Food and Feed Safety and Nutritional Assessment of MON 87427 Maize with Tissue-Selective Glyphosate Tolerance Facilitating the Production of Hybrid Maize Seed (OECD Unique Identifier MON-87427-7)

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

December 15, 2010

FDA BNF 126

Monsanto 10-CR-215F

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). Additionally this submission complies with the Codex Plant Guidelines (CAC/GL 45-2003) insofar as it is within the FDA's jurisdiction.

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: (a) MON 87427 maize is as safe and nutritious as other commercially available maize; and (b) the intended uses of the food and feed derived from MON 87427 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. Upon request, Monsanto will make relevant data or other information not included in this submission available to FDA either: (a) by allowing FDA to review and copy these data or information at Monsanto's offices in St. Louis, MO, during customary business hours; or (b) 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, that is pertinent to the evaluation of the safety, nutritional, or other regulatory issues that may be associated with MON 87427.

[redacted]

Date: December 15 2010

Regulatory Affairs Manager
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 except to the extent it is requested under the Freedom of Information Act (FOIA), 5 U.S.C., § 552; FDA complies with the provisions of FOIA and FDA's implementation regulations (21 CFR Part 20); and this information is responsive to the specific request. Except in accordance with the Freedom of Information Act, Monsanto does not authorize the release, publication or other distribution of this information (including website posting) without Monsanto's prior notice and consent.

ABBREVIATIONS AND DEFINITIONS[1]

AD_2010	Allergen and gliadin protein sequence database (Release date January 22, 2010)
ADF	acid detergent fiber
ae/A	acid equivalent per acre
BLOCKS	A database of amino acid motifs found in protein families
BLOSUM	BLOcks SUbstitution Matrix, used to score similarities between pairs of distantly related protein or nucleotide sequences
BSA	Bovine serum albumin
bw	Body weight
CFR	Code of Federal Regulations
CMS	Cytoplasmic male sterility
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4
cp4 epsps	Codon-optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein
CTAB	Cetyltrimethylammonium bromide
CTP2	chloroplast transit peptide
dwt	Dry weight
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase enzyme
E-Score	Expectation score
FARRP	Food Allergy Research and Resource Program Database
FASTA	Algorithm used to find local high scoring alignments between a pair of protein or nucleotide sequences
fwt	Fresh weight
GE	Genetically Engineered
GenBank	A public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
GI	Gene Identification number
GLP	Good Laboratory Practice
HEPES	N-[2-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)]
IgE	Immunoglobulin E
kDa	KiloDalton
LOQ	Limit of quantitation
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cutoff

[1] Alred, G.J., C.T. Brusaw, and W.E. Oliu. 2003. Handbook of Technical Writing, 7th edn., pp. 2-7. Bedford/St. Martin's, Boston, MA.

NCBI	National Center of Biotechnology Information at the National Institutes of Health, Bethesda, MD, USA
NDF	neutral detergent fiber
NFDM	non-fat dry milk
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Containing 0.05% (v/v) Tween-20
PCR	Polymerase chain reaction
PRT_2010	GenBank protein database, 175.0 (Release date January 22, 2010)
PVDF	Polyvinylidene Difluoride
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SOP	Standard operating procedure
sp.	Species
T-DNA	Transfer DNA
TDF	Total dietary fiber
Tm	Melting temperature
TOX_2010	Toxin protein sequence database (Release date January 22, 2010)
U.S.	United States of America
UTP	Uridine-5'-triphosphate
v/v	Volume to Volume ratio
w/v	Weight to Volume ratio

NAME AND ADDRESS OF SUBMITTER

The submitter of this safety and nutritional assessment summary for maize MON 87427 is:

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

Communications with regard to this submission should be directed to [redacted], [redacted] Regulatory Affairs Manager, at the Monsanto address listed above, [redacted]

STATUS OF SUBMISSION TO USDA-APHIS

Monsanto requested a Determination of Nonregulated Status for MON 87427, including all progenies derived from crosses between MON 87427 and conventional maize or other maize lines previously deregulated in the United States from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) in October, 2010. Under regulations administered by USDA-APHIS (7 CFR 340), MON 87427 is currently considered a "regulated article." Monsanto will continue to conduct all field tests for MON 87427 in strict compliance with USDA field trial regulations until a Determination of Nonregulated Status is granted for MON 87427. Once MON 87427 is deregulated, authorization for import, interstate movement or environmental release of MON 87427 in the United States will no longer be required.

STATUS OF SUBMISSION TO U.S. EPA

Monsanto submitted amended Supplemental Labeling for Registration Numbers 524-537 (Roundup WeatherMAX) and 524-549 (Roundup PowerMAX), which modifies the current use pattern for glyphosate in hybrid maize seed production systems based on MON 87427 in June, 2010 to the United States Environmental Protection Agency (U.S. EPA). This use of glyphosate and the Supplemental Labels were first approved by EPA in April 2008. The amended labeling refines the use directions and removes the current grazing restriction, which is currently required due to the regulated status of MON 87427 and the potential for maize forage glyphosate residues above the current tolerance. This use of glyphosate does not present any new environmental exposures scenarios not previously evaluated and deemed acceptable by EPA.

STATUS OF SUBMISSIONS TO OTHER GOVERNMENT AGENCIES

MON 87427 regulatory submissions will be made to countries that import significant maize or food and feed products derived from U.S. maize and have functional regulatory review processes in place. These governmental regulatory agencies include, but not limited to Canada, Japan, Mexico, Korea, Taiwan, Philippines, and Colombia, as well as to regulatory authorities in other maize importing countries with functioning regulatory systems. As appropriate, notifications will be made to countries that import significant

quantities of U.S. maize and maize products and do not have a formal regulatory review process for biotechnology-derived crops.

EXECUTIVE SUMMARY

Food and Feed Safety Assessment of MON 87427

MON 87427 Product Description

Monsanto Company has developed biotechnology-derived MON 87427 maize with tissue-selective glyphosate tolerance to facilitate the production of viable hybrid maize seed. MON 87427 produces the same 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein that is produced in commercial Roundup Ready® crop products, via the incorporation of a *cp4 epsps* coding sequence. CP4 EPSPS protein confers tolerance to the herbicide glyphosate, the active ingredient in the family of Roundup® agricultural herbicides. Tissue-selective expression of CP4 EPSPS protein in MON 87427 facilitates an extension of the use of glyphosate tolerant maize to enable its use as a tool for hybrid maize seed production.

MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to drive CP4 EPSPS protein expression in vegetative and female reproductive tissues, conferring tolerance to glyphosate in the leaves, stalk, and root tissues and tissues that develop into seed or grain and silks. This specific promoter and intron combination also results in limited or no production of CP4 EPSPS protein in two key male reproductive tissues: pollen microspores, which develop into pollen grains, and tapetum cells that supply nutrients to the pollen. Thus, in MON 87427, male reproductive tissues critical for male gametophyte (pollen) development are not tolerant to glyphosate. This allows glyphosate-treated MON 87427 containing inbred lines to serve as a female parent in the production of hybrid seed. Two glyphosate applications that are made during maize vegetative growth stages ranging from V8 to V13 to inbreds containing MON 87427 will produce a male sterile phenotype through tissue-selective glyphosate tolerance. This will eliminate or greatly reduce the need for detasseling, which is currently used in the production of hybrid maize seed. In a hybrid maize seed production system, the MON 87427 inbred plants, with glyphosate applied during tassel development, will be pollinated by pollen donor (male) plants. This will result in viable hybrid maize seed carrying the gene for tissue-selective glyphosate tolerance. For weed control in both seed and grain production fields, glyphosate may be applied to MON 87427 at vegetative stages as directed on Roundup agricultural product labels, at the same rates used in previously deregulated Roundup Ready® corn 2 events (NK603 and MON 88017).

Only specifically timed glyphosate applications will produce a male sterile phenotype through tissue-selective glyphosate tolerance in MON 87427. Glyphosate is a systemic herbicide that is readily translocated via the phloem in plants. Once glyphosate is in the phloem, it moves to areas of high meristematic activity, following a typical source to sink distribution. Pollen development in a maize plant takes approximately four weeks to complete. Early tassel growth stages start at the approximate maize vegetative growth

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stage V9, therefore glyphosate applications made at approximately this time allow maximum translocation of glyphosate to the male reproductive tissues, and selectively cause cell death in only those cells that are not tolerant to glyphosate (i.e. tapetum and pollen cells).

The benefits of MON 87427 in the production of hybrid seed include:

- *Increased Flexibility in Hybrid Seed Production*: Each year approximately 0.5 M acres used for hybrid maize seed production must be detasseled in order to meet commercial growers' hybrid maize seed needs and to meet established seed purity criteria in the U.S. The critical time period for detasseling is after the tassel has emerged but prior to pollen shed and silk emergence, and encompasses an average 3 - 4 day window. Current detasseling practices may require up to two passes with mechanical detasseling equipment and up to three passes if hand detasseling is used. Further complicating detasseling activity is the logistical planning required for moving enough labor and resources to the designated hybrid seed production fields at the appropriate time. Glyphosate applications made to MON 87427 during the V8 to V13 vegetative growth stages results in the male sterile phenotype. The two glyphosate applications needed to produce the male sterile phenotype would take place during an approximate 14 day window within these growth stages; a much longer time period compared to an average 3 – 4 day window between tassel emergence and pollen shed and silk emergence. This timing accounts for significantly improved flexibility in hybrid seed production.

- *Economic Benefits for Hybrid Seed Producers:* Seed manufacturers continually seek ways to improve hybrid seed productivity and reduce the inputs and land area used to produce high quality hybrid seed. Agricultural field labor costs continue to make up a large percentage of total costs to produce seed in the U.S. Compounding this increasing cost is population migration towards urban areas that is shrinking the agricultural labor pool, thus reducing a reliable labor pool for this work. Costs associated with labor recruitment and deployments to perform detasseling are some of the largest cost improvement opportunities in hybrid seed production. MON 87427 will decrease hybrid seed production costs primarily from a reduction in direct and associated labor costs.

Molecular Characterization of MON 87427 Verifies the Integrity and Stability of the Inserted DNA

MON 87427 was developed through *Agrobacterium*-mediated transformation of maize immature embryos from line LH198 × HiII utilizing plasmid vector PV-ZMAP1043. PV-ZMAP1043 contains one T-DNA that is delineated by Left and Right border regions. The T-DNA contains one expression cassette consisting of the *cp4 epsps* coding sequence under the regulation of the *e35S* promoter, the *hsp70* intron, the *CTP2* targeting sequence, and the *nos* 3′ nontranslated region. After transformation, a single plant was selected and increased (MON 87427).

MON 87427 was subjected to an extensive molecular characterization. Southern blot analyses demonstrated that a single copy of the T-DNA sequence from PV-ZMAP1043 was integrated into the maize genome at a single locus. These analyses also demonstrated that there were no additional genetic elements, including backbone sequences, from PV-ZMAP1043 detected, linked or unlinked to the intact T-DNA present in MON 87427. The PCR and DNA sequence analyses performed on MON 87427 confirmed the organization of the elements within the insert, assessed potential rearrangements at the insertion site, and resulted in the determination of the complete DNA sequence of the T-DNA and adjacent maize genomic DNA sequence in MON 87427. Furthermore, Southern blot analysis demonstrated that the T-DNA insert in MON 87427 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 87427. Finally, results from segregation analyses demonstrated heritability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA in MON 87427 at a single chromosomal locus.

Data Confirm the Safety of Expression Products in MON 87427

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by FDA. The CP4 EPSPS protein expressed in MON 87427 is identical to the CP4 EPSPS in other Roundup Ready crops. Results from the protein characterization studies included in this petition confirmed the identity of the MON 87427-produced CP4 EPSPS protein and established the equivalence of the MON 87427-produced protein to the *E. coli*-produced CP4 EPSPS protein (Section VI.C.). The safety of CP4 EPSPS proteins present in biotechnology-derived crops has been extensively assessed.

A multistep approach was conducted according to guidelines established by the CODEX Alimentarius Commission and the Organization for Economic Co-operation and Development (OECD) and which embody the principles and guidance of the U.S. Food and Drug Administration's (FDA) 1992 policy on foods from new plant varieties, and was used to characterize the CP4 EPSPS protein in MON 87427 resulting from the genetic modification. This detailed assessment confirms the CP4 EPSPS protein is safe for human and animal consumption. The assessment includes: 1) characterization of the physicochemical and functional properties of CP4 EPSPS protein; 2) quantification of CP4 EPSPS expression in plant tissues; 3) examination of the similarity of CP4 EPSPS protein to known allergens, toxins or other biologically active proteins known to have adverse effects on humans and animals; 4) evaluation of the digestibility of CP4 EPSPS protein in simulated gastrointestinal fluids; 5) evaluation of the stability of CP4 EPSPS protein after heat treatment; 6) documenting the history of safe consumption of CP4 EPSPS protein or its structural and functional homology to proteins that lack adverse effects on human or animal health; 7) investigation of potential mammalian toxicity through animal assays and calculating margins of exposure; and 8) assessment of the potential for allergenicity, toxicity and adverse biological activity of putative polypeptides encoded by the insert and flanking sequences. The safety assessment supports the conclusion that dietary exposure to CP4 EPSPS protein derived from MON 87427 poses no meaningful risk to human or animal health.

Food and Feed Safety Assessments of MON 87427 Demonstrate Equivalence to Conventional Crop

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by FDA. The CP4 EPSPS protein expressed in MON 87427 is identical to the CP4 EPSPS protein in other Roundup Ready crops and the mode of action of CP4 EPSPS protein is well understood. Previous Roundup Ready crops reviewed by the FDA have had no biologically relevant compositional changes identified, and there is no reason to expect the CP4 EPSPS protein in MON 87427 to interact with endogenous metabolites or important nutrients that are present in maize grain or forage.

Detailed compositional analyses in accordance with OECD guidelines were conducted to determine whether levels of key nutrients, anti-nutrients and secondary metabolites in MON 87427 were comparable to levels present in the near-isogenic conventional control and several commercial maize reference hybrids. The maize references were used to establish the natural range of levels of the key nutrients, anti-nutrients, and secondary metabolites in commercial maize hybrids that have a history of safe consumption. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber, amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and vitamins [folic acid, niacin, A (β-carotene), B1, B2, B6, and E] in the grain, and proximates, ADF, NDF, calcium and phosphorus in forage. The anti-nutrients assessed in grain included phytic acid and raffinose. Secondary metabolites assessed in grain included furfural, ferulic acid, and p-coumaric acid.

Combined-site analyses were conducted to determine statistically significant differences (5% level of significance) between MON 87427 and the conventional control on both forage and grain samples. Statistical results from the combined-site data were reviewed using considerations relevant to safety and/or nutritional value. These considerations included assessments of: 1) the relative magnitude of the differences in the mean values of key nutrient, anti-nutrient, and secondary metabolite components of MON 87427 and the conventional control, 2) whether the MON 87427 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of commercial maize reference hybrids grown concurrently, 3) evaluation of the reproducibility of the significant (α=0.05) combined-site component differences at individual sites, and 4) assessing the difference within the context of natural variability of commercial maize composition published in the scientific literature and in the International Life Sciences Institute (ILSI) Crop Composition Database.

The levels of assessed components in MON 87427 were compositionally equivalent to the conventional control and within the range of variability of the commercial reference varieties that were grown concurrently. The results demonstrated that the differences observed in the combined-site analysis were not meaningful to food and feed safety or the nutritional quality of MON 87427 maize and support the overall food and feed safety of MON 87427.

Conclusion

All data support the conclusion that food and feed derived from MON 87427 will be as safe and nutritious as food and feed derived from a conventional maize crop. Therefore, the consumption of MON 87427 and the food and feed derived from it will be fully consistent with FDA's Policy (U.S. FDA, 1992) and in compliance with all applicable requirements of the Federal Food, Drug and Cosmetic Act.

I. DESCRIPTION OF MON 87427

This section provides a description of MON 87427 being presented for food and feed safety and nutritional assessment. The description identifies the crop, the transformation event to be reviewed and the type and purpose of the modification, which aids in understanding the nature of the food and feed products that may be developed from MON 87427. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 22.

I.A. MON 87427 Summary

In accordance with OECD's "Guidance for the Designation of a Unique Identifier for Transgenic Plants" MON 87427 has been assigned the unique identifier MON-87427-7

The maize line LH198 × HiII, a proprietary, conventional maize line developed by Monsanto Company, was used as the recipient for the DNA insertion to create MON 87427.

I.B. Rationale for the Introduction of MON 87427

Maize (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. Planted maize acres in the U.S. have ranged from 78.3 to 93.5 million acres from 2005 to 2009 (USDA-NASS, 2010). Maize differs from other major U.S. crops, such as soybean or cotton, in that it is typically planted as a hybrid, and maize hybrids are utilized on nearly all maize production acres currently planted in the U.S. Significant use of hybrids in U.S. maize production dates to the 1930's (Wych, 1988). Maize hybrids have been, and still are, developed and used based on the positive yield increases and plant vigor associated with heterosis, which is also known as hybrid vigor (Duvick, 2001). Inherent to the cultivation of hybrid plants, seed produced from hybrid plants is typically not used for replanting, due to the loss of hybrid vigor. Therefore, new hybrid seed is used each year for planting.

The seed supply used to plant the U.S. maize acreage is generated via hybrid seed production methods and occurs on approximately 0.5 M acres annually (Jugenheimer, 1976). Modern hybrid maize seed production is based on the use of two maize inbred parents, one designated as a female parent and one as a male parent. Hybrid seed production is accomplished through the combining of genetic material from one inbred parent with that of the other inbred parent. Specifically, pollen from the tassel (male flower) of the male parent is used to fertilize the ear (female flower) of the female parent. Maize is a monoecious plant, having separate male and female flowers on the same plant. Due to this separation of the male and female flowers, there exists a practical opportunity to easily facilitate the combining of genetic material, compared to other plant species that contain both male and female reproductive structures in the same flower. The physical separation of the male and female flowers on maize make it well suited for hybrid seed production.

One issue inherent to the production of hybrid maize seed is that the female parent produces pollen at the same time as the male parent. Therefore, pollen from the female parent must be removed or eliminated in order to assure genetic transfer via pollen only from the male parent to the female parent. Pollen from the female parent is removed or eliminated in one of two ways in current hybrid maize seed production. The current primary option utilized for removal of pollen from the female parent during hybrid maize seed production is detasseling. Detasseling is accomplished by physically removing the male flower (tassels) from the female parent prior to pollen shed. Although detasseling is the primary option for removing pollen from the female parent, there are some negative aspects associated with it. These include the need for a large labor pool to perform physically demanding work under very tight (3-4 day) time constraints, and the need for repeated observations to ensure that only the pollen produced from the male inbred is available for hybrid seed production. The other option for eliminating pollen from the female parent during hybrid maize seed production is through the use of Cytoplasmic Male Sterile (CMS) maize. This is a naturally occurring, maternally inherited trait in maize known to produce male sterile plants (Laughnan and Gabay-Laughnan, 1983). However a resource intensive breeding integration process is necessary to move CMS into a particular inbred background, and incomplete male sterility has been noted with CMS that necessitates some detasseling (Wych, 1988).

Monsanto Company has developed MON 87427 maize, with tissue-selective glyphosate tolerance, to facilitate the production of viable hybrid maize seed. This technology allows for more efficient maize hybrid seed production compared to mechanical detasseling or the use of CMS, while producing seed of the same commercially acceptable standards. MON 87427 produces the CP4 EPSPS protein via the incorporation of a *cp4 epsps* coding sequence. Tissue-selective expression of the CP4 EPSPS protein in MON 87427 facilitates an extension of the use of glyphosate tolerant maize to enable its use as a tool for hybrid maize seed production.

MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to drive CP4 EPSPS protein expression in vegetative and female reproductive tissues, conferring tolerance to glyphosate in the leaves, stalk, and root tissues and tissues that develop into seed or grain and silks. Use of this specific promoter and intron combination also results in limited or no production of CP4 EPSPS protein in two key male reproductive tissues: pollen microspores which develop into pollen grains, and tapetum cells that supply nutrients to the pollen (Goldberg, et al., 1993; Huang, et al., 2009). Thus, in MON 87427, male reproductive tissues critical for male gametophyte development are not tolerant to glyphosate. The limited to no production of CP4 EPSPS in pollen of MON 87427 is not unexpected as both the *e35S* promoter and the *CaMV 35S* promoter, which is the promoter from which *e35S* originated (Kay, et al., 1987; Odell, et al., 1985), have demonstrated limited ability in certain crops to drive expression of a gene of interest in pollen previously (CaJacob, et al., 2004; Hamilton, et al., 1992).

Only specifically timed glyphosate applications beginning just prior to and/or during tassel development stages (approximate maize vegetative growth stages ranging from V8 to V13) will produce a male sterile phenotype through tissue-selective glyphosate tolerance, and will eliminate or greatly reduce the need for detasseling, which is currently

used in the production of hybrid maize seed. Glyphosate is a systemic herbicide that is readily translocated via the phloem in plants (Devine, et al., 1993). Once glyphosate is in the phloem, it moves to areas of high meristematic activity, such as developing reproductive tissues, following a source to sink distribution (Devine et al., 1993). Pollen development in a maize plant takes approximately four weeks to complete (Ma, et al., 2008). Early tassel development stages start at the approximate maize vegetative growth stage V9 (Ritchie, et al., 1997; Tranel, et al., 2008), therefore glyphosate applications made at approximately this time allow maximum translocation of glyphosate to the male reproductive tissues, and selectively causes cell death in only those cells that are not tolerant to glyphosate (i.e. tapetum and pollen cells). Glyphosate applications made during early vegetative stages, consistent with the application timing specified in the current Roundup agricultural product label for weed control purposes, do not affect pollen production of MON 87427 because the sensitive male reproductive tissues are not actively developing at that time. The tissue-selective glyphosate tolerance of MON 87427 allows glyphosate-treated MON 87427 to serve as a female parent inbred in the production of hybrid seed. Pollen from the corresponding male parent inbred line will fertilize MON 87427 resulting in viable hybrid maize seed carrying the gene for tissue-selective glyphosate tolerance. When MON 87427 is present in hybrid seed used by growers for the production of maize grain, it does not impact agronomic performance.

The benefits of MON 87427 in the production of hybrid seed include:

- *Increased Flexibility in Hybrid Seed Production*: Each year approximately 0.5 M acres used for hybrid maize seed production must be detasseled in order to meet commercial growers' hybrid maize seed needs and to meet established seed purity criteria in the U.S. The critical time period for detasseling is after the tassel has emerged but prior to pollen shed and silk emergence, and encompasses an average 3 - 4 day window. Current detasseling practices may require up to two passes with mechanical detasseling equipment and up to three passes if hand detasseling is used. Further complicating detasseling activity is the logistical planning required for moving enough labor and resources to the designated hybrid seed production fields at the appropriate time. Glyphosate applications made to MON 87427 during the V8 to V13 vegetative growth stages results in the male sterile phenotype. The two glyphosate applications needed to produce the male sterile phenotype would take place during an approximate 14 day window within these growth stages; a much longer time period compared to an average 3 – 4 day window between tassel emergence and pollen shed and silk emergence. This timing accounts for significantly improved flexibility in hybrid seed production.

- *Economic Benefits for Hybrid Seed Producers:* Seed manufacturers continually seek ways to improve hybrid seed productivity and reduce the inputs and land area used to produce high quality hybrid seed. Agricultural field labor costs continue to make up a large percentage of total costs to produce seed in the U.S. Compounding this increasing cost is population migration towards urban areas that is shrinking the agricultural labor pool, thus reducing a reliable labor pool for this work. Costs associated with labor recruitment and deployments to perform detasseling are some of the largest cost improvement opportunities in hybrid seed

production. MON 87427 will decrease hybrid seed production costs primarily from a reduction in direct and associated labor costs.

I.C. Applications for Which MON 87427 is Not Suitable

Monsanto Company is aware of no food or feed uses of conventional maize that are not applicable to MON 87427.

II. DESCRIPTION OF THE HOST PLANT AND ITS USES AS FOOD OR FEED

This section includes data and information that provides a comprehensive description of the host plant. It also provides relevant phenotypic information on the host plant and on related species that may have contributed to the genetic background of the host plant. The history of use information provided describes how the plant is typically cultivated, transported and stored, any special processing required to assure the plant is safe to eat, and the plant's usual role in the diet. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraphs 23, 24, and 25.

II.A. Biology of Maize

Zea mays subspecies mays (L.), referred to as maize in this petition, is a versatile crop that provides food, feed, and fuel to the global economy. The biology of maize is well understood and documented. The Organization for Economic Co-operation and Development (OECD) Consensus Document on the Biology of *Zea mays* subsp. *mays* (Maize) (OECD, 2003) provides key information on:

- general description of maize biology, including taxonomy and morphology and use as of maize as a crop plant
- agronomic practices in maize cultivation
- geographic centers of origin
- reproductive biology
- inter-species/genus introgression into relatives and interactions with other organisms
- summary of the ecology of maize

Additional information on the Biology of maize can also be found on the Australian Government Department of Health and Ageing (Office of the Gene Technology Regulator) web site (OGTR, 2008).

In addition, more information about the reproductive biology of maize, specifically on the process of pollen development and gametogenesis in maize, is provided in The Maize Handbook (Bedinger and Russell, 1994).

II.A.1. History of Maize Development

The domestication of maize is known to have occurred in southern Mexico between 7,000 and 10,000 years ago (Goodman and Galinat, 1988). While the putative parents of maize have not been recovered, it is likely that teosinte played an important role in the genetic background of maize. Maize, as we know it today, cannot survive in the wild because the female inflorescence (the ear) restricts seed dispersal. The transformation from a wild, weedy species to one dependent on humans for its survival probably evolved over a long period of time through plant breeding by the indigenous inhabitants of the Western Hemisphere.

The first effective introduction of maize into Europe occurred in 1493 (Goodman and Galinat, 1988). Within two generations after the introduction of maize to Europe, maize

became distributed throughout those regions of the world where it could be cultivated. Today, maize is grown in nearly all areas of the globe, and is the largest cultivated crop in the world followed by wheat (*Triticum sp*.) and rice (*Oryza sativa L*.) in total global metric ton production (FAOSTAT, 2009). However, unlike wheat and rice, the majority of maize produced in the U.S. is consumed as animal feed in the form of grain, forage, or silage.

The original maize-growing areas in the U.S. did not include the North Central region of the United States (U.S. Maize Belt). The highly productive dent maize grown in the U.S. Maize Belt was 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 commercial dent maize grown in the U.S. Maize Belt evolved from the gradual mingling of those colonial settlements that spread north and west from Southeastern North America, and those colonial settlements that spread south and west from Northeastern North America.

There are two major backgrounds of maize that were used to develop the current commercial hybrids grown in the U.S., the northern flints and southern dent maize. Although the origin of northern flints is unclear, maize types found in the highlands of Guatemala have similar ear morphology as northern flints (Goodman and Brown, 1988). Characteristics of northern flints maize include eight-rowed cylindrical ears, early maturity, and short-statured plants with tillers. The southern dent maize grown in the southeast U.S. likely originated from the southeast coast of Mexico. Southern dent maize is characterized as having tall, late maturing, non-tillered, and poorly rooted plants with soft-textured white kernels on many-rowed, tapering ears.

Virtually all the maize grown in the U.S. today is a hybrid. Maize hybrids are developed and used based on the positive yield increases and plant vigor associated with heterosis, also known as hybrid vigor.

The history of hybrid maize dates back to the early 1920s when the first commercial hybrids were produced and sold (Wych, 1988). The first hybrid maize seed developed for the U.S. maize belt was called "Copper Cross" (Crabb, 1947) which was utilized on a very limited basis starting in 1924. During the drought conditions of 1934 and 1936 farmers noticed the enhanced performance of hybrid maize seed over the open pollinated inbred varieties, and eventually began to accept and demand access to these new hybrids (Wych, 1988). Hybrid maize is now used globally in all commercial maize growing regions of North America, Europe, Argentina, China, and Brazil (Duvick, 2001).

Hybrid maize seeds are produced on a large scale through the use of two maize inbred parents, one designated as a female parent and the other as a male parent. Production of hybrid seed is accomplished through the combination of genetic material from one inbred parent to another. Pollen from the tassel (male flower) of the male parent is used to fertilize the ear (female flower) of the female parent after eliminating the potential for pollen flow from the female parent through detasseling. Traditionally, detasseling is accomplished by physically removing the male flower (tassels) from the female parent prior to pollen shed. This method of hybrid production is unique to maize, compared to

other cereal crops, because maize is a monoecious plant, the male and female flowers are spatially separated unlike some other plant species with both male and female reproductive structures in the same flower. This physical separation of the male and female flowers on maize make it well suited for hybrid seed production.

II.B. Characteristics of the Recipient Plant

The maize germplasm that was utilized as the recipient of the transgene to create MON 87427 was LH198 x HiII. This line was used because it responds well to *Agrobacterium*-mediated transformation and tissue regeneration.

The LH198 inbred line was released in 1992 by Holden's Foundation Seeds, Inc of Williamsburg Iowa. LH198 is an inbred related to the stiff-stalk family and was derived from the cross (LH132 × B84) × LH132. LH132 is also a Holden's Foundation Seed inbred and B84 is an inbred released by Iowa State University.

The HiII inbred germplasm was specifically developed for use in maize transformation and is publicly available from the Maize Genetics Stock Center (MaizeGDB, 2010). The HiII germplasm was derived from the cross between two Stiff Stalk inbreds B73 and A188 (Armstrong, et al., 1991).

In developing the data to support this safety summary, appropriate test materials were generated for the molecular characterization (Sections IV and V), safety assessment of the expressed protein (Section VI), and composition analysis (Section VII). Molecular characterization was conducted with the MON 87427 test material generation LH198 BC3F4 (Figure V-8) that was used to initiate commercial breeding efforts. Protein safety assessment and composition analysis were conducted with the MON 87427 test material generation [LH198 BC3F7 × LH287] F1 (Figure V-8).

For purposes of evaluating food and feed safety, there are no practical differences between MON 87427 containing hybrids used for grain production, and MON 87427 inbred maize lines used for seed production. In both instances hybrids and inbreds express the CP4 EPSPS protein and hybrid maize lines contain the genetic material from both parental inbreds. The hybrid generation of MON 87427 ([LH198 BC3F7 × LH287] F1) was used for protein characterization and expression analysis in the protein safety assessment and for composition analysis, because it is representative of commercial hybrid maize, and thus represents the form of MON 87427 that will be most exposed to the consumers and livestock. This reasoning is based on the millions of acres of commercial maize production and the millions of tons of commodity maize grain produced from that acreage, compared to the far smaller number of acres for hybrid seed production and the minimal amount of grain from those acres that enters commodity maize stocks. Therefore, the food and feed safety evaluation that was conducted on MON 87427 hybrids is appropriate and equally applicable to the inbreds.

Conventional control materials were developed for use in the Regulatory studies along side the MON 87427 test materials. These conventional controls were non-transformed maize lines with similar germplasm backgrounds to MON 87427, but did not contain the

cp4 epsps expression cassette, so that the effect of the genetic insert could be assessed in an unbiased manner. The conventional control materials included the original transformation line (LH198 × HiII) used for the molecular characterization; and the hybrid conventional control (LH198 × LH287) which has a similar genetic background to the hybrid MON 87427 test material ([LH198 BC3F7 × LH287] F1). The LH198 × LH287 hybrid was the conventional control used in the compositional analysis (Section VII), and protein safety assessment (Section VI). Where appropriate, commercial reference maize materials (hereafter referred to as commercial references) were used to establish a range of variability or responses representative of commercial maize in the U.S. The commercial references used at each location were selected based on their availability and agronomic fitness.

II.B.1. Known Allergenicity of Recipient Plant

Maize has been a staple of the human diet for centuries, and its processed fractions are consumed in a multitude of food and animal feed products. Maize is not a common allergenic food and there have been few reports of allergenic reactions to the consumption of maize products (OECD, 2002). Although rare, some cases of maize allergenicity have been reported. However, these studies generally involve patients with multiple allergies which complicate diagnosis due to potentially weak and irrelevant cross-reactivity exhibited by skin prick tests and in-vitro IgE binding evaluations (Pasini, et al., 2002; Pastorello, et al., 2000; Pauls and Cross, 1998; Tanaka, et al., 2001).

II.C. Maize as a Feed Source

Maize is the preferred material for the production of livestock feed because of its high nutrient value and relative low cost (OECD, 2002). Almost 43% of maize produced in the United States in 2009 was used for animal feed (USDA-ERS, 2010). Due to its high starch and low fiber contents, maize is considered a valuable energy source in animal feed for live stocks, such as cattle, pigs and poultry. Whole maize is usually ground and mixed with a high-protein feed compound and with vitamin and mineral supplements to balance the ratio according to the nutritional requirements of the animals being fed (Leath and Hill, 1987). In addition, maize-derived feed can also be produced as a by product of the dry and wet milling processes (Section II.D), or as a whole plant silage.

The maize milling industry produces several livestock feed products such as maize gluten feed which is mostly fed to ruminants such as cattle, and maize gluten meal, which is mostly included in poultry diets, especially gluten meal derived from yellow maize. The entire maize plant can also be harvested and used as livestock feed, as seen in whole plant maize silage. During the whole plant maize silage process, whole plant maize is harvested, chopped, and ensiled (stored in a silo). About 50% of the available energy present in livestock feed produced from whole maize silage is contained in the grain portion of the maize plant. The other 50% is mostly contained in the stalks, leaves, and cobs. Therefore, in order to produce high energy silage, it is important that the maize plant be well developed before harvest (Perry, 1988).

II.D. Maize as a Food Source

Maize grain contains 82% endosperm, 12% germ, 5% bran, and 1% tip cap. In addition, 2.2% of the bran fraction is made up of crude fiber (Earle, et al., 1946; Perry, 1988).

Maize processing methods include wet milling, dry milling, and fermentation. The milling process separates the maize kernel into three basic parts; endosperm, pericarp, and the germ (Watson, 1988).

Products from wet milling: The majority of the maize used for food and industrial purposes is processed by wet milling to produce starch and sweetener products for use in foodstuffs. Starch is used as a food ingredient in: dairy and ice cream; batters and breading; baked goods; soups, sauces and gravies; salad dressings; meat and poultry; confections; and, in drinks. Starch can also be converted to a variety of sweetener and fermentation products including high fructose maize syrup and ethanol (Watson, 1988).

Products from dry milling: The primary food products derived from the dry milling process are maize grits, maize meal, and maize flours. Maize grits are derived from endosperm of the maize kernel, with less than 1 % oil content. Maize grits are consumed in the U.S. as side dish for breakfast. Maize meal, however, has larger particles than maize grits and is often enriched with thiamine, riboflavin, niacin, and iron to produce baked products such as maize bread and muffins. Maize flour consists of fine endosperm particles, and is often used as a binder in processed meats, as well as in producing several snack foods (Rooney and Serna-Saldivar, 1987).

Products from fermentation: Starch produced from the wet milling process can also be used in producing ethanol and distilled beverages through fermentation (Rooney and Serna-Saldivar, 1987).

III. DESCRIPTION OF THE DONOR ORGANISMS

This section describes the donor organism for the introduced protein. It contains information describing if the donor organism exhibit characteristics of pathogenicity or toxin production, is a known allergenic source, or have other traits that affect food and feed safety. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraph 26.

III.A. Identity and Sources of the Genetic Material Introduced into MON 87427

The donor organism, *Agrobacterium* sp. strain CP4, was isolated based on its tolerance to glyphosate brought about by the production of a naturally glyphosate-tolerant EPSPS protein (Padgette, et al., 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection (ATCC) as an *Agrobacterium* species. This identification was made based on morphological and biochemical characteristics of the isolate and its similarity to a reference strain of *Agrobacterium*. The taxonomy of *Agrobacterium* sp. is:

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Alphaproteobacteria

Order: Rhizobiales

Family: Rhizobiaceae

Genus: Agrobacterium

Agrobacterium sp. strain CP4 is related to microbes commonly present in the soil and in the rhizosphere of plants. *Agrobacterium* species are not known for human or animal pathogenicity, and are not commonly allergenic. According to FAO/WHO there is no known population of individuals sensitized to bacterial proteins (FAO, 2001).

MON 87427 was developed through *Agrobacterium*-mediated transformation using the binary plasmid vector PV-ZMAP1043. PV-ZMAP1043 is approximately 8.9 kb and contains one T-DNA that is delineated by Left and Right Border regions. The T-DNA contains one expression cassette consisting of the *cp4 epsps* coding sequence under the regulation of the *e35S* promoter, the *hsp70* intron, the *CTP2* targeting sequence, and the *nos* 3′ nontranslated region. As described in Tables IV-1 and V-2, the *e35S* promoter, which directs transcription in plant cells, contains the duplicated enhancer region from the cauliflower mosaic virus (CaMV) 35S RNA. The *hsp70* intron is the first intron from the maize heat shock protein 70 gene. The *CTP2* targeting sequence is the targeting sequence from the *ShkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS that directs transport of the CP4 EPSPS protein to the chloroplast. The *nos* 3' nontranslated region is the 3' nontranslated region of the *nopaline*

synthase (nos) gene from *Agrobacterium tumefaciens* that terminates transcription and directs polyadenylation.

There is no evidence of any safety issues related to the use of MON 87427, and there is no evidence of human or animal pathogenicity for any of the donor organisms that provided the coding and non coding DNA sequences present in MON 87427. Finally, *A. tumefaciens* has not been reported to be a source of allergens. DNA has always been present in food and, upon consumption, is quickly degraded to nucleic acids by restriction nucleases present in the gastrointestinal tract of humans and animals (Jonas, et al., 2001). According to the U.S. FDA (U.S. FDA, 1992), nucleic acids are present in the cells of every living organism, do not raise concerns as a component of food, and are generally recognized as safe. Results from an International Life Sciences Institute (ILSI) workshop on safety considerations of DNA in food were reported (Jonas et al., 2001) and confirmed that: 1) all DNA including recombinant DNA is composed of the same four nucleotides; 2) there are no changes to the chemical characteristics or the susceptibility to degradation by chemical or enzymatic hydrolysis of recombinant DNA as compared to non recombinant DNA; and 3) there is no evidence that DNA from dietary sources has ever been incorporated into the mammalian genome.

IV. DESCRIPTION OF THE GENETIC MODIFICATION

This section provides a description of the transformation process and plasmid vector used in the development of MON 87427. Molecular analyses are an integral part of the characterization of crop products with new traits introduced by methods of biotechnology. Vectors and methods are selected for transformation to achieve high probability of obtaining the trait of interest and integration of the introduced DNA into a single locus in the plant genome. This helps ensure that only the intended DNA encoding the desired trait(s) is integrated into the plant genome and facilitates the molecular characterization of the product. Information provided here allows for the identification of the genetic material present in the transformation vector delivered to the host plant and for an analysis of the data supporting the characterization of the DNA inserted in the plant found in Section V. The information provided in this section also addresses the Codex Plant Guidelines, Section 4, paragraphs 27, 28, and 29.

MON 87427 was developed through *Agrobacterium*-mediated transformation of maize immature embryos from line LH198 × HiII utilizing PV-ZMAP1043. This section describes the plasmid vector, the donor gene, and the regulatory elements used in the development of MON 87427 as well as the deduced amino acid sequence of the CP4 EPSPS protein produced in MON 87427. In this section, transfer DNA (T-DNA) refers to DNA that is transferred to the plant during transformation. An expression cassette is comprised of sequences to be transcribed and the regulatory elements necessary for the expression of those sequences.

IV.A. Plasmid Vector PV-ZMAP1043

PV-ZMAP1043 was used in the transformation of maize to produce MON 87427 and is shown in Figure IV-1, and the elements included in this vector are described in Table IV-1. PV-ZMAP1043 is approximately 8.9 kb and contains one T-DNA that is delineated by Left and Right Border sequences. The T-DNA contains one expression cassette consisting of the *cp4 epsps* coding sequence under the regulation of the *e35S* promoter, the *hsp70* intron, the *CTP2* targeting sequence, and the *nos* 3′ non-translated region.

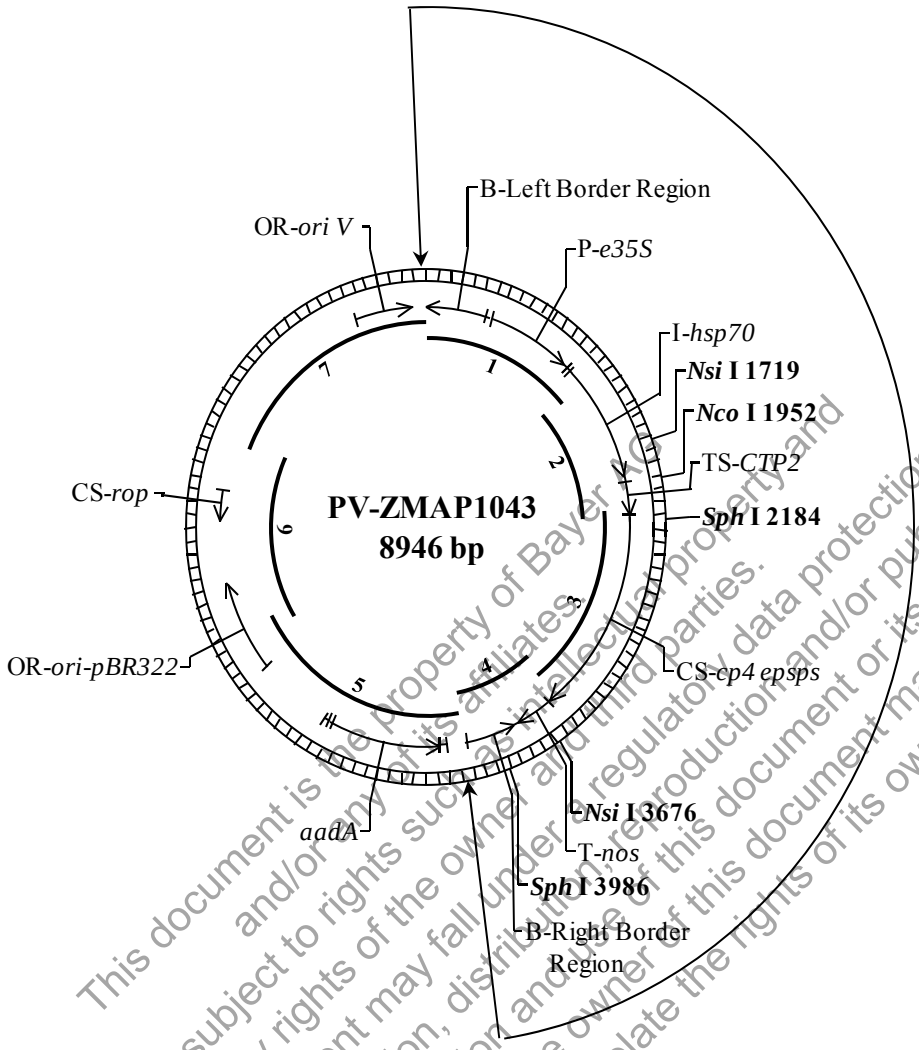
The backbone region of PV-ZMAP1043, located outside of the T-DNA, contains two origins of replication for maintenance of the plasmid vector in bacteria (*ori V*, *ori-pBR322*), a bacterial selectable marker gene (*aadA*), and a coding sequence for repressor of primer protein for maintenance of plasmid vector copy number in *E. coli* (*rop*). A description of the genetic elements and their prefixes (e.g., B-, P-, I-, TS-, CS-, T-, and OR-) in PV-ZMAP1043 is provided in Table IV-1.

IV.B. Description of the Transformation System

MON 87427 was developed through *Agrobacterium*-mediated transformation of immature maize embryos based on the method described by Sidorov and Duncan (2009), utilizing PV-ZMAP1043. Immature embryos were excised from a post-pollinated maize ear of LH198 × HiII. After co-culturing the excised immature embryos with

Agrobacterium carrying the plasmid vector, the immature embryos were placed on selection medium containing glyphosate and carbenicillin disodium salt in order to inhibit the growth of untransformed plant cells and excess *Agrobacterium*. Once transformed callus developed, the callus was placed on media conducive to shoot and root development. Rooted R_0 plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R_0 plants generated through the transformation process described above had already been exposed to glyphosate in the selection medium and demonstrated glyphosate tolerance. Starting from a single R_0 plant, LH198 was then used as the recurrent parent through four backcrossing generations. Backcross progeny generations were evaluated for tolerance to glyphosate using a rate of 0.75 lb ae/A (0.84 kg ae/ha), a representative commercial application rate and timing. Surviving plants were then selfed to produce homozygous plants, which were identified through a quantitative polymerase chain reaction (PCR) analysis. MON 87427 was selected as the lead event based on superior phenotypic characteristics and comprehensive molecular profile. Regulatory studies on MON 87427 were initiated to further characterize the genetic insertion and the expressed protein, and to establish the food, feed, and environmental safety relative to commercial maize. The major steps involved in the development of MON 87427 are depicted in Figure IV-2.



Probe	DNA Probe	Start Position (bp)	End Position (bp)	Total Length (~kb)
1	T-DNA Probe 1	1	1200	1.2
2	T-DNA Probe 2	1150	2150	1.0
3	T-DNA Probe 3	2100	3550	1.5
4	T-DNA Probe 4	3500	4192	0.7
5	Backbone Probe 5	4193	5942	1.8
6	Backbone Probe 6	5864	7368	1.5
7	Backbone Probe 7	7290	8946	1.7

Figure IV-1. Circular Map of Plasmid Vector PV-ZMAP1043 Showing Probes 1-7
A circular map of the plasmid vector PV-ZMAP1043 used to develop MON 87427 is shown. Genetic elements and restriction sites used in Southern analyses (with positions relative to the size of the plasmid vector) are shown on the exterior of the map. The probes used in the Southern analyses are shown on the interior of the map. PV-ZMAP1043 contains a single T-DNA.

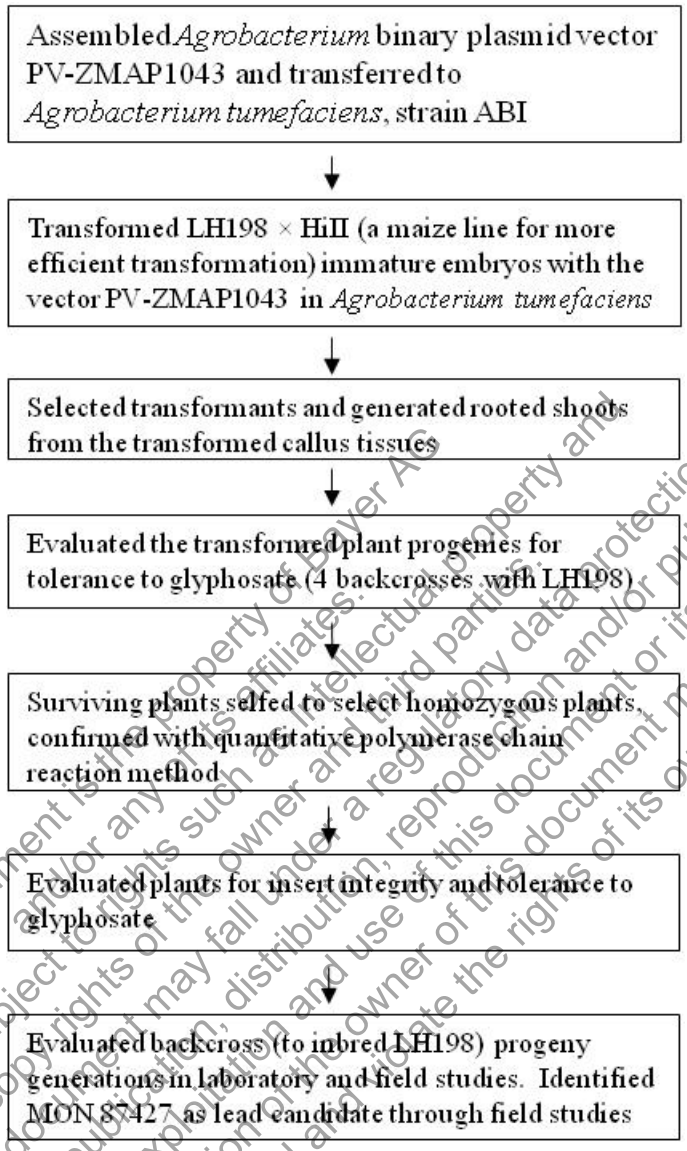


Figure IV-2. Schematic of the Development of MON 87427

IV.C. The *cp4 epsps* Coding Sequence and the CP4 EPSPS Protein (T-DNA)

The *cp4 epsps* expression cassette, also referred to as transfer DNA (T-DNA) in this petition, encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Figure IV-3) (Padgette et al., 1996). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry, et al., 2001; Padgette et al., 1996). The CP4 EPSPS protein is similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Barry et al., 2001; Padgette et al., 1996).

IV.D. Regulatory Sequences

The *cp4 epsps* coding sequence in MON 87427 is under the regulation of the *e35S* promoter, the *hsp70* intron, the *CTP2* targeting sequence, and the *nos* 3′ nontranslated region. The *e35S* promoter, which directs transcription in plant cells, contains the duplicated enhancer region (Kay et al., 1987) from the cauliflower mosaic virus (CaMV) 35S RNA promoter (Odell et al., 1985). The *hsp70* intron is the first intron from the maize heat shock protein 70 gene (Brown and Santino, 1997). The *CTP2* targeting sequence is the targeting sequence from the *ShkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS (Herrmann, 1995; Klee, et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast. The *nos* 3' nontranslated region is the 3' nontranslated region of the *nopaline synthase* (nos) gene from *Agrobacterium tumefaciens* that terminates transcription and directs polyadenylation (Bevan, et al., 1983).

