0.33)	(0.31 - 0.32)	
	Site NE	0.34	0.36	
		(0.34 - 0.35)	(0.34 - 0.36)	
Methionine	All Sites	0.24	0.24	(0.17 - 0.26)
		(0.24 - 0.25)	(0.23 - 0.27)	
	Site IA	0.24	0.24	
		(0.24 - 0.25)	(0.24 - 0.25)	
	Site IL	0.24	0.24	
		(0.24 - 0.25)	(0.23 - 0.25)	
	Site NE	0.25	0.25	
		(0.24 - 0.25)	(0.24 - 0.27)	
Phenylalanine	All Sites	0.64	0.63	(0.44 - 0.69)
		(0.59 - 0.68)	(0.56 - 0.75)	
	Site IA	0.61	0.57	
		(0.59 - 0.64)	(0.56 - 0.59)	
	Site IL	0.63	0.62	
		(0.63 - 0.63)	(0.59 - 0.66)	
	Site NE	0.67	0.70	
		(0.66 - 0.68)	(0.65 - 0.75)	

Table 24 (cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % DW				
Proline	All Sites	1.16	1.14	(0.80 - 1.20)
		(1.09 - 1.26)	(1.05 - 1.31)	
	Site IA	1.14	1.09	
		(1.09 - 1.19)	(1.06 - 1.11)	
	Site IL	1.12	1.10	
		(1.09 - 1.14)	(1.05 - 1.18)	
	Site NE	1.21	1.24	
		(1.19 - 1.26)	(1.16 - 1.31)	
Serine	All Sites	0.59	0.60	(0.46 - 0.66)
		(0.55 - 0.63)	(0.53 - 0.72)	
	Site IA	0.59	0.55	
		(0.57 - 0.61)	(0.53 - 0.57)	
	Site IL	0.58	0.59	
		(0.55 - 0.61)	(0.57 - 0.62)	
	Site NE	0.62	0.67	
		(0.62 - 0.63)	(0.64 - 0.72)	
Threonine	All Sites	0.40	0.40	(0.32 - 0.42)
		(0.37 - 0.42)	(0.36 - 0.45)	
	Site IA	0.40	0.37	
		(0.39 - 0.41)	(0.36 - 0.38)	
	Site IL	0.38	0.39	
		(0.37 - 0.40)	(0.38 - 0.40)	
	Site NE	0.41	0.43	
		(0.41 - 0.42)	(0.42 - 0.45)	
Tryptophan	All Sites	0.066	0.067	(0.052 - 0.080)
		(0.061 - 0.072)	(0.054 - 0.083)	
	Site IA	0.067	0.067	
		(0.061 - 0.072)	(0.064 - 0.068)	
	Site IL	0.067	0.070	
		(0.062 - 0.072)	(0.062 - 0.083)	
	Site NE	0.065	0.064	
		(0.063 - 0.067)	(0.054 - 0.071)	

Table 24 (cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of dry weight

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % DW				
Tyrosine	All Sites	0.41	0.42	(0.23 - 0.48)
		(0.28 - 0.47)	(0.34 - 0.52)	
	Site IA	0.43	0.40	
		(0.42 - 0.45)	(0.39 - 0.41)	
	Site IL	0.35	0.41	
		(0.28 - 0.41)	(0.41 - 0.42)	
	Site NE	0.46	0.44	
		(0.46 - 0.47)	(0.34 - 0.52)	
Valine	All Sites	0.59	0.58	(0.43 - 0.61)
		(0.53 - 0.63)	(0.52 - 0.67)	
	Site IA	0.57	0.54	
		(0.53 - 0.59)	(0.52 - 0.55)	
	Site IL	0.58	0.57	
		(0.57 - 0.59)	(0.55 - 0.61)	
	Site NE	0.62	0.63	
		(0.62 - 0.63)	(0.59 - 0.67)	