IV.E. T-DNA Borders

PV-ZMAP1043 contains Right Border and Left Border regions (Figure IV-1 and Table IV-1) that were derived from *Agrobacterium tumefaciens* (Barker, et al., 1983; Depicker, et al., 1982; Zambryski, et al., 1982). The border regions each contain a 24-25 bp nick site that is the site of DNA exchange during transformation. The border regions separate the T-DNA from the plasmid backbone region and are involved in their efficient transfer into the maize genome.

IV.F Genetic Elements Outside of the T-DNA Borders

Genetic elements that exist outside of the T-DNA borders are those that are essential for the maintenance or selection of PV-ZMAP1043 in bacteria. The origin of replication *ori V* is required for the maintenance of the plasmid in *Agrobacterium* and is derived from the broad host plasmid RK2 (Stalker, et al., 1981). The origin of replication *ori-pBR322* is required for the maintenance of the plasmid in *E. coli* and is derived from the plasmid vector pBR322 (Sutcliffe, 1979). Coding sequence *rop* is the coding sequence of the repressor of primer (ROP) protein and is necessary for the maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989). The selectable marker *aadA* is a bacterial promoter and coding sequence for an enzyme from transposon Tn*7* that confers spectinomycin and streptomycin resistance (Fling, et al., 1985) in *E. coli* and *Agrobacterium* during molecular cloning. Because these elements are outside the border regions, they are not expected to be transferred into the maize genome. The absence of the backbone sequence in MON 87427 has been confirmed by Southern blot analyses (see Section V.B).

Table IV-1. Summary of Genetic Elements in Plasmid Vector PV-ZMAP1043

Genetic Element	Location in Plasmid Vector	Function (Reference)
T-DNA		
B[1]-Left Border Region	1-442	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	443-483	Sequences used in DNA cloning
P[2]-*e35S*	484-1104	Promoter for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells
Intervening Sequence	1105-1125	Sequences used in DNA cloning
I[3]-*hsp70*	1126-1929	First intron from the maize heat shock protein 70 gene (Brown and Santino, 1997)
Intervening Sequence	1930-1953	Sequences used in DNA cloning
TS[4]-*CTP2*	1954-2181	Targeting sequence from the *ShkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS[5]-*cp4 epsps*	2182-3549	Codon-optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgette et al., 1996)
Intervening Sequence	3550-3555	Sequences used in DNA cloning
T[6]-*nos*	3556-3808	3' nontranslated region of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* that terminates transcription and directs polyadenylation (Bevan et al., 1983)
Intervening Sequence	3809-3835	Sequences used in DNA cloning
B-Right Border Region	3836-4192	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)

Table IV-1 (continued). Summary of Genetic Elements in Plasmid Vector PV-ZMAP1043

Vector Backbone		
Intervening Sequence	4193-4328	Sequences used in DNA cloning
aadA	4329-5217	Bacterial promoter, coding sequence, and 3′ untranslated region for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyltransferase from the transposon Tn*7* (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	5218-5747	Sequences used in DNA cloning
OR[7]-*ori-pBR322*	5748-6336	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	6337-6763	Sequences used in DNA cloning
CS-*rop*	6764-6955	Coding sequence for repressor of primer protein from the ColE1 plasmid for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	6956-8463	Sequences used in DNA cloning
OR-*ori V*	8464-8860	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	8861-8946	Sequences used in DNA cloning

[1] B, Border
[2] P, Promoter
[3] I, Intron
[4] TS, Targeting Sequence
[5] CS, Coding Sequence
[6] T, Transcription Termination Sequence
[7] OR, Origin of Replication

```
  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
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Figure IV-3. Deduced Amino Acid Sequence of the MON 87427 CP4 EPSPS Precursor Protein

The amino acid sequence of the CP4 EPSPS precursor protein was deduced from the full-length coding nucleotide sequence present in PV-ZMAP1043. The 76 amino acid CTP2, the transit peptide of the *Arabidopsis thaliana* EPSPS protein (CTP2), is underlined. CTP2 targets CP4 EPSPS protein to the chloroplasts. At the chloroplast the CTP2 is cleaved producing the mature 455 amino acid CP4 EPSPS protein that begins with the methionine at position 77.

V. CHARACTERIZATION OF THE GENETIC MODIFICATION

This section contains a comprehensive molecular characterization of the genetic modification present in MON 87427. It provides information on the DNA insertion(s) into the plant genome of MON 87427, and additional information relative to the arrangement and stability of the introduced genetic material. The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 30, 31, 32, and 33.

Characterization of the DNA insert in MON 87427 was conducted by Southern blot analyses, PCR and DNA sequencing. The results of this characterization demonstrate that MON 87427 contains a single copy of the *cp4 epsps* expression cassette, also referred to in this petition as transfer DNA (T-DNA) that is stably integrated at a single locus and is inherited according to Mendelian principles over multiple generations. These conclusions were based on several lines of evidence; 1) Southern blot analyses assayed the entire maize genome for the presence of DNA derived from PV-ZMAP1043, and demonstrated that only a single copy of the T-DNA was inserted at a single site; 2) DNA sequencing analyses determined the exact sequence of the inserted DNA and allowed a comparison to the T-DNA sequence in the plasmid vector confirming that only the expected sequences were integrated; and 3) compared the DNA flanking the insert to the sequence of the insertion site in conventional maize to identify any rearrangements that may have occurred at the insertion site during transformation. Taken together, the characterization of the genetic modification demonstrates that a single copy of the T-DNA was inserted at a single locus of the genome. The results confirm that no plasmid vector backbone sequences are present in MON 87427.

Southern blot analyses were used to determine the number of copies and insertion sites of the integrated DNA as well as the presence or absence of plasmid vector backbone sequences. The Southern blot strategy was designed to ensure that all potential transgenic segments would have been identified. The entire maize genome was assayed with probes that spanned the complete plasmid vector to detect the presence of the insertion as well as confirm the absence of any plasmid vector backbone sequences. This was accomplished by using probes that were less than 2 kb in length to ensure a high level of sensitivity. This high level of sensitivity was demonstrated for each blot by detection of a positive control added at 0.1 copy per genome equivalent. Two restriction enzymes were specifically chosen to fully characterize the T-DNA and detect any potential fragments of the T-DNA. This two enzyme design also maximizes the possibility of detecting an insertion elsewhere in the genome that could be overlooked if that band co-migrated with an expected band. One of the restriction enzymes had a cleavage site in the 5' flanking sequence, and the other had a cleavage site in the 3' flanking sequence. Together the enzymes result in overlapping segments covering the entire insert. Therefore, at least one segment for each flank is of a predictable size and overlaps with another predictable size segment. This strategy confirms that the entire insert sequence is identified in a predictable hybridization pattern.

To determine the number of copies and insertion sites of the T-DNA, and the presence or absence of the plasmid vector backbone sequences, duplicated samples that consisted of equal amounts of digested DNA were run on the agarose gel. One set of samples was run for a longer period of time (long run) than the second set (short run). The long run allows for greater resolution of large molecular weight DNA, whereas the short run allows the detection of small molecular weight DNA. The molecular weight markers on the left of the figures were used to estimate the sizes of the bands present in the long run lanes of the Southern blots, and the molecular weight markers on the right of the figures were used to estimate the sizes of bands present in the short run lanes of the Southern blots (Figures V-2 to V-4).

The DNA sequencing analyses complemented the Southern analyses. Southern blot results determined that MON 87427 contains a single copy of the T-DNA at a single insertion site. Sequencing of the insert and flanking genomic DNA confirmed the organization of the elements within the insert and determined the 5' and 3' insert-to-plant junctions, as well as the complete DNA sequence of the insert and adjacent maize genomic DNA. In addition, DNA sequencing analyses confirmed the DNA sequences flanking the 5' and 3' ends of the insert in MON 87427, each genetic element in the insert is intact and the sequence of the insert matches the corresponding sequence in PV-ZMAP1043. Furthermore, genomic organization at the insertion site was assessed by comparing the insert and flanking sequence to the insertion site in conventional maize.

The stability of the T-DNA present in MON 87427 across multiple generations was demonstrated by Southern blot fingerprint analyses. Genomic DNA from five generations of MON 87427 (Figure V-8) was digested with one of the enzymes used for the insert and copy number analysis and was hybridized with two probes that detect restriction segments that encompass the entire insert. This fingerprint strategy consists of two border segments and one segment internal to the T-DNA that assess not only the stability of the insert, but also the stability of the DNA directly adjacent to the insert.

The results of these analyses of MON 87427 demonstrated that a single copy of the T-DNA was inserted at a single locus of the genome, and no additional genetic elements, including backbone sequences, from PV-ZMAP1043 were detected in MON 87427. Generational stability analysis demonstrated that an expected Southern blot fingerprint of MON 87427 was maintained through five generations of the breeding history, thereby confirming the stability of T-DNA in MON 87427. Results from segregation analyses showed heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA at a single chromosomal locus (Table V-3).

The Southern blot analyses confirmed that the T-DNA reported in Figure V-1 represents the only detectable insert in MON 87427. Figure V-1 is a linear map depicting restriction sites within the insert as well as within the known maize genomic DNA immediately flanking the insert in MON 87427. The circular map of PV-ZMAP1043 annotated with the probes used in the Southern blot analysis is presented in Figure IV-1. Based on the linear map of the insert and the plasmid map, a table summarizing the expected DNA segments for Southern analyses is presented in Table V-1. The genetic elements

integrated in MON 87427 are summarized in Table V-2. The generations used in the generational stability analysis are depicted in the breeding history shown in Figure V-8. Materials and methods used for the characterization of the insert in MON 87427 are found in Appendix A.

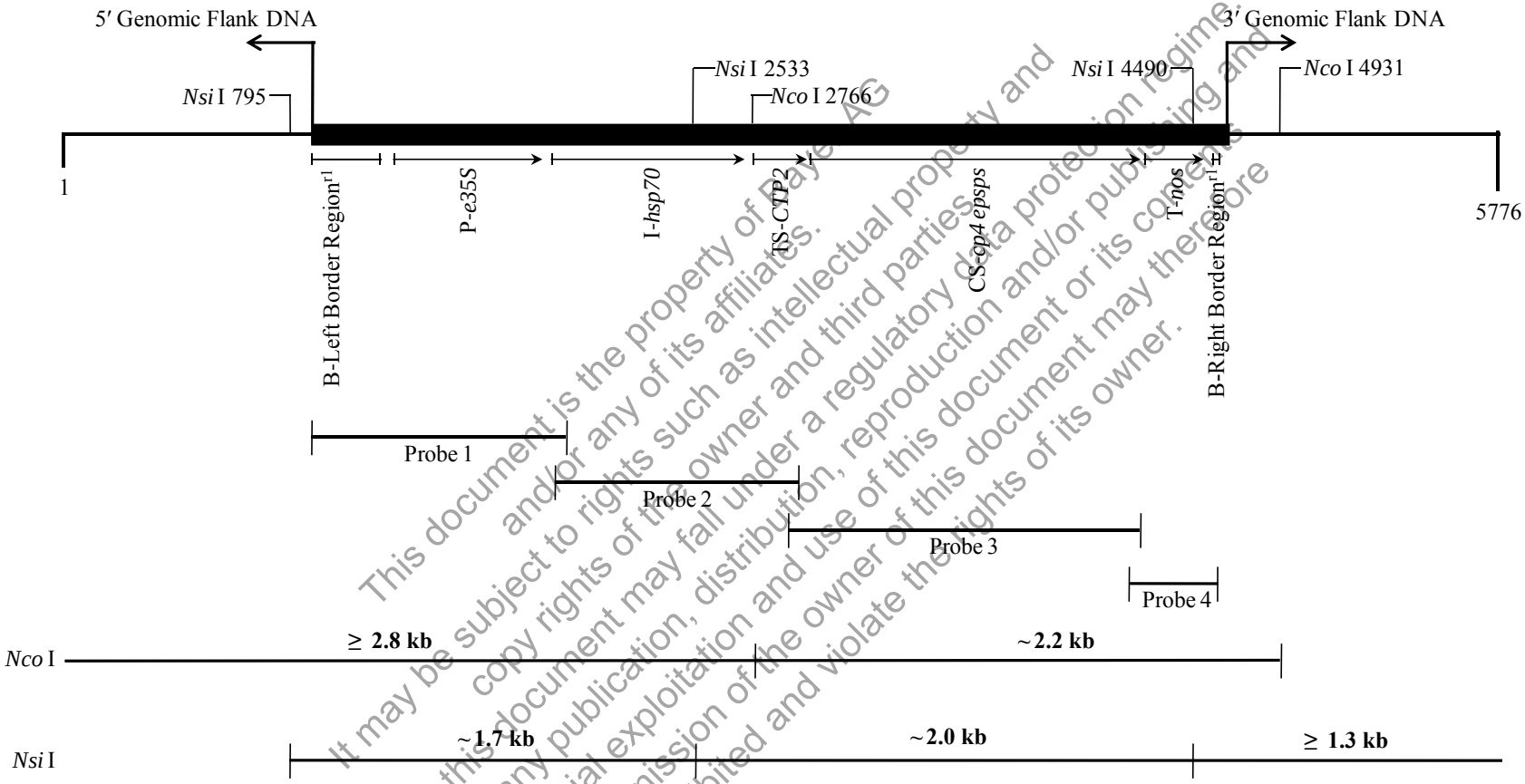


Figure V-1. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87427

A linear map showing DNA derived from the T-DNA of PV-ZMAP1043 and integrated into MON 87427 is shown. Right-angled arrows indicate the ends of the integrated DNA and the beginning of maize genomic flanking sequence. Identified on the map are genetic elements within the insert, as well as restriction sites with positions relative to the size of the DNA sequence (genomic flank and insert) represented by the linear map for enzymes used in the Southern analyses. Also indicated are the relative sizes and locations of the T-DNA probes and the expected sizes of restriction segments labeled by the probes. This schematic figure is not drawn to scale. Locations of genetic elements, restriction sites, and T-DNA probes are approximate. Probes are described in Figure IV-1.

Table V-1. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in MON 87427 Analyses

Southern Blot Figure		**V-2**	**V-3**	**V-4**	**V-5**	**V-9**
Probes Used		1, 4	2	3	5, 6, 7	1, 4
Probing Target	**Digestion Enzyme**	**Expected Band Sizes on Each Southern Blot**				
PV-ZMAP1043	*Sph* I	~7.1 kb ~1.8 kb	~7.1 kb	~7.1 kb ~1.8 kb	~7.1 kb	~7.1 kb ~1.8 kb
Probe Template Spikes[1]		~1.2 kb ~0.7 kb	~1.0 kb	~1.5 kb	~1.8 kb ~1.5 kb ~1.7 kb	~1.2 kb ~0.7 kb
MON 87427	*Nco* I	≥ 2.8 kb ~2.2 kb	≥ 2.8 kb ~2.2 kb	~2.2 kb	No band	--[2]
	Nsi I	~1.7 kb ~2.0 kb ≥ 1.3 kb	~2.0 kb ~1.7 kb	~2.0 kb	No band	~1.7 kb ~2.0 kb ≥ 1.3 kb

[1] probe template spikes were used as positive hybridization controls in Southern blot analyses
[2] '--' indicates that the particular restriction enzyme or the combination of the enzymes was not used in the analysis.

Table V-2. Summary of Genetic Elements in MON 87427

Genetic Element	Location in Sequence	Function (Reference)
Sequence flanking 5' end of the insert	1-1003	DNA sequence adjacent to the 5' end of the insertion site
B[1]-Left Border Region[r1]	1004-1255	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983)
Intervening Sequence	1256-1296	Sequences used in DNA cloning
P[2]-*e35S*	1297-1917	Promoter for the cauliflower mosaic virus (CaMV) 35S RNA (Odell et al., 1985) containing the duplicated enhancer region (Kay et al., 1987) that directs transcription in plant cells
Intervening Sequence	1918-1938	Sequences used in DNA cloning
I[3]-*hsp70*	1939-2742	First intron from the maize heat shock protein 70 gene (Brown and Santino, 1997)
Intervening Sequence	2743-2766	Sequences used in DNA cloning
TS[4]-*CTP2*	2767-2994	Targeting sequence from the *ShkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS[5]-*cp4 epsps*	2995-4362	Codon-optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgette et al., 1996)
Intervening Sequence	4363-4368	Sequences used in DNA cloning
T[6]-*nos*	4369-4621	3' nontranslated region of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* which terminates transcription and directs polyadenylation (Bevan et al., 1983)
Intervening Sequence	4622-4648	Sequences used in DNA cloning
B-Right Border Region[r1]	4649-4684	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Sequence flanking 3' end of the insert	4685-5776	DNA sequence adjacent to the 3' end of the insertion site

[1] B, Border
[2] P, Promoter
[3] TS, Targeting Sequence
[4] I, Intron
[5] CS, Coding Sequence
[6] T, Transcription Termination Sequence
[r1] Superscripts in Left and Right Border Regions indicate that the sequences in MON 87427 were truncated compared to the sequences in PV-ZMAP1043

V.A. Insert and Copy Number of T-DNA in MON 87427

The copy number and insertion sites of the T-DNA were assessed by digesting MON 87427 genomic DNA with the restriction enzymes *Nco* I or *Nsi* I and hybridizing Southern blots with probes that span the T-DNA (Figure IV-1). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table V-1) and any additional integration sites would produce a different banding pattern with additional bands.

The restriction enzyme *Nco* I cut once within the T-DNA and once within the known genomic DNA flanking the 3′ end of the T-DNA (Figure V-1). Therefore, if T-DNA sequences were present at a single integration site in MON 87427, the digestion with *Nco* I was expected to generate two border segments with expected sizes of greater than 2.8 kb and ~2.2 kb (Figure V-1 and Table V-1). The greater than 2.8 kb restriction segment contains genomic DNA flanking the 5′ end of the insert, the Left Border region, the *e35S* promoter, and the *hsp70* intron. The ~2.2 kb restriction segment contains the *CTP2* targeting sequence, the *cp4 epsps* coding sequence, the *nos* 3′ nontranslated sequence, the Right Border region, and genomic DNA flanking the 3′ end of the insert.

The restriction enzyme *Nsi* I cut twice within the T-DNA and once within the known genomic DNA flanking the 5′ end of the T-DNA (Figure V-1). Therefore, if T-DNA sequences are present at a single integration site in MON 87427, the digestion with *Nsi* I was expected to generate two border segments with expected sizes of ~1.7 kb and greater than 1.3 kb, and one segment internal to the T-DNA insert with an expected size of ~2.0 kb (Figure V-1 and Table V-1). The ~1.7 kb restriction segment contains genomic DNA flanking the 5′ end of the insert, the Left Border region, the *e35S* promoter, and a portion of the *hsp70* intron. The ~2.0 kb restriction segment contains a portion of the *hsp70* intron, the *CTP2* targeting sequence, the *cp4 epsps* coding sequence, and a portion of the *nos* 3' nontranslated sequence. The greater than 1.3 kb restriction segment contains a portion of the *nos* 3′ non-translated sequence, the Right Border region, and genomic DNA flanking the 3′ end of the insert.

In the Southern blot analyses performed, each Southern blot contained a negative and several positive control. The conventional control LH198 × HiII was a non-transformed maize line that incorporated similar background genetics of MON 87427 (LH198 BC3F4) but did not contain the *cp4 epsps* expression cassette (Refer to Section II). Conventional control genomic DNA digested with either the restriction enzymes *Nco* I or *Nsi* I was used as a negative control to determine if the probes hybridized to any endogenous maize sequences. Conventional control genomic DNA digested with the appropriate restriction enzyme and spiked with either PV-ZMAP1043 DNA digested with the restriction enzyme *Sph* I, or probe template(s) served as positive controls. The positive hybridization control was spiked at 0.1 and 1 genome equivalents to demonstrate sufficient sensitivity of the Southern blot. Individual Southern blots were hybridized with the following probes: Probe 1, Probe 2, Probe 3, and Probe 4 (Figure IV-1 and Table V-1). The results of these analyses are shown in Figure V-2 through Figure V-4.

V.A.1. Probes 1 and 4

Conventional control genomic DNA digested with *Nco* I (Figure V-2, lane 1 and lane 8) and hybridized with Probe 1 and Probe 4 (Figure IV-1) produced endogenous hybridization bands of ~6.1 kb and ~4.1 kb. Conventional control genomic DNA digested with *Nsi* I (Figure V-2, lane 3 and lane 10) and hybridized with Probe 1 and Probe 4 (Figure IV-1) produced endogenous hybridization bands of ~9.8 kb and ~ 4.3 kb. These signals were present in all lanes, and most likely resulted from hybridization with the endogenous maize *hsp70* intron sequence, because Probe 1 contains a small portion of the *hsp70* intron (Figure IV-1). Since the region of Probe 1 corresponding to the *hsp70* intron sequence was small, the hybridization signals were relatively weak, and are not specific to the inserted DNA in MON 87427.

PV-ZMAP1043 digested with the restriction enzyme *Sph* I and mixed with conventional control genomic DNA pre-digested with the restriction enzyme *Nco* I (Figure V-2, lane 7) produced the two expected bands at ~7.1 kb and ~1.8 kb (Figure IV-1 and Table V-1) in addition to the endogenous hybridization bands listed above. Probe templates generated from PV-ZMAP1043 (Figure IV-1) were mixed with conventional control genomic DNA pre-digested with the restriction enzyme *Nco* I (Figure V-2, lane 5 and lane 6) produced the expected bands at ~1.2 kb and ~0.7 kb (Figure IV-1 and Table V-1) in addition to the endogenous hybridization bands listed above. These results indicate that the probes hybridized to their target sequences.

MON 87427 genomic DNA digested with the restriction enzyme *Nco* I and hybridized with Probe 1 and Probe 4 (Figure IV-1) produced two bands in addition to the endogenous hybridization bands (Figure V-2, lane 2 and lane 9) listed above. The ~5.5 kb band represents the 5' end of the inserted T-DNA and the adjacent flanking DNA, which correlates with the expected border segment size of greater than 2.8 kb (Figure V-1). The ~2.2 kb band represents the 3' end of the inserted T-DNA and the adjacent DNA flanking the 3' end of the insert, which correlates with the expected border segment size of ~2.2 kb (Figure V-1).

MON 87427 genomic DNA digested with *Nsi* I (Figure V-2, lane 4 and lane 11) and hybridized with Probe 1 and Probe 4 produced three bands (Table V-1) in addition to the endogenous hybridization bands listed above. The ~1.7 kb band represents the 5' end of the inserted T-DNA and a small amount of adjacent flanking DNA, which correlates with the expected border segment size of ~1.7 kb (Figure V-1). The ~2.0 kb band contains an internal portion of the inserted DNA; which correlates with the expected segment size of ~2.0 kb (Figure V-1). The ~6.4 kb band represents the 3' end of the inserted T-DNA and the adjacent DNA flanking the 3' end of the insert, which correlates with the expected border segment size of greater than 1.3 kb (Figure V-1).

No additional bands were detected using Probe 1 and Probe 4 other than those listed above. Based on the results presented in Figure V-2, it was concluded that T-DNA sequences covered by Probe 1 and Probe 4 reside at a single integration locus in MON 87427.

V.A.2. Probe 2

Conventional control genomic DNA digested with *Nco* I (Figure V-3, lane 1 and lane 8) and hybridized with Probe 2 (Figure IV-1) produced an endogenous hybridization band of ~4.1 kb. Conventional control genomic DNA digested with *Nsi* I (Figure V-3, lane 3 and lane 10) and hybridized with Probe 2 (Figure IV-1) produced endogenous hybridization bands of ~5.2 kb and ~4.2 kb. These signals were present in all lanes, and most likely resulted from hybridization with the endogenous maize *hsp70* intron sequence because Probe 2 encompasses the majority of the *hsp70* intron in PV-ZMAP1043 (Figure IV-1). Since the region of Probe 2 corresponding to the *hsp70* intron sequence was large, the hybridization signals were relatively strong, but are considered to be endogenous background hybridization and are not specific to the inserted DNA in MON 87427.

PV-ZMAP1043 digested with the restriction enzyme *Sph* I and mixed with conventional control genomic DNA predigested with the restriction enzyme *Nco* I (Figure V-3, lane 7) produced the expected band of ~7.1 kb (Figure IV-1 and Table V-1) in addition to the endogenous hybridization bands listed above. Probe template generated from PV-ZMAP1043 (Figure IV-1) was mixed with conventional control genomic DNA predigested with the restriction enzyme *Nco* I (Figure V-3, lane 5 and lane 6) produced the expected band of~1.0 kb (Figure IV-1 and Table V-1) in addition to the endogenous hybridization bands listed above. These results indicate that the probe hybridized to its target sequence.

MON 87427 genomic DNA digested with *Nco* I and hybridized with Probe 2 (Figure V-3, lane 2 and lane 9) produced two bands in addition to the endogenous hybridization bands listed above. The ~5.5 kb band represents the 5' end of the inserted T-DNA and the adjacent flanking DNA; which correlates with the expected border segment size of greater than 2.8 kb (Figure V-1). The ~2.2 kb band represents the 3' end of the inserted DNA and the adjacent DNA flanking the 3' end of the insert; which correlates with the expected border segment size of ~2.2 kb (Figure V-1).

MON 87427 genomic DNA digested with *Nsi* I (Figure V-3, lane 4 and lane 11) and hybridized with Probe 2 produced two bands in addition to the endogenous hybridization bands listed above. The ~1.7 kb band represents the 5' end of the inserted T-DNA and a small amount of adjacent flanking DNA, which correlates with the expected border segment size of~1.7 kb (Figure V-1). The ~2.0 kb band represents an internal portion of the inserted T-DNA, which correlates with the expected segment size of ~2.0 kb (Figure V-1).

No additional bands were detected using Probe 2 other than those listed above. Based on the results presented in Figure V-3, it was concluded that the T-DNA sequences covered by Probe 2 reside at a single integration locus in MON 87427.

V.A.3. Probe 3

Conventional control genomic DNA digested with the restriction enzyme *Nco* I (Figure V-4, lane 1 and lane 8) or *Nsi* I (Figure V-4, lane 3 and lane 10) and hybridized with Probe 3 (Figure IV-1) showed no detectable hybridization bands. PV-ZMAP1043 DNA digested with the restriction enzyme *Sph* I and mixed with conventional control DNA predigested with the restriction enzyme *Nco* I (Figure V-4, lane 7) produced one band at ~ 1.8 kb (Figure IV-1 and Table V-1). Although the other *Sph* I segment from the plasmid vector (~ 7.1 kb) contains a small portion of the Probe 3 sequence, it was not detected under these assay conditions. Probe template generated from PV-ZMAP1043 (Figure IV-1) was mixed with conventional control DNA predigested with the restriction enzyme *Nco* I (Figure V-4, lane 5 and lane 6) produced the expected band at ~1.5 kb (Figure IV-1 and Table V-1). These results indicate that the probe hybridized to its target sequence.

MON 87427 genomic DNA digested with the restriction enzyme *Nco* I and hybridized with Probe 3 (Figure IV-1) produced one band (Figure V-4, lane 2 and lane 9) of ~2.2 kb. The ~2.2 kb band represents the 3' end of the inserted DNA and the adjacent DNA flanking the 3' end of the insert, which correlates with the expected border segment size of ~2.2 kb (Figure V-1).

MON 87427 genomic DNA digested with the restriction enzyme *Nsi* I and hybridized with Probe 3 (Figure IV-1) produced one band (Figure V-4, lane 4 and lane 11) of ~2.0 kb. The ~2.0 kb band represents an internal portion of the inserted DNA, which correlates with the expected size of ~2.0 kb (Figure V-1).

No additional bands were detected using Probe 3 other than those listed above. Based on the results presented in Figure V-4, it was concluded that the sequence covered by Probe 3 resides at a single integration locus in MON 87427.

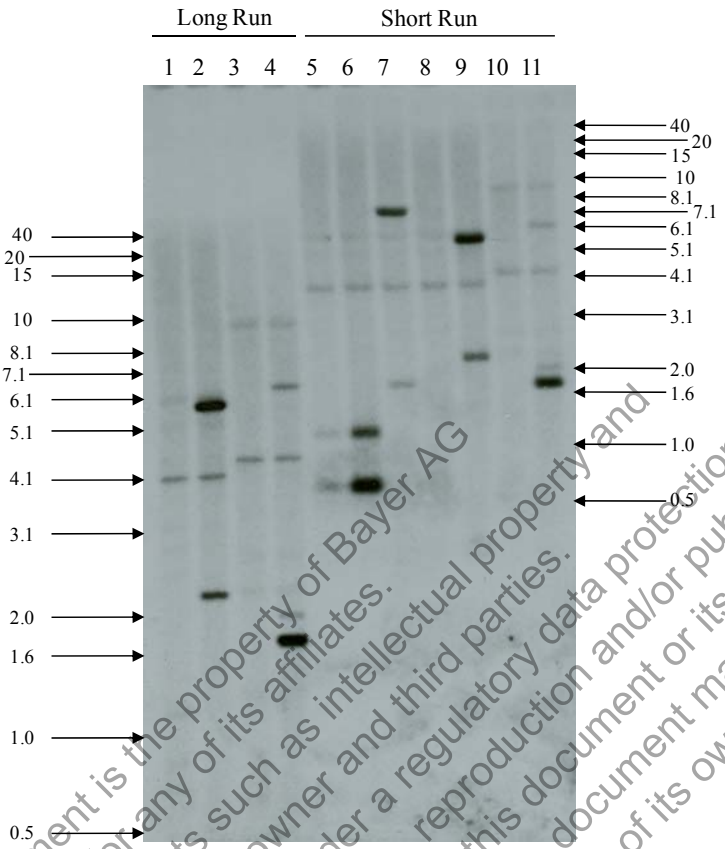


Figure V-2. Southern Blot Analysis to Determine Insert and Copy Number of the T-DNA in MON 87427: Probe 1 and Probe 4

The blot was hybridized with two ^{32}P-labeled probes that spanned portions of the T-DNA sequence (Figure IV-1, Probe 1 and Probe 4). Each lane contains ~10 µg of digested genomic DNA isolated from maize seed. Lane designations are as follows:

Lane

1 Conventional control (*Nco* I)
2 MON 87427 (*Nco* I)
3 Conventional control (*Nsi* I)
4 MON 87427 (*Nsi* I)
5 Conventional control (*Nco* I) spiked with Probe 1 and Probe 4 [~0.1 genome equivalent]
6 Conventional control (*Nco* I) spiked with Probe 1 and Probe 4 [~1.0 genome equivalent]
7 Conventional control (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
8 Conventional control (*Nco* I)
9 MON 87427 (*Nco* I)
10 Conventional control (*Nsi* I)
11 MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

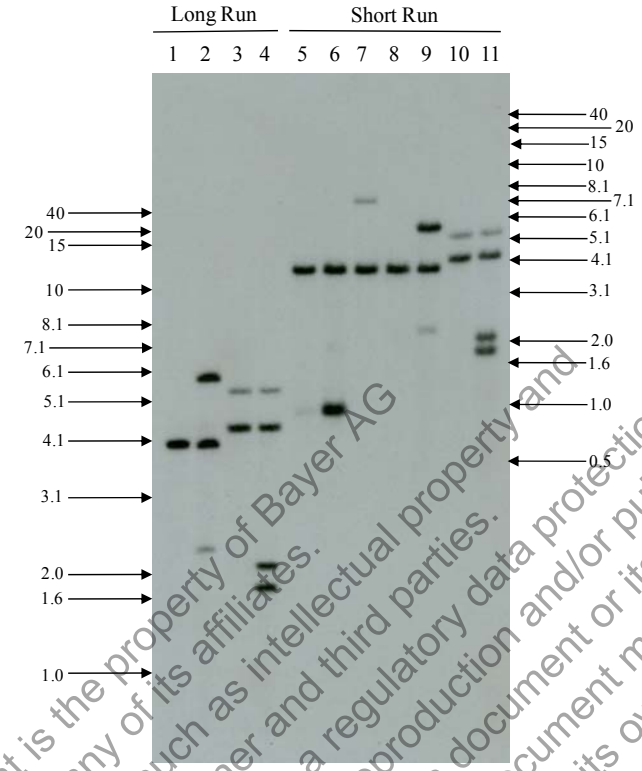


Figure V-3. Southern Blot Analysis to Determine Insert and Copy Number of the T-DNA in MON 87427: Probe 2

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA sequence (Figure IV-1, Probe 2). Each lane contains ~10 µg of digested genomic DNA isolated from maize seed. Lane designations are as follows:

Lane

1 Conventional control (*Nco* I)
2 MON 87427 (*Nco* I)
3 Conventional control (*Nsi* I)
4 MON 87427 (*Nsi* I)
5 Conventional control (*Nco* I) spiked with Probe 2 [~0.1 genome equivalent]
6 Conventional control (*Nco* I) spiked with Probe 2 [~1.0 genome equivalent]
7 Conventional control (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
8 Conventional control (*Nco* I)
9 MON 87427 (*Nco* I)
10 Conventional control (*Nsi* I)
11 MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

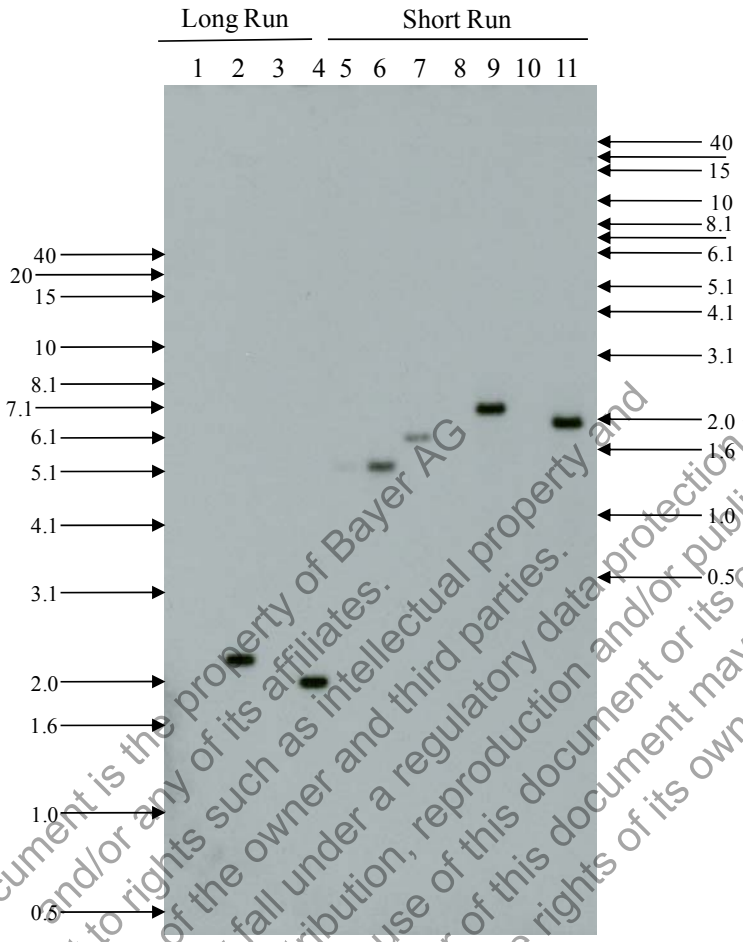


Figure V-4. Southern Blot Analysis to Determine Insert and Copy Number of the T-DNA in MON 87427: Probe 3

The blot was hybridized with one ^{32}P-labeled probe that spanned a portion of the T-DNA sequence (Figure IV-1, Probe 3). Each lane contains ~10 µg of digested genomic DNA isolated from maize seed. Lane designations are as follows:

Lane	
1	Conventional control (*Nco* I)
2	MON 87427 (*Nco* I)
3	Conventional control (*Nsi* I)
4	MON 87427 (*Nsi* I)
5	Conventional control (*Nco* I) spiked with Probe 3 [~0.1 genome equivalent]
6	Conventional control (*Nco* I) spiked with Probe 3 [~1.0 genome equivalent]
7	Conventional control (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
8	Conventional control (*Nco* I)
9	MON 87427 (*Nco* I)
10	Conventional control (*Nsi* I)
11	MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

V.B. Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector PV-ZMAP1043 Backbone Sequences in MON 87427

To determine the presence or absence of PV-ZMAP1043 backbone sequences, MON 87427 and conventional control genomic DNA were digested with the restriction enzymes *Nco* I or *Nsi* I and the Southern blots were hybridized with overlapping probes spanning the entire backbone sequence of PV-ZMAP1043 (Figure IV-1, Probe 5, Probe 6, and Probe 7). Digested PV-ZMAP1043 and probe templates generated from PV-ZMAP1043 were used as positive controls on the Southern blots. Approximately 1 genome equivalent of PV-ZMAP1043 digested with the restriction enzyme *Sph* I was mixed with predigested conventional control DNA. As an additional positive control, approximately 0.1 and 1 genome equivalents of probe templates (Figure IV-1, Probe 5, Probe 6, and Probe 7) generated from PV-ZMAP1043 were mixed with predigested conventional control DNA. If backbone DNA sequences are present in MON 87427, then hybridizing with backbone probes should result in hybridizing bands. The results of this analysis are shown in Figure V-5.

V.B.1. Plasmid Vector Backbone Probes 5, 6, 7

Conventional control genomic DNA digested with *Nco* I (Figure V-5, lane 1 and lane 10) or *Nsi* I (Figure V-5, lane 3 and lane 12) and hybridized simultaneously with overlapping probes spanning the plasmid vector backbone of PV-ZMAP1043 (Figure IV-1, Probe 5, Probe 6, and Probe 7) showed no detectable hybridization bands. PV ZMAP1043 digested with the restriction enzyme *Sph* I and mixed with conventional control genomic DNA predigested with the restriction enzyme *Nco* I (Figure V-5, lane 9) produced one expected band of ~7.1 kb (Figure IV-1 and Table V-1). Probe templates generated from PV-ZMAP1043 (Figure IV-1, Probe 5 and Probe 6) were mixed with conventional control DNA predigested with the restriction enzyme *Nco* I (Figure V-5, lane 5 and lane 6) produced two expected bands at ~1.8 kb and ~1.5 kb, respectively (Figure IV-1 and Table V-1). Probe template generated from PV-ZMAP1043 (Figure IV-1, Probe 7) was mixed with conventional control DNA predigested with the restriction enzyme *Nco* I (Figure V-5, lane 7 and lane 8) produced the expected band at ~1.7 kb. These results indicate that the probes are hybridizing to their target sequences.

MON 87427 genomic DNA digested with *Nco* I (Figure V-5, lane 2 and lane 11) or *Nsi* I (Figure V-5, lane 4 and lane 13) and hybridized with Probe 5, Probe 6, and Probe 7 produced no detectable bands. Based on the results presented in Figure V-5, it was concluded that MON 87427 contains no detectable backbone sequences from PV-ZMAP1043.

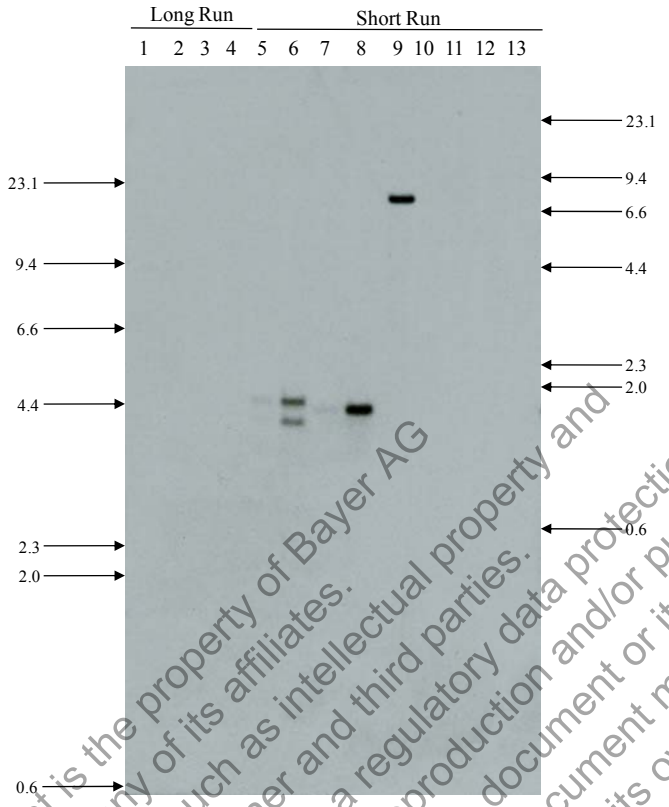


Figure V-5. Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector PV-ZMAP1043 Backbone Sequences in MON 87427: Probes 5, 6, and 7

The blot was hybridized with three ^{32}P-labeled probes that spanned portions of the T-DNA sequence (Figure IV-1, Probe 5, Probe 6, and Probe 7). Each lane contains ~10 µg of digested genomic DNA isolated from maize seed. Lane designations are as follows:

Lane

1 Conventional control (*Nco* I)
2 MON 87427 (*Nco* I)
3 Conventional control (*Nsi* I)
4 MON 87427 (*Nsi* I)
5 Conventional control (*Nco* I) spiked with Probe 5 and Probe 6 [~0.1 genome equivalent]
6 Conventional control (*Nco* I) spiked with Probe 5 and Probe 6 [~1.0 genome equivalent]
7 Conventional control (*Nco* I) spiked with Probe 7 [~0.1 genome equivalent]
8 Conventional control (*Nco* I) spiked with Probe 7 [~1.0 genome equivalent]
9 Conventional control (*Nco* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
10 Conventional control (*Nco* I)
11 MON 87427 (*Nco* I)
12 Conventional control (*Nsi* I)
13 MON 87427 (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the λDNA/*Hind* III Fragments (Invitrogen) on the ethidium bromide stained gel.

V.C. Organization and Sequence of the Insert and Adjacent Genomic DNA in MON 87427

The organization of the elements within the T-DNA was confirmed using DNA sequence analysis. PCR primers were designed to amplify three overlapping regions of the genomic DNA that span the entire length of the insert (Figure V-6). The amplified PCR products were subjected to DNA sequencing analyses. The insert in MON 87427 is 3681 bp and matches the sequence of PV-ZMAP1043 as described in Table IV-1.

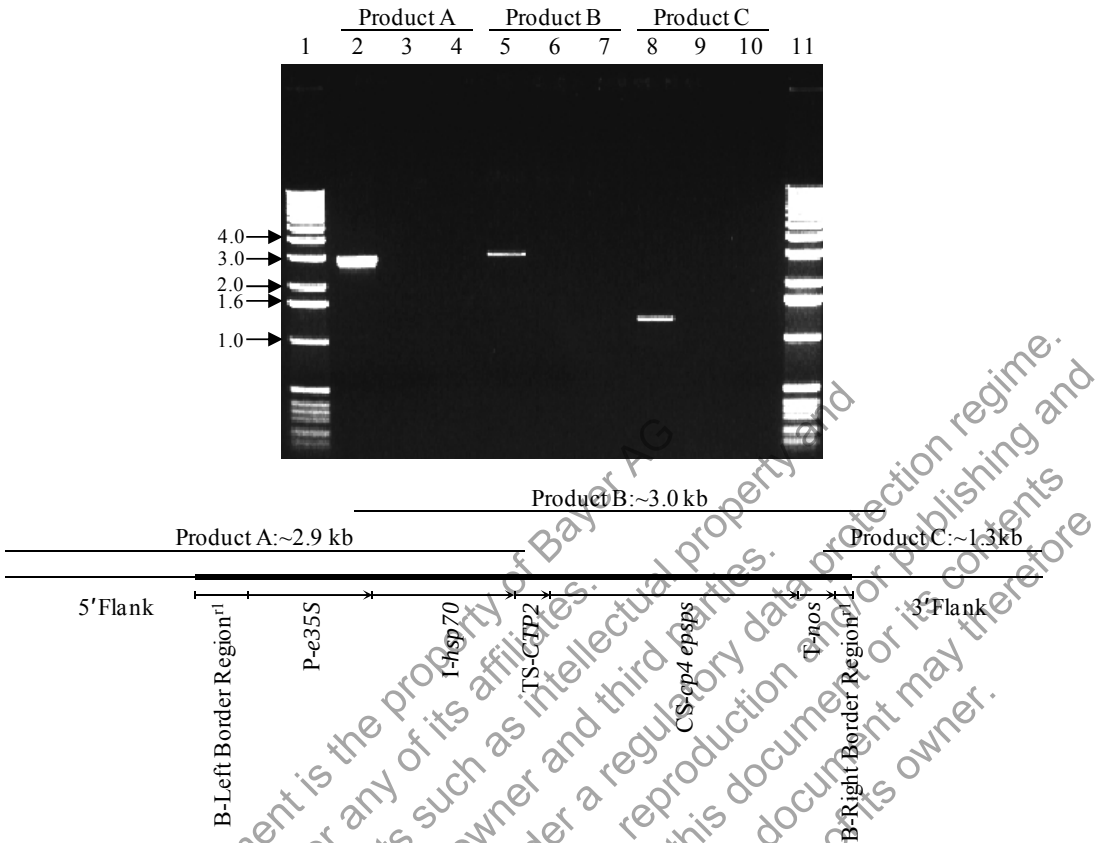


Figure V-6. Overlapping PCR Analysis Across the Insert in MON 87427

PCR was performed on conventional control genomic DNA and MON 87427 genomic DNA extracted from seed tissue. Only lanes containing PCR reactions are shown in the figure. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 87427 that appears at the bottom of the figure. Four to nine microliters of each of the PCR reactions were loaded on the gel. PCR products reported in this figure are representative of the study data.

Lane 1	1 Kb DNA Ladder
2	MON 87427
3	Conventional control
4	No template DNA control
5	MON 87427
6	Conventional control
7	No template DNA control
8	MON 87427
9	Conventional control
10	No template DNA control
11	1 Kb DNA Ladder

The arrows on the agarose gel photograph denote size of DNA, in kilobase pairs, obtained from the 1 Kb DNA Ladder on the ethidium bromide stained gel.

V.D. PCR and DNA Sequence Analyses to Examine the MON 87427 Insertion Site

PCR and sequence analysis were performed on genomic DNA extracted from MON 87427 and the conventional control to examine the insertion site in conventional maize. The PCR was performed with one primer specific to the genomic DNA sequence flanking the 5' end of the insert paired with a second primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure V-7). A sequence comparison between the PCR product generated from the conventional control and the sequence generated from the 5' and 3' flanking sequences of MON 87427 indicates there was a 41 base pair insertion just 5' to the MON 87427 insert, a 24 base pair insertion just 3' to the MON 87427 insert, and a 140 base pair deletion that occurred during integration of the T-DNA sequences. Such changes are quite common during plant transformation; these changes presumably resulted from double-stranded break repair mechanisms in the plant during the *Agrobacterium*-mediated transformation process (Salomon and Puchta, 1998).

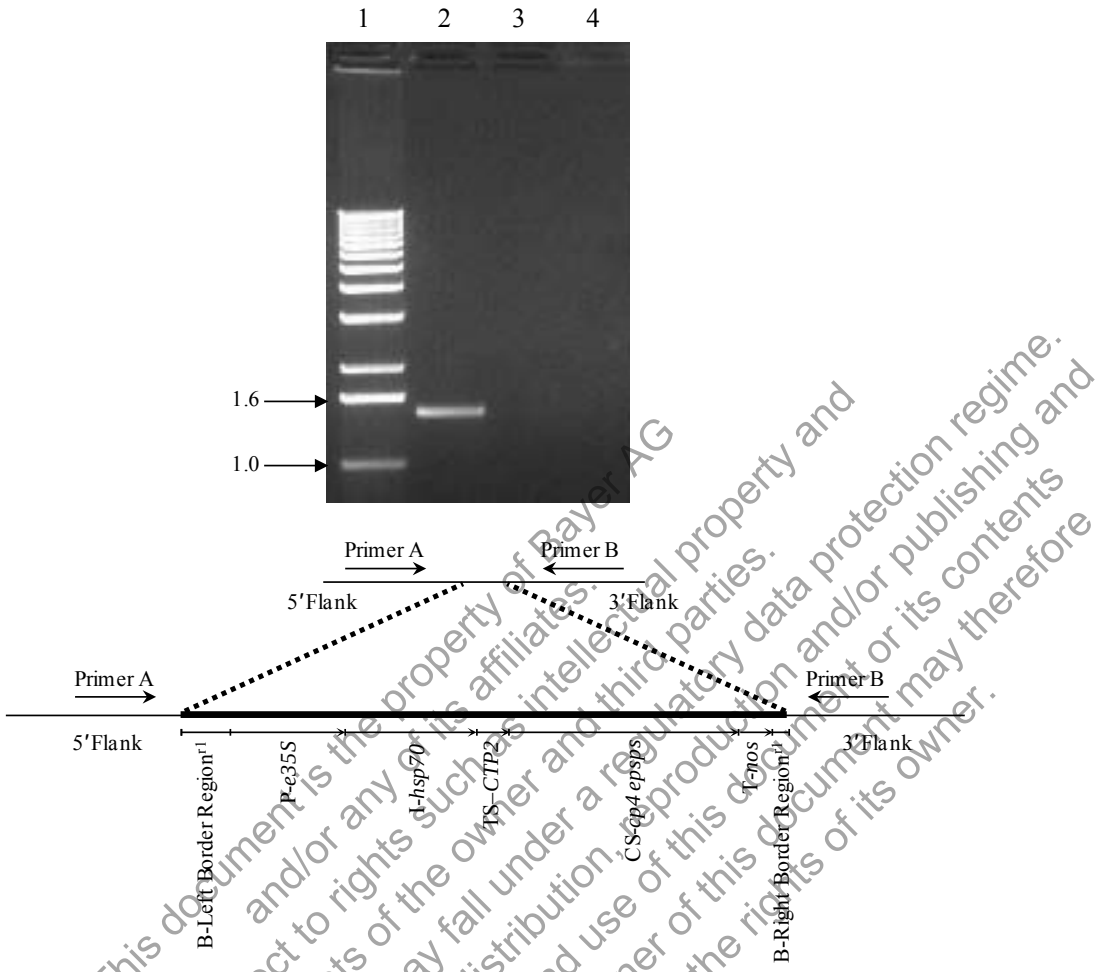


Figure V-7. PCR Amplification of the MON 87427 Insertion Site in Conventional Maize

PCR was performed on conventional control genomic DNA and MON 87427 genomic DNA extracted from seed tissue. Only lanes containing PCR reactions are shown in the figure. Lanes are marked to show which product has been loaded and is visualized on the agarose gel. Depiction of the MON 87427 insertion site in conventional control (upper panel) and the MON 87427 insert (lower panel). PCR amplification was performed using Primer A in the 5' flanking sequence and Primer B in the 3' flanking sequence of the insert in MON 87427. Five microliters of each of the PCR reactions were loaded on the gel. Lane designations are as follows:

Lane	
1	1 Kb DNA Ladder
2	Conventional control
3	MON 87427
4	No template DNA control

The arrows on the agarose gel photograph denote size of DNA, in kilobase pairs, obtained from the 1 Kb DNA Ladder on the ethidium stained gel.

V.E. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87427

In order to demonstrate the stability of the T-DNA present in MON 87427 through multiple generations, Southern blot analysis was performed using DNA obtained from five breeding generations of MON 87427. The breeding history of MON 87427 is presented in Figure V-8, and the specific generations tested are indicated in the legend of Figure V-9. The LH198 BC3F4 generation was used for the molecular characterization analyses shown in Figure V-2 through Figure V-5. To assess stability, four additional generations were evaluated by Southern analysis and compared to the fully characterized MON 87427 LH198 BC3F4 generation. The conventional control materials used for the generational stability analysis included LH198 × HiII, which included similar background genetics of the LH198 BC3F4 generation including the original transformation line, and LH198 × LH287, a hybrid with a similar germplasm background to the MON 87427 [LH198 BC3F7 × LH287] F1 hybrid. Genomic DNA isolated from each of the selected generations of MON 87427 and conventional controls was digested with the restriction enzyme *Nsi* I (Figure V-1) and hybridized with Probe 1 and Probe 4 (Figure IV-1). Probe 1 and Probe 4 will detect both border segments generated by the *Nsi* I digestion. Any instability associated with the T-DNA would be detected as novel bands within the fingerprint on the Southern blot. The Southern blot has the same positive hybridization controls as described in Section V.A. The results are shown in Figure V-9.

V.E.1. Probes 1 and 4

Conventional control genomic DNA digested with *Nsi* I (Figure V-9) and hybridized with Probe 1 and Probe 4 (Figure V-1) produced hybridization signals resulting from endogenous targets residing in the maize genome. Each hybridization signal was produced in a conventional control lane, and a lane containing MON 87427 genomic DNA; therefore, these signals are considered to be endogenous background hybridization and are not specific to the inserted DNA in MON 87427. Conventional control genomic DNA (Figure V-9, lane 4) digested with *Nsi* I and hybridized with Probe 1 and Probe 4 displayed an endogenous hybridization band of ~4.3 kb. Conventional control genomic DNA (Figure V-9, lane 9) digested with *Nsi* I and hybridized with Probe 1 and Probe 4 displayed the endogenous hybridization bands of ~4.4 kb and ~4.3 kb. The endogenous doublet hybridization bands in the conventional control LH198 × LH287 and MON 87427 [LH198 BC3F7 × LH287] F1 genomic DNA (Figure V-9, lane 9 and lane 10), appeared faint on the blot, although they were visible on a longer exposure.

PV-ZMAP1043 digested with the restriction enzyme *Sph* I and mixed with conventional control DNA predigested with the restriction enzyme *Nsi* I (Figure V-9, lane 3) produced the two expected bands at ~7.1 kb and ~1.8 kb (Figure IV-1 and Table V-1) in addition to the endogenous hybridization bands. Probe templates generated from PV-ZMAP1043 (Figure V-1) were mixed with conventional control DNA predigested with the restriction enzyme *Nsi* I (Figure V-9, lane 1 and lane 2) produced the expected bands at ~1.2 kb and ~0.7 kb (Figure V-1 and Table V-1) in addition to the endogenous hybridization bands. These results indicate that the probes hybridized to their target sequences.

MON 87427 DNA extracted from generations MON 87427 LH198 BC3F3, MON 87427 LH198 BC3F4, MON 87427 LH198 BC3F6, MON 87427 LH198 BC3F7, and MON 87427 [LH198 BC3F7 × LH287] F1, digested with *Nsi* I, and hybridized with Probe 1 and Probe 4 (Figure V-9, lane 5, lane 6, lane 7, lane 8, and lane 10) produced three bands (Table V-1) in addition to the endogenous hybridization bands listed above. The ~1.7 kb band represents the 5' end of the inserted T-DNA and a small amount of adjacent flanking DNA, which correlates with the expected border segment size of ~1.7 kb (Figure V-1). The ~2.0 kb band contains an internal portion of the inserted T-DNA (Figure V-1). The ~6.4 kb band represents the 3' end of the inserted T-DNA and the adjacent DNA flanking the 3' end of the insert, which correlates with the expected border segment size of greater than 1.3 kb (Figure V-1). The fingerprint of the Southern signals from the four generations MON 87427 LH198 BC3F3, MON 87427 LH198 BC3F6, MON 87427 LH198 BC3F7, and MON 87427 [LH198 BC3F7 × LH287] F1 (Figure V-9, lane 5, lane 7, lane 8, and lane 10) is consistent with that from the fully characterized generation MON 87427 LH198 BC3F4 (Figure V-2, lane 4 and lane 11; Figure V-9, lane 6). No unexpected bands were detected, indicating that MON 87427 contains one copy of the T-DNA that is stably maintained across multiple generations.

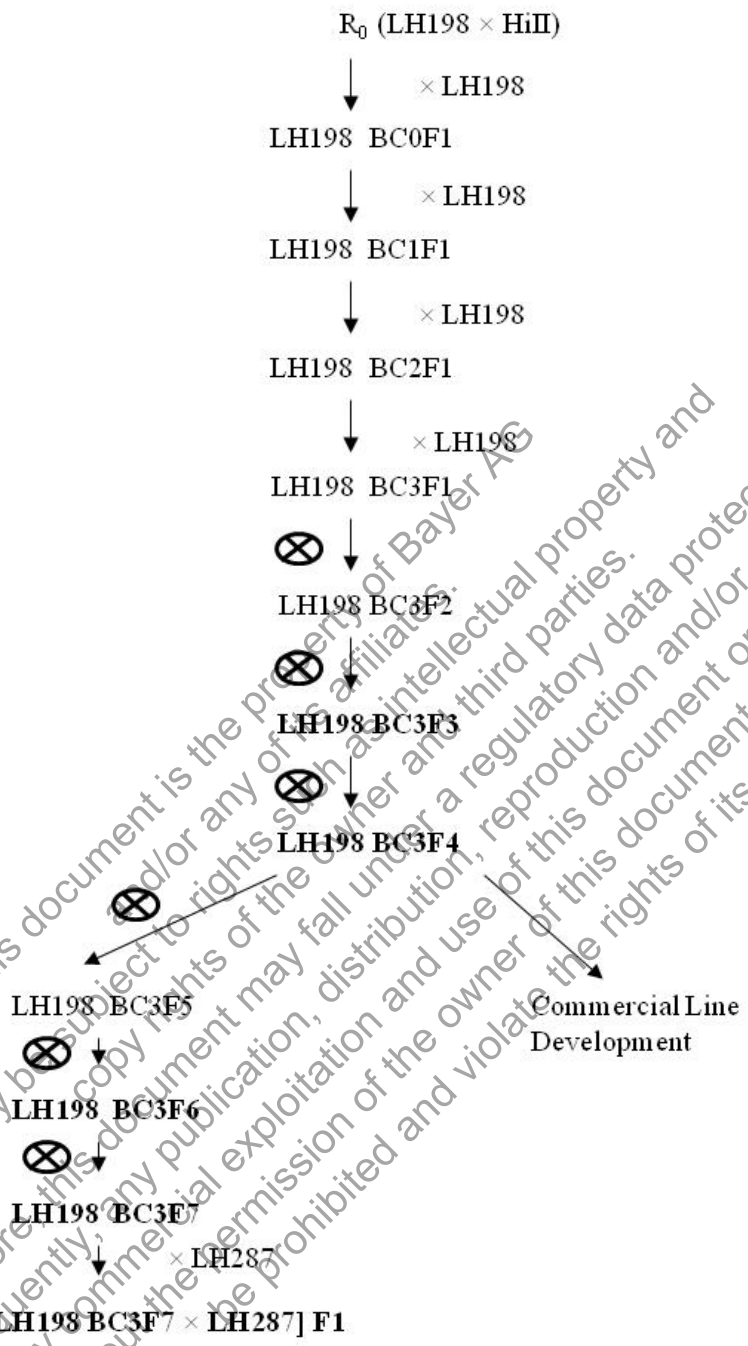


Figure V-8. Breeding History of MON 87427

The LH198 BC3F4 generation was used for the molecular characterization of MON 87427. Generations used for generational stability are indicated in bold text. R_0 corresponds to the transformed plant. F# is the filial generation. ⊗ designates self-pollination. BC# is the backcross generation. The [LH198 BC3F7 × LH287] F1 generation was used for expression, composition and phenotypic, agronomic and environmental interaction analyses.

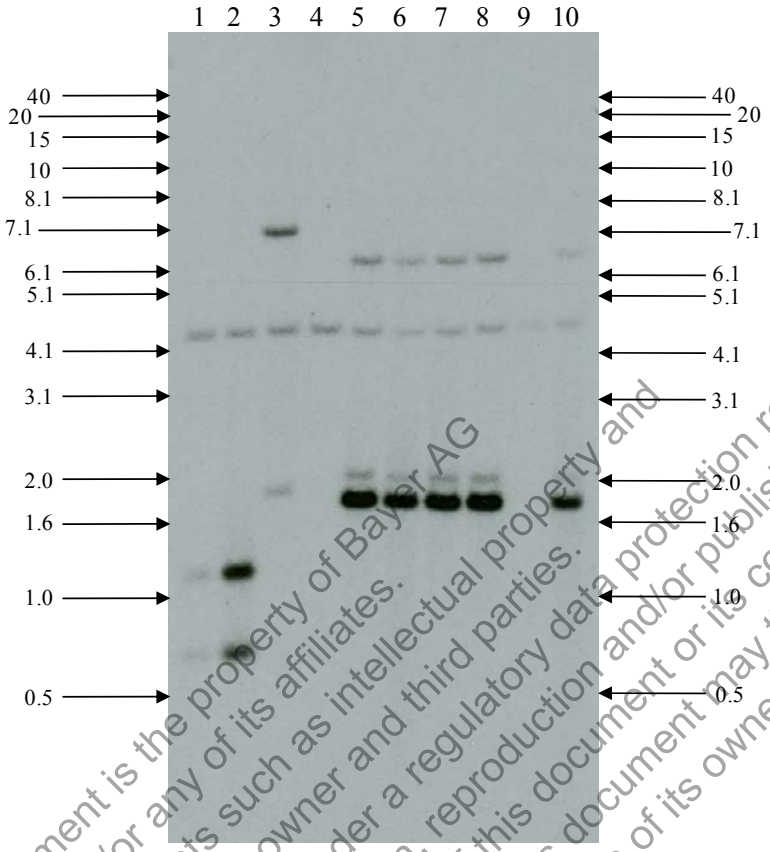


Figure V-9. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87427: Probes 1 and 4

The blot was hybridized with two ^{32}P-labeled probes that spanned portions of the T-DNA sequence (Figure IV-1, Probe 1 and Probe 4). Each lane contains ~10 µg of digested genomic DNA isolated from maize seed, with the exception of MON 87427 LH198 BC3F3, MON 87427 LH198 BC3F6, and MON 87427 LH198 BC3F7, which were isolated from maize leaf tissue. Lane designations are as follows:

Lane

1 Conventional control LH198 × HiII (*Nsi I*) spiked with Probe 1 and Probe 4 [~0.1 genome equivalent]
2 Conventional control LH198 × HiII (*Nsi* I) spiked with Probe 1 and Probe 4 [~1.0 genome equivalent]
3 Conventional control LH198 × HiII (*Nsi* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
4 Conventional control LH198 × HiII (*Nsi* I)
5 MON 87427 (LH198 BC3F3) (*Nsi* I)
6 MON 87427 (LH198 BC3F4) (*Nsi* I)
7 MON 87427 (LH198 BC3F6) (*Nsi* I)
8 MON 87427 (LH198 BC3F7) (*Nsi* I)
9 Conventional control LH198 × LH287 (*Nsi* I)
10 MON 87427([LH198 BC3F7 × LH287] F1) (*Nsi* I)

Arrows denote the size of the DNA, in kilobase pairs, obtained from the 1 Kb DNA Extension Ladder (Invitrogen) on the ethidium bromide stained gel.

V.F. Inheritance of the Genetic Insert in MON 87427

During development of MON 87427, segregation data were recorded to assess the heritability and stability of the *cp4 epsps* cassette present in MON 87427. Chi square analysis was performed over several generations to confirm the segregation and stability of the MON 87427 insert. The Chi square analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 87427 breeding path for generating segregation data is described in Figure V-10. The transformed R_0 plant was crossed several times with LH198 conventional maize through the LH198 BC3F1 generation. The LH198 BC0F1 generation consisted of five plants that were positive for the tissue selective glyphosate tolerance trait. LH198 was then used as the recurrent parent through three backcrossing generations. Heterozygous LH198 BC3F1 plants were self-pollinated to produce LH198 BC3F2, which demonstrated the expected 3:1 (positive:negative) segregation ratio for the tissue selective glyphosate tolerance trait. One surviving LH198 BC3F2 plant was identified and self-pollinated to produce LH198 BC3F3 plants, from which homozygous plants were identified and self-pollinated to produce LH198 BC3F4 plants. Endpoint Taqman analysis was used to confirm homozygosity on both LH198 BC3F3 and LH198 BC3F4 generations.

LH198 BC3F4 seed was used in trait integration and further commercial development, and was crossed with a recurrent parent (RP) that did not contain the *cp4 epsps* expression cassette to produce [RP × LH198 BC3F4] BC0F1 heterozygous seed. The resulting [RP × LH198 BC3F4] BC0F1 plants were crossed with the same recurrent parent to produce BC1F1 seed. The subsequent BC1F1 plants were tested for the presence of the CP4 EPSPS protein by glyphosate spray treatment. Surviving BC1F1 plants were again crossed with the same recurrent parent to produce BC2F1 seed. The subsequent BC2F1 plants were tested for the presence of the CP4 EPSPS protein by glyphosate spray treatment, and then self-pollinated to produce BC2F2 seed. The BC2F2 plants were also tested for the presence of the CP4 EPSPS protein by glyphosate spray application, and demonstrated the expected 3:1 segregation ratio for the MON 87427 trait. The heritability of the tissue selective glyphosate tolerance trait and *cp4 epsps* expression cassette in MON 87427 was demonstrated in the BC1F1, BC2F1, and BC2F2 generations.

A Chi-square (χ^2) analysis was used to compare the observed segregation ratios to the expected ratios according to Mendelian inheritance principles. The Chi-square was calculated as:

$$\chi 2 = \sum [(| o - e |)2 / e]$$

where o = observed frequency of the genotype or phenotype and e = expected frequency of the genotype or phenotype. The level of statistical significance was predetermined to be 5% ($p \leq 0.05$). The results of the χ^2 analysis of the segregating progeny of MON 87427 are presented in Table V-3. The χ^2 value in the BC1 generation indicated no statistically significant difference between the observed and expected 1:1, 1:1, and 3:1

(positive:negative) segregation ratios, respectively, for the tissue selective glyphosate tolerance trait in MON 87427. The observed segregation ratios in the BC1F1, BC2F1, and BC2F2 generations confirm that the tissue selective glyphosate tolerance trait in MON 87427 was fixed in the earlier LH198 BC3F4 generation that was used to initiate commercial inbred line development. These results support the conclusion that the *cp4 epsps* expression cassette in MON 87427 resides at a single locus within the maize genome and is inherited according to Mendelian inheritance principles. These results are also consistent with the molecular characterization data that indicate MON 87427 contains a single intact copy of the *cp4 epsps* expression cassette that was inserted into the maize genome at a single locus.

Table V-3. Segregation of the Tissue-selective Glyphosate Tolerance Trait During the Development of MON 87427

The plants were evaluated for the presence or absence of the glyphosate tolerance phenotype.

Generation	Number of plants	Observed Positives	Observed Negatives	Expected Positives	Expected Negatives	χ^2	Probability
BC1F1	238	109	129	119	119	1.6807	>0.05
BC2F1[1]	290	145	145	145	145	0	>0.05
BC2F2[2]	1107	820	287	830	277	0.5062	>0.05

[1,2]The BC1F1 and BC2F2 generations listed here are those from the trait integration breeding pathway as shown in Figure V-10.

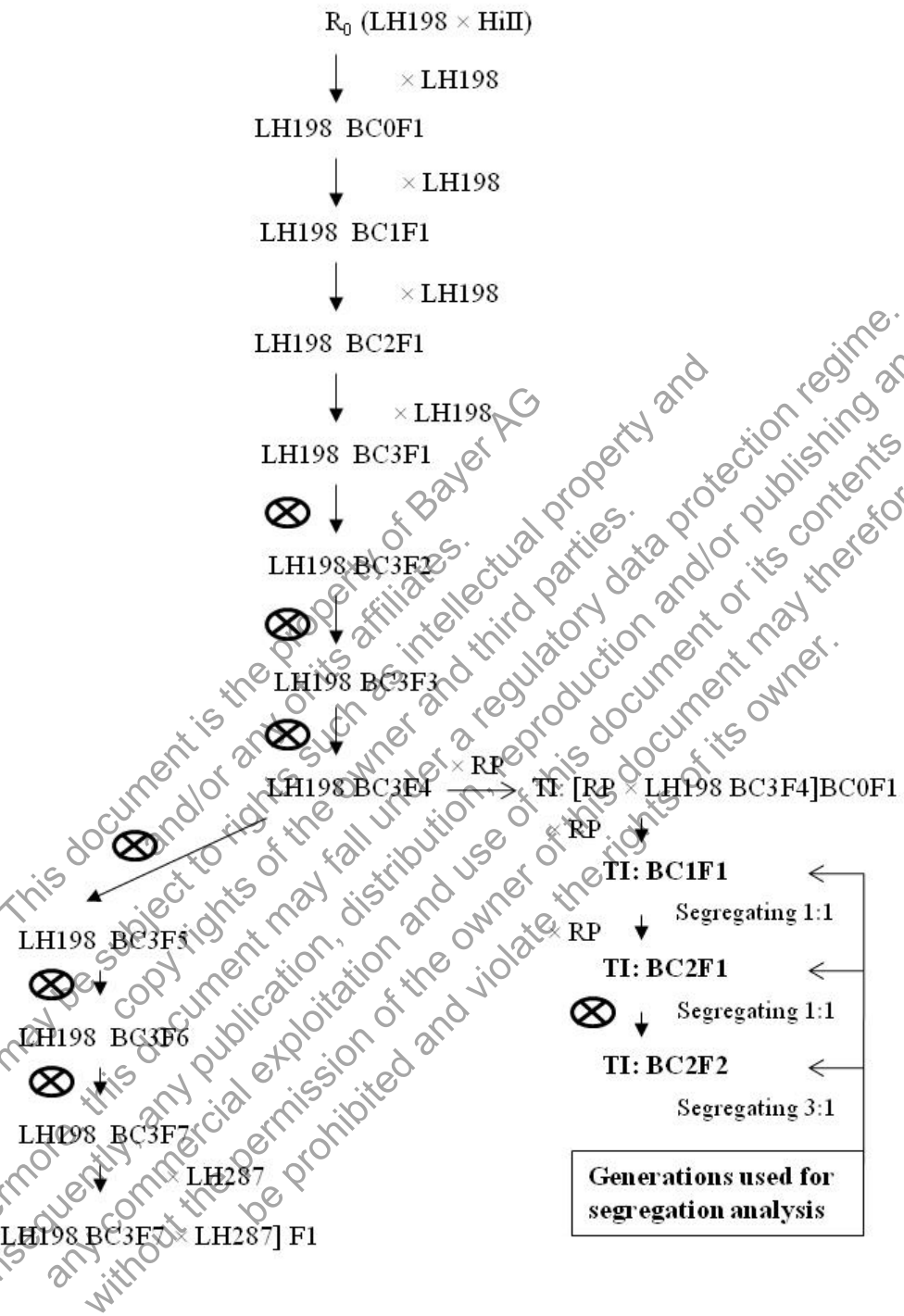


Figure V-10. Breeding Path for Generating Segregation Data for MON 87427
Chi square analysis conducted on segregation data from the BC1F1, BC2F1, and the BC2F2 generations (shown above in bold). R_0 corresponds to the transformed plant. F# is the filial generation. ⊗ designates self-pollination. BC# is the backcross generation, and TI corresponds to trait integration for commercial seed development.

V.G. Characterization of the Genetic Modification Summary and Conclusion

Molecular characterization of MON 87427 by Southern blot analyses demonstrated that a single copy of the T-DNA sequence from PV-ZMAP1043 was integrated into the maize genome at a single locus. There were no additional genetic elements, including backbone sequences, from PV-ZMAP1043 detected, linked or unlinked to the intact T-DNA present in MON 87427.

The PCR and DNA sequence analyses performed on MON 87427 confirmed the organization of the elements within the insert, assessed potential rearrangements at the insertion site, and resulted in the complete DNA sequence of the T-DNA and adjacent maize genomic sequence in MON 87427. Analysis of the T-DNA insertion site indicates that there was a 140 base pair deletion of genomic DNA at the insertion site in MON 87427. Additionally, a 41 base pair insertion was identified in the 5′ flanking sequence of MON 87427, and a 24 base pair insertion was identified in the 3′ flanking sequence of MON 87427.

Generational stability analysis by Southern blot demonstrated that MON 87427 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 87427. Results from segregation analyses show heritability and stability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA in MON 87427 at a single chromosomal locus.

VI. SAFETY ASSESSMENT OF EXPRESSED PRODUCTS

Numerous Roundup Ready crops including Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready corn 2, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton and Roundup Ready alfalfa that produce the CP4 EPSPS protein have been reviewed by FDA. The CP4 EPSPS protein expressed in MON 87427 is identical to the CP4 EPSPS in other Roundup Ready crops. Further, Harrison et al., (1996) has published on the safety of CP4 EPSPS proteins present in biotechnology-derived crops, and concluded that CP4 EPSPS does not pose any important concerns from an allergenicity or toxicity perspective.

A multistep approach was conducted according to guidelines established by the CODEX Alimentarius Commission and OECD and which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties, and was used to characterize the CP4 EPSPS protein in MON 87427 resulting from the genetic modification. These steps include: 1) characterization of the physicochemical and functional properties of CP4 EPSPS; 2) quantification of CP4 EPSPS expression in plant tissues; 3) examination of the similarity of CP4 EPSPS to known allergens, toxins or other biologically active proteins known to have adverse effects on mammals; 4) evaluation of the digestibility of CP4 EPSPS in simulated gastrointestinal fluids; 5) evaluation of the stability of the CP4 EPSPS protein after heat treatment; 6) documenting the history of safe consumption of CP4 EPSPS or its structural and functional homology to proteins that lack adverse effects on human or animal health; 7) investigation of potential mammalian toxicity through animal assays and calculating margins of exposure; and 8) assessment of the potential for allergenicity, toxicity and adverse biological activity of putative polypeptides encoded by the insert and flanking sequences.

The purified CP4 EPSPS protein produced in MON 87427 was characterized to demonstate the equivalence between MON 87427- and *E. coli*-produced CP4 EPSPS proteins. The MON 87427-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein purified from both sources was shown to be biochemically, structurally, and functionally equivalent. As the *E. coli*-produced CP4 EPSPS protein has been used previously in a number of safety assessment studies, including the simulated gastric fluid (SGF), simulated intestinal fluid (SIF), and acute mouse gavage, demonstration of protein equivalence between *E. coli*- and MON 87427-produced CP4 EPSPS proteins allows utilization of the existing data to confirm the safety of the CP4 EPSPS protein in MON 87427.

The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 34, 35, 36, 37, 38, 39, and 40 for assessment of possible toxicity and paragraphs 41, 42, and 43 and Annex 1 for assessment of possible allergenicity.

VI.A. Mode-of-Action of CP4 EPSPS Protein

The 5-enolpyruvylshikimate-3-phosphate synthase family of enzymes is found in plants and microorganisms and their properties have been extensively studied (Harrison, et al.,