Table 25. Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of total protein

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % Total Protein				
Alanine	All Sites	7.43	7.52	(7.06 - 8.33)
		(7.18 - 7.75)	(7.04 - 8.05)	
	Site IA	7.26	7.23	
		(7.18 - 7.37)	(7.04 - 7.48)	
	Site IL	7.38	7.52	
		(7.29 - 7.55)	(7.45 - 7.62)	
	Site NE	7.66	7.82	
		(7.60 - 7.75)	(7.55 - 8.05)	
Arginine	All Sites	4.35	4.27	(3.74 - 5.13)
		(4.02 - 4.70)	(4.10 - 4.52)	
	Site IA	4.59	4.42	
		(4.51 - 4.70)	(4.30 - 4.52)	
	Site IL	4.09	4.19	
		(4.02 - 4.20)	(4.10 - 4.28)	
	Site NE	4.38	4.19	
		(4.28 - 4.50)	(4.17 - 4.22)	
Aspartic Acid	All Sites	6.13	6.23	(6.04 - 6.95)
		(5.83 - 6.35)	(6.00 - 6.41)	
	Site IA	6.14	6.07	
		(5.95 - 6.26)	(6.02 - 6.10)	
	Site IL	5.95	6.23	
		(5.83 - 6.12)	(6.00 - 6.38)	
	Site NE	6.30	6.38	
		(6.19 - 6.35)	(6.36 - 6.41)	
Cystine	All Sites	2.11	2.14	(1.85 - 2.51)
		(1.95 - 2.24)	(2.01 - 2.33)	
	Site IA	2.13	2.22	
		(2.07 - 2.24)	(2.11 - 2.33)	
	Site IL	2.13	2.15	
		(2.10 - 2.16)	(2.08 - 2.20)	
	Site NE	2.06	2.06	
		(1.95 - 2.20)	(2.01 - 2.12)	

Table 25 (cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of total protein

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial (Range)
Amino Acid % Total Protein				
Glutamic Acid	All Sites	20.09	20.36	(18.68 - 22.02)
		(19.62 - 20.78)	(19.07 - 21.71)	
	Site IA	19.79	19.66	
		(19.62 - 20.00)	(19.07 - 20.30)	
	Site IL	19.94	20.39	
		(19.63 - 20.47)	(20.19 - 20.60)	
	Site NE	20.55	21.03	
		(20.35 - 20.78)	(20.35 - 21.71)	
Glycine	All Sites	3.40	3.43	(3.18 - 4.23)
		(3.25 - 3.59)	(3.28 - 3.55)	
	Site IA	3.50	3.49	
		(3.39 - 3.59)	(3.46 - 3.53)	
	Site IL	3.28	3.41	
		(3.25 - 3.32)	(3.28 - 3.55)	
	Site NE	3.42	3.40	
		(3.38 - 3.45)	(3.32 - 3.45)	
Histidine	All Sites	2.94	2.94	(2.46 - 3.16)
		(2.84 - 3.08)	(2.81 - 3.07)	
	Site IA	3.03	3.03	
		(2.95 - 3.08)	(2.99 - 3.07)	
	Site IL	2.85	2.86	
		(2.84 - 2.87)	(2.81 - 2.93)	
	Site NE	2.94	2.93	
		(2.90 - 2.97)	(2.85 - 2.98)	
Isoleucine	All Sites	3.53	3.55	(3.33 - 3.79)
		(3.40 - 3.64)	(3.36 - 3.84)	
	Site IA	3.47	3.46	
		(3.40 - 3.51)	(3.36 - 3.57)	
	Site IL	3.52	3.50	
		(3.46 - 3.57)	(3.38 - 3.64)	
	Site NE	3.62	3.69	
		(3.57 - 3.64)	(3.43 - 3.84)	