1996; Klee et al., 1987; Schonbrunn, et al., 2001; Steinrucken and Amrhein, 1984). EPSPS enzymes generally have a molecular weight of 44-51 kDa and are mono-functional (Franz, et al., 1997; Kishore, et al., 1988), and they catalyze one of the key steps in the shikimate pathway for the biosynthesis of aromatic amino acids (phenylalanine, tryptophan and tyrosine) as well as other aromatic molecules and are the target of the broad spectrum herbicide, glyphosate. Specifically, EPSPS enzymes catalyze the transfer of the enolpyruvyl group from phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (EPSP) (Alibhai and Stallings, 2001). In conventional plants, glyphosate blocks the biosynthesis of EPSP by binding to EPSPS protein thereby depriving plants of essential amino acids and resulting in cell death (Steinrucken and Amrhein, 1980).

The EPSPS transgene (*cp4 epsps*) in MON 87427 is derived from *Agrobacterium* sp. strain CP4. The *cp4 epsps* coding sequence encodes a 47.6 kDa EPSPS protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The CP4 EPSPS protein is functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgette et al., 1996). In conventional plants, glyphosate binds to the endogenous EPSPS enzyme and blocks the biosynthesis of EPSP thereby depriving the plant of essential amino acids (Steinrucken and Amrhein, 1980). In Roundup Ready plants, requirements for aromatic amino acids and other metabolites are met by the continued action of the CP4 EPSPS enzyme in the presence of glyphosate (Padgette et al., 1996). MON 87427 produces the same CP4 EPSPS protein that is produced in numerous commercial Roundup Ready crop products. MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to drive CP4 EPSPS protein expression in vegetative and female reproductive tissues, conferring tolerance to glyphosate in the leaves, stalk, and root tissues and tissues that develop into seed or grain and silks. This specific promoter and intron combination also results in limited or no production of CP4 EPSPS protein in two key male reproductive tissues: pollen microspores which develop into pollen grains, and tapetum cells that supply nutrients to the pollen. Thus, in MON 87427, male reproductive tissues critical for male gametophyte development are not tolerant to glyphosate. Only specifically timed glyphosate applications beginning just prior to and/or during tassel development stages (approximate maize vegetative growth stages ranging from V8 to V13) will produce a male sterile phenotype in MON 87427. Glyphosate applications made during early vegetative stages for weed control, consistent with the application timing specified in the current Roundup agricultural product label, do not affect pollen production of MON 87427 because the sensitive male reproductive tissues are not actively developing at that time.

VI.B. History of Safe Use of CP4 EPSPS Protein

EPSPS enzymes are ubiquitous in plants and microorganisms and have been isolated from both sources (Harrison et al., 1996; Haslam, 1993; Klee et al., 1987; Schonbrunn et al., 2001; Steinrucken and Amrhein, 1984). While the shikimate pathway and the EPSPS enzyme are absent in mammals, fish, birds, reptiles, and insects (Alibhai and Stallings, 2001), the EPSPS enzyme and its activity are found widely in food and feed derived from

plant and microbial sources. Genes for EPSPSs from numerous sources have been cloned, and the expressed catalytic domains of this group of proteins are highly conserved (Padgette et al., 1996). Bacterial EPSPS enzymes have been well characterized with respect to their three dimensional crystal structures (Stallings, et al., 1991) as well as their kinetic and chemical mechanisms (Anderson and Johnson, 1990). The CP4 EPSPS protein thus represents one of many different EPSPSs found in nature; the CP4 EPSPS and native plant EPSPS enzymes are functionally equivalent except for their tolerance to glyphosate (Padgette et al., 1996).

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by FDA. The CP4 EPSPS protein expressed in MON 87427 is identical to the CP4 EPSPS proteins in other Roundup Ready crops including Roundup Ready soybeans, Roundup Ready 2 Yield soybeans, Roundup Ready corn 2, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton and Roundup Ready alfalfa. Results from the protein characterization studies included in this summary confirmed the identity of the MON 87427-produced CP4 EPSPS protein and established the equivalence of MON 87427-produced protein to the *E. coli*-produced CP4 EPSPS protein (Section VI.C.) used in several of the safety studies. The safety of CP4 EPSPS proteins present in numerous Roundup Ready crops have been extensively assessed (Harrison et al., 1996). The Environmental Protection Agency (EPA) also reviewed the safety of the CP4 EPSPS protein and has established a tolerance exemption for the protein and the genetic material necessary for its production either in or on all raw agricultural commodities (U.S. EPA, 1996). This exemption on CP4 EPSPS protein was based on a safety assessment that included rapid digestion in simulated gastric fluids, lack of homology to known toxins and allergens, and lack of toxicity in an acute oral mouse gavage study. A history of safe use is supported by the lack of any documented reports of adverse effects since the introduction of Roundup Ready crops in 1996.

VI.C. Characterization of the CP4 EPSPS Protein from MON 87427

VI.C.1. CP4 EPSPS Identity and Equivalence

The safety assessment of crops derived through biotechnology includes characterization of the physicochemical and functional properties of the protein(s) produced from the inserted DNA, and confirmation of the safety of the protein(s). The safety of a CP4 EPSPS protein produced in and purified from *E. coli* has been assessed previously and the results of these studies summarized by Harrison et al. (1996). For the existing CP4 EPSPS safety data set to be applied to a new biotechnology-derived crop/event expressing CP4 EPSPS, the equivalence of the plant- and *E. coli*-produced protein must first be established. The equivalence of the plant- and *E. coli*-produced CP4 EPSPS proteins has been confirmed previously for Roundup Ready crops such as soybean, cotton, sugar beet, canola, alfalfa and maize. To assess the equivalence between MON 87427-produced and *E. coli*-produced CP4 EPSPS protein, a small quantity of the CP4 EPSPS protein was purified from harvested MON 87427 grain. The equivalence of the physicochemical characteristics and functional activity between the MON 87427-produced and *E. coli*-produced CP4 EPSPS proteins was assessed by a panel of analytical tests, including: (1) N-terminal sequence analysis of the MON 87427-produced

CP4 EPSPS protein to establish its identity, (2) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of peptides derived from tryptic digested MON 87427-produced CP4 EPSPS and of intact MON 87427-produced CP4 EPSPS to establish identity and determine the intact mass, respectively, (3) western blot analysis to establish identity and immunoreactive equivalence between MON 87427-produced protein and the *E. coli*-produced protein using an anti-CP4 EPSPS antibody, (4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to establish equivalence of the apparent molecular weight between MON 87427-produced protein and the *E. coli*-produced protein, (5) glycosylation analysis to determine the glycosylation status of MON 87427-produced CP4 EPSPS and establish the equivalence of glycosylation status between MON 87427-produced and *E. coli*-produced protein, and (6) CP4 EPSPS enzymatic activity analysis to demonstrate functional equivalence between MON 87427-produced and the *E. coli*-produced protein. The details of the materials and methods for the panel of analytical tests used to evaluate and compare the properties of the plant- and *E. coli*-produced proteins are described in Appendix B. A summary of the data obtained to support a conclusion of protein equivalence is below.

A comparison of the MON 87427-produced CP4 EPSPS to the *E. coli*-produced CP4 EPSPS protein confirmed the identity of the MON 87427-produced CP4 EPSPS protein and established the equivalence of the two proteins. The identity of the CP4 EPSPS protein isolated from the grain of MON 87427 was confirmed by N-terminal sequencing, MALDI-TOF MS analysis of peptides produced after trypsin digestion, and by western blot analysis using anti-CP4 EPSPS polyclonal antibodies. The N-terminus of the MON 87427-produced CP4 EPSPS protein was consistent with the predicted amino acid sequence translated from the *cp4 epsps* coding sequence. In addition, the MALDI-TOF mass spectrometric analysis yielded peptide masses consistent with the expected peptide masses from the translated *cp4 epsps* coding sequence and an intact mass consistent with the expected mass of MON 87427-produced CP4 EPSPS protein. The CP4 EPSPS protein isolated from MON 87427 was detected on a western blot probed with antibodies specific for CP4 EPSPS protein. Furthermore, the immunoreactive properties and electrophoretic mobility of the MON 87427-produced CP4 EPSPS protein were shown to be equivalent to those of the *E. coli*-produced CP4 EPSPS protein by immunoblot and SDS-PAGE. Finally, the MON 87427-produced CP4 EPSPS protein and *E. coli*-produced CP4 EPSPS protein were found to be equivalent based on the lack of glycosylation and functional activities. Taken together, these data provide a detailed characterization of the CP4 EPSPS protein isolated from MON 87427 and establish its equivalence to the *E. coli*-produced CP4 EPSPS protein. Furthermore, because CP4 EPSPS proteins isolated from other Roundup Ready crops have been demonstrated previously to be equivalent to the *E. coli*-produced CP4 EPSPS protein, by inference, the MON 87427-produced CP4 EPSPS protein is equivalent to the CP4 EPSPS proteins expressed in other Roundup Ready crops, all of which have been the subject of consultations with the U.S. FDA.

VI.C.1.1 Results of the N-terminal Sequence Analysis

N-terminal sequencing of the first 15 amino acids performed on MON 87427-produced CP4 EPSPS protein resulted in the sequence expected for the CP4 EPSPS protein (Figure VI-1) with the exception of the N-terminal methionine, which was not detected. This result is expected as removal of the N-terminal methionine, catalyzed by methionine aminopeptidase, is a common modification that occurs co-translationally before completion of the nascent protein chain (Giglione and Meinnel, 2001). The N-terminal sequence information, therefore, confirms the identity of the CP4 EPSPS protein isolated from the grain of MON 87427.

Amino acid residue # from the N-terminus	→	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Expected Sequence	→	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence	→	-	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S

Figure VI-1. N-Terminal Sequence of the MON 87427 CP4 EPSPS Protein.
The expected amino acid sequence of the N-terminus of CP4 EPSPS was deduced from the *cp4 epsps* coding region present in MON 87427. The experimental sequence obtained from CP4 EPSPS was compared to the expected sequence. (-) indicates the residue was not observed.

VI.C.1.2. Results of MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 87427-produced CP4 EPSPS protein was also confirmed by MALDI-TOF mass spectrometric analysis of tryptic peptide fragments prepared from the MON 87427-produced CP4 EPSPS protein. The ability to identify a protein using this method is dependent upon a match of a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by proteolytic peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen, et al., 1997).

There were 26 unique peptides identified that corresponded to the expected masses of peptides produced from trypsin-digested CP4 EPSPS (Table VI-1). The identified masses were used to assemble a mass fingerprint map of the entire CP4 EPSPS protein (Figure VI-2). The experimentally determined mass coverage of the CP4 EPSPS protein

was 70.3% (320 out of 455 amino acids). This analysis serves as additional identity confirmation for the MON 87427-produced CP4 EPSPS protein.

Table VI-1. Summary of the Tryptic Masses Identified for the MON 87427-produced CP4 EPSPS Protein Using MALDI-TOF MS

Matrix									
α-Cyano	α-Cyano	DHB	DHB	Sinapinic acid	Sinapinic acid	Expected Mass[1]	Diff.[2]	AA Position[3]	Fragment
Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract 2				
506.08						506.22	0.14	354-357	ESDR
599.17						599.33	0.16	29-33	SISHR
616.17	616.32				615.67	616.34	0.17	128-132	RPMGR
629.16						629.29	0.13	201-205	DHTEK
629.16						629.34	0.18	383-388	GRPDGK
711.26	711.43	711.30				711.45	0.19	133-138	VLNPLR
835.17						835.39	0.22	62-69	AMQAMGAR
863.23						863.46	0.23	15-23	SSGLSGTVR
872.21		872.29				872.45	0.24	313-320	GVTVPEDR
872.21		872.29				872.52	0.31	358-366	LSAVANGLK
948.26	948.48	948.32	948.44			948.52	0.26	161-168	TPTPITYR
991.29						991.55	0.26	14-23	KSSGLSGTVR
1115.27		1115.36		1114.83		1115.57	0.30	295-305	LAGGEDVADLR
1357.32	1357.65	1357.44				1357.71	0.39	146-157	SEDGDRLPVTLR
1359.27	1359.58	1359.39	1359.56	1358.90		1359.72	0.45	354-366	ESDRLSAVANGLK
1359.27	1359.58	1359.39	1359.56	1358.90		1359.64	0.37	34-46	SFMLGGLASGETR
		1558.50	1558.65			1558.83	0.35	47-61	ITGLLEGEDVINTGK
1646.34	1646.70	1646.52	1646.92			1646.84	0.50	389-405	GLGNASGAAVATHLDHR
1763.29						1763.81	0.52	367-382	LNGVDCDEGETSLVVR
1993.38	1993.80	1993.60	1993.68	1993.21		1993.97	0.59	206-224	MLQGFGANLTVETDADGVR
2182.54	2183.00	2182.77	2182.92	2182.40	2182.84	2183.17	0.63	275-294	TGLILTLQEMGADIEVINPR
2366.61	2367.14	2366.86	2366.96	2366.66		2367.33	0.72	178-200	SAVLLAGLNTPGITTVIEPIMTR
				2449.44		2450.23	0.79	24-46	IPGDKSISHRSFMFGGLASGETR
				2449.44		2450.22	0.78	105-127	LTMGLVGVYYDFDSTFIGDASLTK
3250.78(AVE)		3251.23(AVE)		3250.80(AVE)	3252.37(AVE)	3251.75	0.97	321-351	APSMIDEYPILAVAAAFAEGATVMNGLEELR
		4190.17(AVE)		4190.98(AVE)	4190.14(AVE)	4180.89	0.72	234-274	LTGQVIDVPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The difference between the expected mass and the first column mass. Other masses shown within a row are also within 1 Da of the expected mass.
[3]AA position refers to amino acid residues within the predicted CP4 EPSPS sequence as depicted in Figure VI-2.
AVE = mass average

001 MLHGASSRPA TARKSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL

051 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

Figure VI-2. MALDI-TOF MS Coverage Map of the MON 87427 CP4 EPSPS Protein.
The amino acid sequence of the mature CP4 EPSPS protein was deduced from the *cp4 epsps* coding sequence present in MON 87427. Boxed regions correspond to tryptic peptides that were identified from the MON 87427-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 70.3% (320 of 455 total amino acids) of the expected protein sequence was identified.

VI.C.1.3. Results of MALDI-TOF Intact Mass Analysis of MON 87427-produced Protein

The intact mass of the MON 87427-produced CP4 EPSPS protein was determined by MALDI-TOF MS analysis. The average obtained from three measurements of the intact mass of the MON 87427-produced CP4 EPSPS protein was 47552 Da. The theoretical mass of the full-length protein without the N-terminal methionine is 47481 Da. The difference between the measured and theoretical masses is less than 0.15% and within the accuracy window (± 0.4%) of the MALDI-TOF MS instrument. This analysis confirmed the identity of the MON 87427-produced CP4 EPSPS protein.

VI.C.1.4. Results of Western Blot Analysis of CP4 EPSPS Protein Isolated from the Grain of MON 87427 and Immunoreactivity Comparison to *E. coli*-Produced CP4 EPSPS Protein

A western blot analysis was conducted using goat anti-CP4 EPSPS polyclonal antibody to 1) confirm the identity of the CP4 EPSPS protein isolated from the grain of MON 87427 and 2) to determine the relative immunoreactivity of the MON 87427-

produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein. The results demonstrated that the anti-CP4 EPSPS antibody recognized the MON 87427-produced CP4 EPSPS protein that migrated to an identical position as the *E. coli*-produced CP4 EPSPS protein (Figure VI-3). Furthermore, the immunoreactive signal increased with increasing levels of CP4 EPSPS protein loaded.

Densitometric analysis was conducted to compare the immunoreactivity of MON 87427- and *E. coli*-produced CP4 EPSPS proteins. The averaged band intensity of the signal from the MON 87427-produced CP4 EPSPS lanes was 9.6% more than the averaged band intensity of the signal from the *E. coli*-produced CP4 EPSPS lanes (Table VI-2). This observed difference was within the preset acceptance criteria for immunoreactivity (±35%). Thus, the immunoblot analysis established identity of the MON 87427-produced CP4 EPSPS protein and demonstrated that the MON 87427- and *E. coli*-produced CP4 EPSPS proteins have equivalent immunoreactivity with a CP4 EPSPS-specific antibody.

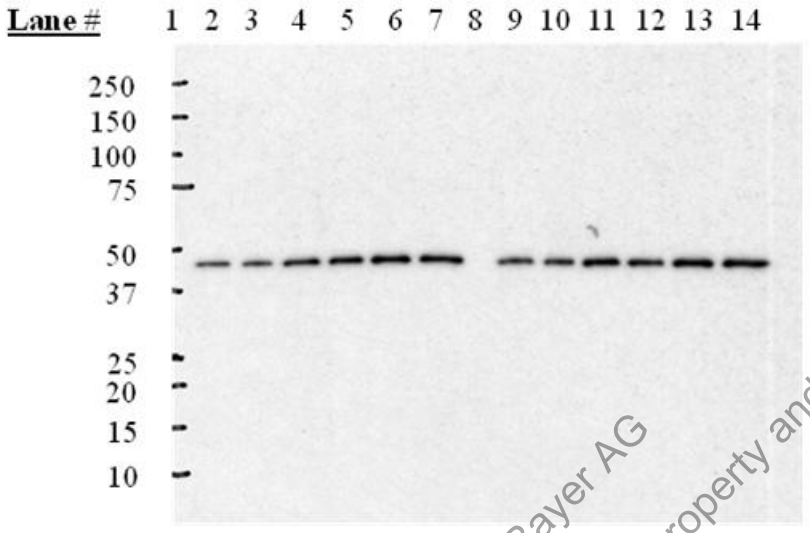


Figure VI-3. Western blot Analysis of MON 87427- and *E. coli* -produced CP4 EPSPS Protein

Aliquots of the MON 87427-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with anti-CP4 EPSPS antibodies and immunoreactive bands were visualized using an ECL system and film. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lane 1. The 5 min exposure is shown.

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color	-
2	*E. coli*-produced CP4 EPSPS protein	1
3	*E. coli*-produced CP4 EPSPS protein	1
4	*E. coli*-produced CP4 EPSPS protein	2
5	*E. coli*-produced CP4 EPSPS protein	2
6	*E. coli*-produced CP4 EPSPS protein	3
7	*E. coli*-produced CP4 EPSPS protein	3
8	Empty	
9	MON 87427-produced CP4 EPSPS protein	1
10	MON 87427-produced CP4 EPSPS protein	1
11	MON 87427-produced CP4 EPSPS protein	2
12	MON 87427-produced CP4 EPSPS protein	2
13	MON 87427-produced CP4 EPSPS protein	3
14	MON 87427-produced CP4 EPSPS protein	3

Table VI-2. Comparison of Immunoreactive Signals Between MON 87427- and *E. coli*-produced CP4 EPSPS Proteins.

Sample	*Gel lane*	*Amount (ng)*	*Contour Quantity*	*Average Contour Quantity*[1]	*Percent difference*[2] *(%)*	*Average Difference*[3] *(%)*
E. coli CP4 EPSPS	2	1	1.201	1.106	14.96	
E. coli CP4 EPSPS	3	1	1.011			
MON 87427 CP4 EPSPS	9	1	1.346	1.3005		
MON 87427 CP4 EPSPS	10	1	1.255			
E. coli CP4 EPSPS	4	2	2.130	2.308	6.46	9.6
E. coli CP4 EPSPS	5	2	2.486			
MON 87427 CP4 EPSPS	11	2	2.829	2.4675		
MON 87427 CP4 EPSPS	12	2	2.106			
E. coli CP4 EPSPS	6	3	3.310	3.388	7.37	
E. coli CP4 EPSPS	7	3	3.466			
MON 87427 CP4 EPSPS	13	3	3.433	3.6575		
MON 87427 CP4 EPSPS	14	3	3.882			

[1]Average Contour Quantity =∑(Contour Quantity)/2; contour quantity is average pixel density × band area

[2]Percent Difference (%)= ((|Average Contour Quantity plant-Average Contour Quantity *E.coli*|)/(Average Density plant))× 100%

[3]Average difference (%) =∑〖% difference〗/3

VI.C.1.5. Results of the determination of the MON 87427 CP4 EPSPS Protein Molecular Weight and Purity

For molecular weight and purity analysis, the MON 87427-produced CP4 EPSPS protein was separated using SDS-PAGE. The gel was stained with Brilliant Blue G Colloidal stain and analyzed by densitometry (Figure VI-4). The MON 87427-produced CP4 EPSPS protein (Figure VI-4, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS protein standard (Figure VI-4, lane 2) and had an apparent molecular weight of 44.1 kDa (Table VI-3). The apparent molecular weight of the *E. coli*-produced CP4 EPSPS protein standard as reported on the Certificate of Analysis was 43.8 kDa. The difference in apparent molecular weight between the MON 87427- and *E. coli*-produced CP4 EPSPS proteins was 0.7% (Table VI-3). Because the experimentally determined difference in apparent molecular weights met the preset acceptance criteria (+/-10%) and the proteins migrated to similar positions on the polyacrylamide gel, the MON 87427- and *E. coli*-produced CP4 EPSPS proteins were determined to have equivalent apparent molecular weights.

The purity of the MON 87427-produced CP4 EPSPS protein was calculated based on the average of six loads on the gel (Figure VI-4, lanes 3 to 8). The average purity was determined to be 96%.

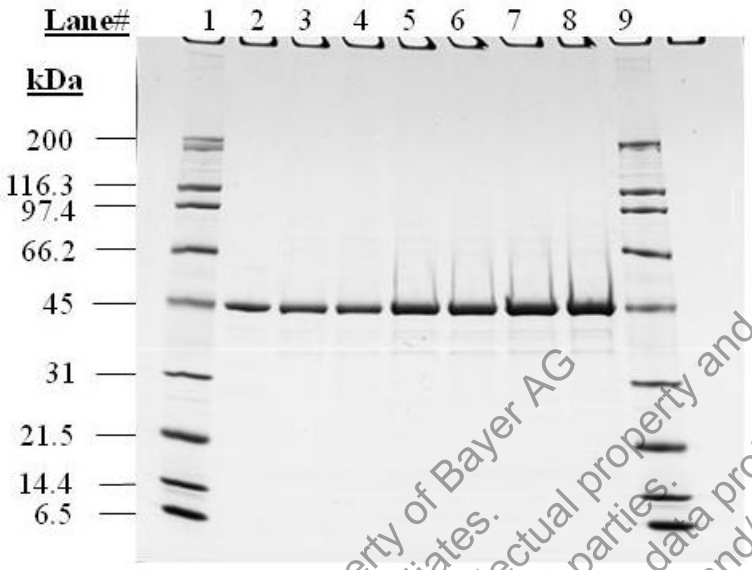


Figure VI-4. Molecular Weight and Purity Analysis of the MON 87427-produced CP4 EPSPS Protein

Aliquots of the MON 87427- and the *E. coli*-produced CP4 EPSPS proteins were separated on a 4-20% Tris glycine polyacrylamide gradient gel and then stained with Brilliant Blue G-Colloidal stain. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 9. An empty lane on the right of the gel was partially cropped.

Lane	Sample	Amount (µg)
1	Broad Range MW markers	4.5
2	*E. coli*-produced CP4 EPSPS protein	0.75
3	MON 87427-produced CP4 EPSPS protein	0.75
4	MON 87427-produced CP4 EPSPS protein	0.75
5	MON 87427-produced CP4 EPSPS protein	1.5
6	MON 87427-produced CP4 EPSPS protein	1.5
7	MON 87427-produced CP4 EPSPS protein	2.25
8	MON 87427-produced CP4 EPSPS protein	2.25
9	Broad Range MW markers	4.5
10	Empty Lane	

Table VI-3. Molecular Weight Comparison of the MON 87427-Produced and *E. coli*-Produced CP4 EPSPS Proteins Based on SDS-PAGE

Molecular Weight of MON 87427-Produced CP4 EPSPS Protein	Molecular Weight of *E. coli*-Produced CP4 EPSPS Protein	% Difference from *E. coli*-Produced CP4 EPSPS Protein[1]
44.1 kDa	43.8 kDa	0.7%

[1]Percent difference =

$$\frac{|(\text{avg. MW of MON 87427} - \text{produced Protein}) - (\text{avg. MW of } E.\,coli - \text{produced protein})|}{(\text{avg. MW of MON 87427} - \text{produced Protein})} \times 100$$

VI.C.1.6. CP4 EPSPS Glycosylation Equivalence

Some eukaryotic proteins are post-translationally modified by the addition of carbohydrate moieties (Rademacher, et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures, simple oligosaccharides or monosaccharides. In contrast, the non-virulent *E. coli* strains used for cloning and expression purposes lack the necessary biochemical synthetic capacity required for protein glycosylation. Therefore, determining whether the MON 87427-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS protein requires an investigation of its glycosylation status.

To test whether CP4 EPSPS protein was glycosylated when expressed in the grain of MON 87427, the MON 87427-produced CP4 EPSPS protein was analyzed for glycosylation using a Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Eugene, OR). Two naturally glycosylated proteins, transferrin and horseradish peroxidase, were used as positive controls in the assay. The results of this analysis are presented in Figure VI-5. The positive controls were clearly detected at the expected molecular weight and the bands increased with increasing protein concentration (Figure VI-5 lanes 2-5). Faint signals at a level slightly above the background noise were observed for the *E. coli*-produced protein as well as the MON 87427-produced CP4 EPSPS protein at the molecular weight expected for CP4 EPSPS protein (Figure VI-5 lanes 6-9). The *E. coli*-produced CP4 EPSPS protein has previously shown to be free of glycosylation (Harrison et al., 1996), therefore, the weak signal observed for both the *E. coli*-produced CP4 EPSPS protein as well as the MON 87427-produced CP4 EPSPS protein are not indicative of glycosylated species. Other data reported here corroborate the absence of glycosylation of the MON 87427-produced CP4 EPSPS protein. In particular, glycosylation would result in an increase in the protein mass relative to the theoretically calculated mass. The agreement of the observed protein mass of the MON 87427-produced CP4 EPSPS protein (47552 Da) as detected by MALDI-TOF mass spectrometric analysis to the theoretical mass (47481 Da) does not support the

existence of a glycosylated species, as the addition of even a single sugar would increase the mass by at least 160 Da.

Finally, to confirm that sufficient MON 87427- and *E. coli*-produced CP4 EPSPS proteins were present for carbohydrate detection and glycosylation analysis, the membrane was stained with Coomassie Brilliant Blue R-250 stain to detect proteins (Figure VI-5, Panel B). Both the MON 87427- and *E. coli*-produced CP4 EPSPS proteins were detected on the membrane (Figure VI-5, Panel B, Lanes 6-9). These data indicate that the MON 87427-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS protein and is not glycosylated.

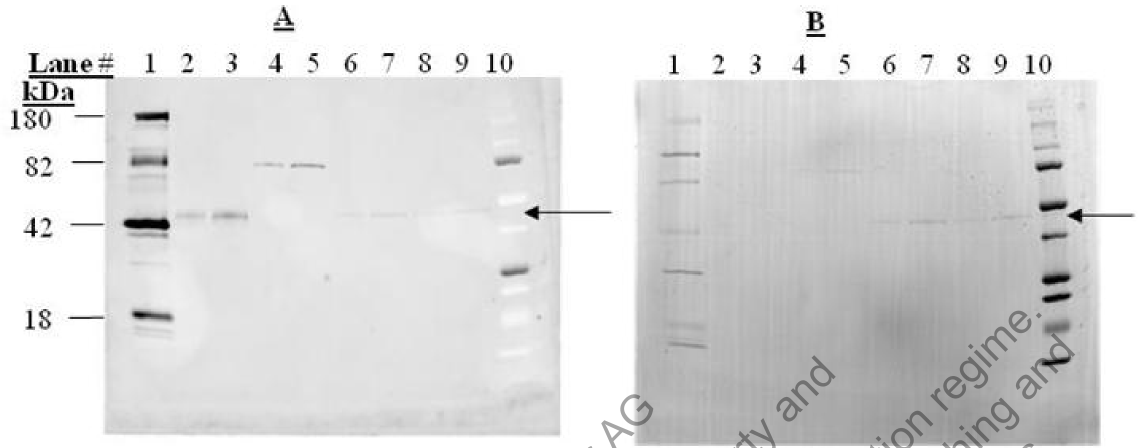


Figure VI-5. Glycosylation Analysis of the MON 87427 CP4 EPSPS Protein.
Aliquots of the MON 87427-produced CP4 EPSPS protein, *E. coli*-produced CP4 EPSPS protein (negative control), horseradish peroxidase (positive control) and transferrin (positive control) were separated by SDS-PAGE (4-20%) and electrotransferred to a PVDF membrane. (Panel A) Where present, periodate-oxidized protein-bound carbohydrate moieties reacted with Pro-Q Emerald 488 glycoprotein stain and emitted a fluorescent signal at 488 nm. The signal was captured using a Bio-Rad Molecular Imager FX. (Panel B) The same blot was stained with Coomassie Brilliant Blue R-250 to confirm the presence of proteins. The signal was captured using a Bio-Rad GS800 with Quantity One software (version 4.4.0). Approximate MWs (kDa) correspond to the glycosylated markers loaded in Lane 1 and the dual color markers (used to verify transfer) in Lane 10. Arrows indicate the band corresponding to CP4 EPSPS protein.

Lane	Sample	Amount (ng)
1	CandyCane Glycoprotein MW standards	-
2	Horseradish Peroxidase (positive control)	30
3	Horseradish Peroxidase (positive control)	60
4	Transferrin (positive control)	30
5	Transferrin (positive control)	60
6	MON 87427-produced CP4 EPSPS protein	30
7	MON 87427-produced CP4 EPSPS protein	60
8	*E. coli*-produced CP4 EPSPS protein(negative control)	30
9	*E. coli*-produced CP4 EPSPS protein(negative control)	60
10	Precision Plus ProteinTM Standards Dual color	-

VI.C.1.7. CP4 EPSPS Functional Activity Equivalence

The functional activities of the *E. coli*- and MON 87427-produced CP4 EPSPS proteins were estimated using an assay that measures the EPSPS-catalyzed formation of inorganic phosphate (P_i) and 5-enolpyruvylshikimate-3-phosphate (EPSP) from shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). In this assay, protein specific activity is expressed as units per milligram of protein (U/mg), where a unit is defined as one µmole of inorganic phosphate released from PEP per min at 25 °C. The *E. coli*- and MON 87427-produced CP4 EPSPS proteins were considered functionally equivalent if the specific activity of one protein was within 50% of the other. The specific activity of the plant-produced CP4 EPSPS protein was determined using a phosphate release assay. This end-point colorimetric assay measures the release of inorganic phosphate from one of the substrates, PEP, by the action of the CP4 EPSPS enzyme.

The results of the specific activity assay are presented in Table VI-4. The specific activity of MON 87427- and *E. coli*-produced CP4 EPSPS proteins was measured to be 8.67 U/mg and 5.41 U/mg of CP4 EPSPS protein, respectively. Because the specific activity of the MON 87427-produced CP4 EPSPS protein falls within the preset acceptance limits (Table VI-4), the MON 87427-produced CP4 EPSPS protein is considered to have equivalent functional activity to that of the *E. coli*-produced CP4 EPSPS protein.

Table VI-4. CP4 EPSPS Functional Assay

MON 87427-produced CP4 EPSPS PROTEIN[1] (U/mg)	*E. coli*-produced CP4 EPSPS PROTEIN[1] (U/mg)	Previously set acceptance limits[2] (U/mg)
8.67 ± 0.23	5.41 ± 0.37	2.71-10.82

[1]Value refers to mean and standard deviation calculated based on n = 6 which includes three replicate assays spectrophotometrically analyzed at 660 nm in duplicate.
[2]Within 2-fold (50% difference) of the *E. coli*-produced CP4 EPSPS protein specific activity (5.41 ÷ 2 U/mg to 5.41 × 2 U/mg).

VI.C.2. CP4 EPSPS Protein Identity and Equivalence Conclusion

A panel of analytical techniques was used to characterize the MON 87427-produced CP4 EPSPS protein purified from grain of MON 87427. Identity of the MON 87427-produced CP4 EPSPS protein was confirmed by identification of the first 15 amino acids of the N-terminus by amino acid sequencing, mapping of tryptic peptides that yielded a 70.3% overall coverage of the expected protein sequence, and recognition with anti-CP4 EPSPS antibodies. MALDI-TOF mass spectrometry analysis of the intact protein resulted in an average mass of 47.552 kDa, reflecting the expected mass of the protein

minus the N-terminal methionine. The purity and apparent molecular weight of the MON 87427-produced CP4 EPSPS protein was 96% and 44.1 kDa, respectively.

The equivalence of the MON 87427- and *E. coli*-produced CP4 EPSPS proteins was evaluated by comparing their apparent molecular weight, immunoreactivity with anti-CP4 EPSPS antibodies, glycosylation status, and functional activity. The results obtained demonstrate that the MON 87427-produced CP4 EPSPS protein is equivalent to the *E. coli*-produced CP4 EPSPS protein. This equivalence justifies the use of protein safety studies conducted previously in which the *E. coli*-produced CP4 EPSPS protein was used as a test substance.

VI.D. Expression Levels of CP4 EPSPS Protein in MON 87427

CP4 EPSPS protein levels in various tissues of MON 87427 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Tissues of MON 87427 were collected from three replicates during the 2008 growing season from the following five field sites in the U.S.: Jackson County, Arkansas; Jefferson County, Iowa; Stark County, Illinois; Parke County, Indiana; and York County, Nebraska. These field sites were representative of maize producing regions suitable for commercial production. Over-season leaf (OSL1-4), grain, pollen, silk, forage, stover, over-season root (OSR1-4), forage-root, senescent root and over-season whole plant (OSWP1-4) tissue samples were collected from each replicated plot at all field sites.

CP4 EPSPS protein levels were determined in all nineteen tissue types. The results obtained from ELISA analysis are summarized in Table VI-5 and the details of the materials and methods are described in Appendix C. CP4 EPSPS protein levels in MON 87427 across tissue types ranged from below the limit of detection (LOD) to 940 µg/g dwt. The mean CP4 EPSPS protein levels across the five sites were highest in OSL (ranging from OSL3 290 µg/g dwt to OSL1 680 µg/g dwt), followed by OSWP (ranging from OSWP4 240 µg/g dwt to OSWP1 500 µg/g dwt), OSR (ranging from OSR3 73 µg/g dwt to OSR1 140 µg/g dwt), forage (120 µg/g dwt), silk (100 µg/g dwt), forage root (72 µg/g dwt), senescent root (72 µg/g dwt), stover (43 µg/g dwt), and grain (4.2 µg/g dwt). CP4 EPSPS protein levels in MON 87427 pollen across the sites were either <LOD, had a very low level just above LOQ (mean of 0.87 µg/g dwt) of CP4 EPSPS protein, or were not able to be determined (Table VI-5).

The CP4 EPSPS protein expression data from MON 87427 is consistent with the MON 87427 product concept. As discussed in Section I, MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to drive CP4 EPSPS protein expression in vegetative and female reproductive tissues, conferring tolerance to glyphosate in these tissues. The specific promoter and intron combination used in MON 87427 also drives little or no CP4 EPSPS protein production in the tapetum and microspores cells, precursors of pollen, thus these cells in MON 87427 are not tolerant to glyphosate. CP4 EPSPS protein was quantified in the vegetative (leaf, whole plant, forage, stover, and root) and female reproductive tissues (Table VI-5). The low concentration of CP4 EPSPS protein found in pollen samples might be attributed to the presence of anther

tissue collected with the pollen from MON 87427. Alternatively, a low amount of CP4 EPSPS protein in MON 87427 pollen may be inherent to this product due to the use of the e35S promoter (CaJacob et al., 2004).

Table VI-5. Summary of CP4 EPSPS Protein Levels in Tissues from MON 87427 Grown in 2008 US Field Trials

Tissue Type[1]	Development Stage[2]	Days after planting (DAP)	Mean (SD) Range (µg/g fwt)[3]	Mean (SD) Range (µg/g dwt)[4]	LOD/LOQ[5] (µg/g fwt)
OSL1	V2-V5	20-28	100 (21) 75 – 140	680 (170) 400 – 940	0.069/0.137
OSL2	V6-V8	32-46	83 (25) 30 –110	410 (130) 130 – 560	0.069/0.137
OSL3	V10-V12	41-67	61 (19) 35 –95	290 (74) 210 – 410	0.069/0.137
OSL4	VT	54-73	95 (30) 17 – 140	370 (120) 70 – 520	0.069/0.137
Grain	R6	118-182	3.6 (0.73) 2.6 – 5.3	4.2 (0.89) 2.8 – 6.2	0.16/0.228
Pollen[6]	At Pollination	58-81	< LOD (NA) NA 0.49 (0.36) 0.18 – 1.1	<LOD (NA) NA 0.87 (0.70) 0.25 – 2.2	0.099/0.137
Silk	During Pollination	58-76	9.4 (0.97) 8.1 – 11	100 (12) 90 – 120	0.121/0.137
Forage	R5	83-116	38 (14) 8.3 – 57	120 (48) 21 – 200	0.069/0.137
Stover	R6	124-180	14 (6.3) 5.9 – 26	43 (27) 13 – 98	0.069/0.137
OSR1	V2-V5	22-28	18 (5.3) 8.1 – 27	140 (46) 58 – 210	0.033/0.068
OSR2	V6-V8	32-46	16 (6.8) 8.3 –29	110 (62) 48 – 240	0.033/0.068
OSR3	V10-V12	41-67	12 (4.3) 4.9 –19	73 (28) 22 – 110	0.033/0.068

Table VI-5. (continued) Summary of CP4 EPSPS Protein Levels in Maize Tissues from MON 87427 Grown in 2008 U.S. Field Trials

Tissue Type[1]	Development Stage[2]	Days after planting (DAP)	Mean (SD) Range (µg/g fwt)[3]	Mean (SD) Range (µg/g dwt)[4]	LOD/LOQ[5] (µg/g fwt)
OSR4	VT	54-73	15 (5.7) 5.6 – 23	83 (36) 23 – 140	0.033/0.068
Forage-Root	R5	83-116	15 (5.2) 8.6 – 24	72 (23) 39 – 100	0.033/0.068
Senescent Root	R6	124-180	16 (8.3) 5.9 – 29	72 (37) 26 – 130	0.033/0.068
OSWP1	V2-V5	22-28	50 (8.3) 37 – 66	500 (190) 310 – 840	0.069/0.137
OSWP2	V6-V8	32-46	46 (7.6) 33 – 58	360 (42) 300 – 420	0.069/0.137
OSWP3	V10-V12	41-67	43 (7.1) 28 – 56	380 (78) 230 – 500	0.069/0.137
OSWP4	VT	54-73	37 (6.3) 23 – 47	240 (42) 160 – 340	0.069/0.137

[1]OSL= over-season leaf; OSR= over-season root; OSWP= over-season whole plant.

[2]The maize development stage each tissue was collected.

[3]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fwt). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites (n=14 for all tissues, except forage root where n=11 and pollen (see footnote [6])). NA: Not Applicable.

[4]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dwt). The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factor obtained from moisture analysis data. NA: Not Applicable.

[5]LOQ=limit of quantitation; LOD=limit of detection.

[6]CP4 EPSPS protein levels in MON 87427 pollen across the sites were either < LOD µg/g dwt (n=6), or had a very low level of CP4 EPSPS protein (n=6). Two pollen samples were not included in calculations due to inconclusive results.

VI.E. Generational Stability of CP4 EPSPS Protein Expression in MON 87427

In order to confirm the presence of the CP4 EPSPS protein in MON 87427 across multiple generations, western blot analysis of CP4 EPSPS protein in MON 87427 was conducted on leaf tissue collected from generation LH198 BC3F3 and seed tissue collected from generations LH198 BC3F4, LH198 BC3F6, LH198 BC3F7, and (LH198 BC3F7 × LH287)F1 and on seed tissue of a conventional control (LH198 × HiII).

The five breeding generations of MON 87427 as well as an appropriate control, as described above, and a reference substance, were included in the analysis (Figure VI-6). The *E. coli*-produced CP4 EPSPS protein standard (2 ng) was used as a reference for the identification of the CP4 EPSPS protein. The presence of the CP4 EPSPS protein in MON 87427 tissue samples was determined by visual comparison of the bands produced in five breeding generations (Figure VI-6, lanes 4-8) to the CP4 EPSPS protein reference standard (Figure VI-6, lane 3). As shown in Figure VI-6, lane 4, LH198 BC3F3 produced a band of greater intensity than the other generations, which was expected given the higher expression of CP4 EPSPS protein in leaf tissue relative to grain (Table VI-5).

CP4 EPSPS protein was present in all five generations of MON 87427 tissue samples, as expected. The MON 87427-produced CP4 EPSPS protein migrated with mobility indistinguishable from that of the E. coli-produced protein standard analyzed on the same western blot. As expected, the CP4 EPSPS protein was not detected in the conventional control seed extract (Figure VI-6, lane 10).

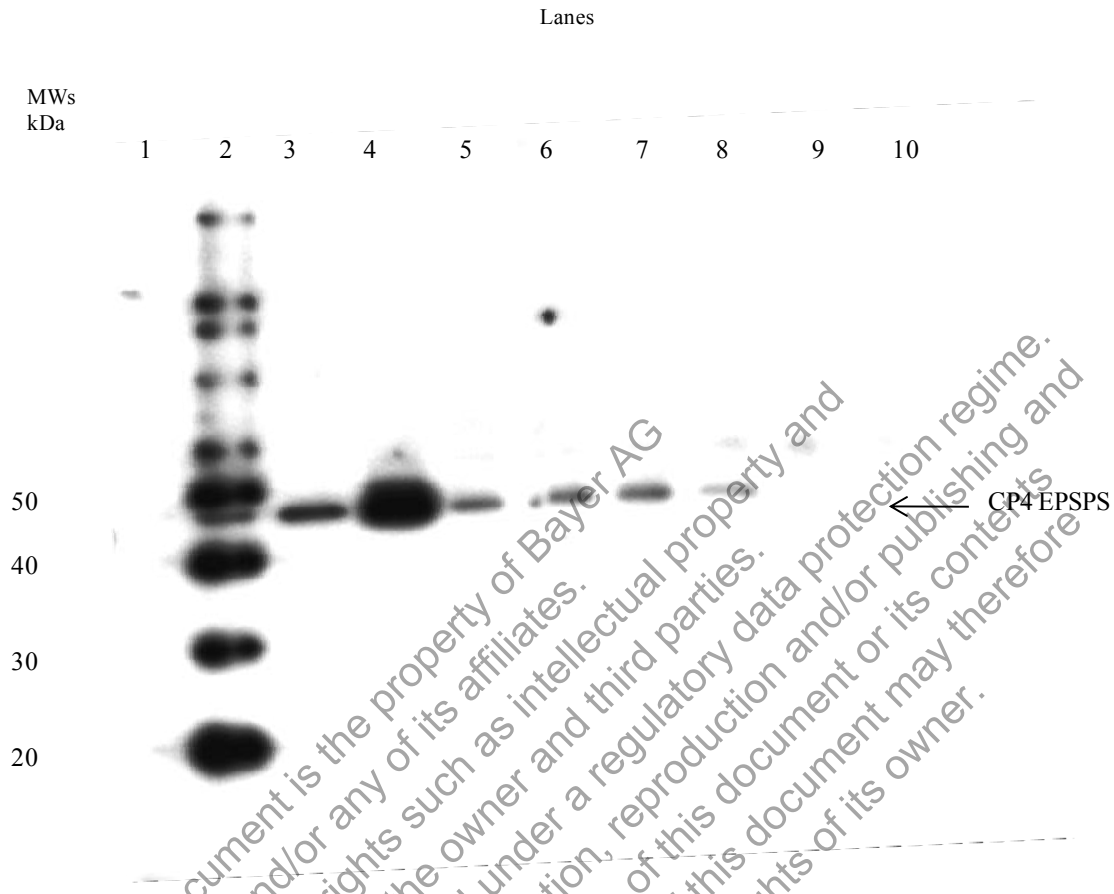


Figure VI-6. Presence of CP4 EPSPS Protein in Multiple Generations of MON 87427

Aliquots of extracts from four generations of MON 87427 seed tissues, one generation of leaf tissue, and molecular weight markers were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with goat anti-CP4 EPSPS antibody and immunoreactive bands visualized through the use of chemiluminescent reagents. The image represents a one minute exposure.

Lane	Description	Amount Loaded on Gel
1	Blank	NA
2	Magic Marker Molecular Weight Marker	0.5 µl
3	*E. coli*-produced CP4 EPSPS protein (2 ng)	2 ng
4	Test Substance, LH198 BC3F3 (leaf)	20 µl
5	Test Substance, LH198 BC3F4 (seed)	20 µl
6	Test Substance, LH198 BC3F6 (seed)	20 µl
7	Test Substance, LH198 BC3F7 (seed)	20 µl
8	Test Substance, (LH198 BC3F7 × LH287)F1 (seed)	20 µl
9	Blank	NA
10	Conventional control LH198 × HiII (11214241-004)	20 µl

VI.F. Assessment of the Potential Allergenicity, Toxicity and Dietary Safety of the CP4 EPSPS Protein

History of safe use of the introduced protein is a key consideration for the assessments of allergenicity and toxicity potential and dietary safety.

Additionally, according to guidelines adopted by the Codex Alimentarius Commission (Codex Alimentarius, 2003a) for the assessment of potential allergenicity of introduced proteins, the allergenic potential of an introduced protein is assessed by comparing the biochemical characteristics of the introduced protein to characteristics of known allergens (Codex Alimentarius, 2003a). A protein is not likely to be associated with allergenicity if: 1) the protein is from a nonallergenic source, 2) the protein represents only a very small portion of the total plant protein, 3) the protein does not share structural similarities based on the amino acid sequence to known allergens, 4) the protein is rapidly digested in mammalian gastrointestinal systems, and 5) the protein is not stable to heat treatment. The CP4 EPSPS protein in MON 87427 has been assessed for its potential allergenicity according to these safety assessment guidelines.

The assessment of the potential toxicity of an introduced protein is based on comparing the biochemical characteristics of the introduced protein to characteristics of known toxins. These biochemical characteristics are assessed by determining: 1) if the protein has structural similarity to known toxins or other biologically-active proteins that could cause adverse effects in humans or animals; 2) if the protein is rapidly digested in mammalian gastrointestinal systems; 3) if the protein is stable to heat treatment; 4) if the protein exerts any acute toxic effects in mammals; and 5) the anticipated exposure levels for humans and animals. The CP4 EPSPS protein in MON 87427 has been assessed for its potential toxicity based on these criteria.

VI.F.1. Assessment of Potential Allergenicity of CP4 EPSPS Protein

VI.F.1.1. Safety of the Donor Organism

The donor organism, *Agrobacterium* sp. strain CP4, was isolated based on its tolerance to glyphosate brought about by the production of a naturally glyphosate-tolerant EPSPS protein (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 for human or animal pathogenicity, and are not commonly allergenic. According to FAO/WHO, there is no known population of individuals sensitized to bacterial proteins (FAO, 2001). Furthermore, *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding Roundup Ready soybean, Roundup Ready canola, Roundup Ready cotton, Roundup Ready Corn 2, Roundup Ready sugar beet, Roundup Ready Flex cotton, and Roundup Ready 2 Yield soybean.

VI.F.1.2. The CP4 EPSPS Protein as a Proportion of Total Protein

The CP4 EPSPS protein was detected in all plant tissues assayed, at a number of time points during the growing season (Table VI-5) with the expected exception of certain pollen samples. Among tested tissues of MON 87427, harvested grain is the most relevant to the assessment of food allergenicity. The mean level of CP4 EPSPS protein in harvested grain is 4.2 μg/g dwt. The mean percent dry weight of total protein in harvested grain from MON 87427 is 10.05 % (or 100500 μg/g). The percentage of CP4 EPSPS in MON 87427 harvested grain is calculated as follows:

$(4.2\ \mu g/g \div 100500\ \mu g/g) \times 100\% \approx 0.004\%$ of total maize protein

Therefore, the CP4 EPSPS protein represents a very small portion of the total protein in harvested grain of MON 87427.

VI.F.1.3. Structural Similarity of CP4 EPSPS to Known Allergens

The Codex guidelines for the evaluation of the allergenicity potential of introduced proteins (Codex Alimentarius, 2003b) are based on the comparison of amino acid sequences between introduced proteins and allergens, where allergenic cross-reactivity may exist if the introduced protein is found to have at least 35% amino acid identity with an allergen over any segment of at least 80 amino acids. The Codex guideline also recommends that a sliding window search with a scientifically justified peptide size could be used to identify immunologically relevant peptides in otherwise unrelated proteins. Therefore, the extent of sequence similarities between the CP4 EPSPS protein present in MON 87427 and known allergens, gliadins, and glutenins was assessed using the FASTA sequence alignment tool and an eight-amino acid sliding window search (Codex Alimentarius, 2003b; Thomas, et al., 2005). The data generated from these analyses confirm that the CP4 EPSPS protein does not share any amino acid sequence similarities with known allergens, gliadins, or glutenins.

The FASTA program directly compares amino acid sequences (i.e., primary, linear protein structure). This alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins usually have common secondary structures, and three-dimensional configuration, and, consequently, may share similar functions. The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from Food Allergy Research and Resource Program Database (FARRP_2010) (http://www.allergenonline.com) and was used for the evaluation of sequence similarities shared between the CP4 EPSPS protein and all proteins. The AD_2010 database contains 1,471 sequences. When used to align the sequence of the introduced protein to each protein in the database, the FASTA algorithm produces an *E*-score (expectation score) for each alignment. The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a low degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences which have an *E*-score of less than or equal

to 1×10^{-5} are considered to have significant homology. Results indicate that the CP4 EPSPS protein sequence does not share significant similarity with sequences in the allergen database. No alignment met nor exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2003) or had an *E*-score of less than or equal to 1×10^{-5}.

A second bioinformatic tool, an eight-amino acid sliding window search, was used to specifically identify short linear polypeptide matches to known allergens. It is possible that proteins structurally unrelated to allergens, gliadins, and glutenins may contain smaller immunologically significant epitopes. An amino acid sequence may have allergenic potential if it has an exact sequence identity of at least eight linearly contiguous amino acids with a potential allergen epitope (Hileman, et al., 2002; Metcalfe, et al., 1996). Using a sliding window of less than eight amino acids can produce matches containing significant uncertainty depending on the length of the query sequence (Silvanovich, et al., 2006) and are not useful to the allergy assessment process (Thomas et al., 2005). No eight contiguous amino acid identities were detected when the CP4 EPSPS protein sequence was compared to the proteins in the AD_2010 sequence database.

Results indicate there were no similarities to allergens when the CP4 EPSPS protein sequence was used as a query for a FASTA search of the AD_2010 database. Furthermore, no short (eight amino acid) polypeptide matches were shared between the CP4 EPSPS protein sequence and proteins in the allergen database. These data indicate that the CP4 EPSPS protein sequence lacks both structurally and immunologically relevant sequence similarities to known allergens.

VI.F.1.4. Digestive Fate of the CP4 EPSPS Protein

A correlation between digestive stability in simulated gastric fluid (SGF) and the allergenicity of a protein has been previously reported (Astwood, et al., 1996), but this correlation is not absolute (Fu, et al., 2002). The SGF assay serves as a tool to compare the relative susceptibility of novel proteins to digestion in pepsin. The SGF assay protocol has been standardized based on results obtained from an international, multi-laboratory ring study (Thomas, et al., 2004). This study showed that the standardized protocol provides reproducibility and consistency for determining the digestive stability of a protein. Using this standardized protocol, the digestive stability of CP4 EPSPS was analyzed (Appendix F) and a summary of the results is reported below.

Harrison et al. (1996) demonstrated that the *E. coli*-produced CP4 EPSPS protein is rapidly degraded under simulated digestive conditions. Based on Western blot analysis, CP4 EPSPS protein was undetectable within 15 seconds under simulated gastric conditions greatly minimizing the potential for this protein to be absorbed in the intestinal mucosa. In addition, CP4 EPSPS protein was undetectable within 10 minutes under simulated intestinal conditions. Therefore, if any of the CP4 EPSPS protein were to survive in the gastric system, it is expected that it would be rapidly degraded in the intestine. As a comparison, it has been estimated that 50% of solid food was digested in the human stomach within two hours, while 50% of liquid was digested within 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 confirmed the *in vitro* digestibility of the CP4 EPSPS protein in simulated gastric fluid (SGF) using the standardized method published by the International Life Science Institute (ILSI) (Thomas et al., 2004). *E. coli*-produced CP4 EPSPS protein, shown to be physiochemically and functionally equivalent to the CP4 EPSPS protein produced in MON 87427 (Section VI.C), was utilized in these experiments. Similar to the results reported by Harrison et al. (1996), greater than 98% of the CP4 EPSPS protein was digested within 15 seconds, based on the results of visual inspection of colloidal blue stained SDS-PAGE gels (Figure VI-7). Western blot analysis confirmed that greater than 95% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 seconds (Figure VI-8). In summary, the results of these experiments confirmed that the *E. coli*-produced CP4 EPSPS protein was rapidly digested after incubation in SGF and is therefore unlikely to pose a human health concern.

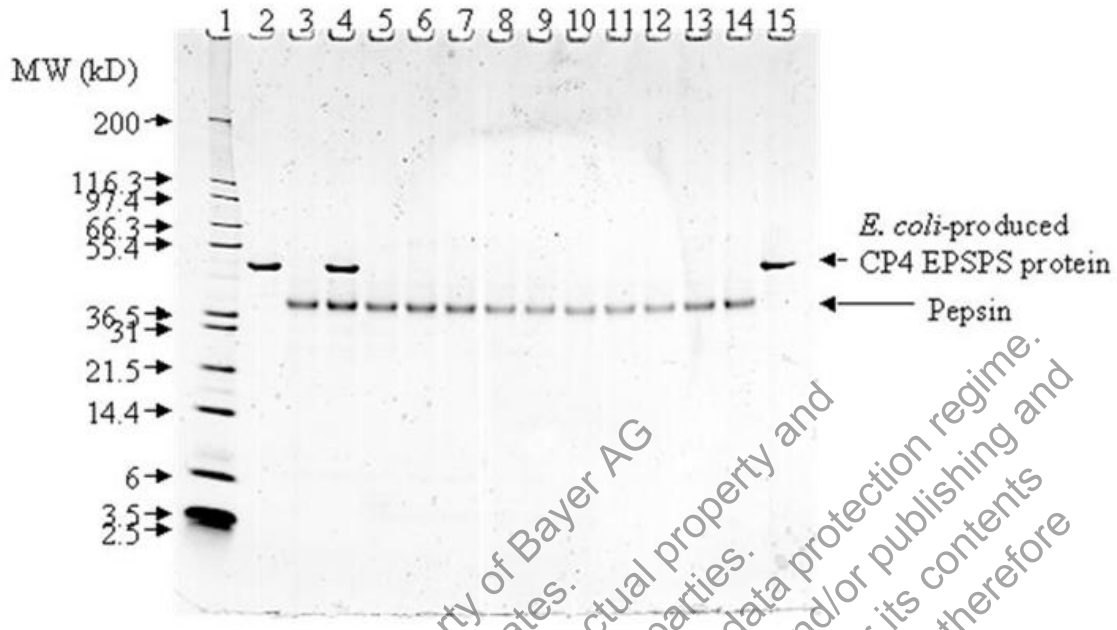


Figure VI-7. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid

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 concentrations.

Lane	Description	Incubation Time
1	Molecular weight markers	
2	Experimental control without pepsin	0 s
3	Experimental control without CP4 EPSPS	0 s
4	CP4 EPSPS protein in SGF	0 s
5	CP4 EPSPS protein in SGF	15 s
6	CP4 EPSPS protein in SGF	30 s
7	CP4 EPSPS protein in SGF	1 min
8	CP4 EPSPS protein in SGF	2 min
9	CP4 EPSPS protein in SGF	4 min
10	CP4 EPSPS protein in SGF	8 min
11	CP4 EPSPS protein in SGF	15 min
12	CP4 EPSPS protein in SGF	30 min
13	CP4 EPSPS protein in SGF	60 min
14	Experimental control without CP4 EPSPS	60 min
15	Experimental control without pepsin	60 min

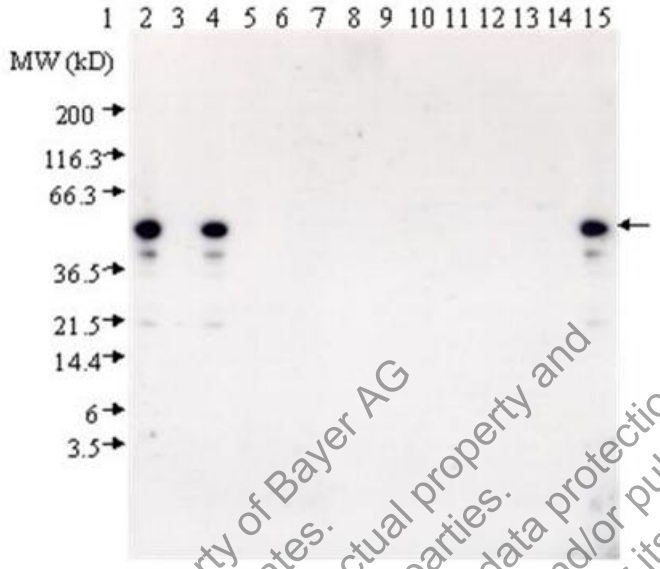


Figure VI-8. Western Blot Analysis of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid

Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a tricine buffered gel, electroblotted, and probed with anti-CP4 EPSPS antibody. *E. coli*-produced CP4 EPSPS protein was loaded at 1 ng per lane based on 90% purity and pre-digestion concentrations. Arrow at right indicates the band corresponding to CP4 EPSPS protein.

Lane	Description	Incubation Time
1	Molecular weight markers	
2	Experimental control without pepsin	0 s
3	Experimental control without CP4 EPSPS	0 s
4	CP4 EPSPS protein in SGF	0 s
5	CP4 EPSPS protein in SGF	15 s
6	CP4 EPSPS protein in SGF	30 s
7	CP4 EPSPS protein in SGF	1 min
8	CP4 EPSPS protein in SGF	2 min
9	CP4 EPSPS protein in SGF	4 min
10	CP4 EPSPS protein in SGF	8 min
11	CP4 EPSPS protein in SGF	15 min
12	CP4 EPSPS protein in SGF	30 min
13	CP4 EPSPS protein in SGF	60 min
14	Experimental control without CP4 EPSPS	60 min
15	Experimental control without pepsin	60 min

VI.F.1.5. Heat Stability of CP4 EPSPS Protein

Heat treatment is widely used in maize grain processing and in the preparation of foods containing components derived from maize grain. The effect of heat treatment on the activity of CP4 EPSPS protein was evaluated using the *E. coli*-produced CP4 EPSPS protein heated at 25, 37, 55, 75, and 95 °C for 30 minutes. Heat-treated samples and an un-heated control sample of CP4 EPSPS protein samples were analyzed: (1) using a functional assay to assess the impact of temperature on the enzymatic activity of CP4 EPSPS protein and (2) using SDS-PAGE to assess the impact of temperature on protein integrity.

The effect of heating on the functional activity of the *E. coli*-produced CP4 EPSPS protein is presented in Table VI-6. CP4 EPSPS protein retained functional activity following the heat treatments conducted at 25 ºC and 37 ºC. A significant decrease in functional activity (25 % of the un-heated control activity), was observed after heating at 55 ºC. CP4 EPSPS protein activity was below the limit of detection (LOD) following heat treatment at either 75 ºC or 95 ºC. SDS-PAGE analysis of heat treated samples demonstrated equivalent electrophoretic mobility of both the reference and the no heat control samples with no significant loss in stained band intensity (Figure VI-9). These data demonstrate that the CP4 EPSPS protein is not functionally active at elevated temperatures and therefore, is not thermostable.

Table VI-6. Specific Activity of CP4 EPSPS Protein Following Heat Treatment

Temperature	Specific Activity Units/mg CP4 EPSPS Protein (Mean[1] ± SD[2])	Relative activity[3]
No Heat Control	2.8 ± 0.26	100%
25 ºC	3.1 ± 0.23	110%
37 ºC	2.5 ± 0.05	88%
55 ºC	0.70 ± 0.09	25%
75 ºC	<LOD[4]	0.00%
95 ºC	<LOD[4]	0.00%

[1]Value refers mean calculated based on n= 3.
[2]SD : standard deviation
[3]No Heat Control assigned as 100% activity
[4]LOD : limit of detection (level of LOD)

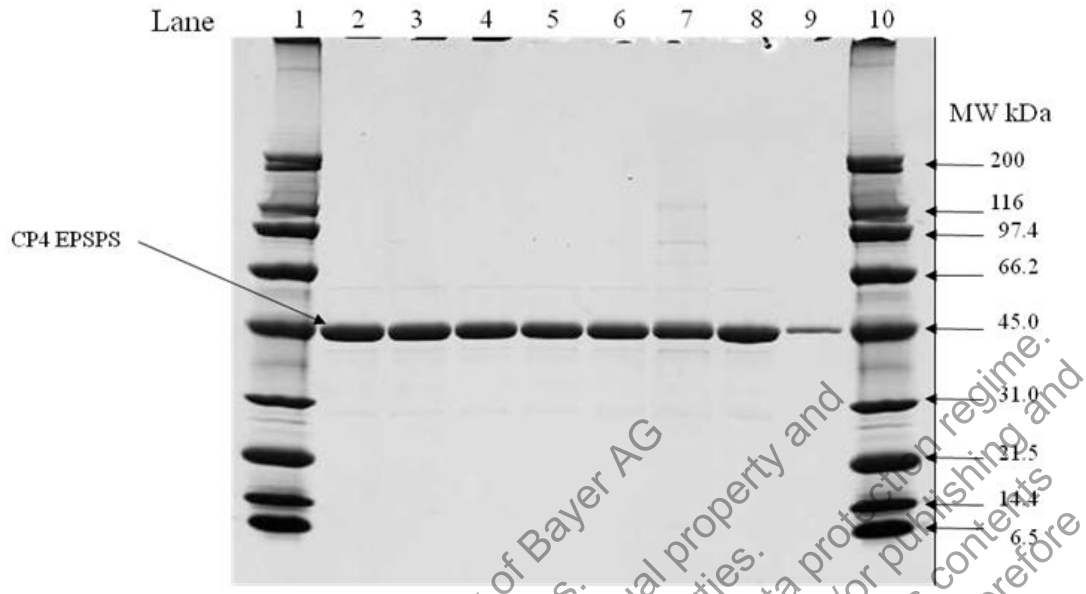


Figure VI-9. SDS-PAGE of CP4 EPSPS Protein Demonstrating the Effect of Elevated Temperatures on Protein Stability.
Heated samples of CP4 EPSPS protein (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular weights (kDa) are shown on the right and correspond to molecular weight markers in lanes 1 and 10.

Lane	Sample	Amount
1	Broad Range Molecular Weight Markers	4.5 µg
2	CP4 EPSPS protein 25 ºC	3.2 µg
3	CP4 EPSPS protein 37 ºC	3.2 µg
4	CP4 EPSPS protein 55 ºC	3.2 µg
5	CP4 EPSPS protein 75 ºC	3.2 µg
6	CP4 EPSPS protein 95 ºC	3.2 µg
7	CP4 EPSPS protein no-heat control	3.2 µg
8	CP4 EPSPS Reference	3.2 µg
9	CP4 EPSPS Reference	0.32 µg
10	Broad Range Molecular Weight Markers	4.5 µg

VI.F.2. Assessment for the Potential for Toxicity of the CP4 EPSPS Protein

VI.F.2.1. Structural Similarity of CP4 EPSPS Protein to Known Toxins

The assessment of the potential for protein toxicity includes bioinformatic analysis of the amino acid sequence of the introduced protein. The goal of the bioinformatic analysis is to ensure that the introduced protein does not share homology to known toxins or anti-nutritional proteins associated with adverse health effects.

Potential structural similarities shared between the CP4 EPSPS protein and sequences in a protein database were evaluated using the FASTA sequence alignment tool. The FASTA program directly compares amino acid sequences (i.e., primary, linear protein structure) and the alignment data may be used to infer shared higher order structural similarities between two sequences (i.e., secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire sequence are often homologous. Homologous proteins usually have common secondary structures, common three-dimensional configuration, and, consequently, may share similar functions.

FASTA bioinformatic alignment searches using the CP4 EPSPS amino acid sequence were performed with the toxin database to identify possible homology with proteins that may be harmful to human and animal health. The toxin database, TOX_2010, is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. The TOX_2010 database contains 8,448 sequences.

An *E*-score acceptance criteria of 1×10^{-5} or less for any alignment was used to identify proteins from the TOX_2010 database with potential for significant shared structural similarity and function with CP4 EPSPS protein. As described above, the *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of 1×10^{-5} or less to be considered to have sufficient sequence similarity to infer homology. The results of the search comparisons showed that no relevant alignments were observed against proteins in the TOX_2010 database. No FASTA alignment displayed an *E*-score of 1×10^{-5} or less.

The results of the bioinformatic analyses demonstrated that no structurally relevant similarity exists between the CP4 EPSPS protein and any known toxic or other biologically active proteins that would be harmful to human or animal health.

VI.F.2.2. Heat Stability and Digestability of the CP4 EPSPS Protein

The stability of a protein to heat or its degradation in simulated mammalian gastrointestinal fluids is a key consideration in the assessment of its potential toxicity. Exposure to heat during food processing or cooking, and to digestive fluids is likely to have a profound effect on the structure and function of proteins. The effect of heat treatment on the activity of CP4 EPSPS protein was evaluated using a functional assay to assess the impact of temperature on enzymatic activity, and using SDS-PAGE to assess

the impact of temperature on protein integrity. The results show that CP4 EPSPS protein was completely deactivated by heating at temperatures above 75ºC (Section VI.F.1.5.). The digestability of CP4 EPSPS protein was evaluated by incubation with simulated gastric fluid, and the results show that CP4 EPSPS protein was readily digested (Section VI.F.1.4.). Therefore, it is anticipated that exposure to functionally active CP4 EPSPS protein from the consumption of MON 87427 or foods derived from MON 87427 will be negligible.

VI.F.2.3. Acute Oral Toxicity Study with the CP4 EPSPS Protein

Most known protein toxins act through acute mechanisms to exert toxicity (Hammond and Fuchs, 1998; Pariza and Johnson, 2001; Sjoblad, et al., 1992). The primary exceptions to this rule consist of certain anti-nutritional proteins such as lectins and protease inhibitors, which manifest toxicity in a short-term (three-week) feeding study (Liener, 1994). The amino acid sequence of the CP4 EPSPS protein produced in MON 87427 is not similar to any of these anti-nutritional proteins or to any other known protein toxin. Therefore, an acute oral mouse toxicity study was considered sufficient to evaluate the potential toxicity of the CP4 EPSPS protein.

CP4 EPSPS protein was administered as a single dose by gavage to three groups of 10 male and 10 female CD-1 mice at dose levels up to 572 mg/kg body weight (bw) (Harrison et al., 1996). The CP4 EPSPS protein was produced by *E. coli* but shown to be physicochemically and functionally equivalent to the CP4 EPSPS protein produced in MON 87427. Additional groups of mice were administered comparable levels of either the buffer or bovine serum albumin (BSA) to serve as vehicle or protein controls. Following dosing, all mice were observed twice daily for mortality or signs of toxicity. Food consumption was measured daily. Body weights were measured prior to dosing and at study day 7. All animals were sacrificed on day 8 or 9 and subjected to a gross necropsy. There were no treatment-related effects on survival, clinical observations, body weight gain, food consumption or gross pathology. Therefore, the No Observable Adverse Effect Level (NOAEL) for CP4 EPSPS protein was considered to be 572 mg/kg bw, the highest dose tested.

VI.F.3. Dietary Risk Assessment of the CP4 EPSPS Protein

VI.F.3.1. Estimated Human Exposure to the CP4 EPSPS Protein from MON 87427

MON 87427 is intended for use in field maize and may also be used in vegetable maize and popcorn. Thus all three types of maize were used to estimate potential exposure to CP4 EPSPS proteins from MON 87427. Acute exposure estimates were conducted using the Dietary Exposure Evaluation Model (DEEM-FCID version 2.03, Exponent Inc.). DEEM-FCID utilizes U.S. food consumption data from the 1994-1996 and 1998 USDA Continuing Surveys of Food Intakes by Individuals (CSFII). Human exposures to CP4 EPSPS protein from MON 87427 in the U.S. were estimated using a reasonable worst case scenario of the 95th percentile of acute maize consumption on an "eater-only" basis.

DEEM-FCID separates field maize into six fractions: flour, meal, bran, starch, oil and syrup. However, maize oil and maize syrup were excluded from the assessment because they are essentially devoid of protein and would thus not contain significant amounts of CP4 EPSPS. Maize starch was included in the assessment but, because of the very low protein content, any contribution from maize starch is expected to be minimal. Field maize is a blended commodity that is used primarily as animal feed and is processed before being consumed by humans. Popcorn and some forms of vegetable maize (all except corn-on-the cob) are also blended commodities. Thus, except for corn-on-the-cob, most MON 87427 grain entering the human food supply would likely be blended with other grain before being processed and/or consumed. However, the exposure calculations herein make the conservative assumption that grain from MON 87427 is not blended with other grain varieties prior to consumption; i.e., for the purposes of this assessment, 100% of the maize-derived food products consumed were assumed to be derived from MON 87427. This is a very conservative assumption because MON 87427 will likely represent only a portion of the total maize consumed.

For the purposes of this assessment, the concentration of the CP4 EPSPS protein in flour, meal, bran, starch, vegetable maize and popcorn was assumed to be equal to the mean expression level in whole MON 87427 grain grown in the 2008 U.S. field trials, which was 3.6 µg/gram (ppm) fresh weight. These protein expression estimates are conservative because they assume that there is no loss of the CP4 EPSPS protein during storage, processing and/or cooking of the grain or food. Based on these assumptions, the 95th percentiles for acute dietary intake of CP4 EPSPS are estimated to be 8.23 x 10^{-3} and 18.53 x 10^{-3} mg/kg for the general population and children 1-6 years of age, respectively.

VI.F.3.1.1. Dietary Exposure Assessment: Margin of Exposure for the CP4 EPSPS Protein Derived from MON 87427

A common approach used to assess potential health risks from chemicals or other potentially toxic products is to calculate a Margin of Exposure (MOE) between the lowest NOAEL from an appropriate animal toxicity study and an estimate of human exposure. No adverse health effects were observed when male or female mice were administered a dose of 572 mg/kg bw of CP4 EPSPS protein (Harrison et al., 1996). Therefore, based on an apparent absence of hazard, a dietary risk assessment for this protein would normally not be considered necessary. Nevertheless, a dietary risk assessment for CP4 EPSPS protein was conducted to provide further safety assurance.

Potential health risks from the acute dietary intake of CP4 EPSPS protein from consumption of food derived from MON 87427 were evaluated by calculating the MOE based on the acute mouse NOAEL for CP4 EPSPS and the 95th percentile "eater-only" estimates of acute dietary exposure from DEEM-FCID. The MOEs for acute dietary intake of CP4 EPSPS protein were estimated to be 70,000 and 31,000 for the general population and children (1-6 years of age), respectively (Table VI-7). These very large MOEs indicate that there are no meaningful risks to human health from dietary exposure to CP4 EPSPS protein derived from MON 87427.

Table VI-7. Acute (95th Percentile, "eater-only") Dietary Intake and Margins of Exposure for MON 87427-produced CP4 EPSPS Protein from Consumption of MON 87427 Maize Grain-Derived Food Products in the U.S. [1]

Population	Protein Intake [2] (mg/kg/day x 10^{-3})	Margins of Exposure[3]
	CP4 EPSPS protein	CP4 EPSPS protein
General Population	8.23	70,000
Children (1-6 yrs)	18.53	31,000

[1]Estimated using DEEM-FCID version 2.03, Exponent Inc., utilizing food consumption data from the 1994-1996 and 1998 USDA Continuing Surveys of Food Intakes by Individuals (CSFII). Assumes 100% of maize products (excluding maize oil and syrup) consumed in the U.S. are derived from MON 87427.
[2]Based on average expression levels of 3.6 µg/gram fresh weight for grain of MON 87427 (Table VI-5).
[3]Calculated by dividing the NOAEL from the CP4 EPSPS acute mouse gavage study (572 mg/kg) by estimated dietary intake of MON 87427-produced CP4 EPSPS protein. MOEs were rounded to the nearest thousand.

VI.F.3.2. Estimated Animal Exposure to the CP4 EPSPS Protein from MON 87427

In the United States almost 43% of the maize crop produced from September 2008 to August 2009 went to feed animals (USDA-ERS, 2010). Maize is the primary grain fed to poultry, pigs, beef cattle, and lactating dairy cattle in the U.S. The daily consumption of maize grain for the young pig is 40 g/kg body weight (bwt)/day (assuming 60% dietary inclusion rate) and 24.6 g/kg bw/day for the finishing pig (assuming 80% dietary inclusion rate) (NRC, 1998). The four-week old broiler consumes 60 g/kg bw/day when the inclusion rate of maize grain is 65% of the diet (NRC, 1994). The lactating dairy cow (550 kg bw) producing 33 kg of milk per day consumes about 4.3 kg of maize grain per day and 10.4 kg of maize silage per day or 7.8 g/kg bw/day and 18.9 g/kg bw/day, respectively (Ouellet, et al., 2003).

VI.F.3.2.1. Animal Dietary Intake of CP4 EPSPS Protein

Animals will be exposed to the CP4 EPSPS protein through dietary intake of feed derived from MON 87427 maize The quantity of maize consumed on a daily basis by poultry and livestock, as well as the levels of CP4 EPSPS protein in MON 87427 are necessary to derive an estimate of daily dietary intake (DDI). Ruminants may consume both maize grain and forage. DDI is computed as follows:

$$\text{DDI} = \text{Daily maize consumption (g)} \times \text{CP4 EPSPS protein concentration } (\mu g/g)$$

The intake calculations make the conservative assumption that there is no loss of the CP4 EPSPS protein during the processing of maize grain or forage into animal feed. It also assumes that 100% of the maize grain or forage ending up in animal feed is derived from MON 87427, which could be the case for farmers that produced the maize that was

fed to their livestock. However, larger livestock operations purchase commodity maize that is a blend of many different hybrids.

The potential dietary intake of CP4 EPSPS protein from the consumption of MON 87427 can be estimated by multiplying the consumption of each commodity by the levels of the protein in that commodity.

For the purpose of this dietary intake calculation the highest expression of the CP4 EPSPS protein reported for MON 87427 grain and forage were used, which represents the highest exposure of animals to CP4 EPSPS protein expressed in MON 87427. The mean and maximum values of the CP4 EPSPS protein levels in grain used in this assessment were from maize hybrids containing MON 87427 grown in the U.S. (Table VI-8). The mean level of CP4 EPSPS protein in MON 87427 grain is 4.2 µg/g on a dry-weight (dwt) basis (range 2.8 – 6.2 µg/g dwt) and forage is 120 µg/g dwt (range 21 – 200 µg/g dwt) (Table VI-5). Maize silage contains about 45% grain and 55% forage on a dry matter basis (Schroeder, 2004), so maize silage would contain approximately 67.9 µg/g dwt of CP4 EPSPS protein ((4.2 µg/g dwt in maize grain × 45%) + (120 µg/g dwt maize in forage × 55%)) when using the mean level of CP4 EPSPS protein for the grain and forage or 120 µg/g dwt CP4 EPSPS ((6.2 µg/g dwt in maize grain × 45%) + (200 µg/g dwt maize in forage × 55%)) when using the high end of the range.

The estimated mean and maximum daily intake of the CP4 EPSPS protein by poultry and livestock are shown in Table VI-8. The broiler chicken, young pig, finishing pig, and lactating dairy cow would typically consume 18 g dietary protein/kg bw (NRC, 1994), 14 g dietary protein/kg bwt (NRC, 1998), 4 g dietary protein/kg bw (NRC, 1998), and 6 g dietary protein/kg bwt (NRC, 2001), respectively. Due to consumption of forage, which has the highest CP4 EPSPS protein expression levels, the highest percentage of CP4 EPSPS protein (g/kg bw) per total protein consumed was in the dairy cow, 0.036% (g/g) of the total dietary protein intake (0.00218 g CP4 EPSPS/kg bw divided by 6 g dietary protein, which is the total dietary protein intake for the cow). The chicken and pig percentages of the CP4 EPSPS protein consumed as part of the daily protein intake are much less than for the dairy cow.

Table VI-8. Mean and maximum daily intakes of the CP4 EPSPS protein in poultry and livestock (g/kg body wt/day)[1]

Species	Total Consumption of Maize (g/kg of body weight/day dwt[1])	CP4 EPSPS Protein Intake (g/kg of body weight/day dwt)	
		Mean	Highest Level
Chicken broiler[2]	60.0	0.00025	0.00037
Young pig[2]	40.0	0.00017	0.00025
Finishing pig[2]	24.6	0.00010	0.00015
Lactating dairy cow[3]	26.7	0.00132	0.00218

[1] dwt = dry weight

[2] Maize grain consumed × concentration of CP4 EPSPS protein in the grain.

[3] (Maize grain consumed × concentration of CP4 EPSPS protein in the grain) + (maize silage consumed × concentration of CP4 EPSPS protein in the maize silage).

VI.F.4. Potential Allergenicity or Toxicity of CP4 EPSPS Protein Produced in MON 87427 Summary and Conclusion

MON 87427 CP4 EPSPS protein possesses a strong safety profile. Its donor organism, *Agrobacterium* sp. strain CP4, is ubiquitous in the environment, is not known for human or animal pathogenicity, and is not commonly allergenic. Furthermore, *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding other approved Roundup Ready crops. MON 87427 CP4 EPSPS protein is present at a very low level in the harvested grain of MON 87427, and therefore, constitutes a very small portion of the total protein present in food and feed derived from MON 87427. MON 87427 CP4 EPSPS protein lacks structural similarity to known allergens or toxins known to have adverse effects on mammals. MON 87427 CP4 EPSPS protein was rapidly digested in SGF and SIF. MON 87427 CP4 EPSPS protein lost activity upon heating and demonstrated no oral toxicity in mice at the level tested. In addition, the overall animal and human exposure as a percent of total protein is small and large MOEs have been demonstrated for the consumption of CP4 EPSPS protein derived from MON 87427 for the U.S. general population and for non-nursing infants, the highest exposed sub-population.

Based on the above information, the consumption of CP4 EPSPS protein from MON 87427 grain or products derived from MON 87427 is considered safe for humans and animals.

VI.G. Bioinformatic Assessment of Putative Open Reading Frames (ORFs) of MON 87427 Insert and Flanking Sequences

The 2003 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2003b) includes an assessment

element on the identification and evaluation of "open reading frames within the inserted DNA or created by the insertion with contiguous plant genomic DNA". These assessments examine the potential homology of any putative polypeptides or proteins that could be produced from open reading frames (ORFs) in the insert or at the plant-insert junction to known toxins or allergens. These analyses are conducted even if there is no evidence that such ORFs at the plant-insert junction or alternative reading frames in the insert are capable of being transcribed or translated into a protein. Results from these bioinformatics analyses demonstrate that any putative polypeptides in MON 87427 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

In addition to the bioinformatic analysis conducted on MON 87427 CP4 EPSPS (see Sections VI.F.1.3 and VI.F.2.1) bioinformatic analyses were also performed on the MON 87427 insert and flanking genomic DNA sequences to assess the potential for allergenicity, toxicity, or biological activity of putative polypeptides encoded by all six reading frames present in the MON 87427 insert DNA as well as ORFs present in the 5' and 3' inserted DNA-5' and 3' flanking sequence junctions. These various bioinformatic evaluations are depicted in Figure VI-10. ORFs spanning the 5' flanking sequence DNA-inserted DNA junctions, and 3' flanking sequence DNA-inserted DNA junctions were translated from stop codon to stop codon in all six reading frames (three forward reading frames and three reading frames in reverse complement orientation). Putative peptides/polypeptides from each reading frame were then compared to toxin, allergen and all proteins databases using bioinformatic tools. Similarly, the entire MON 87427 insert DNA sequence was translated in all six reading frames (three forward reading frames and three reading frames in reverse complement orientation) and the resulting amino acid sequence was subjected to bioinformatic analyses. There are no analytical data that indicate any putative polypeptides/proteins subjected to bioinformatic evaluation other than the MON 87427 CP4 EPSPS which is part of the insert DNA sequence analysis are produced. Moreover, the data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than MON 87427 CP4 EPSPS was derived from frames 1 to 6 of the insert DNA, or the ORFs spanning the insert junctions, they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Therefore, there is no evidence for concern regarding the putative polypeptides for MON 87427 relatedness to known toxins, allergens, or biologically active putative peptides.

VI.G.1. Bioinformatics Assessment of Insert DNA Reading Frames

Bioinformatic analyses were performed to assess the potential of toxicity, allergenicity or biological activity of any putative peptides encoded by translation of reading frames 1 through 6 of the inserted DNA in MON 87427 (Figure VI-10).

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and any protein sequences in the AD_2010, TOX_2010, and PRT_2010 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent

identity and alignment length as 35% or greater identity in 80 or greater amino acids (to ascertain if alignments exceeded Codex (Codex Alimentarius, 2003b) thresholds for FASTA searches of the AD_2010 database), and the *E*-score. Alignments having *E*-score less than 1×10^{-5} are deemed significant because they may reflect shared structure and function among sequences (Ladics, et al., 2007). In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope (Silvanovich et al., 2006) and evaluated against the AD_2010 database.

Using the FASTA algorithm to search the AD_2010 database, frame 2 showed an alignment with glutenin that resulted in a significant *E*-score of 8.2e-06. Inspection of this alignment revealed that it was punctuated with a stop codon and contained several gaps. As a result, it is unlikely that this alignment reflects conserved structure or function. No alignments with the other five query sequences and the AD_2010 database generated an *E*-score of less than or equal to 1e-5. The results of the search comparisons also showed that no relevant structural similarity to toxins were observed for any of the putative polypeptides when compared to proteins in the toxin (TOX_2010) database. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database.

When used to search the PRT_2010 database, translations of all frames yielded alignments with *E*-scores less than or equal to a 1×10^{-5} threshold. Inspections of frame 1, 2, 4, 5 and 6 alignments revealed that they were punctuated with numerous stop codons in the query sequence and required numerous gaps to optimize the alignment. As a result, it is unlikely these alignments reflect conserved structure. When used as a query in a FASTA search of the PRT_2010 database, the translation of frame 3 yielded numerous alignments with *E*-scores less than or equal to the 1×10^{-5} threshold. The top alignment yielding the most significant *E*-score, 7.9×10^{-179}, displayed 88.7% identity over 531 amino acids with 5-enol-pyruvylshikimate-3-phosphate synthase. The next two high scoring alignments displayed an *E*-score of 4.4×10^{-176} reflecting 100% identity over 455 amino acids with 5-enolpyruvylshikimate-3-phosphate synthase These frame 3 alignments positively identify MON 87427 CP4 EPSPS and are consistent with the known structure of protein coding sequence contained in the MON 87427 inserted DNA.

Taken together, these data demonstrate the lack of relevant similarities between known allergens or toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of MON 87427. As a result, in the unlikely event that a translation product other than MON 87427 CP4 EPSPS was derived from reading frames 1 to 6, these putative polypeptides are not expected to be cross-reactive allergens, toxins, or display adverse biological activity.

VI.G.2. Insert Junction Open Reading Frame Bioinformatics Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the MON 87427 inserted DNA were performed using a bioinformatic

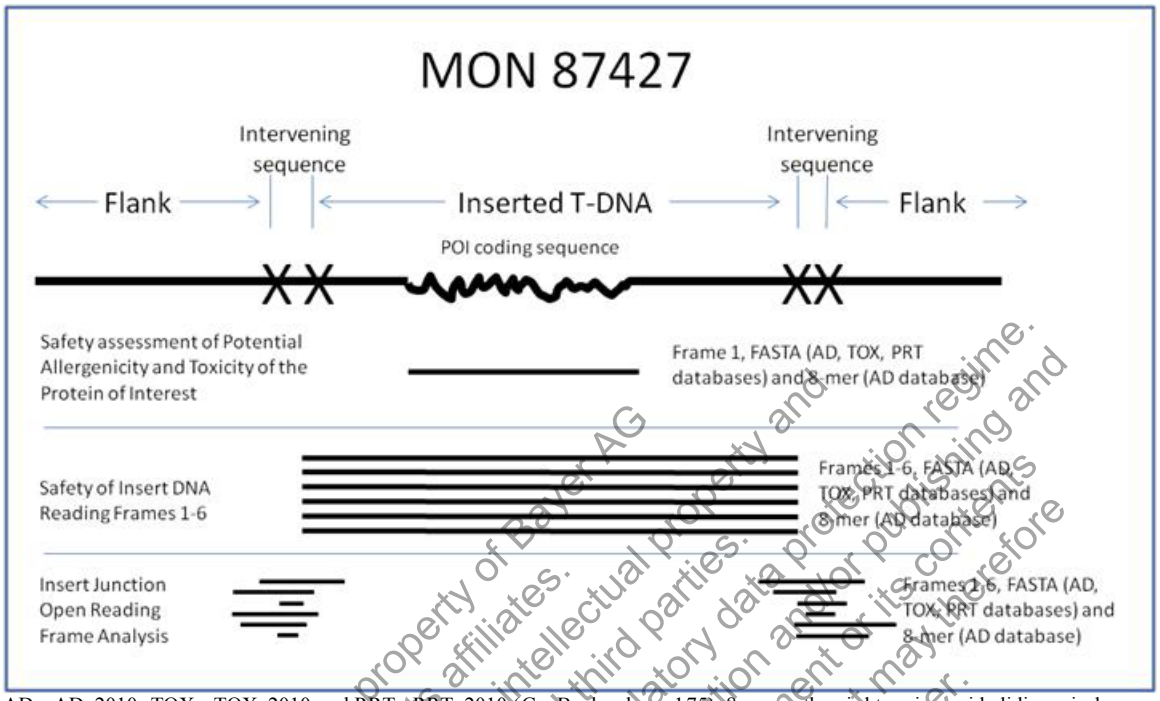
comparison strategy (Figure VI-10). The purpose of the assessment is to evaluate the potential for novel open reading frames (ORFs) that may have homology to known allergens, toxins, or proteins that display adverse biological activity. Sequences spanning the 5' flanking sequence DNA-inserted DNA and the inserted DNA-3' flanking sequence DNA (Figure VI-10) were translated from stop codon (TGA, TAG, TAA) to stop codon in all six reading frames. The resulting putative polypeptides from each reading frame, that were eight amino acids or greater in length, were compared to AD_2010, TOX_2010, and PRT_2010 databases using FASTA and to the AD_2010 database using an eight amino acid sliding window search.

The FASTA sequence alignment tool was used to assess structural relatedness between the query sequences and protein sequences in the AD_2010, TOX_2010, and PRT_2010 databases. Structural similarities shared between each putative polypeptide with each sequence in the database were examined. The extent of structural relatedness was evaluated by detailed visual inspection of the alignment, the calculated percent identity and the alignment length as 35% or greater identity in 80 or greater amino acids (to ascertain if alignments exceeded CODEX (Codex Alimentarius, 2003b) thresholds for FASTA searches of the AD_2010 database), and the *E*-score. In addition to structural similarity, each putative polypeptide was screened for short polypeptide matches using a pair-wise comparison algorithm. In these analyses, eight contiguous and identical amino acids were defined as immunologically relevant, where eight represents the typical minimum sequence length likely to represent an immunological epitope, and evaluated against the AD_2010 database.

No biologically relevant structural similarity to known allergens or toxins was observed for any of the putative polypeptides. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. As a result, in the unlikely event that a translation product was derived from DNA spanning the 5' or 3' genomic DNA-insert DNA junctions of MON 87427, these putative polypeptides are not expected to be cross-reactive allergens, toxins, or display adverse biological activity.

VI.G.3. Bioinformatic Assessment of Allergenicity, Toxicity, and Adverse Biological Activity Potential of MON 87427 Polypeptides Putatively Encoded by the Insert and Flanking Sequences Summary and Conclusions

A conservative bioinformatic assessment of potential allergenicity, toxicity and adverse biological activity for putative polypeptides that span the 5' and 3' insert junctions or were derived from different reading frames of the entire insert was conducted for MON 87427. The data generated from these analyses confirm that even in the highly unlikely occurrence that a translation product other than MON 87427 CP4 EPSPS derived from frames 1 to 6 of the insert DNA, or the insert junctions, they would not share a sufficient degree of sequence similarity with other proteins to indicate they would be potentially allergenic, toxic, or have other safety implications. Furthermore, no short (eight amino acid) polypeptide matches were shared between any of the putative polypeptides and proteins in the allergen database. Therefore, there is no evidence for concern regarding health implications of putative polypeptides for MON 87427.



AD= AD_2010; TOX= TOX_2010 and PRT= PRT_2010 (GenBank release 175); 8-mer = the eight amino acid sliding window search

Figure VI-10. Schematic Summary of MON 87427 Bioinformatic Analyses

VI.H. Safety Assessment of Expressed Products Summary and Conclusion

The data and information provided in this section address the questions important to the assessment of allergenic potential of the CP4 EPSPS protein and the potential allergenicity and toxicity of putative polypeptides potentially encoded by the insert and flanking sequences.

To summarize, there are no reports of allergies to the CP4 EPSPS donor organism, *Agrobacterium* sp.; thus, the CP4 EPSPS protein is not from a known allergenic source. The CP4 EPSPS protein represents only approximately 0.004% of the total protein in maize grain of MON 87427. Since the CP4 EPSPS protein represents only a small portion of the total protein in grain of MON 87427, it is not expected to be an allergenic protein. The updated bioinformatic analysis confirmed that the CP4 EPSPS protein did not share significant amino acid sequence similarities with known allergens glutenins, or gliadins. Therefore, it is unlikely that CP4 EPSPS contains allergenic epitopes. In addition, analyses using *E. coli*-produced CP4 EPSPS protein demonstrated that it was rapidly digested in simulated digestive fluids, a characteristic shared among proteins with a history of safe consumption. As the CP4 EPSPS protein equivalence from MON 87427- and *E. coli*-derived sources has been established, the digestibility of the MON 87427-produced CP4 EPSPS can be inferred. Heat stability studies demonstrated that the CP4 EPSPS protein is not thermostable. In addition, bioinformatics analyses demonstrate the lack of relevant similarities between known toxins and CP4 EPSPS protein and between known allergens and toxins and all putative peptides derived from all six reading frames from the entire inserted DNA sequence of MON 87427. Taken together, the updated assessment on allergenic potential reaffirms the earlier conclusion that the CP4 EPSPS protein expressed in MON 87427, as in other previously deregulatated Roundup Ready crops, does not pose a significant allergenic risk. Finally, in the unlikely event that translation products other than the CP4 EPSPS protein were to be produced, they pose no allergenic or toxic risk.

VII. COMPOSITIONAL ASSESSMENT OF MON 87427

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by FDA. The CP4 EPSPS protein expressed in MON 87427 is identical to the CP4 EPSPS protein in other Roundup Ready crops and the mode of action of CP4 EPSPS protein is well understood. Previous Roundup Ready crops reviewed by the FDA have had no biologically relevant compositional changes identified, and there is no reason to expect the CP4 EPSPS protein in MON 87427 to interact with endogenous metabolites or important nutrients that are present in maize grain or forage.

Safety assessments of biotechnology-derived crops typically include comparisons of the composition of forage and grain of the GE crop to that of conventional counterparts (Codex Alimentarius, 2003b). Compositional assessments were performed using the principles and analytes outlined in the OECD consensus document for maize composition (OECD, 2002).

A recent review of compositional assessments conducted according to OECD guidelines that encompassed a total of seven GE crops, nine countries and 11 growing seasons concluded that incorporation of biotechnology-derived agronomic traits has had little impact on natural variation in crop composition; most compositional variation is attributable to growing region, agronomic practices, and genetic background (Harrigan, et al., 2010). Compositional quality therefore implies a very broad range of endogenous levels of individual constituents. Numerous scientific publications have further documented the extensive variability in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites that reflects the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan, et al., 2007; OECD, 2002; Reynolds, et al., 2005; Ridley, et al., 2004).

Compositional equivalence between biotechnology-derived and conventional crops provides an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 2002). The OECD consensus documents emphasize quantitative measurements of essential nutrients, known anti-nutrients and secondary metabolites. This is based on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential safety and anti-nutritional concerns. Levels of the components in forage and grain of the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, grown concurrently, under field conditions, and 2) natural ranges generated from an evaluation of commercial references grown concurrently and from data published in the scientific literature.

The comparison to data published in the literature places any potential differences between the assessed crop and its comparator in the context of the natural variation in the concentrations of crop nutrients, anti-nutrients, and secondary metabolites.

This section provides analyses of concentrations of key nutrients, anti-nutrients, and secondary metabolites of MON 87427 compared with equivalent analyses of a

conventional counterpart grown and harvested under the same conditions. The production of materials for the compositional analyses used field designs (randomized complete block with three replicates) to allow accurate assessments of compositional characteristics over a range on environmental conditions under which MON 87427 is expected to be grown.

The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses.

VII.A. Compositional Equivalence of MON 87427 Forage and Grain to Conventional Maize

Compositional analysis of MON 87427 and comparison to the conventional control (LH198 × LH287) and commercial references demonstrated that MON 87427 is compositionally equivalent to conventional maize. Forage and grain samples were collected from MON 87427 and the conventional control from a 2008 U.S. field production. The background genetics of the conventional control were similar to that of MON 87427, but it did not contain the *cp4 epsps* expression cassette. Four different commercial references were included at each site of the field production to provide data on natural variability of each compositional component analyzed. The samples utilized for compositional analysis were obtained from three sites: Jefferson County, Iowa, Stark County, Illinois, and Jackson County, Arkansas. The sites were planted in a randomized complete block design with three blocks per site. MON 87427, the conventional control, and commercial references were treated with conventional weed control programs. In addition, MON 87427 plots were treated with glyphosate herbicide at a target rate of 1.0 lb ai/acre (1.13 kg ai/ha).

Compositional analyses were conducted to assess whether levels of key nutrients, anti-nutrients, and secondary metabolites in MON 87427 were different from the levels in the conventional control and to the composition of commercial references. A description of nutrients, anti-nutrients, and secondary metabolites present in maize is provided in the OECD consensus document on compositional considerations for maize (OECD, 2002). Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and vitamins [folic acid, niacin, A (β-carotene), B_1, B_2, B_6, and E] in the grain, and proximates, ADF, NDF, calcium and phosphorus in forage. The anti-nutrients assessed in grain included phytic acid and raffinose. Secondary metabolites assessed in grain included furfural, ferulic acid, and p-coumaric acid. In all, 78 different analytical components were measured (9 in forage, 69 in grain). Of these, 16 components (15 nutrients and one anti-nutrient) in grain had more than 50% of the observations below the assay limit of quantitation (LOQ) and, as a result, were excluded from the statistical analysis. Therefore, 62 components were statistically assessed using a mixed model analysis of variance method. Values for all components were expressed on a dry weight basis with the exception of moisture, which was expressed as percent fresh weight and fatty acids, which were expressed as percent of total fatty acids.

For MON 87427, four statistical comparisons to the conventional control were conducted for each component. One comparison was based on compositional data combined across all three field sites (combined-site analysis) and three separate comparisons were conducted on data from each of the individual field sites. Statistically significant differences were identified at a 5% level of significance (α=0.05). Data from the commercial references were combined across all sites and used to calculate a 99% tolerance interval for each compositional component to define the natural variability of each component in maize hybrids that have a history of safe consumption and that were grown concurrently with MON 87427 and the conventional control.

For the combined-site analysis, significant differences in nutrient, anti-nutrient, and secondary metabolite components were further evaluated using considerations relevant to the safety and nutritional quality of MON 87427 when compared to the conventional counterpart with a history of safe consumption: 1) the relative magnitude of the differences in the mean values of nutrient, anti-nutrient, and secondary metabolite components of MON 87427 and the conventional control, 2) whether the MON 87427 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of commercial references grown concurrently, 3) evaluation of the reproducibility of the significant (α=0.05) combined-site component differences at individual sites, and 4) assessing the difference within the context of natural variability of commercial maize composition published in the scientific literature and in the International Life Sciences Institute (ILSI) Crop Composition Database

This analysis provides a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients, and secondary metabolites in grain and of key nutrients in forage of MON 87427 and the conventional control, discussed in the context of natural variability of commercial maize. Results of the comparison indicate that the composition of the forage and grain of MON 87427 is compositionally equivalent to conventional maize with a demonstrated history of safe use.

VII.A.1. Nutrient Levels in Maize Grain

Grain was analyzed for 64 compositional nutrients including: protein, moisture, fat, ash, carbohydrates, ADF, NDF, TDF, amino acids (18), fatty acids (22), vitamins [A (β-carotene), B_1, B_2, B_6, E, niacin, folic acid], and minerals (9). Fifteen nutrients were below the limit of quantitation. In the combined-site analysis of grain, no significant differences were observed between MON 87427 and the conventional control for 43 nutrients. Significant differences included mean values for 16:0 palmitic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 20:0 arachidic acid, and total fat (Tables VII-1 and VII-2).

The significant differences in nutrients were evaluated using considerations relevant to the nutritional quality of MON 87427 when compared to the conventional control:

1) All nutrient component differences observed in the combined-site analysis, whether reflecting increased or decreased MON 87427 mean values with respect to the

conventional control, were small. Relative magnitudes of differences (mean difference as % of control) ranged from 1.96% to 5.09%.

2) MON 87427 mean values for these nutrient components were within the 99% tolerance interval established from the commercial references grown concurrently. Therefore, the MON 87427 mean values were within the range of natural variability of commercial maize hybrids with a history of safe consumption (Tables VII-1 and VII-2).

3) Assessment of reproducibility for the combined-site significant differences at the three individual sites demonstrated significant differences (α=0.05) for 18:0 stearic acid and 20:0 arachidic acid at one individual site and significant differences for 16:0 palmitic acid, 18:1 oleic acid, and 18:2 linoleic acid at all three sites. No significant difference was observed for total fat at any of the individual sites. Individual site mean values of MON 87427 for all nutrient components with significant differences fell within the 99% tolerance interval established from the commercial references grown concurrently and were, therefore, within the range of natural variability of that component in commercial maize hybrids with a history of safe consumption.

4) All of the compositional components identified as significantly different from the conventional control were within the natural variability of these components in commercial maize composition as published in the scientific literature and available in the ILSI Crop Composition Database (ILSI, 2009); (Table VII-6).

The six combined-site significant differences (α=0.05) between MON 87427 and the conventional control were attributable to five fatty acids (all expressed as percent total FA) and total fat. The relative magnitude of differences between the mean values for MON 87427 and conventional control were small in the combined-site analysis for 16:0 palmitic acid (3.52% increase), 18:0 stearic acid (3.67% increase), 18:1 oleic acid, (3.22% increase), 18:2 linoleic acid (1.96% decrease), 20:0 arachidic acid (4.00% increase) and total fat (5.09% decrease) and at the three individual sites (all were approximately 5% or less) (Tables VII-2, H-3, H-7, and H-11). The observed significant differences between MON 87427 and conventional control for 16:0 palmitic acid, 18:1 oleic acid, 18:2 linoleic acid 18:0 stearic acid, 20:0 arachidic acid, and total fat are markedly less than differences in hybrids developed through conventional breeding (Harrigan, et al., 2009; Reynolds et al., 2005). Harrigan et al. (2009) and the ILSI Crop Composition Database (ILSI, 2009) highlight the extensive natural variability in compositional component levels in maize, as presented in Table VII-6. All of the compositional components identified as significantly different from the conventional control were within the natural variability of these components in maize based upon published literature data and the ILSI-CCD (Table VII-6). Therefore, these significant differences are not meaningful to food and feed safety and nutrition.

In summary, the statistical analysis identified six significant differences that were all small in magnitude. Of these significant differences, only 16:0 palmitic acid, 18:1 oleic acid, and 18:2 linoleic acid were observed as consistently at all of the individual sites. All of the components identified as significantly different were within the natural variability of commercial maize defined by the 99% tolerance interval and published

literature ranges. These findings support the conclusion that with regard to nutrients in grain, MON 87427 is compositionally equivalent to conventional maize.

VII.A.2. Anti-Nutrient Levels in Maize Grain

Maize grain contains two main anti-nutrients according to OECD (OECD, 2002), phytic acid and raffinose. Phytic acid is present in maize grain, where it chelates mineral nutrients, including calcium, magnesium, potassium, iron, and zinc, rendering them biologically unavailable to mono-gastric animals consuming the grain (Liener, 2000). Raffinose is a low molecular weight non-digestible carbohydrate present in maize grain that is considered to be an anti-nutrient due to the gas production and resulting flatulence caused by consumption (Liener, 2000).

In the combined-site analysis, a statistically significant difference (α=0.05) between MON 87427 and conventional control (Tables VII-1 and VII-3) was identified for phytic acid. No significant difference was observed for raffinose.

1) The phytic acid component difference observed in the combined-site analysis was small in relative magnitude, a decrease of 5.92% in MON 87427 with respect to the conventional control.

2) The MON 87427 mean phytic acid value from the combined-site analysis was within the 99% tolerance interval established from the commercial references grown concurrently and was therefore within the range of natural variability of this component in commercial maize hybrids with a history of safe consumption (Tables VII-1 and VII-3)

3) No significant differences for phytic acid were observed at any of the individual sites. Mean values for phytic acid in MON 87427 at the individual sites were within the 99% tolerance interval established from the commercial references.

4) The difference in phytic acid was also within the range of the natural variability of commercial maize composition as published in the scientific literature and available in the ILSI Crop Composition Database (ILSI, 2009).

In summary, the statistical analyses found a significant difference in phytic acid that was small in magnitude and not consistently observed at all of the individual sites. The mean phytic acid values for MON 87427 were within the natural variability of commercial maize defined by the 99% tolerance interval and published literature ranges. Thus, an evaluation of anti-nutrient components in grain supports the conclusion that MON 87427 is compositionally equivalent to conventional maize.

VII.A.3. Secondary Metabolites in Maize Grain

Maize grain contains three main secondary metabolites according to OECD, furfural, ferulic acid, and p-coumaric acid (OECD, 2002). The non-starch polysaccharide pentosans are a major source of furfural (Adams, et al., 1997). Ferulic acid and p-coumaric acid are derived from the aromatic amino acids, phenylalanine and tyrosine (Buchanan, et al., 2000), and serve as precursors for a large group of phenylpropanoid

compounds. There were no combined-site significant differences (α=0.05) observed in secondary metabolites when the grain mean values from MON 87427 were compared to the conventional control and furfural was not detected in MON 87427, the conventional control, or commercial references. Thus, an evaluation of secondary metabolite components in grain supports the conclusion that MON 87427 is compositionally equivalent to conventional maize.

VII.A.4. Nutrient Levels in Maize Forage

Maize forage was analyzed for nine compositional nutrients (protein, moisture, fat, ash, carbohydrates, ADF, NDF, calcium, and phosphorus). There were no combined-site significant differences (α=0.05) observed when the forage mean values from MON 87427 were compared to the conventional control. Thus, an evaluation of nutrient components in forage supports the conclusion that MON 87427 is compositionally equivalent to conventional maize.

Table VII-1. Summary of Differences (α=0.05) for the Comparison of Maize Component Levels for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87427 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87427 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Grain Proximate (% dw)						
Total Fat	3.50	3.69	-5.09	0.036	3.13 - 3.83	2.12, 5.35
Grain Fatty Acid (% Total FA)						
16:0 Palmitic	10.91	10.54	3.52	<0.001	10.44 - 11.52	6.42, 15.23
18:0 Stearic	1.97	1.90	3.67	0.038	1.81 - 2.17	0.87, 2.88
18:1 Oleic	24.28	23.52	3.22	0.010	22.84 - 26.62	11.30, 43.27
18:2 Linoleic	60.84	62.06	-1.96	0.002	57.61 - 62.70	41.35, 74.78
20:0 Arachidic	0.42	0.41	4.00	0.005	0.37 - 0.48	0.15, 0.67
Grain Anti-nutrient (% dw)						
Phytic Acid	0.96	1.02	-5.92	0.008	0.87 - 1.04	0.73, 1.23

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Maize Component Levels for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87427 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87427 minus Control) Significance (p-Value)	MON 87427 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Grain Fatty Acid (% total FA)						
16:0 Palmitic Site ARNE	11.49	10.99	4.53	<0.001	11.47 - 11.52	6.42, 15.23