Table 25 (cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of total protein

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % Total Protein				
Leucine	All Sites	13.07	13.26	(11.85 - 14.65)
		(12.57 - 13.57)	(12.22 - 14.72)	
	Site IA	12.74	12.68	
		(12.57 - 12.83)	(12.22 - 13.17)	
	Site IL	13.07	13.22	
		(12.94 - 13.27)	(13.02 - 13.55)	
	Site NE	13.41	13.89	
		(13.22 - 13.57)	(13.19 - 14.72)	
Lysine	All Sites	2.65	2.65	(2.35 - 3.28)
		(2.37 - 2.85)	(2.47 - 2.76)	
	Site IA	2.73	2.67	
		(2.61 - 2.85)	(2.59 - 2.71)	
	Site IL	2.55	2.60	
		(2.37 - 2.64)	(2.47 - 2.72)	
	Site NE	2.66	2.69	
		(2.60 - 2.70)	(2.61 - 2.76)	
Methionine	All Sites	1.95	2.00	(1.73 - 2.41)
		(1.87 - 2.03)	(1.85 - 2.22)	
	Site IA	1.99	2.12	
		(1.94 - 2.03)	(2.02 - 2.22)	
	Site IL	1.94	1.97	
		(1.89 - 1.99)	(1.92 - 2.00)	
	Site NE	1.92	1.91	
		(1.87 - 1.95)	(1.85 - 1.94)	
Phenylalanine	All Sites	5.10	5.12	(4.62 - 5.53)
		(4.91 - 5.26)	(4.80 - 5.42)	
	Site IA	5.02	4.99	
		(4.91 - 5.10)	(4.80 - 5.27)	
	Site IL	5.09	5.11	
		(5.05 - 5.12)	(4.94 - 5.27)	
	Site NE	5.18	5.26	
		(5.13 - 5.26)	(5.12 - 5.42)	

Table 25(cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of total protein

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % Total Protein				
Proline	All Sites	9.24	9.30	(8.14 - 9.55)
		(8.84 - 9.66)	(8.65 - 9.90)	
	Site IA	9.36	9.45	
		(9.20 - 9.47)	(9.14 - 9.90)	
	Site IL	9.01	9.08	
		(8.84 - 9.22)	(8.65 - 9.53)	
	Site NE	9.36	9.37	
		(9.22 - 9.66)	(9.12 - 9.51)	
Serine	All Sites	4.76	4.89	(4.67 - 5.20)
		(4.50 - 4.98)	(4.58 - 5.20)	
	Site IA	4.80	4.76	
		(4.54 - 4.98)	(4.68 - 4.85)	
	Site IL	4.65	4.87	
		(4.50 - 4.94)	(4.58 - 5.02)	
	Site NE	4.82	5.04	
		(4.80 - 4.84)	(4.86 - 5.20)	
Threonine	All Sites	3.17	3.23	(3.02 - 3.53)
		(2.99 - 3.35)	(3.03 - 3.38)	
	Site IA	3.25	3.20	
		(3.08 - 3.35)	(3.18 - 3.23)	
	Site IL	3.08	3.23	
		(2.99 - 3.23)	(3.03 - 3.38)	
	Site NE	3.20	3.27	
		(3.15 - 3.24)	(3.26 - 3.28)	
Tryptophan	All Sites	0.53	0.55	(0.44 - 0.81)
		(0.49 - 0.58)	(0.41 - 0.67)	
	Site IA	0.55	0.58	
		(0.52 - 0.58)	(0.57 - 0.61)	
	Site IL	0.54	0.58	
		(0.50 - 0.58)	(0.53 - 0.67)	
	Site NE	0.50	0.48	
		(0.49 - 0.52)	(0.41 - 0.52)	

Table 25 (cont.) Amino acid content in MON88017 and control LH198xLH59 corn calculated as percent of total protein

Component	Location	MON88017 Mean (Range)	LH198xLH59 Mean (Range)	Commercial corn (Range)
Amino Acid % Total Protein				
Tyrosine	All Sites	3.30	3.41	(2.22 - 3.78)
		(2.23 - 3.63)	(2.60 - 3.77)	
	Site IA	3.54	3.50	
		(3.43 - 3.63)	(3.38 - 3.69)	
	Site IL	2.81	3.41	
		(2.23 - 3.35)	(3.26 - 3.54)	
	Site NE	3.56	3.32	
		(3.53 - 3.59)	(2.60 - 3.77)	
Valine	All Sites	4.71	4.72	(4.33 - 5.26)
		(4.56 - 4.87)	(4.50 - 4.89)	
	Site IA	4.66	4.65	
		(4.56 - 4.72)	(4.57 - 4.82)	
	Site IL	4.65	4.73	
		(4.60 - 4.69)	(4.50 - 4.89)	
	Site NE	4.82	4.79	
		(4.77 - 4.87)	(4.62 - 4.89)	