16:0 Palmitic Site IARL	10.72	10.44	2.66	0.007	10.58 - 10.85	6.42, 15.23
16:0 Palmitic Site ILWY	10.54	10.21	3.25	<0.001	10.44 - 10.65	6.42, 15.23
18:1 Oleic Site ARNE	26.34	25.35	3.93	<0.001	26.16 - 26.62	11.30, 43.27
18:1 Oleic Site IARL	22.91	21.95	4.41	0.002	22.84 - 22.98	11.30, 43.27
18:1 Oleic Site ILWY	23.58	23.24	1.44	0.043	23.29 - 23.78	11.30, 43.27
18:2 Linoleic Site ARNE	57.94	59.56	-2.72	<0.001	57.61 - 58.13	41.35, 74.78
18:2 Linoleic Site IARL	62.57	63.90	-2.09	<0.001	62.49 - 62.70	41.35, 74.78

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Maize Component Levels for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87427 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87427 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Grain Fatty Acid (% total FA)						
18:2 Linoleic Site ILWY	62.01	62.72	-1.13	0.005	61.68 - 62.32	41.35, 74.78
Grain Amino Acid (% dw)						
Methionine Site ARNE	0.29	0.27	6.48	0.043	0.28 - 0.29	0.11, 0.29
Methionine Site IARL	0.23	0.25	-7.29	0.018	0.22 - 0.23	0.11, 0.29
Grain Fatty Acid (% total FA)						
18:3 Linolenic Site ARNE	1.15	1.19	-3.92	0.033	1.13 - 1.17	0.78, 1.52
18:3 Linolenic Site IARL	1.24	1.20	3.35	0.014	1.22 - 1.26	0.78, 1.52
Grain Vitamin (mg/kg dw)						
Vitamin B2 Site ARNE	3.27	2.36	38.30	0.004	3.05 - 3.56	0, 4.47
Vitamin B2 Site IARL	1.41	1.93	-26.71	0.042	1.17 - 1.60	0, 4.47

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Maize Component Levels for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87427 minus Control) Mean Difference (% of Control)	Mean Difference (MON 87427 minus Control) Significance (p-Value)	MON 87427 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Individual Site						
Grain Proximate (% dw)						
Carbohydrates Site IARL	84.24	83.11	1.36	0.047	83.60 - 84.96	80.77, 89.46
Moisture (% fw) Site IARL	10.93	10.40	5.13	0.043	10.90 - 11.00	7.56, 14.80
Protein Site IARL	10.60	11.73	-9.64	0.019	9.91 - 11.35	5.79, 13.43
Grain Fiber (% dw)						
Acid Detergent Fiber Site ILWY	3.78	3.05	23.75	0.020	3.33 - 4.27	1.84, 4.39
Grain Amino Acid (% dw)						
Arginine Site IARL	0.48	0.53	-9.19	0.033	0.45 - 0.49	0.24, 0.68
Cystine Site IARL	0.24	0.26	-5.95	0.012	0.24 - 0.25	0.14, 0.30
Serine Site IARL	0.49	0.56	-11.21	0.037	0.46 - 0.51	0.24, 0.66

Table VII-1 (continued) . Summary of Differences (α=0.05) for the Comparison of Maize Component Levels for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87427 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87427 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Individual Site						
Grain Amino Acid (% dw)						
Tryptophan Site ARNE	0.062	0.052	19.32	0.006	0.059 - 0.064	0.032, 0.069
Grain Fatty Acid (% total FA)						
18:0 Stearic Site ARNE	2.17	2.04	6.43	0.002	2.16 - 2.17	0.87, 2.88
20:0 Arachidic Site ARNE	0.48	0.46	4.63	0.002	0.47 - 0.48	0.15, 0.67
22:0 Behenic Site ARNE	0.21	0.19	11.00	0.007	0.21 - 0.23	0, 0.32
Grain Mineral						
Calcium (% dw) Site ARNE	0.0077	0.0067	14.03	0.024	0.0075 - 0.0079	0.0019, 0.0076
Zinc (mg/kg dw) Site IARL	23.54	26.51	-11.20	0.010	22.45 - 24.61	11.46, 30.37
Grain Vitamin (mg/kg dw)						
Folic Acid Site IARL	0.36	0.45	-19.59	0.020	0.31 - 0.40	0.11, 0.61

Table VII-1 (continued). Summary of Differences (α=0.05) for the Comparison of Maize Component Levels for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean[3]	Control[4] Mean	Mean Difference (MON 87427 minus Control) Mean Difference (% of Control)	Significance (p-Value)	MON 87427 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Individual Site						
Grain Anti-nutrient (% dw)						
Raffinose Site ARNE	0.11	0.13	-18.51	0.031	0.11 - 0.11	0.024, 0.29
Forage Proximate (% dw)						
Carbohydrates Site IARL	86.46	84.12	2.78	0.029	86.21 - 86.75	80.13, 94.05
Moisture (% fw) Site IARL	69.90	74.71	-6.44	0.008	67.70 - 71.20	51.70, 86.22
Protein Site IARL	7.03	8.63	-18.59	0.037	6.75 - 7.40	1.34, 11.57

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 87427 treated with glyphosate.
[3]Mean = least-square mean.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table VII-2. Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	1.58 (0.036) (1.43 - 1.81)	1.56 (0.038) (1.48 - 1.67)	0.013 (0.042) (-0.14 - 0.14)	-0.074, 0.099	0.765	1.13, 1.97 (1.18 - 1.82)
Carbohydrates	84.88 (0.56) (83.60 - 86.33)	84.51 (0.57) (82.96 - 85.76)	0.37 (0.33) (-0.87 - 1.63)	-0.40, 1.14	0.305	80.77, 89.46 (82.26 - 87.17)
Moisture (% fw)	11.62 (0.46) (10.90 - 13.30)	11.41 (0.46) (10.20 - 12.40)	0.22 (0.21) (-0.30 - 1.10)	-0.27, 0.71	0.337	7.56, 14.80 (9.31 - 12.70)
Protein	10.05 (0.63) (8.46 - 11.35)	10.26 (0.63) (8.62 - 11.92)	-0.21 (0.38) (-1.50 - 1.20)	-1.08, 0.66	0.594	5.79, 13.43 (8.07 - 12.13)
Total Fat	3.50 (0.13) (3.13 - 3.83)	3.69 (0.13) (3.47 - 3.98)	-0.19 (0.075) (-0.52 - 0.11)	-0.36, -0.015	0.036	2.12, 5.35 (2.90 - 4.30)
Fiber (% dw)						
Acid Detergent Fiber	3.37 (0.23) (2.67 - 4.27)	3.19 (0.23) (2.80 - 3.54)	0.18 (0.27) (-0.27 - 1.09)	-0.43, 0.79	0.521	1.84, 4.39 (2.29 - 4.27)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	10.00 (0.51) (9.17 - 10.97)	10.12 (0.51) (9.21 - 11.27)	-0.12 (0.24) (-0.90 - 0.98)	-0.68, 0.43	0.628	5.69, 11.81 (7.06 - 10.66)
Total Dietary Fiber	13.00 (0.37) (12.13 - 14.35)	13.05 (0.37) (12.64 - 13.75)	-0.044 (0.24) (-0.67 - 1.07)	-0.53, 0.44	0.854	8.67, 15.32 (10.25 - 14.30)
Amino Acid (% dw)						
Alanine	0.75 (0.061) (0.61 - 0.89)	0.76 (0.061) (0.55 - 0.90)	-0.0061 (0.033) (-0.15 - 0.080)	-0.082, 0.069	0.857	0.32, 1.12 (0.58 - 0.98)
Arginine	0.48 (0.024) (0.40 - 0.55)	0.49 (0.025) (0.39 - 0.56)	-0.010 (0.015) (-0.079 - 0.065)	-0.040, 0.020	0.501	0.24, 0.68 (0.34 - 0.57)
Aspartic Acid	0.64 (0.041) (0.54 - 0.71)	0.64 (0.042) (0.48 - 0.73)	-0.0025 (0.025) (-0.099 - 0.064)	-0.059, 0.054	0.920	0.34, 0.92 (0.52 - 0.78)
Cystine	0.24 (0.010) (0.21 - 0.27)	0.24 (0.010) (0.21 - 0.26)	-0.0022 (0.0068) (-0.015 - 0.020)	-0.018, 0.013	0.750	0.14, 0.30 (0.18 - 0.26)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Glutamic Acid	1.87 (0.15) (1.53 - 2.24)	1.89 (0.15) (1.38 - 2.28)	-0.020 (0.077) (-0.35 - 0.20)	-0.20, 0.16	0.801	0.77, 2.84 (1.46 - 2.49)
Glycine	0.38 (0.018) (0.34 - 0.43)	0.38 (0.018) (0.31 - 0.42)	0.0012 (0.0098) (-0.038 - 0.033)	-0.021, 0.024	0.906	0.23, 0.52 (0.32 - 0.43)
Histidine	0.30 (0.013) (0.27 - 0.34)	0.30 (0.013) (0.23 - 0.34)	-0.0014 (0.0081) (-0.045 - 0.033)	-0.018, 0.015	0.867	0.16, 0.39 (0.22 - 0.33)
Isoleucine	0.35 (0.026) (0.29 - 0.42)	0.36 (0.027) (0.26 - 0.42)	-0.0018 (0.014) (-0.081 - 0.039)	-0.035, 0.032	0.901	0.16, 0.53 (0.27 - 0.46)
Leucine	1.23 (0.11) (0.97 - 1.52)	1.25 (0.11) (0.89 - 1.56)	-0.022 (0.060) (-0.29 - 0.13)	-0.16, 0.12	0.725	0.43, 1.95 (0.93 - 1.69)
Lysine	0.30 (0.012) (0.27 - 0.33)	0.30 (0.013) (0.25 - 0.33)	-0.0020 (0.0072) (-0.024 - 0.026)	-0.018, 0.014	0.782	0.19, 0.40 (0.26 - 0.34)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Methionine	0.24 (0.019) (0.20 - 0.29)	0.24 (0.019) (0.20 - 0.27)	0.00043 (0.0094) (-0.015 - 0.024)	-0.021, 0.022	0.964	0.11, 0.29 (0.17 - 0.25)
Phenylalanine	0.51 (0.040) (0.40 - 0.60)	0.52 (0.040) (0.38 - 0.61)	-0.0088 (0.023) (-0.10 - 0.052)	-0.063, 0.045	0.714	0.23, 0.75 (0.39 - 0.66)
Proline	0.90 (0.067) (0.74 - 1.08)	0.90 (0.067) (0.65 - 1.06)	-0.0045 (0.032) (-0.15 - 0.12)	-0.078, 0.069	0.889	0.40, 1.24 (0.66 - 1.07)
Serine	0.47 (0.033) (0.38 - 0.52)	0.48 (0.033) (0.36 - 0.58)	-0.011 (0.022) (-0.063 - 0.052)	-0.062, 0.040	0.625	0.24, 0.66 (0.38 - 0.59)
Threonine	0.35 (0.020) (0.29 - 0.39)	0.35 (0.020) (0.28 - 0.39)	-0.0022 (0.013) (-0.042 - 0.033)	-0.032, 0.028	0.871	0.20, 0.46 (0.28 - 0.41)
Tryptophan	0.054 (0.0032) (0.045 - 0.064)	0.053 (0.0033) (0.042 - 0.065)	0.00070 (0.0032) (-0.015 - 0.013)	-0.0067, 0.0081	0.835	0.032, 0.069 (0.039 - 0.063)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Tyrosine	0.29 (0.029) (0.18 - 0.38)	0.30 (0.029) (0.21 - 0.39)	-0.0041 (0.026) (-0.12 - 0.11)	-0.057, 0.048	0.874	0.077, 0.45 (0.11 - 0.43)
Valine	0.48 (0.029) (0.41 - 0.55)	0.49 (0.029) (0.37 - 0.56)	-0.0015 (0.017) (-0.089 - 0.049)	-0.040, 0.037	0.930	0.25, 0.67 (0.38 - 0.58)
Fatty Acid (% total FA)						
16:0 Palmitic	10.91 (0.26) (10.44 - 11.52)	10.54 (0.26) (10.15 - 11.08)	0.37 (0.065) (0.14 - 0.59)	0.22, 0.52	<0.001	6.42, 15.23 (9.13 - 12.33)
18:0 Stearic	1.97 (0.091) (1.81 - 2.17)	1.90 (0.091) (1.77 - 2.07)	0.070 (0.028) (-0.028 - 0.18)	0.0048, 0.13	0.038	0.87, 2.88 (1.54 - 2.38)
18:1 Oleic	24.28 (0.92) (22.84 - 26.62)	23.52 (0.92) (21.74 - 25.71)	0.76 (0.23) (0.13 - 1.20)	0.23, 1.28	0.010	11.30, 43.27 (21.39 - 34.71)
18:2 Linoleic	60.84 (1.28) (57.61 - 62.70)	62.06 (1.28) (59.18 - 64.09)	-1.22 (0.29) (-1.69 - -0.46)	-1.88, -0.55	0.002	41.35, 74.78 (49.38 - 63.16)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% total FA)						
18:3 Linolenic	1.20 (0.014) (1.13 - 1.26)	1.20 (0.014) (1.18 - 1.22)	-0.0012 (0.015) (-0.088 - 0.043)	-0.035, 0.033	0.935	0.78, 1.52 (0.97 - 1.35)
20:0 Arachidic	0.42 (0.030) (0.37 - 0.48)	0.41 (0.030) (0.37 - 0.46)	0.016 (0.0043) (-0.0022 - 0.034)	0.0063, 0.026	0.005	0.15, 0.67 (0.32 - 0.53)
20:1 Eicosenoic	0.21 (0.0080) (0.19 - 0.23)	0.21 (0.0080) (0.20 - 0.23)	-0.00097 (0.0017) (-0.0049 - 0.0033)	-0.0049, 0.0029	0.583	0.12, 0.36 (0.21 - 0.31)
22:0 Behenic	0.17 (0.018) (0.14 - 0.23)	0.16 (0.018) (0.14 - 0.20)	0.0076 (0.0050) (-0.0099 - 0.031)	-0.0039, 0.019	0.167	0, 0.32 (0.057 - 0.23)
Mineral						
Calcium (% dw)	0.0060 (0.00063) (0.0048 - 0.0079)	0.0055 (0.00063) (0.0046 - 0.0076)	0.00049 (0.00033) (-0.00037 - 0.0017)	-0.00027, 0.0013	0.176	0.0019, 0.0076 (0.0038 - 0.0068)
Copper (mg/kg dw)	1.63 (0.11) (1.21 - 2.07)	1.71 (0.12) (1.49 - 1.99)	-0.085 (0.11) (-0.42 - 0.18)	-0.33, 0.16	0.458	0.17, 3.48 (1.10 - 2.62)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Iron (mg/kg dw)	23.61 (0.78) (22.21 - 25.84)	23.03 (0.79) (20.66 - 25.57)	0.58 (0.61) (-2.12 - 2.11)	-0.82, 1.98	0.368	11.42, 28.01 (16.55 - 24.10)
Magnesium (% dw)	0.13 (0.0033) (0.13 - 0.14)	0.13 (0.0033) (0.12 - 0.14)	-0.00021 (0.0034) (-0.0062 - 0.010)	-0.0080, 0.0076	0.952	0.080, 0.16 (0.11 - 0.15)
Manganese (mg/kg dw)	7.91 (1.06) (5.52 - 9.40)	8.07 (1.06) (4.89 - 9.82)	-0.16 (0.27) (-0.83 - 0.83)	-0.71, 0.39	0.567	0, 12.67 (4.00 - 9.17)
Phosphorus (% dw)	0.34 (0.0034) (0.32 - 0.35)	0.34 (0.0036) (0.33 - 0.35)	-0.0071 (0.0050) (-0.020 - 0.0053)	-0.018, 0.0040	0.185	0.24, 0.42 (0.28 - 0.37)
Potassium (% dw)	0.40 (0.0074) (0.38 - 0.42)	0.40 (0.0077) (0.38 - 0.43)	-0.0045 (0.0073) (-0.029 - 0.021)	-0.019, 0.010	0.546	0.24, 0.54 (0.33 - 0.46)
Zinc (mg/kg dw)	22.67 (1.06) (20.99 - 25.42)	23.99 (1.07) (21.65 - 28.08)	-1.32 (1.00) (-5.63 - 3.29)	-3.62, 0.99	0.225	11.46, 30.37 (17.30 - 25.45)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Folic Acid	0.36 (0.025) (0.28 - 0.43)	0.39 (0.025) (0.29 - 0.49)	-0.030 (0.030) (-0.097 - 0.078)	-0.099, 0.040	0.347	0.11, 0.61 (0.24 - 0.57)
Niacin	27.22 (2.15) (22.56 - 33.37)	27.71 (2.18) (22.61 - 33.26)	-0.48 (1.34) (-3.30 - 2.66)	-3.22, 2.26	0.722	7.89, 49.83 (20.63 - 43.08)
Vitamin A	1.01 (0.050) (0.88 - 1.21)	0.96 (0.051) (0.76 - 1.16)	0.057 (0.043) (-0.094 - 0.21)	-0.029, 0.14	0.186	0.38, 1.68 (0.58 - 1.50)
Vitamin B1	2.97 (0.19) (2.58 - 3.41)	2.88 (0.20) (2.48 - 3.41)	0.084 (0.16) (-0.44 - 0.45)	-0.28, 0.45	0.606	2.21, 3.65 (2.41 - 3.48)
Vitamin B2	2.09 (0.37) (1.17 - 3.56)	1.93 (0.37) (1.32 - 2.58)	0.16 (0.33) (-0.72 - 1.23)	-0.59, 0.92	0.630	0, 4.47 (1.28 - 3.29)
Vitamin B6	7.48 (0.60) (5.91 - 8.69)	7.71 (0.60) (5.67 - 9.61)	-0.23 (0.41) (-1.40 - 1.76)	-1.16, 0.70	0.589	2.57, 12.07 (5.24 - 10.29)

Table VII-2 (continued). Summary of Combined-Site Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Vitamin E	13.14 (2.09) (7.04 - 17.44)	13.46 (2.10) (10.13 - 18.10)	-0.31 (0.86) (-6.54 - 4.52)	-2.05, 1.43	0.718	0, 25.61 (6.67 - 17.34)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table VII-3. Summary of Combined-Site Grain Anti-nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dw)						
Phytic Acid	0.96 (0.031) (0.87 - 1.04)	1.02 (0.031) (0.94 - 1.12)	-0.060 (0.022) (-0.12 - 0.032)	-0.10, -0.016	0.008	0.73, 1.23 (0.82 - 1.07)
Raffinose	0.14 (0.028) (0.098 - 0.21)	0.15 (0.029) (0.11 - 0.21)	-0.0054 (0.0082) (-0.028 - 0.025)	-0.024, 0.013	0.524	0.024, 0.29 (0.092 - 0.21)

[1]dw = dry weight.
[2]MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table VII-4. Summary of Combined-Site Grain Secondary Metabolites for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Secondary Metabolite (µg/g dw)						
Ferulic Acid	2348.63 (58.17) (2188.55 - 2559.19)	2387.92 (60.24) (2236.10 - 2500.00)	-39.29 (81.45) (-171.29 - 209.93)	-221.69, 143.10	0.640	1070.41, 2955.86 (1588.35 - 2630.98)
p-Coumaric Acid	204.94 (17.45) (166.11 - 260.43)	205.00 (17.54) (162.58 - 252.26)	-0.060 (8.82) (-28.53 - 32.92)	-20.17, 20.05	0.994	58.74, 313.97 (124.16 - 250.30)

[1]dw = dry weight.
[2]MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table VII-5. Summary of Combined-Site Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.73 (0.23) (4.39 - 5.13)	4.86 (0.23) (3.99 - 5.84)	-0.13 (0.19) (-0.74 - 0.66)	-0.53, 0.27	0.508	2.66, 6.48 (3.70 - 5.95)
Carbohydrates	87.23 (0.90) (86.21 - 89.23)	86.69 (0.91) (83.80 - 88.92)	0.54 (0.49) (-1.59 - 2.61)	-0.46, 1.54	0.277	80.13, 94.05 (83.23 - 90.37)
Moisture (% fw)	68.71 (2.30) (62.70 - 73.10)	69.76 (2.32) (64.10 - 75.00)	-1.05 (1.06) (-5.90 - 5.70)	-3.50, 1.40	0.350	51.70, 86.22 (61.00 - 76.00)
Protein	6.44 (0.75) (4.48 - 7.40)	6.78 (0.76) (5.17 - 8.94)	-0.34 (0.39) (-2.00 - 1.26)	-1.25, 0.57	0.413	1.34, 11.57 (4.37 - 9.31)
Total Fat	1.60 (0.17) (1.09 - 1.85)	1.69 (0.18) (0.58 - 2.28)	-0.092 (0.25) (-1.11 - 1.18)	-0.65, 0.46	0.720	0.44, 3.33 (0.78 - 3.16)
Fiber (% dw)						
Acid Detergent Fiber	24.96 (0.97) (21.08 - 29.00)	26.74 (1.03) (20.27 - 32.16)	-1.78 (1.42) (-8.15 - 3.58)	-4.65, 1.09	0.216	14.84, 38.51 (21.33 - 35.92)

Table VII-5 (continued). Summary of Combined-Site Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	39.79 (1.32) (36.14 - 43.70)	38.12 (1.38) (33.07 - 43.43)	1.67 (1.76) (-1.55 - 4.79)	-2.32, 5.65	0.368	25.12, 54.99 (29.68 - 60.16)
Mineral						
Calcium (% dw)	0.19 (0.010) (0.14 - 0.22)	0.19 (0.011) (0.15 - 0.25)	-0.0083 (0.011) (-0.063 - 0.036)	-0.031, 0.014	0.455	0.075, 0.29 (0.10 - 0.24)
Phosphorus (% dw)	0.24 (0.021) (0.20 - 0.31)	0.24 (0.021) (0.19 - 0.31)	-0.0050 (0.013) (-0.074 - 0.038)	-0.032, 0.022	0.708	0.063, 0.37 (0.16 - 0.31)

[1]dw = dry weight; fw = fresh weight.
[2]MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table VII-6. Literature and ILSI Ranges for Components in Maize Forage and Grain

Grain Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Grain Nutrients		
Proximates (% dw)		
Ash	1.17 – 2.01[a]; 1.14 – 1.63[b]	0.616 – 6.282
Carbohydrates by calculation	81.31 – 87.06[a]; 82.10 – 86.65[b]	77.4 – 89.5
Fat, total	2.95 – 4.40[a]; 3.16 – 4.23[b]	1.742 – 5.823
Moisture (% fw)	8.74 – 11.30[a]; 11.00 – 13.20[b]	6.1 – 40.5
Protein	8.27 – 13.33[a]; 8.55 – 12.19[b]	6.15 – 17.26
Fiber (% dw)		
Acid detergent fiber	1.82 – 4.48[a]; 1.14 – 4.41[b]	1.82 – 11.34
Neutral detergent fiber	6.51 –12.28[a]; 6.08 – 10.36[b]	5.59 – 22.64
Total dietary fiber	10.65 – 16.26[a]; 10.24 – 14.56[b]	8.82 – 35.31
Amino Acids (% dw)		
Alanine	0.60 – 1.04[a]; 0.63 – 0.96[b]	0.439 – 1.393
Arginine	0.34 – 0.52[a]; 0.32 – 0.50[b]	0.119 –0.639
Aspartic acid	0.52 – 0.78[a]; 0.56 – 0.77[b]	0.335 – 1.208
Cystine	0.19 – 0.26[a]; 0.20 – 0.26[b]	0.125 – 0.514
Glutamic acid	1.54 – 2.67[a]; 1.62 – 2.44[b]	0.965 – 3.536
Glycine	0.33 – 0.43[a]; 0.31 – 0.42[b]	0.184 – 0.539
Histidine	0.25 – 0.37[a]; 0.24 – 0.34[b]	0.137 – 0.434
Isoleucine	0.30 – 0.48[a]; 0.30 – 0.44[b]	0.179 – 0.692
Leucine	1.02 – 1.87[a]; 1.06 – 1.65[b]	0.642 – 2.492
Lysine	0.26 – 0.33[a]; 0.25 – 0.31[b]	0.172 – 0.668
Methionine	0.17 – 0.26[a]; 0.16 – 0.30[b]	0.124 – 0.468
Phenylalanine	0.43 – 0.72[a]; 0.43 – 0.63[b]	0.244 – 0.930
Proline	0.74 – 1.21[a]; 0.72 – 1.11[b]	0.462 – 1.632
Serine	0.39 – 0.67[a]; 0.40 – 0.60[b]	0.235 – 0.769
Threonine	0.29 – 0.45[a]; 0.29 – 0.39[b]	0.224 – 0.666
Tryptophan	0.047 – 0.085[a]; 0.040 – 0.070[b]	0.0271 – 0.215
Tyrosine	0.13 – 0.43[a]; 0.12 – 0.41[b]	0.103 – 0.642
Valine	0.42 – 0.62[a]; 0.41 – 0.58[b]	0.266 – 0.855
Fatty Acids (% Total FA)		
16:0 Palmitic	8.80 – 13.33[a]; 9.53 – 12.33[b]	7.94 – 20.71
18:0 Stearic	1.36 – 2.14[a]; 1.28 – 2.13[b]	1.02 – 3.40
18:1 Oleic	19.50 – 33.71[a]; 19.59 – 31.09[b]	17.4 – 40.2
18:2 Linoleic	49.31 – 64.70[a]; 55.17 – 65.65[b]	36.2 – 66.5
18:3 Linolenic	0.89 – 1.56[a]; 1.00 – 1.38[b]	0.57 – 2.25
20:0 Arachidic	0.30 – 0.49[a]; 0.29 – 0.42[b]	0.279 – 0.965
20:1 Eicosenoic	0.17 – 0.29[a]; 0.17 – 0.31[b]	0.170 – 1.917
22:0 Behenic	0.069 – 0.28[a]; 0.059 – 0.33[b]	0.110 – 0.349
Minerals		
Calcium (% dw)	0.0036 – 0.0068[a]; 0.0032 – 0.0070[b]	0.00127 – 0.02084
Copper (mg/kg dw)	1.14 – 3.43[a]; 1.29 – 4.16[b]	0.73 – 18.50
Iron (mg/kg dw)	14.17 – 23.40[a]; 14.37 – 24.66[b]	10.42 – 49.07
Magnesium (% dw)	0.091 – 0.14[a]; 0.095 – 0.14[b]	0.0594 – 0.194
Manganese (mg/kg dw)	4.83 – 8.34[a]; 4.55 – 9.35[b]	1.69 – 14.30
Phosphorous (% dw)	0.24 – 0.37[a]; 0.26 – 0.38[b]	0.147 – 0.533
Potassium (% dw)	0.29 – 0.39[a]; 0.32 – 0.45[b]	0.181 – 0.603
Zinc (mg/kg dw)	16.78 – 28.17[a]; 18.12 – 30.44[b]	6.5 – 37.2

Table VII-6 (continued). Literature and ILSI Ranges for Components in Maize Forage and Grain

Grain Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Vitamins (mg/kg DW)		
Folic acid	0.19 – 0.35[a]; 0.22 – 0.42[b]	0.147 – 1.464
Vitamin A [β–Carotene]	Not Available	0.19 – 46.81
Vitamin B_1 [Thiamine]	2.33 – 4.17[a]; 2.71 – 4.78[b]	1.26 – 40.00
Vitamin B_2 [Riboflavin]	0.94 – 2.42[a]; 1.46 – 2.81[b]	0.50 – 2.36
Vitamin B_3 [Niacin]	15.07 – 32.38[a]; 13.64 – 42.60[b]	10.37 – 46.94
Vitamin B_6 [Pyridoxine]	4.93 – 7.53[a]; 4.01 – 8.27[b]	3.68 – 11.32
Vitamin E [α–Tocopherol]	5.96 – 18.44[a]; 2.83 – 15.53[b]	1.5 – 68.7
Grain Anti–Nutrients (%DW)		
Phytic acid	0.69 – 1.09[a]; 0.58 – 0.97[b]	0.111 – 1.570
Raffinose	0.079 – 0.22[a]; 0.028 – 0.15[b]	0.020 – 0.320
Grain Secondary Metabolites (µg/g DW)		
Ferulic acid	1205.75 – 2873.05[a]; 820.14 – 2539.86[b]	291.9 – 3885.8
p–Coumaric acid	94.77 – 327.39[a]; 64.03 – 259.68[b]	53.4 – 576.2

Forage Tissue Components[1]	Literature Range[2]	ILSI Range[3]
Forage Nutrients		
Proximates (% dw)		
Ash	2.67–8.01[a]; 3.88 – 6.90[b]	1.527 – 9.638
Carbohydrates by calculation	81.88 – 89.26[a]; 84.11 – 89.52[b]	76.4 – 92.1
Fat, total	1.28 – 3.62[a]; 0.20 – 2.33[b]	0.296 – 4.570
Moisture (% FW)	64.20 – 75.70[a]; 71.40 – 78.00[b]	49.1 – 81.3
Protein	5.80 – 10.24[a]; 5.56 – 9.14[b]	3.14 – 11.57
Fiber (% dw)		
Acid detergent fiber	19.11 – 30.49[a]; 20.73 – 33.39[b]	16.13 – 47.39
Neutral detergent fiber	27.73 – 49.62[a]; 31.81 – 50.61[b]	20.29 – 63.71
Minerals (% dw)		
Calcium	0.12 – 0.33[a]; 0.17 – 0.41[b]	0.0714 – 0.5768
Phosphorous	0.090 – 0.26[a]; 0.13 – 0.21[b]	0.0936 – 0.3704

[1]dw=dry weight; fw=fresh weight, FA = fatty acids.
[2]Literature range references: a(Harrigan et al., 2009)[US 2006], b(Harrigan et al., 2009)[Chile 2006/2007].
[3]ILSI range is from ILSI Crop Composition Database (ILSI, 2009).

VII.B. Compositional Assessment of MON 87427 Summary and Conclusion

Analyses of nutrient, anti-nutrient, and secondary metabolite levels in MON 87427 and the conventional control were conducted to assess compositional equivalence. The tissues analyzed included forage and grain harvested from plants grown at three field sites in the U.S. during the 2008 field season. The composition analysis, conducted in accordance with OECD guidelines, also included measurement of nutrients, anti-nutrients, and secondary metabolites in commercial maize reference hybrids that have a history of safe consumption to establish the natural range of variability. MON 87427, the conventional control, and commercial references were treated with conventional weed control programs. In addition, MON 87427 plots were treated with glyphosate herbicide at a target rate of 1.0 lb ai/acre (1.13 kg ai/ha).

There were no significant differences identified for grain secondary metabolites or forage nutrients. The significant differences (α=0.05) in nutrient and anti-nutrient content were evaluated using considerations relevant to the safety and nutritional quality of MON 87427 when compared to the conventional control:

1) All nutrient and anti-nutrient component significant differences observed in the combined-site analysis, whether reflecting increased or decreased MON 87427 mean values with respect to the conventional control were small. Relative magnitude of differences ranged from 1.96% to 5.92%.

2) Mean values for these nutrient and anti-nutrient components from the combined-site analysis of MON 87427 fell within the 99% tolerance interval established from the commercial references grown concurrently and were, therefore, within the range of natural variability of that component in commercial maize hybrids with a history of safe consumption (Tables VII-1 - VII-3).

3) Assessment of the reproducibility of the combined-site differences at the three individual sites showed significant differences (α=0.05) for 18:0 stearic acid and 20:0 arachidic acid at one individual site and differences for 16:0 palmitic acid, 18:1 oleic acid, and 18:2 linoleic acid differed across all three sites. No difference was observed for total fat and phytic acid at any of the individual sites. Individual site mean values of MON 87427 for all components with significant differences were within the 99% tolerance interval established from the commercial references grown concurrently and were, therefore, within the range of natural variability of that component in commercial maize hybrids with a history of safe consumption.

4) All of the compositional components identified as significantly different from the conventional control were within the natural variability of these components in commercial commercial maize composition as published in the scientific literature and available in the ILSI Crop Composition Database.

This analysis provides a comprehensive comparative assessment of the levels of key nutrients, anti-nutrients, and secondary metabolites in grain and of key nutrients in forage of MON 87427 and the conventional control, discussed in the context of natural

variability of commercial maize. Results of the comparison indicate that the composition of the forage and grain of MON 87427 is compositionally equivalent to conventional maize with a demonstrated history of safe use.

Food and Feed uses of conventional maize is discussed in Section II. The processing of MON 87427 is not expected to be any different from that of conventional maize. As described in this section, detailed compositional analyses of key components of MON 87427 have been performed and have demonstrated that MON 87427 is compositionally equivalent to conventional maize. Additionally, the mode of action of CP4 EPSPS protein, as described in Section VI.C., is well understood, and there is no reason to expect interactions with endogenous metabolites or important nutrients that may be present in maize. Therefore, when MON 87427 is used on a commercial scale as a source of food or feed, these products are not expected to be different from the equivalent foods or feeds originating from conventional maize.

VIII. USE OF ANTIBIOTIC RESISTANCE MARKER GENES

The information provided in this section addresses the relevant factors in Codex Plant Guidelines, Section 5, paragraphs 55 through 58.

VIII.A. Presence of Genes that Encode Resistance to Antibiotics

No genes that encode resistance to an antibiotic marker were inserted into the crop genome during the development of MON 87427. Molecular characterization data presented in Section V demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 87427.

IX. SUMMARY OF FOOD AND FEED SAFETY ASSESSMENTS

This section provides a concluding discussion of the safety assessment and addresses the relevant factors in Codex Plant Guidelines, Section 5, paragraph 59.

IX.A. Donor Organism

As described in detail in Section III, the *cp4 epsps* coding sequence is derived from the soil bacterium *Agrobacterium* sp. strain CP4, which is related to microbes commonly present in the soil and in the rhizosphere of plants. *Agrobacterium* species are not known for human or animal pathogenicity, and are not commonly allergenic. According to FAO/WHO there is no known population of individuals sensitized to bacterial proteins (FAO, 2001).

IX.B. Genetic Insert

MON 87427 was produced by *Agrobacterium* -mediated transformation of maize with PV-ZMAP1043, as described in detail in Sections IV and V. The plasmid vector contains a single T-DNA delineated by Left and Right Border regions and has one expression cassette consisting of the *cp4 epsps* coding sequence under the regulation of the *e35S* promoter, the *hsp70* intron, the *CTP2* targeting sequence, and the *nos* 3' nontranslated region (Figure IV-1). The *cp4 epsps* expression cassette encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Figure IV-3) (Padgette et al., 1996). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS protein (Barry et al., 2001; Padgette et al., 1996). Subsequent to plant transformation, backcross progeny generations were evaluated for tolerance to glyphosate and surviving plants were then selfed to produce homozygous plants, which were identified through quantitative PCR analysis.

MON 87427 was subjected to an extensive molecular characterization. Southern blot analyses demonstrated that a single copy of the T-DNA sequence from PV-ZMAP1043 was integrated into the maize genome at a single locus. These analyses also demonstrated that there were no additional genetic elements, including backbone sequences from PV-ZMAP1043 detected, linked or unlinked to the intact T-DNA present in MON 87427. The PCR and DNA sequence analyses performed on MON 87427 confirmed the organization of the elements within the insert, assessed potential rearrangements at the insertion site, and resulted in the complete DNA sequence of the T-DNA and adjacent maize genomic DNA sequence in MON 87427. Furthermore, Southern blot analysis demonstrated that the T-DNA insert in MON 87427 has been maintained through five breeding generations, thereby confirming the stability of the T-DNA in MON 87427. Finally, results from segregation analyses demonstrate heritability of the insert occurred as expected across multiple generations, which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA in MON 87427 at a single chromosomal locus.

IX.C. CP4 EPSPS Protein

As described in detail in Section VI, a multistep approach was conducted according to guidelines established by the CODEX Alimentarius Commission and OECD and which embody the principles and guidance of the FDA's 1992 policy on foods from new plant varieties, and was used to characterize the CP4 EPSPS protein expressed in MON 87427 resulting from the genetic modification. This detailed characterization confirms the CP4 EPSPS protein is safe for human and animal consumption. The assessment involved: 1) characterization of the physicochemical and functional properties of CP4 EPSPS protein; 2) quantification of CP4 EPSPS expression in plant tissues; 3) examination of the similarity of CP4 EPSPS protein to known allergens, toxins or other biologically active proteins known to have adverse effects on humans and animals; 4) evaluation of the digestibility of CP4 EPSPS protein in simulated gastrointestinal fluids; 5) evaluation of the stability of CP4 EPSPS protein after heat treatment; 6) documenting the history of safe consumption of CP4 EPSPS protein or its structural and functional homology to proteins that lack adverse effects on human or animal health; 7) investigation of potential mammalian toxicity through animal assays and calculating margins of exposure; and 8) assessment of the potential for allergenicity, toxicity and adverse biological activity of putative polypeptides encoded by the insert and flanking sequences. The safety assessment supports the conclusion that dietary exposure to CP4 EPSPS protein derived from MON 87427 poses no meaningful risk to human or animal health.

The CP4 EPSPS protein expressed in MON 87427 is identical to the CP4 EPSPS protein in other Roundup Ready crops, and the *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during Monsanto consultations with the FDA regarding Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready corn 2, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton and Roundup Ready alfalfa. Furthermore, the U.S. EPA has established an exemption from the requirement of a tolerance for residues of CP4 EPSPS protein and the genetic material necessary for its production in all plants (U.S. EPA, 1996). Because the MON 87427-produced CP4 EPSPS protein is equivalent to the exempted CP4 EPSPS protein a similar conclusion can be reached that the MON 87427-produced CP4 EPSPS protein is safe for human and animal consumption. Using the guidance provided by the FDA in its 1992 Policy Statement regarding the evaluation of New Plant Varieties, a conclusion of "no concern" is reached for the donor organism and the CP4 EPSPS protein. The food and feed products containing MON 87427 or derived from MON 87427 are as safe as maize currently on the market for human and animal consumption.

IX.D. Composition

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by FDA. The CP4 EPSPS protein expressed in MON 87427 is identical to the CP4 EPSPS protein in other Roundup Ready crops and the mode of action of CP4 EPSPS protein is well understood. Previous Roundup Ready crops reviewed by the FDA have had no biologically relevant compositional changes identified, and there is no reason to

expect the CP4 EPSPS protein in MON 87427 to interact with endogenous metabolites or important nutrients that are present in maize grain or forage.

Detailed compositional analyses in accordance with OECD guidelines were conducted to determine whether levels of key nutrients, anti-nutrients and secondary metabolites in MON 87427 were comparable to levels present in the near-isogenic conventional control and several commercial maize reference hybrids. The maize references were used to establish the natural range of levels of the key nutrients, anti-nutrients, and secondary metabolites in commercial maize hybrids that have a history of safe consumption. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber, amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and vitamins [folic acid, niacin, A (β-carotene), B1, B2, B6, and E] in the grain, and proximates, ADF, NDF, calcium and phosphorus in forage. The anti-nutrients assessed in grain included phytic acid and raffinose. Secondary metabolites assessed in grain included furfural, ferulic acid, and p-coumaric acid.

Combined-site analyses were conducted to determine statistically significant differences (5% level of significance) between MON 87427 and the conventional control on both forage and grain samples. Statistical results from the combined-site data were reviewed using considerations relevant to safety and/or nutritional value. These considerations included assessments of: 1) the relative magnitude of the differences in the mean values of key nutrient, anti-nutrient, and secondary metabolite components of MON 87427 and the conventional control, 2) whether the MON 87427 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of commercial maize reference hybrids grown concurrently, 3) evaluation of the reproducibility of the significant (α=0.05) combined-site component differences at individual sites, and 4) assessing the difference within the context of natural variability of commercial maize composition published in the scientific literature and in the International Life Sciences Institute (ILSI) Crop Composition Database.

Analytical results support the overall conclusion that MON 87427 grain and forage was compositionally equivalent to conventional maize in accordance with OECD guidelines. This conclusion is supported by data from three sites in the U.S. that are representative of typical agronomic practices in the U.S. Maize Belt, and extends to the foods and feeds produced from MON 87427.

IX.E. Summary of Food and Feed Safety Assessment of MON 87427

Collectively, these data and a history of safe use of the host organism, maize, 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 (U.S. FDA, 1992) and shown in Figure IX-1. MON 87427 is not materially different in composition, safety or nutrition from conventional maize other than its tissue-selective expression of CP4 EPSPS protein that is useful for the production of hybrid maize seed. Sales and consumption of maize grain and processed products derived from

MON 87427 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 maize hybrids and biotechnology traits.

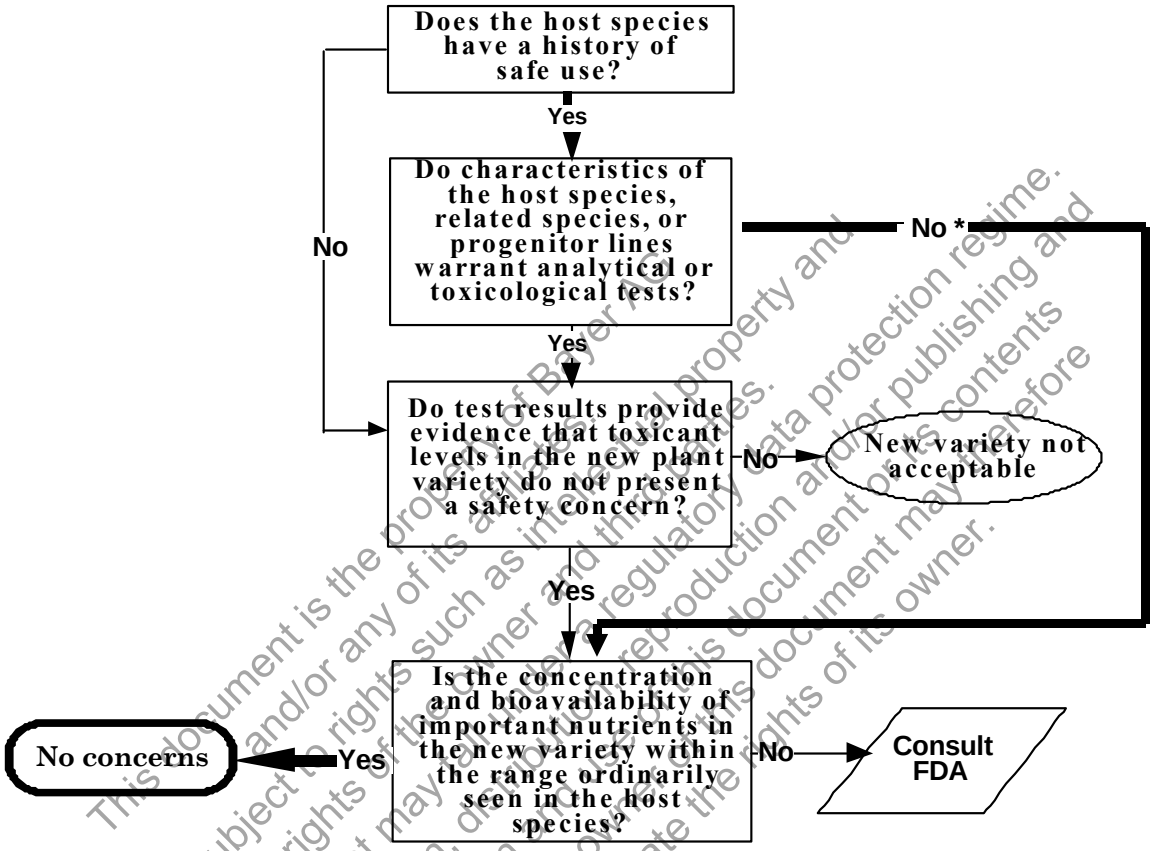


Figure IX-1. Safety Assessment of New Varieties: The Host Plant

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APPENDICES

Appendix A: Materials and Methods Used for Molecular Analyses of MON 87427

A.1. Materials

The genomic DNA used in molecular analyses was isolated from seed of MON 87427 LH198 BC3F4 and the conventional control LH198 × HiII. For generational stability analysis, genomic DNA was extracted from seed of the [LH198 BC3F7 × LH287] F1 generation of MON 87427, both conventional controls (LH198 × HiII and LH198 × LH287), and from leaf tissue of the LH198 BC3F3, LH198 BC3F6, and LH198 BC3F7 generations, which were harvested from production plan PPN-09-218. The reference substance, PV-ZMAP1043 (Figure IV-1), was used as a positive hybridization control in Southern analyses. Probe templates generated from PV-ZMAP1043 were used as additional positive hybridization controls. As additional reference standards, the 1 Kb DNA Extension Ladder and λ DNA/Hind III Fragments from Invitrogen (Carlsbad, CA) were used for size estimations on Southern blots and agarose gels. The 1 Kb DNA Ladder from Invitrogen was used for size estimations on agarose gels for PCR analyses.

A.2. Characterization of the Materials

The identities of the source materials were verified by methods used in molecular characterization to confirm presence or absence of MON 87427. The stability of the genomic DNA was confirmed by observation of interpretable signals from digested DNA samples on ethidium bromide stained agarose gels and/or specific PCR products, and the samples did not appear visibly degraded on the ethidium bromide stained gels.

A.3. DNA Isolation for Southern Blot and PCR Analyses

Genomic DNA was isolated from MON 87427 maize seed according to a hexadecyltrimethylammonium bromide (CTAB) based method. First, the seed was processed to a fine powder using a Harbil paint shaker for three minutes. Briefly, approximately 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 10 µl of RNase (10 mg/ml, Roche) were added to approximately 6 grams of the processed seed. The samples were incubated at 65ºC for ~35 minutes with intermittent mixing and then allowed to cool to room temperature. Approximately 16 ml of chloroform:isoamyl alcohol (CIA) (24:1 (v/v)) was added to the samples, 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 tube. The CIA extraction was repeated twice. Approximately 1/10 volume (~1.6 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)] was added to the aqueous phase, which was then mixed for 5 minutes. To separate the phases, the samples were centrifuged at ~16,000 x g for 5 minutes at room temperature. 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 hour. The samples were centrifuged at ~16,000 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 (10 mM Tris-HCl pH 8.0, 11 mM EDTA pH 8.0, and 1 M NaCl) at 60°C for approximately 15 minutes. Approximately 1/10 volume (0.2 ml) of 3 M NaOAc (pH 5.2) and 2 volumes (~4 ml relative to the supernatant) of 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 extracted DNA was stored in a 4ºC refrigerator.

Genomic DNA was also isolated from MON 87427 leaf tissue using a hexadecyltrimethylammonium bromide (CTAB) based method. First, the leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle. Briefly, 10 ml of CTAB buffer (1.5% w/v CTAB, 75 mM Tris HCl, 100 mM EDTA, 1.05 M NaCl, and 0.75% w/v PVP) and 200 µg RNase A were added to approximately 2 ml of ground leaf tissue and incubated at 60-70°C for 40-50 minutes with intermittent mixing. The samples were allowed to come to room temperature and split into two 13 ml tubes. Five ml of chloroform were added to the samples. The samples were mixed by hand for 2-3 minutes, then centrifuged at 10,300 x g for 8-10 minutes at room temperature. The upper aqueous phase was transferred to a clean tube and the chloroform step was repeated twice. After the last chloroform step, the aqueous phase was transferred to a clean tube and the DNA was precipitated with 5 ml of 100% ethanol. The precipitated DNA was spooled into a tube with 5-6 ml of 70% ethanol to wash the DNA pellet. The samples were centrifuged at 5,100 x g for 5 minutes at room temperature to pellet the DNA. DNA pellets were vacuum dried, then re-suspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH8.0). The extracted DNA was stored in a 4ºC refrigerator.

A.4. Quantification of Genomic DNA

Genomic DNA was quantified using a DyNA Quant 200 Fluorometer (Hoefer, Inc., Holliston, MA). Molecular Size Marker IX (Roche, Indianapolis, IN) was used as the calibration standard.

A.5. Restriction Enzyme Digestion of Genomic DNA

Approximately ten micrograms (µg) of genomic DNA extracted from MON 87427 and the conventional controls were digested with the restriction enzymes *Nco* I or *Nsi* I (New England Biolabs (Ipswich, MA). All digests were conducted in 1X NEBuffer 3 (New England Biolabs) at 37°C in a total volume of ~500 µl using ~20 units or ~50 units of the appropriate enzyme. For the purpose of running positive hybridization controls, ~10 µg of genomic DNA extracted from the control substance was digested, and the appropriate positive hybridization control(s) were added to these digests.

A.6. Agarose Gel Electrophoresis

Digested DNA was resolved on 0.8% (w/v) agarose gels. For all Southern blot analyses except for generational stability, individual digests containing ~10 µg each of MON 87427 and conventional control DNA were loaded on the same gel in a long run/short run format. The long run allows for greater resolution of large molecular

weight DNA whereas the short run allows for the detection of small molecular weight DNA. The positive hybridization controls were only run in the short run format to ensure that the fragments would be retained on the gel. For the generational stability analysis, individual digests of ~10 µg each of genomic DNA extracted from seed or leaf tissue across five generations of MON 87427 and the conventional controls were loaded on the agarose gel in a single run format.

A.7. DNA Probe Preparation for Southern Blot Analyses

Probe templates were prepared by PCR amplification using PV-ZMAP1043 as the template and purified by agarose gel electrophoresis. The probe templates were designed based on the nucleotide composition (% GC) of the sequence in order to optimize the detection of DNA sequences during hybridization. Where possible, probes possessing similar Tms were combined in the same Southern blot hybridization. Approximately 25 ng of each probe template were radiolabeled with either [α^{32}P] deoxycytidine triphosphate (dCTP) or [α^{32}P] deoxyadenosine triphosphate (dATP) (6000 Ci/mmol) using the random priming method (RadPrime DNA Labeling System, Invitrogen, Carlsbad, CA). Probe locations relative to the genetic elements in PV-ZMAP1043 are depicted in Figure IV-1.

A.8. Southern Blot Analyses of Genomic DNA

Digested genomic DNA isolated from MON 87427 and from the conventional maize controls was evaluated using Southern blot analyses. PV-ZMAP1043 DNA digested with *Sph* I was added to the conventional control genomic DNA pre-digested with *Nco* I to serve as a positive hybridization control. When multiple probes were hybridized simultaneously to one Southern blot, the appropriate probe templates generated from PV-ZMAP1043 were mixed with pre-digested conventional control genomic DNA to serve as additional positive hybridization controls (Figure IV-1). The digested DNA was then separated by agarose gel electrophoresis and transferred onto a nylon membrane. Southern blots were hybridized and washed at 55°C, 60°C, or 65°C, depending on the melting temperature (Tm) of the probes. Table A-1 lists the radiolabeling conditions and hybridization temperatures of the probes used in this study. Multiple exposures of each blot were then generated using Kodak Biomax MS film in conjunction with one or two Kodak Biomax MS intensifying screen(s) in a -80ºC freezer.

Table A-1. Hybridization Conditions of Utilized Probes

Probe	DNA Probe	Element Sequence Spanned by DNA Probe	Probe labeled with dNTP (^{32}P)	Hybridization/Wash Temperature (°C)
1	T-DNA Probe 1	B-Left Border, P-*e35S*, I-*hsp70* (portion)	dATP	60
2	T-DNA Probe 2	I-*hsp70* (portion), TS-*CTP2* (portion)	dATP	55
3	T-DNA Probe 3	TS-*CTP2* (portion), CS-*cp4 epsps* (portion)	dCTP	65
4	T-DNA Probe 4	CS-*cp4 epsps* (portion), T-*nos*, B-Right Border	dATP	60
5	Backbone Probe	Backbone sequence	dCTP	60
6	Backbone Probe	Backbone sequence	dCTP	60
7	Backbone Probe	Backbone sequence	dCTP	60

A.9. DNA Sequence Analyses of the Insert

Overlapping PCR products were generated that span the insert and adjacent 5' and 3' flanking genomic DNA sequences in MON 87427. These products were sequenced using BigDye® terminator chemistry to determine the nucleotide sequence of the insert in MON 87427 as well as that of the DNA flanking the 5' and 3' ends of the insert.

The PCR analyses for product A and product B were conducted using 50 ng of genomic DNA template in a 50 µl reaction volume containing a final concentration of 2 mM $MgSO_4$, 0.2 µM of each primer, 0.2 mM each dNTP, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA). A primary PCR reaction (product C′) was used in a secondary (nested) reaction to generate product C in order to acquire an adequate amount of template for sequencing. The primary PCR reaction for product C' was conducted using 50 ng of genomic DNA template in a 25 µl reaction volume containing a final concentration of 2 mM $MgSO_4$, 0.2 µM of each primer, 0.2 mM each dNTP, 10% DMSO, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity. The secondary (nested) reaction was conducted using 1 µl of a 1:10 or 1:100 dilution of product C' as genomic DNA template in a 50 µl reaction volume containing a final concentration of 2 mM $MgSO_4$, 0.2 µM of each primer, 0.2 mM each dNTP, 10% DMSO, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity.

The amplification of product A and product B were performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 15 seconds,

60°C for 30 seconds, 68°C for 3.25 minutes; and one cycle at 68°C for 5 minutes. The amplification of product C′ was performed under the following touchdown cycling conditions: one cycle at 94°C for 2 minutes; 16 cycles at 94°C for 20 seconds, 62°C decreasing 1°C per cycle for 30 seconds, 68°C for 2 minutes; 20 cycles at 94°C for 20 seconds, 45°C for 30 seconds, 68°C for 2 minutes; and one cycle at 68°C for 7 minutes. The amplification of product C was performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1.5 minutes; and one cycle at 68°C for 5 minutes.

Aliquots of each PCR product were separated on 1.0% (w/v) agarose gels and visualized by ethidium bromide staining to verify that the products were of the expected size prior to sequencing. To concentrate DNA prior to sequencing, some of the PCR reactions for product B and product C were combined separately and purified with the QIAquick PCR Purification Kit following the manufacturer's instructions (Qiagen, Valencia, CA). The PCR products were sequenced using multiple primers, including primers used for PCR amplification. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye® terminator chemistry (Applied Biosystems, Foster City, CA).

A.10. PCR and DNA Sequence Analysis to Examine the MON 87427 Insertion Site

To characterize the MON 87427 insertion site in conventional maize, PCR analysis was performed on genomic DNA from both MON 87427 and the conventional control. The product resulting from the PCR analysis on the conventional control was sequenced. The primers used in this analysis were designed from the DNA sequences flanking the insert in MON 87427. One primer specific to the 5' flanking end of the insert was paired with a second primer specific to the 3' flanking end of the insert in the genomic DNA sequence.

The PCR analyses were conducted using 50 ng of MON 87427 and conventional control genomic DNA template in separate 50 µl reactions containing a final concentration of 2 mM $MgSO_4$, 0.2 µM of each primer, 0.2 mM each dNTP, 10% DMSO, and 0.02 units/µl of Accuprime Taq DNA Polymerase High Fidelity (Invitrogen). The amplification of the product was performed under the following cycling conditions: one cycle at 94°C for 2 minutes; 30 cycles at 94°C for 15 seconds, 64°C for 30 seconds, 68°C for 1.5 minutes; and one cycle at 68°C for 5 minutes.

Aliquots of each PCR product were separated on 1.0% (w/v) agarose gels and visualized by ethidium bromide staining to verify that the product was of the expected size prior to sequencing. To concentrate DNA prior to sequencing, some of the PCR reactions were purified using the QIAquick PCR Purification Kit following the manufacturer's instructions (Qiagen), and eluates were dried down using a vacufuge. The PCR products were sequenced using multiple primers, including primers used for PCR amplification and primers designed internal to the amplified sequences. All sequencing was performed by the Monsanto Genomics Sequencing Center using BigDye® terminator chemistry (Applied Biosystems, Foster City, CA).

Appendix B: Materials and Methods for Characterization of CP4 EPSPS Protein Produced in MON 87427

B.1. Materials

The MON 87427-produced CP4 EPSPS protein (lot 11243843) purified from MON 87427 grain (lot 10007650) was used as the test substance. The MON 87427-produced CP4 EPSPS protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM dithiothreitol (DTT), 1 mM benzamidine-HCl, and 25% glycerol.

The *E. coli*-produced CP4 EPSPS protein (lot 10000739, historical lot 20-100015) was used as the reference substance. The CP4 EPSPS protein reference substance was generated from cell paste produced by large-scale fermentation of *E. coli* containing the pMON21104 expression plasmid. The coding sequence for cp4 epsps contained on the expression plasmid (pMON21104) was confirmed prior to and after fermentation. The E. coli-produced CP4 EPSPS protein was previously characterized.

B.2. Description of Assay Controls

Protein molecular weight standards (Precision Plus Protein™ Standards Dual color; Bio-Rad, Hercules, CA) were used to calibrate some SDS-PAGE gels and verify protein transfer to polyvinylidene difluoride (PVDF) membranes. Broad range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA) were used to generate a standard curve for the apparent molecular weight estimation of the MON 87427-produced CP4 EPSPS protein. The *E. coli*-produced CP4 EPSPS reference standard was used to construct a standard curve for the estimation of total protein concentration using a Bio-Rad protein assay. A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) 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 peptide mixture (Sequazyme™ Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass and a bovine serum albumin (BSA) standard (NIST, Gaithersburg, MD) was used to calibrate the MALDI-TOF mass spectrometer for intact mass analysis. Transferrin (Amersham Biosciences, Piscataway, NJ) and horseradish peroxidase (Sigma-Aldrich, St. Louis, MO) were used as positive controls for glycosylation analysis. CandyCane™ glycoprotein molecular weight standards (Molecular Probes, Eugene, OR) were used as molecular weight markers, as well as, additional positive and negative controls for glycosylation analysis.

B.3. Protein Purification

The plant-produced CP4 EPSPS protein was purified from grain of MON 87427. The purification procedure was not performed under a GLP plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. The CP4 EPSPS protein was purified at ~4 °C from an extract of ground grain using a combination of ammonium sulfate fractionation, hydrophobic interaction

chromatography, anion exchange chromatography, and cellulose phosphate affinity chromatography. The purification procedure is briefly described below.

Approximately 400 g of grain of MON 87427 was mixed with 400 g of dry ice and then ground using a laboratory mill (Perten Instruments, model 3100). The ground powder (~400 g) was stored in a -80 ºC freezer until used for extraction of the CP4 EPSPS protein. The ground powder was mixed with extraction buffer (100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM benzamidine-HCl, 4 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1% polyvinylpolypyrrolidone and 10% glycerol) for 1 h at a sample weight (g) to buffer volume (ml) of approximately 1:10. The slurry was centrifuged at 15,000 x *g* for 1 h at ~4 ºC. The supernatant (3.8 liters) was collected and brought to 40% ammonium sulfate saturation by slow addition of 859 g of ammonium sulfate at ~4 ºC. The solution was stirred for ~1 h at ~4 ºC and then centrifuged at 15,000 x *g* for 45 min. The supernatant (3.8 liters) was again collected and 710 g of ammonium sulfate was added to bring the solution to 70% ammonium sulfate saturation. The solution was stirred for 1 h at ~4 ºC and the pellet was collected by centrifugation at 15,000 x *g* for 1 hr. The pellet was re-suspended in 750 ml of PS(A) buffer [50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol (v/v), 1.5 M ammonium sulfate]. The sample was loaded onto a 471 ml column (5 cm x 24 cm) of Phenyl Sepharose™ Fast Flow (GE Healthcare, Piscataway, NJ) equilibrated with PS(A) buffer. Proteins were eluted with a linear salt gradient that decreased from 1.5 M to 0 M ammonium sulfate over a volume of 2400 ml. Fractions containing the CP4 EPSPS protein, identified based on Western blot analysis, were pooled to a final volume of ~225 ml. The pooled sample was desalted by dialysis against 4 liters of QS(A) buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM benzamidine-HCl, 4 mM DTT) at ~4 °C with one additional 4 liters buffer change using a dialysis tubing [Spectrum Laboratories, Inc., Rancho Dominguez, CA; Molecular Weight Cutoff (MWCO): 3.5 kDa] for a total of 24 h.

The desalted sample (310 ml) was loaded onto a 48 ml column (2.6 cm x 9 cm) of Q Sepharose™ Fast Flow anion exchange resin (GE Healthcare, Piscataway, NJ) equilibrated with QS(A) buffer. The bound CP4 EPSPS protein was eluted with a linear salt gradient that increased from 0 M to 0.4 M KCl in QS(A) buffer over 600 ml. Fractions containing CP4 EPSPS, identified by Western blot analysis, were pooled to a final volume of ~ 110 ml. The pooled sample was dialyzed against 2 liters CP2(A) buffer (10 mM sodium citrate, pH 5.0, 1 mM benzamidine-HCl, 2 mM DTT) for a total of 36 h at ~4 °C with 2 additional 2 liters buffer changes using a dialysis tubing (Spectrum Laboratories, Inc. Rancho Dominguez, CA; MWCO: 3.5 kDa).

The dialyzed sample (120 ml) was then loaded onto a 32 ml column (2.6 x 6 cm) of cellulose phosphate P11 cation exchange (Whatman) pre-equilibrated with CP2(A) buffer. After an initial wash with 300 ml of CP2(A), the column was washed with a linear gradient that increased from 0 to 100% UGN50 buffer (10 mM sodium citrate, 1 mM benzamidine, 50 mM NaCl, 0.3 mM UTP, 0.3 mM glucose-1-phosphate, and 4 mM DTT, pH 5.0) over 32 ml and was held at 100% for ~70 ml. The column was further washed with a linear gradient that increased from 0 to 100% PEP buffer (10 mM sodium citrate, 1 mM benzamidine, 50 mM NaCl, 0.3 mM phosphoenolpyruvate (PEP), 4 mM DTT, pH 5.3) over 32 ml and was held at 100% for ~140 ml. The bound CP4 EPSPS

protein was eluted with a linear gradient that increased from 0-100% PEP/S3P buffer (10 mM sodium citrate, 1 mM benzamidine, 50 mM NaCl, 0.5 mM PEP, and 0.5 mM shikimate-3-phosphate (S3P), 4 mM DTT, pH 5.7) over 32 ml and was held at 100% for ~130 ml. Fractions containing CP4 EPSPS protein, based on SDS PAGE analysis and confirmed by Western blot analysis, were pooled (~27 ml). The pooled sample was divided between four iCon™ Concentrators (MWCO: 20 kDa; size: 7 ml; Pierce, Rockford, IL) and concentrated by centrifugation at 4,000 x *g* for 30 min at ~4 °C. Buffer exchange was carried out in the same units by the addition of ~6.5 ml FSB buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl) followed by centrifugation at 4,000 x *g* for 30 min at ~4 °C. The exchange was conducted a total of four times, and during the final exchange, the sample was concentrated to ~0.2 ml per unit. The samples were pooled (~0.8 ml) and mixed with 0.8 ml FSB buffer (containing 50% glycerol) to final volume of 1.6 ml. Final buffer composition of the sample was: 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine-HCl and 25% glycerol. The concentration of the MON 87427-produced CP4 EPSPS produced was determined to be 0.1 mg/ml based on the Bio-Rad protein assay. The CP4 EPSPS protein purified from the grain of MON 87427 was aliquoted and stored in a -80°C freezer.

B.4. N-Terminal Sequencing

N-terminal sequencing, carried out by automated Edman degradation chemistry, was used to confirm the identity of the MON 87427-produced CP4 EPSPS protein.

MON 87427-produced CP4 EPSPS protein was separated by SDS-PAGE and transferred to PVDF membrane. The blot was stained using Coomassie Brilliant Blue R-250 (Bio-rad, Hercules, CA). The major band at ~44 kDa containing the test protein was excised from the blot and was used for N-terminal sequence analysis. The analysis was performed for 15 cycles using automated Edman degradation chemistry (Hunkapillar, et al., 1983). An Applied Biosystems 494 Procise® Sequencing System with 140C Microgradient pump and 785 Programmable Absorbance Detector was controlled with Procise Control (version 1.1a) software. Chromatographic data were collected using Atlas 2003 software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A control protein (10 picomoles of β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the sequence analysis of the CP4 EPSPS protein to verify that the sequencer met performance criteria for repetitive yield and sequence identity. Identity was established if ≥8 amino acids, consistent with the predicted sequence of the N-terminus of the MON 87427-produced CP4 EPSPS protein, were observed during analysis.

B.5. MALDI-TOF Tryptic Mass Map Analysis

MALDI-TOF tryptic mass fingerprint analysis was used to confirm the identity of the MON 87427-produced CP4 EPSPS protein. MON 87427-produced CP4 EPSPS protein was subjected to SDS-PAGE and the gel was stained using Brilliant Blue G Colloidal stain. Each ~44 kDa band was excised, transferred to a microcentrifuge tube, and destained with 40% methanol/10% glacial acetic acid followed by10% acetonitrile in 25 mM ammonium bicarbonate. The gel bands were washed in 100 mM ammonium

bicarbonate and then, to reduce the protein in each, gel bands were incubated in 100 µl of 10 mM DTT at ~37°C for 2 h. The protein was then alkylated in the dark for 2 h with 100 µl of 20 mM iodoacetic acid and washed with 200 µl of 25 mM ammonium bicarbonate for 1 h once and for 15 min twice. Gel bands were dried with a Speed-Vac® concentrator and then rehydrated with 20 µl of trypsin solution (20 µg/ml). After 1 h, excess liquid was removed and the gel was incubated at 37.6 °C for 16 h in 40 µl of 10% acetonitrile in 25 mM ammonium bicarbonate. To elute proteolytic fragments, gel bands were sonicated for 5 min. The resulting extracts were transferred to new microcentrifuge tubes labeled Extract 1 and dried using Speed-Vac concentrator. The gel bands were re-extracted twice with 30 µl of a 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% β-octyl-glucopyranoside solution and sonicated for 5 min. Both 60% acetonitrile, 0.1% trifluoroacetic acid, 0.1% β-octyl-glucopyranoside extracts were pooled into a new tube labeled Extract 2 and dried with a Speed-Vac concentrator. A solution of 0.1% trifluoroacetic acid (TFA) was added to all Extract 1 and 2 tubes and they were dried as before. To acidify the extracts, a solution of 50% acetonitrile, 0.1% trifluoroacetic acid was added to each tube and all were sonicated for 5 min. Each extract (0.3 µl) was spotted to three wells on an analysis plate. For each extract, 0.75 µl of 2, 5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (α-Cyano), or 3, 5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) (Waters Corp., Milford, MA) was added to one of the spots. The samples in DHB matrix were analyzed in the 300 to 7500 Dalton (Da) range. Samples in α-Cyano and Sinapinic acid were analyzed in the 500 to 5000 and 500 to 7500 Da range, respectively. Protonated (MH+) peptide masses were monoisotopically resolved in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). Calibration mixture 2 was used as the external calibrant (Sequazyme™ Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) for the analysis. GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical trypsin digest of the CP4 EPSPS protein sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Known autocatalytic fragments from trypsin digestion and apparent modifications were identified in the raw data. The list of experimental masses was then compared to the theoretical list from the GPMAW software. Those experimental masses within 1 Da of a theoretical mass were matched. All matching masses were tallied and a coverage map was generated for the mass fingerprint. The tryptic mass fingerprint coverage was considered acceptable if ≥ 40 % of the protein sequence was identified by matching experimental masses observed for the tryptic peptide fragments to the expected masses for the fragments.

B.6. MALDI-TOF Mass Analysis of MON 87427-produced CP4 EPSPS Protein

MALDI-TOF mass spectrometry was used to further characterize the MON 87427-produced CP4 EPSPS protein. Prior to MALDI-TOF MS analysis, an ethanol precipitation was performed to concentrate the MON 87427-produced CP4 EPSPS protein sample and remove buffer components that interfere with the MALDI-TOF MS analysis. The precipitated protein was re-suspended in 5 µl 60% formic acid. A portion of the MON 87427-produced CP4 EPSPS protein sample, and a BSA protein standard (0.3 µl each), were spotted on an analysis plate, mixed with 0.75 µl of Sinapinic acid solution containing 0.3% TFA and air-dried. Mass spectral analysis of the MON 87427-produced CP4 EPSPS protein was performed using an Applied Biosystems Voyager DE™ Pro

Biospectrometry™ Workstation MALDI-TOF MS instrument with the supplied Data Explorer software (version 4.0.0.0, Foster City, CA). Mass calibration of the instrument was performed using the BSA protein standard. The sample was analyzed in the 2,000 to 100,000 Da range using 150 shots at a laser intensity setting of 3316 (unit-less MALDI-TOF instrument specific value). Average protonated (MH+) protein masses were observed in linear mode (Aebersold, 1993). GPMAW32 software (Lighthouse Data, Odense M, Denmark) was used to generate a theoretical mass of the expected CP4 EPSPS protein sequence based upon the nucleotide sequence. The mass of the MON 87427-produced CP4 EPSPS protein was reported as an average of three separate mass spectral acquisitions.

B.7. Immunoblot Analysis-Immunoreactivity

Western blot analysis was performed to confirm the identity of the CP4 EPSPS protein purified from grain of MON 87427 and to compare the immunoreactivity of the MON 87427- and *E. coli*-produced proteins.

The MON 87427- and *E. coli*-produced CP4 EPSPS proteins were analyzed concurrently on the same gel using three loadings of 1, 2, and 3 ng. Loadings of the three concentrations of the test and reference proteins were made in duplicate on the gel. Aliquots of each protein were diluted in water and 5X Laemmli Buffer (LB) [312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8], heated at ~99°C for 3 min, and applied to a 15 well pre-cast Tris-glycine 4 - 20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA). Pre-stained molecular weight markers (Precision Plus Protein Standards Dual color; Bio-Rad, Hercules, CA) were loaded in parallel to verify electrotransfer of the proteins to the membrane and to estimate the size of the immunoreactive bands observed. Electrophoresis was performed at a constant 150 V for 1.5 h. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 100 min at a constant 25 V. After electrotransfer, the membrane was blocked for 1 h with 5% (w/v) non-fat dried milk (NFDM) in 1X phosphate buffered saline containing 0.05% (v/v) Tween-20 (PBST). The membrane was then probed with a 1:1000 dilution of goat anti-CP4 EPSPS antibody (lot 10000787) in 5% (w/v) NFDM in PBST for 1 h. Excess antibody was removed using three 10 min washes with PBST. Finally, the membrane was probed with horseradish peroxidase-conjugated rabbit anti-goat IgG (Thermo, Rockford, IL) at a dilution of 1:10,000 in 5% (w/v) NFDM in PBST for 1 h. Excess horseradish peroxidase-conjugate was removed using three 10 min washes with PBST. All incubations were performed at room temperature. Immunoreactive bands were visualized using the Amersham ECL™ Western Blotting Detection Reagents (GE, Healthcare, Piscataway, NJ) with exposure (1, 3, and 5 min) to Amersham Hyperfilm ECL™ (GE, Healthcare, Piscataway, NJ). The film was developed using a Konica SRX-101A automated film processor (Tokyo, Japan).

Quantification of the bands on the blot was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One® software (version 4.4.0, Hercules, CA) using the lane finding and contour tool. The signal intensities of the immunoreactive bands observed for the MON 87427-produced CP4 EPSPS and *E. coli*-produced

CP4 EPSPS proteins migrating at the expected position on the blot film were quantified as "contour quantity" values. The raw data was exported to a Microsoft Excel (2007) file for the pair wise comparison of the average of the load replicates. An average difference was calculated for each comparison to assess the immunoreactivity equivalence.

B.8. Molecular Weight and Purity Estimation SDS-PAGE

An aliquot of the MON 87427-produced CP4 EPSPS protein was mixed with 5X LB to a final total protein concentration of 0.08 µg/µl. Molecular weight markers (Bio-Rad broad-range) and *E. coli*-produced CP4 EPSPS protein were diluted to a final total protein concentration of 0.9 and 0.15 µg/µl, respectively. The MON 87427-produced CP4 EPSPS protein was analyzed in duplicate at 0.75, 1.5, and 2.25 µg protein per lane. The E. coli-produced CP4 EPSPS protein was analyzed at 0.75 µg total protein in a single lane. All samples were heated at ~100 °C for 3 min and loaded onto a 10-well pre-cast Tris glycine 4 - 20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant 150 volts (V) for 95 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) acetic acid for 30 min, stained for 16 h and 40 min with Brilliant Blue G-Colloidal stain (Sigma-Aldrich, St. Louis, MO). The gels were destained for 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and for 6 h and 15 min with 25% (v/v) methanol. Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). The apparent molecular weight of each observed band was estimated from a standard curve generated by the Quantity One software which was based on the molecular weights of the markers and their migration distance on the gel. All visible bands within each lane were quantified using Quantity One software. Apparent molecular weight and purity were reported as an average of all six lanes containing the MON 87427-produced CP4 EPSPS protein.

B.9. Glycosylation Analysis

Glycosylation analysis was used to determine whether the MON 87427-produced CP4 EPSPS protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 87427-produced CP4 EPSPS protein, the *E. coli*-produced CP4 EPSPS protein, and the positive controls, transferrin (GE Healthcare, Piscataway, NJ) and horseradish peroxidase (Sigma-Aldrich, St Louis, MO), were each diluted with water and mixed with 5X LB. These samples were heated at ~98 °C for 3 min, cooled, and each was loaded at approximately 30 and 60 ng per lane on a Tris-glycine 10-well 4 - 20% polyacrylamide gradient mini-gel (Invitrogen, Carlsbad, CA). Precision Plus Protein Dual color Standards (Bio-Rad, Hercules, CA) were also loaded to verify electrotransfer of the proteins to the membrane and CandyCane™ Glycoprotein Molecular Weight Standards (Molecular Probes, Eugene, OR) were loaded as positive controls and markers for molecular weight. Electrophoresis was performed at a constant 150 V for 80 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 105 min at a constant 25 V.

Carbohydrate detection was performed directly on the PVDF membrane at room temperature using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit

(Molecular Probes, Eugene, OR). With this kit, carbohydrate moieties are detected by fluorescence which is produced when Pro-Q Emerald 488 glycoprotein stain reacts with periodate oxidation carbohydrates conjugated to proteins. An image of the final blot containing the fluorescent-labeled glycoproteins was captured using the Bio-Rad PharosFX™ Molecular Imager® System using the Alexa 488 band pass setting and equipped with Quantity One software (version 4.6).

After glycosylation analysis the blot was stained to visualize the proteins present on the membrane. Proteins were stained for 30 sec to 2 min using Coomassie Brilliant Blue R-250 staining solution (Bio-Rad, Hercules, CA) and then destained with 1X destain solution (Bio-Rad, Hercules, CA) for 5 min. After washing with water, the blot was scanned using Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA).

B.10. Functional Activity Assay

Prior to functional activity analysis, both MON 87427- and *E.coli*-produced proteins were diluted to a purity corrected concentration of ~50 µg/ml with a 50 mM HEPES, pH 7.0 buffer. Assays for both proteins were conducted in triplicate. The reactions were performed in 50 mM HEPES (pH 7.0), 0.1 mM ammonium molybdate, 1 mM PEP and 5 mM potassium fluoride with or without 2 mM S3P for 2 min at ~25 °C. The reactions were initiated by the addition of PEP. After 2 min, the reactions were quenched with malachite green (phosphate assay reagent) and then fixed with 33% (w/v) sodium citrate. A standard curve was prepared using 0 to 10 nmoles of inorganic phosphate in water treated with the phosphate assay reagent and 33% (w/v) sodium citrate. The absorbance of each reaction and each standard was measured in duplicate at 660 nm using a PowerWave™ Xi (BioTek, Richmond, VA) microplate reader. The amount of inorganic phosphate released from PEP in each reaction was determined using the standard curve. For CP4 EPSPS protein, the specific activity was defined in unit per mg of protein (U/mg), where a unit (U) is defined as 1 µmole of inorganic phosphate released from PEP per min at 25 °C. Calculations of the specific activities were performed using Microsoft Excel (2007).

References for Appendix B

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Appendix C: Materials and Methods Used for the Analysis of the Levels of CP4 EPSPS Protein in MON 87427

C.1. Materials

Over-season leaf (OSL1-4), grain, pollen, silk, forage, stover, over-season root (OSR1-4), forage-root, senescent root and over-season whole plant (OSWP1-4) tissue samples from MON 87427 were harvested from five field sites in the U.S. during 2008 from plants grown from starting seed lot 10001857. An *E. coli*-produced CP4 EPSPS protein (lot 20-100015) was used as the analytical reference standard.

C.2. Characterization of the Materials

The identity of MON 87427 was confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of MON 87427 event-specific polymerase chain reaction (PCR) analyses were conducted on the harvested grain from each site. The PCR analyses and the resulting Verification of Identities were archived in the Monsanto Regulatory Archives under the starting seed lot numbers.

C.3. Field Design and Tissue Collection

Field trials were initiated during the 2008 planting season to generate MON 87427 samples at various maize growing locations in the U.S. The OSL1-4, grain, pollen, silk, forage, stover, OSR1-4, forage-root, senescent root and OSWP1-4 tissue samples from the following field sites were analyzed: Jackson County, Arkansas (site code ARNE); Jefferson County, Iowa (site code IARL); Stark County, Illinois (site code ILWY); Parke County, Indiana (site code INRC); and York County Nebraska (site code NEYO). The field sites were representative of maize producing regions suitable for maize commercial production. At the ARNE, IARL and ILWY sites, three replicated plots of plants containing MON 87427 were planted using a randomized complete block field design. The NEYO site contained 3 replicated plots, but was not a randomized complete block field design which has no impact on expression analysis. OSL1-4, grain, pollen, silk, forage, stover, OSR1-4, forage-root, senescent root and OSWP1-4 samples were collected from each replicated plot at all field sites. See Table VI-5 for a detailed description of when samples were harvested.

C.4. Tissue Processing and Protein Extraction

All tissue samples were shipped to Monsanto. The processed tissue samples and unprocessed pollen samples were stored in a -80 °C freezer.

CP4 EPSPS protein was extracted from the tissue samples as described in Table C-1. CP4 EPSPS protein was extracted from all grain tissue samples using a Harbil Mixer with the appropriate amount of Tris-borate buffer with L-ascorbic acid and 10 mM deoxycholic acid (TBA with 10 mM DCA) [0.1 M Tris, 0.1 M $Na_2B_4O_7 \cdot 10H_2O$, 0.005 M $MgCl_2 \cdot 6H_2O$, 0.05% (v:v) Tween®-20 at pH 7.8, 0.2% (w:v) L-ascorbic acid and 10 mM DCA]. CP4 EPSPS protein was extracted from all over season leaf, over season root, forage, pollen, silk, forage root, stover, senescent root, and over season

whole plant tissue samples using a Harbil Mixer with the appropriate amount of a phosphate buffered saline 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 Tween 20 (1× PBST) and 0.1% (w/v) bovine serum albumin (BSA) (1× PBST with 0.1% (w/v) BSA). Insoluble material was removed from all tissue extracts using a serum filter (Fisher Scientific, Pittsburgh, PA). The extracts were aliquotted and stored frozen in a -80 °C freezer until ELISA analysis.

Table C-1. Protein Extraction Methods for Tissue Samples

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf[2]	1:100	1X PBST with 0.1% (w/v) BSA
Grain	1:100	1X TBA with 10 mM DCA
Pollen	1:100	1X PBST with 0.1% (w/v) BSA
Silk	1:100	1X PBST with 0.1% (w/v) BSA
Root[3]	1:50	1X PBST with 0.1% (w/v) BSA
Forage[4]	1:100	1X PBST with 0.1% (w/v) BSA

[1]The CP4 EPSPS protein was extracted from each tissue by adding the appropriate volume of CP4 EPSPS Extraction Buffer, and shaking in a Harbil mixer. The extracted sample was clarified using a serum filter.
[2]Over- season leaf (OSL1, OSL2, OSL3, and OSL4).
[3]Over- season root (OSR1, OSR2, OSR3, and OSR4, forage-root, and senescent root).
[4]Forage, stover, and over-season whole plant (OSWP1, OSWP2, OSWP3, OSWP4)

C.5. CP4 EPSPS Antibodies

Mouse monoclonal antibody clone 39B6.1 (IgG2a isotype, kappa light chain; Monsanto lot 7022111) specific for the CP4 EPSPS protein was purified from mouse ascites fluid using Protein-A Sepharose affinity chromatography and was used as the capture antibody in the CP4 EPSPS ELISA. The concentration of the purified IgG was determined to be 2.3 mg/ml by spectrophotometric methods. Production of the 39B6.1 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 20 mM sodium phosphate, 150 mM sodium chloride, and 15 ppm Proclin 300 (Sigma-Aldrich, St. Louis, MO).

The detection reagent was goat anti-CP4 EPSPS antibody, otherwise known as anti-protein 4 (Sigma-Aldrich, catalog number P-5867) conjugated to horseradish peroxidase (HRP).

C.6. CP4 EPSPS ELISA Method

Mouse anti-CP4 EPSPS antibodies were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) to a final concentration of 2.0 µg/ml, and immobilized onto 96-well microtiter plates followed by incubation in a 4 °C refrigerator for >8 hours. Prior to each step in the assay, plates were washed with 1× PBST. CP4 EPSPS protein standard or sample extract was added at 100 µl per well and

incubated for 1 hour at 37 °C. The captured CP4 EPSPS protein was detected by the addition of 100 µl per well of anti-CP4 EPSPS peroxidase conjugate. Plates were developed by adding 100 µl per well of 3,3',5,5' tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantification of the CP4 EPSPS protein was accomplished by interpolation from a CP4 EPSPS protein standard curve that ranged from 0.456 14.6 ng/ml.

C.7. Moisture Analysis

Tissue moisture content was determined using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO). A homogeneous tissue-specific site pool (TSSP) was prepared consisting of at least three test and control samples of a given tissue type grown at a given site. These pools were prepared for all tissue types in this study. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$\text{DWCF} = 1 - \frac{(\text{Mean \% TSSP Moisture})}{(100)}$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fwt) basis into levels reported on a µg/g dry weight (dwt) basis using the following calculation:

$$\text{Protein Level in Dry Weight} = \frac{(\text{Protein Level Fresh Weight})}{(\text{DWCF})}$$

The protein levels (ng/ml) that were reported to be less than or equal to the limit of detection (LOD) or less than the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

C.8. Data Analyses

All CP4 EPSPS ELISA plates were analyzed on a SPECTRAmax Plus 384 or a SPECTRAmax Plus (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. All protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620-655 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GXP version 5.0.1. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was converted to a "µg/g fwt" basis for data that were greater than or equal to the LOQ. This conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values expressed as "µg/g fwt" were also converted to "µg/g dwt" by applying the DWCF. Microsoft Excel 2007 (Version (12.0.6504.5001) SP1 MSO (12.0.6320.5000) Microsoft, Redmond, WA) was used to calculate the CP4 EPSPS protein level in maize tissues. The sample means, standard deviations, and ranges were also calculated by Microsoft Excel 2007.

Any MON 87427 sample extracts that resulted in unexpectedly negative results by ELISA analysis were re-extracted twice for the protein of interest and re-analyzed by ELISA to confirm the results. Samples with confirmed unexpected results were omitted from all calculations. Samples that were not confirmed to be positive were reported as inconclusive and omitted from all calculations.

Appendix D: Western Blot Analysis of CP4 EPSPS Protein in Mature Seed and Leaf of MON 87427 Across Multiple Generations

D.1. Materials

Seed or leaf tissues of MON 87427 were collected from plants of five breeding generations.

D.2. MON 87427 Materials

A summary of the MON 87427 samples and the starting seed lot numbers are listed in the tables below:

Generation	ORION Number	Tissue Type	Starting Seed Virgo/Midas Number
LH198 BC3F3	11216372-005	Leaf	60014785781
LH198 BC3F4	11214238-004	Seed	60070411411
LH198 BC3F6	10009479-005	Seed	60083347483
LH198 BC3F7	10009480-005	Seed	60082580121
(LH198 BC3F7xLH287)F1	10001857-001	Seed	10000001407453561750000 1

D.3. Control Material

The negative control substance was a conventional control in a similar genetic background to MON 87427 (LH198 × HiII). The conventional control does not contain the *cp4 epsps* coding sequence and is not expected to produce the CP4 EPSPS protein. Seed tissues from the conventional control were analyzed.

Control Substance	ORION Number	Starting Seed Virgo Number
Conventional Maize	11214241-004	60070416877

D.4. Characterization of MON 87427 and Control Materials

The identities of the MON 87427 and the conventional control were confirmed by verifying the chain of custody documentation. The identities of the MON 87427 and the

conventional control were further confirmed by analysis of the DNA by Southern Blot fingerprint analysis and archived in Monsanto Regulatory.

D.5. Reference Material

The *E. coli*–produced CP4 EPSPS protein (lot 20 100015) was used as the analytical reference standard for the western blot analysis.

D.6. Methods

Leaf or seed tissue samples from five generations of MON 87427 were analyzed by western blot to demonstrate the presence of CP4 EPSPS protein in the LH198 BC3F3, LH198 BC3F4, LH198 BC3F6, LH198 BC3F7, and (LH198 BC3F7×LH287) F1 generations. Seed tissue from the conventional control was analyzed by western blot to confirm the expected absence of the CP4 EPSPS protein. The presence or absence of CP4 EPSPS protein was determined using a goat anti-CP4 EPSPS polyclonal antibody (lot 047K6082), and the *E. coli*-produced protein standard (lot 20 100015) was used as a reference for molecular weight comparison.

D.7. MON 87427 Tissue Processing

All samples were processed by the Monsanto Sample Management Team in Saint Louis, Missouri. The processed tissue samples were stored in a –80 °C freezer until transferred on dry ice to the analytical facility.

D.8. Extraction

The CP4 EPSPS protein was extracted from processed leaf and seed tissue samples in a 1× PBST and 0.1% (w/v) bovine serum albumin (BSA) buffer. All processed tissues were kept on dry ice during extract preparation. The CP4 EPSPS protein was extracted from each tissue by adding the appropriate volume of extraction buffer, and shaking in a Harbil mixer. Insoluble material was removed from the extracts by using a serum filter. The extracts were aliquoted and stored in a –80 °C freezer until analysis.

D.9. SDS-PAGE

Prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and immunoblotting, MON 87427 and conventional control extracts were diluted 1:2.5 (v/v) in dilution buffer; 1 × PBST, then 1:2 (v/v) using 2× Laemmli Buffer (Bio-Rad, Hercules, CA). Two nanograms of CP4 EPSPS protein reference standard prepared in 1× PBST was loaded onto the gel along with the MON 87427 and conventional control extracts. Sample extracts were loaded on a Novex 4 20% Tris Glycine gradient gel (Invitrogen, Carlsbad, CA), along with the Precision Plus molecular weight marker (Bio-Rad) to demonstrate the transfer of protein to membrane and the Magic Marker molecular weight marker (Invitrogen) to show the molecular weights of the protein. Electrophoresis was conducted at 125 V for approximately 120 minutes in 1× Novex Tris-Glycine SDS running buffer (Invitrogen) until the dye front reached the end of the gel.

D.10. Western Blot Analysis (Immunoblotting)

Proteins separated by SDS PAGE were electrophoretically transferred to 0.45 µm Invitrolon Polyvinylidene Fluoride PVDF membrane (Invitrogen) using 1× Novex Tris-Glycine transfer buffer (Invitrogen) containing 20% methanol. After transfer, non specific sites on the membrane were blocked using 5% (w/v) non-fat dried milk (NFDM) in 1× PBST. The membrane was probed for the presence of the CP4 EPSPS protein with a 1:4000 dilution of purified goat anti-CP4 EPSPS antibody (Lot 047K6082) in 1× PBST with 2% (w/v) NFDM. The membrane was washed three times for 10 minutes each time in 1× PBST to remove unbound antibody. Bound antibody was probed with a 1:1250 dilution of anti-goat IgG antibody conjugated to horseradish peroxidase (HRP, Thermo Scientific, Rockford, IL) in 1× PBST with 2% (w/v) NFDM. The membrane was washed four times for 10 minutes each time in 1× PBST. The SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL) was added to the membrane according to the manufacturers' instructions. The membrane was exposed using the Kodak Gel Logic 2200 imaging system.

Appendix E: Bioinformatics Evaluation of MON 87427

E.1. Bioinformatics Evaluation of the CP4 EPSPS Protein in MON 87427

E.1.1. Sequence Database Preparation

The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP (2010) and was used as provided. The AD_2010 database contains 1,471 sequences.

GenBank protein database, release 175.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2010 database and contains 17,815,538 sequences.

The toxin database is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2010 database and contains 8,448 sequences.

E.1.2. Sequence Database Searches

FASTA analyses using the AD_2010, PRT_2010 and TOX_2010 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The structural similarity of the translated protein sequences to sequences in each database (AD_2010, TOX_2010 and PRT_2010) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (*k-tuple*), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of 1×10^{-5} or less to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also 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 relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 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 is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of the MON 87427 CP4 EPSPS protein sequence to allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2010). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Goodman, et al., 2002; Hileman, et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

E.1.3. Significance of the Alignment

An *E*-score of 1×10^{-5} was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than 1×10^{-5} was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2010 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids amino acids.

E.2. Bioinformatics Evaluation of the Transfer DNA Insert in MON 87427

E.2.1. Methods

E.2.1.1. Database Assembly

The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP (2010) and was used as provided. The AD_2010 database contains 1,471 sequences.

GenBank protein database, release 175.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2010 database and contains 17,815,538 sequences.

The toxin database is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2010 database and contains 8,448 sequences.

E.2.1.2. Translation of Query Sequences

The DNA insert sequence was translated beginning with nucleotide 1, 2 or 3 through the final nucleotide to yield frames 1, 2 or 3, respectively. Likewise, the reverse complement (anti-sense) strand of the above described sequence was translated beginning with nucleotide 1, 2 or 3 through the final nucleotide to yields frame 4, 5, or 6, respectively. All sequences were translated using standard genetic code with DNAStar, version 8.0.2 (13), 412. The resultant amino acid sequences were used to search the AD_2010, PRT_2010 and TOX_2010 databases.

E.2.1.3. Sequence Database Searches

FASTA analyses using the AD_2010, PRT_2010 and TOX_2010 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The structural similarity of the translated protein sequences to sequences in each database (AD_2010, TOX_2010 and PRT_2010) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (*k-tuple*), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of 1×10^{-5} or less to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also 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 relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 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 is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of each putative polypeptide to allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2010). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Goodman et al., 2002;

Hileman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

E.2.1.4. Significance of the Alignment

An *E*-score of 1×10^{-5} was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than 1×10^{-5} was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2010 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids amino acids.

E.2.2. Bioinformatics Evaluation of DNA Sequences Flanking the 5′ and 3′ Junctions of Inserted DNA in MON 87427: Assessment of Putative Polypeptides

E.2.2.1. Sequence Database Preparation

The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from FARRP (2010) and was used as provided. The AD_2010 database contains 1,471 sequences.

GenBank protein database, release 175.0 was downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to herein as the PRT_2010 database and contains 17,815,538 sequences.

The toxin database is a subset of sequences derived from the PRT_2010 database, that was selected using a keyword search and filtered to remove likely non-toxin proteins and proteins that are not relevant to human or animal health. It is referred to herein as the TOX_2010 database and contains 8,448 sequences.

E.2.2.2. Translation of Putative Polypeptides

DNA sequence spanning the 5′ and 3′ junctions of the MON 87427 insertion site was analyzed for translational stop codons (TGA, TAG, TAA). All six reading frames originating or terminating within the MON 87427 T-DNA insert and originating or terminating within the intervening sequence were translated using the standard genetic code from stop codon to stop codon using DNAStar, version 8.0.2 (13), 412. A total of twenty sequences of eight amino acids or greater that spanned the junction(s) were analyzed.

E.2.2.3. Sequence Database Searches

FASTA analyses using the AD_2010, TOX_2010 and PRT_2010 databases were performed on a virtual machine loaded with a SUSE LINUX version 10 operating system and FASTA version 3.4t26 July 7, 2006. The DNA sequence was translated to the amino acid sequence with DNAStar, version 8.0.2 (13), 412 or SeqBuilder 8.0.2 (13). The structural similarity of the translated protein sequences to sequences in each database (AD_2010, TOX_2010, and PRT_2010) was assessed using the FASTA algorithm (Lipman and Pearson, 1985; Pearson and Lipman, 1988).

FASTA comparisons are initiated by aligning the first match of a specific wordsize. The alignment is then extended based on the chosen scoring matrix. Default FASTA comparison parameters for wordsize (*k-tuple*), gap creation penalty and gap extension penalty were used. The expectation threshold (*E*-score) limit was set to one. The *E*-score (expectation score) is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences will need to have an *E*-score of 1×10^{-5} or less to be considered to have significant homology. FASTA comparisons were performed using the BLOSUM50 scoring matrix (Henikoff and Henikoff, 1992). Multiple alignments are made between the query sequence and each sequence in the database with a score calculated for each alignment. Only the top scoring alignment is extensively analyzed for each database sequence. The BLOSUM matrix series (Henikoff and Henikoff, 1992) was derived from a set of aligned, ungapped regions from protein families, called the BLOCKS database. Sequences from each block were clustered based on the percent of identical residues in the alignments (Henikoff and Henikoff, 1996). The BLOSUM50 matrix will identify blocks of conserved residues that are at least 50% identical. BLOSUM50 works well for identifying sequence similarities that include gaps, and thus recognizes distant evolutionary relationships (Pearson, 2000).

If two proteins share sufficient linear sequence similarity and identity, they will also 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 relatedness between homologs varies widely, the data need to be carefully evaluated in order to maximize their potential predictive value. The allergenicity assessment is used to identify known allergens or potentially cross-reactive proteins. While related (homologous) proteins may share 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 is rare at 50% identity and typically requires >70% amino acid identity across the full length of the protein sequences (Aalberse, 2000). A conservative approach is currently applied by which related protein sequences are identified as potentially cross-reactive if linear identity is 35% or greater in an 80 amino acid overlap (Thomas et al., 2005). Such levels of identity are readily detected using FASTA. Additionally, proteins closely related to gliadins or glutenins, the proteins that trigger celiac disease, can be easily identified using FASTA.

In addition to the FASTA comparisons of each putative polypeptide to known allergens (to assess overall structural similarity), an eight amino acid sliding window search was performed. An algorithm was developed to identify whether or not a linearly contiguous match of eight amino acids existed between the query sequence and sequences within the allergen database (AD_2010). This program compares the query sequence to each protein sequence in the allergen database using a sliding-window of eight amino acids; that is, with a seven amino acid overlap relative to the preceding window. While there have been recommendations for using a shorter scanning window (Gendel, 1998; Kleter and Peijnenburg, 2002), only a few studies have actually investigated the ability of six, seven, or eight amino acid search windows to identify allergens (Goodman et al., 2002; Hileman et al., 2002; Stadler and Stadler, 2003). In these studies, randomly or specifically selected protein sequences were used as query sequences in FASTA and six, seven, and eight amino acid window searches against allergen databases. The results demonstrated that searches with six and seven amino acid windows led to high rates of false positive matches between non-allergenic query sequences and allergen database sequences. Additionally, searches with a six or seven amino acid window identified apparently random matches between totally unrelated proteins, such that the matched proteins were not likely to share any structural or sequence similarities that could act as cross-reactive epitopes. These studies concluded that six or seven amino acid sliding-window searches yielded such a high rate of false positive hits that they were of no predictive value. Furthermore, Silvanovich et al. (2006) recently demonstrated the lack of value of six or seven amino acid sliding-window searches in a comprehensive analysis of short peptide match frequencies by analyzing the match frequencies of peptides derived from ~1.95 million published protein sequences. In order to provide the best predictive capability to identify potentially cross-reactive proteins, a window of eight contiguous amino acids is used to represent the smallest immunologically significant sequential, or linear IgE binding epitope (Metcalfe et al., 1996).

E.2.2.4. Significance of the Alignment

An *E*-score of 1×10^{-5} was set as an initial high cut-off value for FASTA alignment significance. Although all alignments were inspected visually, any aligned sequence that yielded an *E*-score less than 1×10^{-5} was analyzed further to determine if such an alignment represented significant sequence homology. Furthermore, FASTA alignments with the AD_2010 database were inspected to determine whether they exceeded the CODEX threshold of 35% or greater identity in 80 or greater amino acids amino acids.

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Appendix F: Methods Used in Assessing Stability of Proteins in Simulated Digestive Fluids

F.1. Materials

Purified *E. coli*-produced CP4 EPSPS protein (Lot 5192245) was used as the test substance. The *E. coli*-produced CP4 EPSPS protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT and 50% glycerol. The *E. coli*-produced CP4 EPSPS protein has a purity of 90% and a concentration of 3.96 mg/ml. The protein was diluted 1.68 mg/ml with PBS before use.

Simulated gastric fluid (SGF) contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH of 1.2. The SGF was prepared using a highly purified form of pepsin.

F.2. Digestion of CP4 EPSPS in Simulated Gastric Fluid (SGF) Method

Digestions were initiated by addition of *E. coli*-produced CP4 EPSPS protein to tubes containing simulated gastric fluid (SGF), where 10 units of pepsin activity were used per 1 µg of total protein. Digestions were incubated at 37 ± 2 ˚C in separate tubes for various durations, and the reactions were quenched by addition of a sodium carbonate solution to each tube. The zero incubation time point (T = 0) was quenched by addition of sodium carbonate solution to SGF prior to addition of the *E. coli*-produced CP4 EPSPS protein. The SGF was assayed before conducting the timed incubations to demonstrate that pepsin was active.

Experimental controls were prepared to demonstrate the stability of *E. coli*-produced CP4 EPSPS protein in the system without pepsin. These controls were incubated for 0 and 60 minutes and were designated with the letter "P" (P0 and P9, respectively). Additionally, experimental controls to characterize the system without *E. coli*-produced CP4 EPSPS protein were also included. These experimental controls were prepared by substituting buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM DTT and 25% glycerol) for *E. coli*-produced CP4 EPSPS protein. These controls were incubated for 0 and 60 minutes and were designated with the letter "N" (N0 and N9).

All quenched specimens were stored in a –80 ºC freezer until analyzed. The digestibility of *E. coli*-produced CP4 EPSPS protein in SGF was assessed using SDS-PAGE gel followed by Brilliant Blue G Colloidal dye (Sigma P/N B-2025) staining and western blotting. Limits of detection (LOD) were determined for the gel staining and western blot methods.

Appendix G: Heat Stability of CP4 EPSPS Protein

The previously characterized *E. coli*-produced CP4 EPSPS protein (lot 10000739) was used as both the test substance and reference protein. As reference protein, CP4 EPSPS protein was maintained at -80 °C until the heat treatment samples were ready for analysis. The reference protein was evaluated along with the heat treatment samples in the functional assay and the SDS-PAGE analysis.

G.1. Heat Treatment

The CP4 EPSPS protein was thawed on wet ice and diluted in 50 mM Tris HCl, pH 7.5, 50 mM KCl, 2 mM DTT, 1 mM benzamidine HCl to a final total protein concentration of 1 mg/ml. Aliquots of 200 µl of the diluted CP4 EPSPS protein were transferred to six tubes. The six aliquots in tubes were maintained on wet ice until the heat treatments were initiated. Five tubes were placed in the appropriate heat treatment conditions (25, 37, 55, 75, or 95 °C, each ± 2 °C) and incubated for 30 ± 1 min. The sixth tube was a control treatment, and was maintained on wet ice throughout the heat treatment incubation period. All temperature-treated samples were returned immediately to wet ice following the incubation period.

Following the heat treatments, 20 µl of each temperature treated sample (including the control treatment) was transferred to a clean tube and mixed with 5 µl of 5× LB (0.312 M Tris HCl, pH 6.8, 10% SDS, 50% glycerol, 3.6 M 2-mercaptoethanol, 0.025% Bromophenol Blue) in preparation for SDS-PAGE analysis. The 20 µl samples were heated at 95 ± 5 °C for 3-5 min, quick frozen by placement on dry ice, and stored at -80 °C until analysis. The remainder of each temperature-treated sample (approximately 180 µl each) was maintained on wet ice and used for functional activity assessment.

G.2. Functional Activity Assay

The CP4 EPSPS functional activity of the heat treatment samples, the control treatment sample, and the reference protein were determined using the functional activity assay described in Appendix B.10. All samples were diluted to total protein concentration of 0.05 mg/ml in 50 mM HEPES, pH 7.0 prior to analysis. Two replicates of each diluted protein sample were used for the analysis.

G.3. SDS-PAGE

The samples prepared above (Section G.1) for SDS-PAGE analysis, were thawed, heated at 95 ± 5 °C for 3-5 min, and loaded on one 4-20% polyacrylamide gradient gel at 0.8 mg total protein/ml. The reference protein was loaded on the same gel at 0.8 mg/ml and at 0.08 mg/ml. Following electrophoresis, gels were stained with Brilliant Blue G Colloidal (Sigma, St. Louis, MO).

After staining, the stability of CP4 EPSPS protein at each heat treatment was evaluated qualitatively. The intensity of the major protein band at 43.8 kDa in the heat treatment

lanes was compared visually to the same band in the lanes with the control treatment, 100% reference protein equivalent, and 10% reference protein equivalent.

Appendix H: Materials and Methods Used for Compositional Analysis of MON 87427 Maize Forage and Grain

H.1. Materials

Forage and grain from MON 87427 (Seed Lot Number 10001857) were evaluated in this study. Forage and grain from the conventional control (LH198 × LH287) was evaluated. The conventional control was a conventional maize hybrid (Seed Lot Number 10001859) with background genetics similar to that of MON 87427 but does not produce the CP4 EPSPS protein.

The commercial references were 12 conventional maize hybrids. The commercial references were distributed across sites (Table H-1).

Table H-1. Commercial Reference Maize Hybrids

Material Name	Seed Lot Number	Field Site Code
Crows C6501	10001546	ARNE
Midwest Genetics 87801	10000934	ARNE
Fielder's Choice 7864	10001319	ARNE
Fontanelle 5797	10001548	ARNE
Asgrow RX708	10001564	IARL
Dekalb DKC60-15	10000950	IARL
Midwest Genetics G7944	10001571	IARL
NC + 4443	10001572	IARL
Asgrow RX715	10000952	ILWY
Dekalb DKC61-50	10001328	ILWY
Midland 7B15	10001545	ILWY
NK N69-P9	10001544	ILWY

H.2. Characterization of the Materials

The identities of MON 87427, the conventional control, and commercial references were confirmed by verifying the chain of custody documentation prior to analysis. To further confirm the identities of MON 87427, the conventional control, and commercial references, event-specific polymerase chain reaction (PCR) analyses were conducted on the harvested grain from each site. The PCR analyses and the resulting Verification of Identities were archived in the Monsanto Regulatory Archives under the starting seed lot numbers.

H.3. Field Production of the Samples

Forage and grain from MON 87427, the conventional control, and the commercial references were collected from replicated plots at three field sites during the 2008 U.S.

growing season. MON 87427, the conventional control, and the commercial references were planted in a randomized complete block design with three replicates at field sites in Arkansas (ARNE), Iowa (IARL), and Illinois (ILWY). The MON 87427 plots were treated with glyphosate applications, between the V2 – V6 maize growth stages at a target rate of 1.0 lb ai/acre. All samples at the field sites were grown under normal agronomic field conditions for their respective geographic regions. Forage was collected at the R5 plant growth stage and grain was collected at physiological maturity. Forage samples were shipped on dry ice and grain was shipped at ambient temperature from the field sites to Monsanto Company (St. Louis, MO). Sub-samples were ground to a powder, stored in a freezer set to maintain -20°C located at Monsanto Company (St. Louis, MO), and then shipped on dry ice to Covance Laboratories Inc. (Madison, WI) for analysis.

H.4. Summary of Analytical Methods

Ground forage and grain samples were analyzed by Covance Laboratories Inc. Upon receipt, the samples were stored in a freezer set to maintain -20°C until their use. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and fat), acid detergent fiber (ADF), neutral detergent fiber (NDF), total dietary fiber (TDF), amino acids, fatty acids (C8-C22), minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), and vitamins [folic acid, niacin, A (β-carotene), B_1, B_2, B_6, and E] in the grain, and proximates, ADF, NDF, calcium and phosphorus in forage. The anti-nutrients assessed in grain included phytic acid and raffinose. Secondary metabolites assessed in grain included furfural, ferulic acid, and p-coumaric acid.

H.4.1. 2-Furaldehyde

The ground sample was extracted with 4% trichloroacetic acid and injected directly on a high-performance liquid chromatography system for quantitation of free furfurals by ultraviolet detection (Albala-Hurtado et al., 1997). The quantitation limit was 0.500 ppm.

Reference Standard:

- Acros, 2-Furaldehyde, 99.7%, Lot Number A0219180

H.4.2. Acid Detergent Fiber

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 removed the fats and pigments. The lignocellulose fraction was collected on the frit and determined gravimetrically (USDA, 1970). The limit of quantitation was 0.100%.

H.4.3. Amino Acid Composition

The following 18 amino acids were analyzed:

Total threonine	Total aspartic acid (including asparagine)
Total serine	Total tyrosine
Total phenylalanine	Total glutamic acid (including glutamine)
Total proline	Total histidine
Total glycine	Total lysine
Total alanine	Total arginine
Total valine	Total tryptophan
Total isoleucine	Total methionine
Total leucine	Total cystine (including cysteine)

The sample was assayed by three methods to obtain the full profile. Tryptophan required a base hydrolysis with sodium hydroxide. The sulfur-containing amino acids required an oxidation with performic acid prior to hydrolysis with hydrochloric acid. Analysis of the samples for the remaining amino acids was accomplished through direct acid hydrolysis with hydrochloric acid. Once hydrolyzed, the individual amino acids were then quantitated using an automated amino acid analyzer (AOAC-International, 2005d). The limit of quantitation was 0.100 mg/g.

Reference Standards:

- Thermo Scientific, K18, 2.5 µmol/mL per constituent (except cystine 1.25 µmol/mL), Lot Number JK126327
- Sigma, L-Tryptophan, 100%, Lot Number 076K0075
- Sigma/BioChemika, L-Cysteic Acid Monohydrate, 99.5% (used as 100%), Lot Number 1305674
- Sigma, L-Methionine Sulfone, 100%, Lot Number 047K1321

H.4.4. Ash

The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash (AOAC-International, 2005a). The limit of quantitation was 0.100%.

H.4.5. Beta Carotene

The sample was saponified and extracted with hexane. The sample was then injected on a reverse phase high-performance liquid chromatography system with ultraviolet light detection. Quantitation was achieved with a linear regression analysis (AOAC-International, 2005j; Quackenbush, 1987). The limit of quantitation for β-carotene was approximately 0.0200 mg/100g.

Reference Standard:

- Sigma-Aldrich, Beta Carotene, Type 1, Purity 96.30% and 94.96% (determined spectrophotometrically), Lot Number 068K2561

H.4.6. Carbohydrates

The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation (USDA, 1973):

% carbohydrates = 100 % - (% protein + % fat + % moisture + % ash)

The limit of quantitation was 0.100%.

H.4.7. Fat by Acid Hydrolysis

The sample was hydrolyzed with hydrochloric acid at an elevated temperature. The fat was extracted with ether and hexane. The extract was evaporated on a steambath, re-dissolved in hexane and filtered through a sodium sulfate column. The hexane extract was then evaporated again on a steambath under nitrogen, dried, and weighed (AOAC-International, 2005g). The limit of quantitation was 0.100%.

H.4.8. Fat by Soxhlet Extraction

The sample was weighed into a cellulose thimble containing sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was then evaporated, dried, and weighed (AOAC-International, 2005m). The limit of quantitation was 0.100%.

H.4.9. Fatty Acid Profile with Trans Fat by GC

The lipid was extracted and saponified with 0.5N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in 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 (AOAC-International, 2005e; AOCS, 1997; AOCS, 2001). The limit of quantitation was 0.00400%.

Reference Standards:

- Nu Chek Prep GLC Reference Standard Hazleton No. 1, *, Lot Number AU18-S
- Nu Chek Prep GLC Reference Standard Hazleton No. 2, *, Lot Number M13-O
- Nu Chek Prep GLC Reference Standard Hazleton No. 3, *, Lot Number MA18-S
- Nu Chek Prep GLC Reference Standard Hazleton No. 4, *, Lot Number JA16-T

- Nu Chek Prep Methyl Gamma Linolenate, used as 100%,
- Lot Number U-63M-JY12-R
- Nu Chek Prep Methyl Tridecanoate, used as 100%, Lot Number N-13M-JA16-T

*Overall purity of the sum of the mixture of components was used as 100%

H.4.10. Folic Acid

The sample was hydrolyzed in a potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis by autoclaving, 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 determined by comparing the growth response of the sample, using the bacteria *Lactobacillus casei*, with the growth response of a folic acid standard. This response was measured turbidimetrically (AOAC-International, 2005n; Infant Formula Council, 1985). The limit of quantitation was 0.0600 µg/g.

Reference Standard:

- USP, Folic acid, 98.9%, Lot Number Q0G151

H.4.11. ICP Emission Spectrometry

The sample was dried, precharred, and ignited overnight in a muffle set to maintain 500°C. The resulting ash was dissolved with nitric acid, treated with hydrochloric acid, evaporated to dryness, and put into a solution of 5% hydrochloric acid. The amount of each element was determined at appropriate wavelengths by comparing the emission of the unknown sample, measured on the inductively coupled plasma optical emission spectrometer, with the emission of the standard solutions (AOAC-International, 2005o).

Reference Standards

Inorganic Ventures Reference Standards and Limits of Quantitation:

Mineral	Lot Numbers	Concentration (µg/ml)	Limit of Quantitation (ppm)
Calcium	C2-MEB290078, C2-MEB289124	200, 1000	20.0
Copper	C2-MEB290078, C2-MEB290079	2, 10	0.50
Iron	C2-MEB290078, C2-MEB290080	10, 50	2.00
Magnesium	C2-MEB290078, C2-MEB290079	50, 250	20.0
Manganese	C2-MEB290078, C2-MEB290079	2, 10	0.30

Mineral	Lot Numbers	Concentration (µg/ml)	Limit of Quantitation (ppm)
Phosphorus	C2-MEB290078, C2-MEB289124	200, 1000	20.0
Potassium	C2-MEB290078, BB11-203K*	200, 10000*	100
Sodium	C2-MEB290078, C2-MEB289124	200, 1000	100
Zinc	C2-MEB290078, C2-MEB290079	10, 50	0.40

*Used SPEX standard for potassium (1000 µg/ml)

H.4.12. Moisture

The sample was dried in a vacuum oven at approximately 100°C to a constant weight. The moisture weight loss was determined and converted to percent moisture (AOAC-International, 2005h). The limit of quantitation was 0.100%.

H.4.13. Neutral Detergent Fiber

The sample was placed in a fritted vessel and washed with a neutral boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. An acetone wash removed the fats and pigments. Hemicellulose, cellulose, and lignin fractions were collected on the frit and determined gravimetrically (AACC, 1998; USDA, 1970). The limit of quantitation was 0.100%.

H.4.14. Niacin

The sample was hydrolyzed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus plantarum*, with the growth response of a niacin standard. This response was measured turbidimetrically (AOAC-International, 2005b). The limit of quantitation was 0.300 µg/g.

Reference Standard:

- USP, Niacin, 99.8%, Lot Number I0E295

H.4.15. p Coumeric Acid and Ferulic Acid

The sample was extracted with methanol using ultrasonication, hydrolyzed using 4N sodium hydroxide, buffered using acetic acid/sodium hydroxide, acidified with 3N hydrochloric acid, and filtered. The levels of p-coumaric and ferulic acids in the extract were determined by reverse phase high-performance liquid chromatography with ultraviolet detection (Hagerman and Nicholson, 1982). The limit of quantitation for the p-coumaric acid and ferulic acid assays was 50.0 ppm.

Reference Standards:

- Acros Organics, 4-Hydroxy-3-methoxycinnamic (ferulic acid), 99.4%,

  Lot Number A0248008

- Acros Organics, p-Hydroxycinnamic acid (coumaric acid), 99.4%,

  Lot Number A0236839

H.4.16. Phytic Acid

The sample was extracted using 0.5M HCl with ultrasonication. Purification and concentration were accomplished on a silica-based anion-exchange column. The sample was analyzed on a polymer high-performance liquid chromatography column PRP-1, 5µm (150 x 4.1mm) with a refractive index detector (Lehrfeld, 1989; Lehrfeld, 1994). The limit of quantitation was 0.100%.

Reference Standard:

- Aldrich, Phytic Acid Dodecasodium Salt Hydrate, 98%, Lot Number 068K0755

H.4.17. Protein

Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a mercury catalyst mixture to form ammonia. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 (AOAC-International, 2005l; Bradstreet, 1965a; b; Kalthoff and Sandell, 1948). The limit of quantitation was 0.100%.

H.4.18. Raffinose

The sample was extracted with deionized water and the extract 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 hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector (Brobst, 1972; Mason and Slover, 1971). The limit of quantitation was 0.0500%.

Reference Standard:

- Sigma, D-(+)-Raffinose Pentahydrate, 95.5% after correction for degree of hydration, Lot Number 037K1059

H.4.19. Total Dietary Fiber

Duplicate samples were gelatinized with α-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 (AOAC-International, 2005f). The limit of quantitation was 1.00%.

H.4.20. Vitamin B_1 (Thiamine Hydrochloride)

The sample was autoclaved under weak acid conditions to extract the thiamine. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamine. The solution was purified on a cation-exchange column. An aliquot was reacted with potassium ferricyanide to convert thiamine to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantitated by comparison to a known standard (AOAC-International, 2005k). The limit of quantitation was 0.01 mg/100g. Results are reported as thiamine hydrochloride.

Reference Standard:

- USP, Thiamine hydrochloride, 95.9% after correction for moisture content,

  Lot Number 01F236

H.4.21. Vitamin B_2

The sample was hydrolyzed with dilute hydrochloric acid and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus casei*, with the growth response of multipoint riboflavin standards. The growth response was measured turbidimetrically (AOAC-International, 2005i; USP, 2005). The limit of quantitation was 0.200 µg/g.

Reference Standard:

- USP, Riboflavin, 100%, Lot Number: N0C021

H.4.22. Vitamin B_6 (Pyridoxine Hydrochloride)

The sample was hydrolyzed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the sample, using the yeast *Saccharomyces carlsbergensis*, with the growth response of a pyridoxine standard. The response was measured turbidimetrically (AOAC-International, 2005c; Atkins et al., 1943). Results are reported as pyridoxine hydrochloride. The limit of quantitation was 0.0700 µg/g.

Reference Standard:

- USP, Pyridoxine hydrochloride, 99.8%, Lot Number: Q0G409

H.4.23. Vitamin E

The product was saponified to break down any fat and release vitamin E. The saponified mixture was extracted with ethyl ether and then quantitated by high-performance liquid chromatography using a silica column (Cort et al., 1983; McMurray et al., 1980; Speek et al., 1985). The limit of quantitation was approximately 0.00500 mg/g.

Reference Standard:

- USP, Alpha Tocopherol, 100%, Lot Number M

H.5. Data Processing and Statistical Analysis

After compositional analyses were performed, data spreadsheets containing individual values for each analysis were sent to Monsanto Company for review. Data were then transferred to Certus International where they were converted into the appropriate units and statistically analyzed. The formulas were used for re-expression of composition data for statistical analysis are listed in Table H-2.

Table H-2. Re-expression Formulas for Statistical Analysis of Composition Data

Component	From (X)	To	Formula[1]
Proximates (excluding Moisture), Fiber, Anti-nutrients	% fw	% dw	X/d
Minerals (Calcium, Magnesium, Phosphorus, Potassium, Sodium)	ppm fw	% dw	$(X/d) \times 10^{-4}$
Grain Minerals (Copper, Iron, Manganese, Zinc)	ppm fw	mg/kg dw	X/d
Vitamin A, Vitamin B_1	mg/100g fw	mg/kg dw	$(X/d) \times 10$
Vitamin E	mg/g fw	mg/kg dw	$(X/d) \times 10^{3}$
Folic Acid, Niacin, Vitamin B_2, Vitamin B_6	µg/g fw	mg/kg dw	X/d
Secondary Metabolites	ppm fw	µg/g dw	X/d
Fatty Acids (FA)	% fw	% total fa	$(100)X_j/\Sigma X$, for each FA_j where ΣX is over all the FA
Amino Acids (AA)	mg/g fw	% dw	$(X/d) \times 10^{-1}$

[1] 'X' is the individual sample value; 'd' is the fraction of the sample that is dry matter.

In order to complete a statistical analysis for a compositional constituent, at least 50% of the values for an analyte had to be greater than the assay limit of quantitation (LOQ). Analytes with more than 50% of observations below the assay LOQ were excluded from summaries and analysis. The following 16 analytes with more than 50% of observations below the assay LOQ were excluded from statistical analysis: 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, 16:1 palmitoleic acid, 17:0 heptadecanoic acid, 17:1

heptadecenoic acid, 18:3 gamma linolenic acid, 20:2 eicosadienoic acid, 20:3 eicosatrienoic acid, 20:4 arachidonic acid, sodium, and furfural.

Otherwise, results below the LOQ were assigned a value equal to one-half the quantitation limit. Five observations for 22:0 behenic acid were assigned a value equal to one-half of the LOQ (0.002 % FW.)

The data were assessed for potential outliers using a studentized PRESS residuals calculation. A PRESS residual is the difference between any value and its value predicted from a statistical model that excludes the data point. The studentized version scales these residuals so that the values tend to have a standard normal distribution when outliers are absent. Thus, most values are expected to be between ± 3. Extreme data points that are also outside of the ± 6 studentized PRESS residual range are considered for exclusion, as outliers, from the final analyses. Six fatty acids from two conventional references at the ILWY site had a PRESS residual values outside of ± 6 range. As none of the identified values were the extreme highest or lowest values within the dataset, these values were not removed from the statistical analysis.

All maize compositional components were statistically analyzed using a mixed model analysis of variance with the SAS MIXED procedure. The three replicated sites were analyzed both separately and combined. Individual replicated site analyses used model (1).

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

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

Combined site analyses used model (2).

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

where Y_{ijk} = unique individual observation, U = overall mean, T_i = material effect, L_j = random location effect, $B(L)_{jk}$ = random block within location effect, LT_{ij} = random location by material interaction effect, and e_{ijk} = residual error.

For each component analysis, mean comparison tests of MON 87427 versus the conventional control were conducted.

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 using the commercial references that are expected to contain, with 95% confidence, 99% of the quantities expressed in the population of commercial references. Each estimate was based upon the average of all observations per unique reference. Because negative quantities are not possible, negative calculated lower tolerance bounds were set to zero.

Table H-3. Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	1.51 (0.029) (1.43 - 1.58)	1.51 (0.029) (1.48 - 1.57)	0.00040 (0.039) (-0.047 - 0.042)	-0.090, 0.091	0.992	1.13, 1.97 (1.18 - 1.82)
Carbohydrates	84.46 (0.29) (84.03 - 84.78)	84.73 (0.29) (84.16 - 85.12)	-0.27 (0.41) (-0.87 - 0.62)	-1.22, 0.69	0.537	80.77, 89.46 (82.26 - 87.17)
Moisture (% fw)	11.40 (0.16) (11.20 - 11.70)	11.60 (0.16) (11.30 - 11.90)	-0.20 (0.16) (-0.30 - -0.10)	-0.56, 0.16	0.233	7.56, 14.80 (9.31 - 12.70)
Protein	10.84 (0.33) (10.47 - 11.33)	10.22 (0.33) (9.91 - 10.62)	0.62 (0.47) (-0.15 - 1.20)	-0.45, 1.70	0.217	5.79, 13.43 (8.07 - 12.13)
Total Fat	3.18 (0.11) (3.13 - 3.23)	3.54 (0.11) (3.47 - 3.65)	-0.36 (0.16) (-0.52 - -0.24)	-0.72, 0.0014	0.050	2.12, 5.35 (2.90 - 4.30)
Fiber (% dw)						
Acid Detergent Fiber	3.34 (0.16) (3.15 - 3.49)	3.41 (0.16) (3.27 - 3.54)	-0.064 (0.21) (-0.27 - 0.22)	-0.54, 0.41	0.766	1.84, 4.39 (2.29 - 4.27)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	10.41 (0.22) (10.16 - 10.92)	10.88 (0.22) (10.32 - 11.27)	-0.47 (0.30) (-0.90 - -0.16)	-1.17, 0.23	0.162	5.69, 11.81 (7.06 - 10.66)
Total Dietary Fiber	13.28 (0.18) (13.14 - 13.51)	13.23 (0.18) (12.67 - 13.75)	0.046 (0.25) (-0.24 - 0.52)	-0.53, 0.63	0.860	8.67, 15.32 (10.25 - 14.30)
Amino Acid (% dw)						
Alanine	0.81 (0.029) (0.78 - 0.83)	0.75 (0.029) (0.72 - 0.77)	0.065 (0.042) (0.054 - 0.080)	-0.032, 0.16	0.160	0.32, 1.12 (0.58 - 0.98)
Arginine	0.53 (0.021) (0.49 - 0.55)	0.52 (0.021) (0.48 - 0.56)	0.0067 (0.029) (-0.068 - 0.065)	-0.061, 0.074	0.825	0.24, 0.68 (0.34 - 0.57)
Aspartic Acid	0.70 (0.020) (0.68 - 0.71)	0.65 (0.020) (0.62 - 0.68)	0.049 (0.028) (0.036 - 0.056)	-0.017, 0.11	0.126	0.34, 0.92 (0.52 - 0.78)
Cystine	0.26 (0.0055) (0.25 - 0.27)	0.25 (0.0055) (0.24 - 0.25)	0.013 (0.0078) (0.0054 - 0.020)	-0.0053, 0.031	0.142	0.14, 0.30 (0.18 - 0.26)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Glutamic Acid	1.99 (0.076) (1.90 - 2.04)	1.86 (0.076) (1.78 - 1.92)	0.14 (0.11) (0.12 - 0.17)	-0.11, 0.38	0.244	0.77, 2.84 (1.46 - 2.49)
Glycine	0.43 (0.0087) (0.42 - 0.43)	0.40 (0.0087) (0.39 - 0.42)	0.022 (0.012) (0.011 - 0.032)	-0.0067, 0.050	0.116	0.23, 0.52 (0.32 - 0.43)
Histidine	0.31 (0.0084) (0.30 - 0.31)	0.30 (0.0084) (0.28 - 0.31)	0.011 (0.012) (0.00008 - 0.019)	-0.016, 0.039	0.366	0.16, 0.39 (0.22 - 0.33)
Isoleucine	0.38 (0.015) (0.37 - 0.40)	0.36 (0.015) (0.33 - 0.37)	0.029 (0.021) (0.022 - 0.035)	-0.019, 0.077	0.195	0.16, 0.53 (0.27 - 0.46)
Leucine	1.32 (0.058) (1.24 - 1.36)	1.22 (0.058) (1.16 - 1.27)	0.099 (0.082) (0.078 - 0.13)	-0.091, 0.29	0.264	0.43, 1.95 (0.93 - 1.69)
Lysine	0.33 (0.0055) (0.32 - 0.33)	0.32 (0.0055) (0.31 - 0.33)	0.011 (0.0078) (-0.0011 - 0.019)	-0.0075, 0.029	0.215	0.19, 0.40 (0.26 - 0.34)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Methionine	0.29 (0.0052) (0.28 - 0.29)	0.27 (0.0052) (0.26 - 0.27)	0.017 (0.0073) (0.011 - 0.024)	0.00064, 0.034	0.043	0.11, 0.29 (0.17 - 0.25)
Phenylalanine	0.55 (0.020) (0.53 - 0.57)	0.51 (0.020) (0.49 - 0.53)	0.038 (0.029) (0.026 - 0.049)	-0.028, 0.10	0.220	0.23, 0.75 (0.39 - 0.66)
Proline	0.96 (0.033) (0.91 - 0.98)	0.91 (0.033) (0.86 - 0.95)	0.050 (0.047) (0.029 - 0.073)	-0.059, 0.16	0.318	0.40, 1.24 (0.66 - 1.07)
Serine	0.51 (0.018) (0.49 - 0.52)	0.48 (0.018) (0.46 - 0.50)	0.029 (0.025) (0.015 - 0.052)	-0.029, 0.088	0.280	0.24, 0.66 (0.38 - 0.59)
Threonine	0.38 (0.010) (0.38 - 0.39)	0.36 (0.010) (0.35 - 0.37)	0.026 (0.014) (0.015 - 0.033)	-0.0072, 0.059	0.108	0.20, 0.46 (0.28 - 0.41)
Tryptophan	0.062 (0.0019) (0.059 - 0.064)	0.052 (0.0019) (0.051 - 0.053)	0.010 (0.0027) (0.0061 - 0.013)	0.0037, 0.016	0.006	0.032, 0.069 (0.039 - 0.063)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Tyrosine	0.33 (0.038) (0.25 - 0.38)	0.32 (0.038) (0.25 - 0.36)	0.0090 (0.054) (-0.12 - 0.11)	-0.12, 0.13	0.872	0.077, 0.45 (0.11 - 0.43)
Valine	0.53 (0.017) (0.51 - 0.54)	0.49 (0.017) (0.46 - 0.51)	0.032 (0.023) (0.021 - 0.041)	-0.022, 0.086	0.210	0.25, 0.67 (0.38 - 0.58)
Fatty Acid (% total FA)						
16:0 Palmitic	11.49 (0.056) (11.47 - 11.52)	10.99 (0.056) (10.88 - 11.08)	0.50 (0.080) (0.38 - 0.59)	0.31, 0.68	<0.001	6.42, 15.23 (9.13 - 12.33)
18:0 Stearic	2.17 (0.021) (2.16 - 2.17)	2.04 (0.021) (1.99 - 2.07)	0.13 (0.030) (0.093 - 0.18)	0.063, 0.20	0.002	0.87, 2.88 (1.54 - 2.38)
18:1 Oleic	26.34 (0.14) (26.16 - 26.62)	25.35 (0.14) (25.06 - 25.71)	1.00 (0.17) (0.88 - 1.20)	0.61, 1.38	<0.001	11.30, 43.27 (21.39 - 34.71)
18:2 Linoleic	57.94 (0.16) (57.61 - 58.13)	59.56 (0.16) (59.18 - 59.82)	-1.62 (0.21) (-1.69 - -1.57)	-2.11, -1.13	<0.001	41.35, 74.78 (49.38 - 63.16)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% total FA)						
18:3 Linolenic	1.15 (0.014) (1.13 - 1.17)	1.19 (0.014) (1.18 - 1.22)	-0.047 (0.018) (-0.088 - -0.017)	-0.089, -0.0047	0.033	0.78, 1.52 (0.97 - 1.35)
20:0 Arachidic	0.48 (0.0035) (0.47 - 0.48)	0.46 (0.0035) (0.45 - 0.46)	0.021 (0.0050) (0.0092 - 0.030)	0.0096, 0.033	0.002	0.15, 0.67 (0.32 - 0.53)
20:1 Eicosenoic	0.22 (0.0024) (0.22 - 0.23)	0.23 (0.0024) (0.22 - 0.23)	-0.0013 (0.0034) (-0.0045 - 0.0025)	-0.0091, 0.0065	0.711	0.12, 0.36 (0.21 - 0.31)
22:0 Behenic	0.21 (0.0042) (0.21 - 0.23)	0.19 (0.0042) (0.18 - 0.20)	0.021 (0.0059) (0.0054 - 0.031)	0.0075, 0.035	0.007	0, 0.32 (0.057 - 0.23)
Mineral						
Calcium (% dw)	0.0077 (0.00024) (0.0075 - 0.0079)	0.0067 (0.00024) (0.0060 - 0.0076)	0.00095 (0.00034) (-0.00009 - 0.0017)	0.00016, 0.0017	0.024	0.0019, 0.0076 (0.0038 - 0.0068)
Copper (mg/kg dw)	1.86 (0.074) (1.59 - 2.07)	1.84 (0.074) (1.78 - 1.89)	0.022 (0.10) (-0.19 - 0.18)	-0.22, 0.26	0.835	0.17, 3.48 (1.10 - 2.62)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Iron (mg/kg dw)	24.12 (0.84) (23.45 - 24.69)	23.57 (0.84) (22.10 - 25.57)	0.55 (1.19) (-2.12 - 2.11)	-2.20, 3.29	0.657	11.42, 28.01 (16.55 - 24.10)
Magnesium (% dw)	0.13 (0.0041) (0.13 - 0.14)	0.12 (0.0041) (0.12 - 0.13)	0.0057 (0.0058) (-0.00043 - 0.010)	-0.0076, 0.019	0.348	0.080, 0.16 (0.11 - 0.15)
Manganese (mg/kg dw)	8.74 (0.27) (8.42 - 9.31)	8.86 (0.27) (8.33 - 9.31)	-0.12 (0.38) (-0.46 - 0.092)	-1.00, 0.76	0.760	0, 12.67 (4.00 - 9.17)
Phosphorus (% dw)	0.33 (0.0070) (0.33 - 0.34)	0.34 (0.0070) (0.33 - 0.35)	-0.0060 (0.0099) (-0.020 - 0.0053)	-0.029, 0.017	0.558	0.24, 0.42 (0.28 - 0.37)
Potassium (% dw)	0.40 (0.0086) (0.39 - 0.40)	0.40 (0.0086) (0.39 - 0.41)	-0.0035 (0.012) (-0.013 - 0.0029)	-0.032, 0.024	0.777	0.24, 0.54 (0.33 - 0.46)
Zinc (mg/kg dw)	23.24 (0.63) (21.98 - 25.42)	22.06 (0.63) (21.65 - 22.40)	1.18 (0.89) (-0.41 - 3.29)	-0.89, 3.24	0.224	11.46, 30.37 (17.30 - 25.45)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Folic Acid	0.38 (0.025) (0.34 - 0.43)	0.41 (0.025) (0.35 - 0.47)	-0.030 (0.036) (-0.097 - 0.073)	-0.11, 0.052	0.423	0.11, 0.61 (0.24 - 0.57)
Niacin	25.77 (2.47) (24.92 - 27.18)	26.05 (2.47) (24.52 - 28.52)	-0.28 (3.49) (-3.30 - 2.66)	-8.33, 7.78	0.938	7.89, 49.83 (20.63 - 43.08)
Vitamin A	0.95 (0.056) (0.88 - 0.99)	0.87 (0.056) (0.76 - 0.98)	0.078 (0.079) (0.013 - 0.21)	-0.10, 0.26	0.349	0.38, 1.68 (0.58 - 1.50)
Vitamin B1	2.90 (0.14) (2.83 - 2.93)	2.53 (0.14) (2.48 - 2.60)	0.37 (0.20) (0.33 - 0.45)	-0.085, 0.83	0.097	2.21, 3.65 (2.41 - 3.48)
Vitamin B2	3.27 (0.17) (3.05 - 3.56)	2.36 (0.17) (2.18 - 2.58)	0.91 (0.23) (0.62 - 1.23)	0.38, 1.43	0.004	0, 4.47 (1.28 - 3.29)
Vitamin B6	8.50 (0.31) (8.21 - 8.69)	8.92 (0.31) (8.23 - 9.61)	-0.42 (0.45) (-1.40 - 0.36)	-1.45, 0.60	0.367	2.57, 12.07 (5.24 - 10.29)

Table H-3 (continued). Statistical Summary of Site ARNE Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Vitamin E	16.71 (0.85) (16.01 - 17.44)	17.76 (0.85) (17.47 - 18.10)	-1.06 (1.20) (-2.09 - -0.27)	-3.82, 1.71	0.405	0, 25.61 (6.67 - 17.34)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the non near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table H-4. Statistical Summary of Site ARNE Grain Anti-nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dw)						
Phytic Acid	0.89 (0.029) (0.87 - 0.89)	0.97 (0.029) (0.94 - 1.00)	-0.080 (0.036) (-0.12 - -0.054)	-0.16, 0.0038	0.058	0.73, 1.23 (0.82 - 1.07)
Raffinose	0.11 (0.0066) (0.11 - 0.11)	0.13 (0.0066) (0.13 - 0.14)	-0.024 (0.0094) (-0.028 - -0.023)	-0.046, -0.0028	0.031	0.024, 0.29 (0.092 - 0.21)

[1]dw = dry weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table H-5. Statistical Summary of Site ARNE Grain Secondary Metabolite Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Secondary Metabolite (µg/g dw)						
Ferulic Acid	2475.67 (73.70) (2342.34 - 2559.19)	2416.91 (73.70) (2315.55 - 2500.00)	58.76 (104.23) (-92.83 - 209.93)	-181.59, 299.11	0.588	1070.41, 2955.86 (1588.35 - 2630.98)
p-Coumaric Acid	243.80 (9.78) (227.48 - 260.43)	245.08 (9.78) (233.83 - 252.26)	-1.28 (11.70) (-21.68 - 9.66)	-28.27, 25.71	0.915	58.74, 313.97 (124.16 - 250.30)

[1]dw = dry weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table H-6. Statistical Summary of Site ARNE Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.60 (0.27) (4.39 - 4.76)	4.56 (0.27) (3.99 - 5.09)	0.043 (0.34) (-0.33 - 0.66)	-0.74, 0.82	0.901	2.66, 6.48 (3.70 - 5.95)
Carbohydrates	87.05 (0.78) (86.68 - 87.25)	86.93 (0.78) (85.44 - 88.52)	0.12 (1.11) (-1.27 - 1.24)	-2.43, 2.67	0.915	80.13, 94.05 (83.23 - 90.37)
Moisture (% fw)	70.73 (1.29) (68.50 - 73.10)	69.37 (1.29) (67.40 - 72.30)	1.37 (1.82) (-3.80 - 5.70)	-2.83, 5.57	0.474	51.70, 86.22 (61.00 - 76.00)
Protein	6.84 (0.55) (6.65 - 7.02)	6.40 (0.55) (5.40 - 7.18)	0.44 (0.72) (-0.17 - 1.26)	-1.22, 2.10	0.560	1.34, 11.57 (4.37 - 9.31)
Total Fat	1.52 (0.28) (1.45 - 1.56)	2.12 (0.28) (1.98 - 2.28)	-0.60 (0.35) (-0.74 - -0.42)	-1.40, 0.20	0.120	0.44, 3.33 (0.78 - 3.16)
Fiber (% dw)						
Acid Detergent Fiber	24.14 (1.62) (21.78 - 26.97)	27.26 (1.62) (25.49 - 28.84)	-3.11 (2.13) (-7.07 - -0.46)	-8.02, 1.79	0.181	14.84, 38.51 (21.33 - 35.92)

Table H-6 (continued). Statistical Summary of Site ARNE Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	38.71 (1.43) (37.17 - 41.50)	34.61 (1.43) (33.07 - 36.71)	4.10 (1.92) (3.13 - 4.79)	-0.33, 8.54	0.065	25.12, 54.99 (29.68 - 60.16)
Mineral						
Calcium (% dw)	0.19 (0.011) (0.18 - 0.21)	0.18 (0.011) (0.15 - 0.20)	0.018 (0.013) (-0.0016 - 0.034)	-0.012, 0.047	0.207	0.075, 0.29 (0.10 - 0.24)
Phosphorus (% dw)	0.24 (0.016) (0.20 - 0.27)	0.22 (0.016) (0.19 - 0.23)	0.022 (0.020) (0.013 - 0.038)	-0.025, 0.068	0.316	0.063, 0.37 (0.16 - 0.31)

[1]dw = dry weight; fw = fresh weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table H-7. Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	1.57 (0.069) (1.46 - 1.76)	1.56 (0.084) (1.53 - 1.60)	0.0092 (0.11) (-0.14 - -0.031)	-0.25, 0.27	0.934	1.13, 1.97 (1.18 - 1.82)
Carbohydrates	84.24 (0.32) (83.60 - 84.96)	83.11 (0.39) (82.96 - 83.33)	1.13 (0.47) (0.63 - 1.63)	0.017, 2.25	0.047	80.77, 89.46 (82.26 - 87.17)
Moisture (% fw)	10.93 (0.14) (10.90 - 11.00)	10.40 (0.17) (10.20 - 10.60)	0.53 (0.22) (0.30 - 0.80)	0.020, 1.05	0.043	7.56, 14.80 (9.31 - 12.70)
Protein	10.60 (0.30) (9.91 - 11.35)	11.73 (0.35) (11.41 - 11.92)	-1.13 (0.38) (-1.50 - -0.57)	-2.02, -0.24	0.019	5.79, 13.43 (8.07 - 12.13)
Total Fat	3.60 (0.058) (3.56 - 3.66)	3.65 (0.071) (3.59 - 3.71)	-0.046 (0.092) (-0.055 - 0.0098)	-0.26, 0.17	0.635	2.12, 5.35 (2.90 - 4.30)
Fiber (% dw)						
Acid Detergent Fiber	2.98 (0.22) (2.67 - 3.31)	3.13 (0.27) (3.02 - 3.23)	-0.15 (0.34) (-0.063 - 0.078)	-0.96, 0.67	0.684	1.84, 4.39 (2.29 - 4.27)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	9.34 (0.13) (9.17 - 9.43)	9.26 (0.16) (9.21 - 9.26)	0.079 (0.19) (-0.092 - 0.22)	-0.38, 0.54	0.693	5.69, 11.81 (7.06 - 10.66)
Total Dietary Fiber	12.46 (0.29) (12.13 - 12.68)	12.72 (0.35) (12.64 - 12.81)	-0.26 (0.46) (-0.67 - -0.070)	-1.34, 0.82	0.585	8.67, 15.32 (10.25 - 14.30)
Amino Acid (% dw)						
Alanine	0.82 (0.035) (0.74 - 0.89)	0.90 (0.042) (0.89 - 0.90)	-0.084 (0.051) (-0.15 - -0.017)	-0.21, 0.037	0.143	0.32, 1.12 (0.58 - 0.98)
Arginine	0.48 (0.015) (0.45 - 0.49)	0.53 (0.017) (0.51 - 0.53)	-0.048 (0.018) (-0.079 - -0.016)	-0.091, -0.0051	0.033	0.24, 0.68 (0.34 - 0.57)
Aspartic Acid	0.67 (0.026) (0.62 - 0.71)	0.73 (0.031) (0.72 - 0.73)	-0.061 (0.038) (-0.099 - -0.024)	-0.15, 0.030	0.156	0.34, 0.92 (0.52 - 0.78)
Cystine	0.24 (0.0036) (0.24 - 0.25)	0.26 (0.0042) (0.26 - 0.26)	-0.015 (0.0046) (-0.015 - -0.011)	-0.026, -0.0044	0.012	0.14, 0.30 (0.18 - 0.26)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Glutamic Acid	2.05 (0.092) (1.85 - 2.24)	2.26 (0.11) (2.20 - 2.28)	-0.21 (0.13) (-0.35 - -0.047)	-0.53, 0.11	0.161	0.77, 2.84 (1.46 - 2.49)
Glycine	0.38 (0.012) (0.36 - 0.40)	0.39 (0.014) (0.39 - 0.39)	-0.018 (0.018) (-0.038 - 0.0035)	-0.060, 0.024	0.344	0.23, 0.52 (0.32 - 0.43)
Histidine	0.31 (0.012) (0.29 - 0.34)	0.34 (0.015) (0.33 - 0.34)	-0.022 (0.018) (-0.045 - 0.0030)	-0.065, 0.020	0.251	0.16, 0.39 (0.22 - 0.33)
Isoleucine	0.38 (0.019) (0.34 - 0.42)	0.42 (0.023) (0.41 - 0.42)	-0.036 (0.029) (-0.081 - 0.0093)	-0.10, 0.032	0.249	0.16, 0.53 (0.27 - 0.46)
Leucine	1.38 (0.065) (1.23 - 1.52)	1.55 (0.079) (1.52 - 1.56)	-0.17 (0.095) (-0.29 - -0.042)	-0.40, 0.052	0.112	0.43, 1.95 (0.93 - 1.69)
Lysine	0.29 (0.0093) (0.29 - 0.30)	0.31 (0.011) (0.31 - 0.31)	-0.015 (0.015) (-0.024 - -0.0085)	-0.049, 0.020	0.346	0.19, 0.40 (0.26 - 0.34)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Methionine	0.23 (0.0038) (0.22 - 0.23)	0.25 (0.0046) (0.24 - 0.25)	-0.018 (0.0059) (-0.015 - -0.013)	-0.032, -0.0040	0.018	0.11, 0.29 (0.17 - 0.25)
Phenylalanine	0.55 (0.025) (0.50 - 0.60)	0.61 (0.030) (0.60 - 0.61)	-0.059 (0.038) (-0.10 - -0.011)	-0.15, 0.030	0.162	0.23, 0.75 (0.39 - 0.66)
Proline	1.00 (0.039) (0.91 - 1.08)	1.07 (0.047) (1.06 - 1.06)	-0.074 (0.055) (-0.15 - 0.023)	-0.20, 0.055	0.217	0.40, 1.24 (0.66 - 1.07)
Serine	0.49 (0.017) (0.46 - 0.51)	0.56 (0.021) (0.52 - 0.58)	-0.062 (0.024) (-0.063 - -0.062)	-0.12, -0.0047	0.037	0.24, 0.66 (0.38 - 0.59)
Threonine	0.36 (0.010) (0.34 - 0.37)	0.38 (0.013) (0.38 - 0.39)	-0.029 (0.016) (-0.042 - -0.016)	-0.066, 0.0085	0.109	0.20, 0.46 (0.28 - 0.41)
Tryptophan	0.053 (0.0035) (0.049 - 0.058)	0.057 (0.0043) (0.050 - 0.065)	-0.0049 (0.0056) (-0.015 - 0.0080)	-0.018, 0.0083	0.408	0.032, 0.069 (0.039 - 0.063)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Tyrosine	0.32 (0.023) (0.28 - 0.35)	0.36 (0.029) (0.32 - 0.39)	-0.039 (0.036) (-0.11 - 0.00062)	-0.13, 0.047	0.314	0.077, 0.45 (0.11 - 0.43)
Valine	0.51 (0.022) (0.47 - 0.55)	0.55 (0.027) (0.54 - 0.56)	-0.039 (0.035) (-0.089 - 0.010)	-0.12, 0.043	0.298	0.25, 0.67 (0.38 - 0.58)
Fatty Acid (% total FA)						
16:0 Palmitic	10.72 (0.053) (10.58 - 10.85)	10.44 (0.063) (10.44 - 10.46)	0.28 (0.074) (0.14 - 0.39)	0.10, 0.45	0.007	6.42, 15.23 (9.13 - 12.33)
18:0 Stearic	1.84 (0.018) (1.81 - 1.86)	1.79 (0.022) (1.77 - 1.79)	0.052 (0.027) (0.034 - 0.054)	-0.012, 0.12	0.095	0.87, 2.88 (1.54 - 2.38)
18:1 Oleic	22.91 (0.13) (22.84 - 22.98)	21.95 (0.16) (21.74 - 22.15)	0.97 (0.20) (0.83 - 1.10)	0.49, 1.45	0.002	11.30, 43.27 (21.39 - 34.71)
18:2 Linoleic	62.57 (0.14) (62.49 - 62.70)	63.90 (0.17) (63.72 - 64.09)	-1.34 (0.22) (-1.59 - -1.01)	-1.87, -0.81	<0.001	41.35, 74.78 (49.38 - 63.16)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% total FA)						
18:3 Linolenic	1.24 (0.0078) (1.22 - 1.26)	1.20 (0.0096) (1.20 - 1.20)	0.040 (0.012) (0.019 - 0.043)	0.011, 0.069	0.014	0.78, 1.52 (0.97 - 1.35)
20:0 Arachidic	0.38 (0.0035) (0.37 - 0.39)	0.37 (0.0043) (0.37 - 0.37)	0.011 (0.0055) (0.0017 - 0.010)	-0.0021, 0.024	0.087	0.15, 0.67 (0.32 - 0.53)
20:1 Eicosenoic	0.20 (0.0016) (0.19 - 0.20)	0.20 (0.0020) (0.20 - 0.20)	-0.0016 (0.0026) (-0.0049 - -0.0018)	-0.0077, 0.0045	0.546	0.12, 0.36 (0.21 - 0.31)
22:0 Behenic	0.15 (0.0027) (0.14 - 0.15)	0.15 (0.0033) (0.15 - 0.15)	-0.00002 (0.0040) (-0.0045 - 0.00034)	-0.0094, 0.0093	0.995	0, 0.32 (0.057 - 0.23)
Mineral						
Calcium (% dw)	0.0055 (0.00020) (0.0054 - 0.0057)	0.0049 (0.00024) (0.0046 - 0.0053)	0.00054 (0.00031) (0.00007 - 0.00084)	-0.00019, 0.0013	0.121	0.0019, 0.0076 (0.0038 - 0.0068)
Copper (mg/kg dw)	1.36 (0.18) (1.21 - 1.56)	1.55 (0.23) (1.49 - 1.61)	-0.19 (0.29) (-0.30 - 0.070)	-0.88, 0.50	0.537	0.17, 3.48 (1.10 - 2.62)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Iron (mg/kg dw)	24.21 (0.67) (22.67 - 25.84)	23.52 (0.77) (23.15 - 23.83)	0.70 (0.82) (-0.48 - 2.01)	-1.23, 2.63	0.419	11.42, 28.01 (16.55 - 24.10)
Magnesium (% dw)	0.13 (0.0025) (0.13 - 0.13)	0.13 (0.0030) (0.13 - 0.13)	-0.0050 (0.0036) (-0.0055 - -0.0040)	-0.014, 0.0036	0.208	0.080, 0.16 (0.11 - 0.15)
Manganese (mg/kg dw)	9.35 (0.39) (9.26 - 9.40)	9.78 (0.47) (9.51 - 9.82)	-0.42 (0.56) (-0.43 - -0.11)	-1.76, 0.91	0.477	0, 12.67 (4.00 - 9.17)
Phosphorus (% dw)	0.33 (0.0060) (0.32 - 0.35)	0.34 (0.0073) (0.34 - 0.35)	-0.014 (0.0091) (-0.018 - -0.0067)	-0.035, 0.0076	0.170	0.24, 0.42 (0.28 - 0.37)
Potassium (% dw)	0.38 (0.010) (0.38 - 0.39)	0.40 (0.012) (0.38 - 0.41)	-0.013 (0.016) (-0.029 - -0.0011)	-0.050, 0.023	0.419	0.24, 0.54 (0.33 - 0.46)
Zinc (mg/kg dw)	23.54 (0.55) (22.45 - 24.61)	26.51 (0.67) (24.94 - 28.08)	-2.97 (0.86) (-5.63 - -0.34)	-5.01, -0.93	0.010	11.46, 30.37 (17.30 - 25.45)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Folic Acid	0.36 (0.024) (0.31 - 0.40)	0.45 (0.028) (0.42 - 0.49)	-0.088 (0.029) (-0.088 - -0.058)	-0.16, -0.018	0.020	0.11, 0.61 (0.24 - 0.57)
Niacin	24.74 (1.48) (22.56 - 27.27)	24.08 (1.62) (22.61 - 27.52)	0.65 (1.35) (-0.24 - 1.78)	-2.55, 3.86	0.643	7.89, 49.83 (20.63 - 43.08)
Vitamin A	0.98 (0.042) (0.94 - 1.03)	0.89 (0.052) (0.83 - 0.95)	0.086 (0.067) (-0.0013 - 0.21)	-0.071, 0.24	0.236	0.38, 1.68 (0.58 - 1.50)
Vitamin B1	2.73 (0.17) (2.58 - 3.03)	2.94 (0.21) (2.90 - 3.02)	-0.21 (0.25) (-0.44 - 0.14)	-0.80, 0.38	0.423	2.21, 3.65 (2.41 - 3.48)
Vitamin B2	1.41 (0.13) (1.17 - 1.60)	1.93 (0.16) (1.89 - 1.96)	-0.51 (0.21) (-0.72 - -0.36)	-1.00, -0.024	0.042	0, 4.47 (1.28 - 3.29)
Vitamin B6	7.11 (0.57) (5.91 - 8.29)	7.51 (0.68) (6.51 - 8.14)	-0.39 (0.78) (-0.60 - 0.15)	-2.23, 1.45	0.630	2.57, 12.07 (5.24 - 10.29)

Table H-7 (continued). Statistical Summary of Site IARL Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Vitamin E	11.09 (1.18) (8.48 - 13.58)	10.93 (1.45) (10.67 - 11.19)	0.17 (1.87) (-2.70 - 0.55)	-4.25, 4.59	0.931	0, 25.61 (6.67 - 17.34)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table H-8. Statistical Summary of Site IARL Grain Anti-nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dw)						
Phytic Acid	0.98 (0.025) (0.89 - 1.03)	1.00 (0.030) (0.98 - 1.02)	-0.023 (0.039) (-0.010 - 0.032)	-0.12, 0.070	0.576	0.73, 1.23 (0.82 - 1.07)
Raffinose	0.11 (0.0043) (0.098 - 0.12)	0.11 (0.0051) (0.11 - 0.11)	0.0036 (0.0059) (-0.0073 - 0.013)	-0.010, 0.017	0.560	0.024, 0.29 (0.092 - 0.21)

[1]dw = dry weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table H-9. Statistical Summary of Site IARL Grain Secondary Metabolite Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Secondary Metabolite (µg/g dw)						
Ferulic Acid	2253.00 (92.22) (2188.55 - 2289.56)	2377.76 (112.69) (2293.99 - 2460.85)	-124.75 (141.79) (-171.29 - -13.09)	-460.02, 210.52	0.408	1070.41, 2955.86 (1588.35 - 2630.98)
p-Coumaric Acid	177.78 (10.27) (166.11 - 195.51)	178.58 (12.58) (162.58 - 194.63)	-0.81 (16.14) (-28.53 - 32.92)	-38.97, 37.35	0.961	58.74, 313.97 (124.16 - 250.30)

[1]dw = dry weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table H-10. Statistical Summary of Site IARL Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.81 (0.29) (4.49 - 5.10)	5.58 (0.35) (5.23 - 5.84)	-0.78 (0.43) (-0.74 - -0.74)	-1.81, 0.25	0.116	2.66, 6.48 (3.70 - 5.95)
Carbohydrates	86.46 (0.60) (86.21 - 86.75)	84.12 (0.72) (83.80 - 84.64)	2.34 (0.85) (2.11 - 2.61)	0.32, 4.35	0.029	80.13, 94.05 (83.23 - 90.37)
Moisture (% fw)	69.90 (1.03) (67.70 - 71.20)	74.71 (1.21) (73.60 - 75.00)	-4.81 (1.33) (-5.90 - -4.20)	-7.96, -1.66	0.008	51.70, 86.22 (61.00 - 76.00)
Protein	7.03 (0.40) (6.75 - 7.40)	8.63 (0.49) (8.32 - 8.94)	-1.60 (0.63) (-2.00 - -1.57)	-3.09, -0.12	0.037	1.34, 11.57 (4.37 - 9.31)
Total Fat	1.71 (0.32) (1.57 - 1.82)	1.61 (0.39) (1.19 - 2.04)	0.097 (0.50) (-0.30 - 0.63)	-1.10, 1.29	0.853	0.44, 3.33 (0.78 - 3.16)
Fiber (% dw)						
Acid Detergent Fiber	22.89 (2.31) (21.08 - 24.01)	26.21 (2.83) (20.27 - 32.16)	-3.32 (3.66) (-8.15 - 0.82)	-11.97, 5.32	0.393	14.84, 38.51 (21.33 - 35.92)

Table H-10 (continued). Statistical Summary of Site IARL Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	39.68 (2.27) (37.33 - 42.71)	39.33 (2.78) (38.88 - 39.77)	0.36 (3.59) (-1.55 - -0.76)	-8.12, 8.83	0.923	25.12, 54.99 (29.68 - 60.16)
Mineral						
Calcium (% dw)	0.16 (0.016) (0.14 - 0.18)	0.19 (0.019) (0.18 - 0.20)	-0.033 (0.025) (-0.049 - -0.042)	-0.093, 0.026	0.228	0.075, 0.29 (0.10 - 0.24)
Phosphorus (% dw)	0.27 (0.020) (0.25 - 0.31)	0.29 (0.024) (0.28 - 0.31)	-0.024 (0.031) (-0.063 - -0.031)	-0.098, 0.049	0.456	0.063, 0.37 (0.16 - 0.31)

[1]dw = dry weight; fw = fresh weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table H-11. Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	1.65 (0.053) (1.53 - 1.81)	1.62 (0.053) (1.55 - 1.67)	0.025 (0.067) (-0.051 - 0.14)	-0.13, 0.18	0.716	1.13, 1.97 (1.18 - 1.82)
Carbohydrates	85.94 (0.17) (85.55 - 86.33)	85.58 (0.17) (85.24 - 85.76)	0.36 (0.24) (-0.18 - 1.09)	-0.19, 0.91	0.169	80.77, 89.46 (82.26 - 87.17)
Moisture (% fw)	12.53 (0.21) (12.10 - 13.30)	12.17 (0.21) (11.90 - 12.40)	0.37 (0.29) (-0.30 - 1.10)	-0.31, 1.04	0.245	7.56, 14.80 (9.31 - 12.70)
Protein	8.71 (0.15) (8.46 - 8.86)	8.97 (0.15) (8.62 - 9.19)	-0.26 (0.21) (-0.73 - 0.19)	-0.75, 0.23	0.253	5.79, 13.43 (8.07 - 12.13)
Total Fat	3.72 (0.083) (3.62 - 3.83)	3.84 (0.083) (3.60 - 3.98)	-0.12 (0.12) (-0.33 - 0.11)	-0.39, 0.15	0.333	2.12, 5.35 (2.90 - 4.30)
Fiber (% dw)						
Acid Detergent Fiber	3.78 (0.18) (3.33 - 4.27)	3.05 (0.18) (2.80 - 3.18)	0.73 (0.25) (0.15 - 1.09)	0.15, 1.30	0.020	1.84, 4.39 (2.29 - 4.27)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	10.25 (0.33) (9.77 - 10.97)	10.27 (0.33) (9.99 - 10.59)	-0.014 (0.46) (-0.82 - 0.98)	-1.08, 1.05	0.975	5.69, 11.81 (7.06 - 10.66)
Total Dietary Fiber	13.26 (0.38) (12.63 - 14.35)	13.28 (0.38) (13.13 - 13.44)	-0.022 (0.51) (-0.64 - 1.07)	-1.20, 1.16	0.966	8.67, 15.32 (10.25 - 14.30)
Amino Acid (% dw)						
Alanine	0.62 (0.020) (0.61 - 0.63)	0.63 (0.020) (0.55 - 0.67)	-0.016 (0.029) (-0.067 - 0.075)	-0.082, 0.051	0.603	0.32, 1.12 (0.58 - 0.98)
Arginine	0.42 (0.017) (0.40 - 0.45)	0.43 (0.017) (0.39 - 0.45)	-0.0053 (0.020) (-0.035 - 0.015)	-0.051, 0.040	0.796	0.24, 0.68 (0.34 - 0.57)
Aspartic Acid	0.54 (0.016) (0.54 - 0.55)	0.55 (0.016) (0.48 - 0.59)	-0.0075 (0.022) (-0.049 - 0.064)	-0.059, 0.044	0.745	0.34, 0.92 (0.52 - 0.78)
Cystine	0.22 (0.0041) (0.21 - 0.22)	0.22 (0.0041) (0.21 - 0.23)	-0.0048 (0.0051) (-0.013 - 0.0072)	-0.017, 0.0070	0.375	0.14, 0.30 (0.18 - 0.26)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Glutamic Acid	1.55 (0.053) (1.53 - 1.58)	1.59 (0.053) (1.38 - 1.70)	-0.035 (0.074) (-0.18 - 0.20)	-0.21, 0.14	0.650	0.77, 2.84 (1.46 - 2.49)
Glycine	0.34 (0.0091) (0.34 - 0.35)	0.35 (0.0091) (0.31 - 0.37)	-0.0046 (0.012) (-0.028 - 0.033)	-0.032, 0.023	0.706	0.23, 0.52 (0.32 - 0.43)
Histidine	0.27 (0.0082) (0.27 - 0.27)	0.27 (0.0082) (0.23 - 0.29)	-0.00074 (0.011) (-0.018 - 0.033)	-0.027, 0.025	0.949	0.16, 0.39 (0.22 - 0.33)
Isoleucine	0.29 (0.011) (0.29 - 0.30)	0.30 (0.011) (0.26 - 0.32)	-0.0075 (0.015) (-0.033 - 0.039)	-0.043, 0.028	0.638	0.16, 0.53 (0.27 - 0.46)
Leucine	1.00 (0.036) (0.97 - 1.02)	1.03 (0.036) (0.89 - 1.10)	-0.028 (0.051) (-0.13 - 0.13)	-0.14, 0.088	0.591	0.43, 1.95 (0.93 - 1.69)
Lysine	0.27 (0.0078) (0.27 - 0.27)	0.28 (0.0078) (0.25 - 0.30)	-0.0041 (0.0094) (-0.021 - 0.026)	-0.026, 0.018	0.671	0.19, 0.40 (0.26 - 0.34)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Methionine	0.20 (0.0039) (0.20 - 0.21)	0.20 (0.0039) (0.20 - 0.21)	0.0012 (0.0055) (-0.0089 - 0.012)	-0.011, 0.014	0.829	0.11, 0.29 (0.17 - 0.25)
Phenylalanine	0.42 (0.014) (0.40 - 0.43)	0.43 (0.014) (0.38 - 0.46)	-0.015 (0.020) (-0.063 - 0.052)	-0.062, 0.032	0.474	0.23, 0.75 (0.39 - 0.66)
Proline	0.75 (0.028) (0.74 - 0.77)	0.76 (0.028) (0.65 - 0.83)	-0.015 (0.039) (-0.091 - 0.12)	-0.10, 0.075	0.717	0.40, 1.24 (0.66 - 1.07)
Serine	0.40 (0.014) (0.38 - 0.41)	0.41 (0.014) (0.36 - 0.43)	-0.011 (0.019) (-0.057 - 0.035)	-0.055, 0.033	0.590	0.24, 0.66 (0.38 - 0.59)
Threonine	0.30 (0.0079) (0.29 - 0.30)	0.31 (0.0079) (0.28 - 0.32)	-0.0074 (0.011) (-0.031 - 0.025)	-0.033, 0.018	0.524	0.20, 0.46 (0.28 - 0.41)
Tryptophan	0.047 (0.0030) (0.045 - 0.049)	0.051 (0.0030) (0.042 - 0.056)	-0.0042 (0.0031) (-0.011 - 0.0061)	-0.011, 0.0030	0.215	0.032, 0.069 (0.039 - 0.063)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Tyrosine	0.23 (0.032) (0.18 - 0.28)	0.23 (0.032) (0.21 - 0.24)	0.0053 (0.046) (-0.061 - 0.049)	-0.10, 0.11	0.909	0.077, 0.45 (0.11 - 0.43)
Valine	0.42 (0.014) (0.41 - 0.42)	0.42 (0.014) (0.37 - 0.45)	-0.0077 (0.020) (-0.044 - 0.049)	-0.054, 0.038	0.707	0.25, 0.67 (0.38 - 0.58)
Fatty Acid (% total FA)						
16:0 Palmitic	10.54 (0.054) (10.44 - 10.65)	10.21 (0.054) (10.15 - 10.24)	0.33 (0.056) (0.28 - 0.40)	0.20, 0.46	<0.001	6.42, 15.23 (9.13 - 12.33)
18:0 Stearic	1.90 (0.018) (1.89 - 1.91)	1.88 (0.018) (1.82 - 1.93)	0.021 (0.025) (-0.028 - 0.096)	-0.037, 0.080	0.424	0.87, 2.88 (1.54 - 2.38)
18:1 Oleic	23.58 (0.12) (23.29 - 23.78)	23.24 (0.12) (23.17 - 23.39)	0.34 (0.14) (0.13 - 0.49)	0.012, 0.66	0.043	11.30, 43.27 (21.39 - 34.71)
18:2 Linoleic	62.01 (0.18) (61.68 - 62.32)	62.72 (0.18) (62.45 - 62.92)	-0.71 (0.19) (-0.89 - -0.46)	-1.15, -0.27	0.005	41.35, 74.78 (49.38 - 63.16)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% total FA)						
18:3 Linolenic	1.20 (0.012) (1.17 - 1.22)	1.20 (0.012) (1.19 - 1.21)	0.0051 (0.017) (-0.030 - 0.024)	-0.033, 0.044	0.767	0.78, 1.52 (0.97 - 1.35)
20:0 Arachidic	0.41 (0.0052) (0.40 - 0.42)	0.40 (0.0052) (0.38 - 0.41)	0.015 (0.0071) (-0.0022 - 0.034)	-0.0015, 0.031	0.068	0.15, 0.67 (0.32 - 0.53)
20:1 Eicosenoic	0.21 (0.0019) (0.20 - 0.21)	0.21 (0.0019) (0.21 - 0.21)	0 (0.0027) (-0.0045 - 0.0033)	-0.0062, 0.0062	0.999	0.12, 0.36 (0.21 - 0.31)
22:0 Behenic	0.15 (0.0036) (0.15 - 0.16)	0.15 (0.0036) (0.14 - 0.16)	0.00005 (0.0050) (-0.0099 - 0.016)	-0.011, 0.012	0.992	0, 0.32 (0.057 - 0.23)
Mineral						
Calcium (% dw)	0.0049 (0.00014) (0.0048 - 0.0050)	0.0049 (0.00014) (0.0047 - 0.0052)	0 (0.00019) (-0.00037 - 0.00030)	-0.00045, 0.00045	0.994	0.0019, 0.0076 (0.0038 - 0.0068)
Copper (mg/kg dw)	1.66 (0.093) (1.56 - 1.79)	1.75 (0.093) (1.63 - 1.99)	-0.086 (0.13) (-0.42 - 0.16)	-0.39, 0.22	0.530	0.17, 3.48 (1.10 - 2.62)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Iron (mg/kg dw)	22.51 (0.41) (22.21 - 22.95)	21.84 (0.41) (20.66 - 22.49)	0.66 (0.57) (-0.019 - 1.55)	-0.66, 1.99	0.280	11.42, 28.01 (16.55 - 24.10)
Magnesium (% dw)	0.13 (0.0021) (0.13 - 0.13)	0.13 (0.0021) (0.13 - 0.14)	-0.0017 (0.0028) (-0.0062 - 0.0039)	-0.0082, 0.0047	0.550	0.080, 0.16 (0.11 - 0.15)
Manganese (mg/kg dw)	5.63 (0.32) (5.52 - 5.72)	5.74 (0.32) (4.89 - 6.49)	-0.10 (0.45) (-0.83 - 0.83)	-1.14, 0.94	0.829	0, 12.67 (4.00 - 9.17)
Phosphorus (% dw)	0.34 (0.0033) (0.34 - 0.35)	0.34 (0.0033) (0.34 - 0.35)	-0.0020 (0.0046) (-0.0049 - 0.00002)	-0.013, 0.0086	0.673	0.24, 0.42 (0.28 - 0.37)
Potassium (% dw)	0.41 (0.0074) (0.40 - 0.42)	0.41 (0.0074) (0.40 - 0.43)	0.0028 (0.010) (-0.017 - 0.021)	-0.021, 0.027	0.796	0.24, 0.54 (0.33 - 0.46)
Zinc (mg/kg dw)	21.25 (0.76) (20.99 - 21.56)	23.55 (0.76) (22.61 - 25.00)	-2.31 (1.07) (-3.44 - -1.62)	-4.78, 0.17	0.063	11.46, 30.37 (17.30 - 25.45)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Folic Acid	0.33 (0.025) (0.28 - 0.39)	0.31 (0.025) (0.29 - 0.32)	0.024 (0.036) (-0.039 - 0.078)	-0.059, 0.11	0.529	0.11, 0.61 (0.24 - 0.57)
Niacin	31.16 (0.90) (28.72 - 33.37)	32.07 (0.90) (31.16 - 33.26)	-0.90 (1.27) (-3.06 - 0.23)	-3.83, 2.03	0.497	7.89, 49.83 (20.63 - 43.08)
Vitamin A	1.12 (0.058) (1.07 - 1.21)	1.10 (0.058) (1.07 - 1.16)	0.019 (0.082) (-0.094 - 0.14)	-0.17, 0.21	0.824	0.38, 1.68 (0.58 - 1.50)
Vitamin B1	3.28 (0.11) (3.08 - 3.41)	3.23 (0.11) (3.08 - 3.41)	0.052 (0.16) (-0.33 - 0.27)	-0.32, 0.43	0.755	2.21, 3.65 (2.41 - 3.48)
Vitamin B2	1.60 (0.10) (1.36 - 1.80)	1.51 (0.10) (1.32 - 1.70)	0.091 (0.15) (-0.35 - 0.34)	-0.25, 0.43	0.555	0, 4.47 (1.28 - 3.29)
Vitamin B6	6.83 (0.41) (6.51 - 7.43)	6.90 (0.41) (5.67 - 7.63)	-0.063 (0.58) (-1.11 - 1.76)	-1.40, 1.28	0.915	2.57, 12.07 (5.24 - 10.29)

Table H-11 (continued). Statistical Summary of Site ILWY Grain Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Vitamin (mg/kg dw)						
Vitamin E	11.63 (1.15) (7.04 - 14.65)	11.84 (1.15) (10.13 - 13.58)	-0.20 (1.63) (-6.54 - 4.52)	-3.97, 3.56	0.903	0, 25.61 (6.67 - 17.34)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table H-12. Statistical Summary of Site ILWY Grain Anti-nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient (% dw)						
Phytic Acid	1.02 (0.024) (1.00 - 1.04)	1.09 (0.024) (1.03 - 1.12)	-0.071 (0.034) (-0.11 - -0.032)	-0.15, 0.0081	0.072	0.73, 1.23 (0.82 - 1.07)
Raffinose	0.20 (0.0076) (0.19 - 0.21)	0.20 (0.0076) (0.18 - 0.21)	0.0046 (0.011) (-0.017 - 0.025)	-0.020, 0.029	0.671	0.024, 0.29 (0.092 - 0.21)

[1]dw = dry weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table H-13. Statistical Summary of Site ILWY Grain Secondary Metabolite Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Secondary Metabolite (µg/g dw)						
Ferulic Acid	2317.21 (50.54) (2243.74 - 2354.95)	2368.37 (50.54) (2236.10 - 2500.00)	-51.16 (41.17) (-145.05 - 7.64)	-146.09, 43.77	0.249	1070.41, 2955.86 (1588.35 - 2630.98)
p-Coumaric Acid	193.24 (4.64) (184.51 - 198.39)	191.67 (4.64) (183.88 - 203.20)	1.57 (4.42) (-6.38 - 10.46)	-8.63, 11.76	0.731	58.74, 313.97 (124.16 - 250.30)

[1]dw = dry weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table H-14. Statistical Summary of Site ILWY Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	4.79 (0.16) (4.53 - 5.13)	4.58 (0.16) (4.40 - 4.80)	0.21 (0.22) (-0.0096 - 0.33)	-0.31, 0.72	0.378	2.66, 6.48 (3.70 - 5.95)
Carbohydrates	88.18 (0.46) (87.27 - 89.23)	88.56 (0.46) (87.89 - 88.92)	-0.38 (0.47) (-1.59 - 0.31)	-1.45, 0.70	0.442	80.13, 94.05 (83.23 - 90.37)
Moisture (% fw)	65.50 (1.37) (62.70 - 67.90)	66.00 (1.37) (64.10 - 67.30)	-0.50 (1.54) (-1.40 - 1.30)	-4.04, 3.04	0.753	51.70, 86.22 (61.00 - 76.00)
Protein	5.46 (0.40) (4.48 - 6.17)	5.55 (0.40) (5.17 - 5.96)	-0.082 (0.50) (-1.48 - 0.66)	-1.23, 1.07	0.873	1.34, 11.57 (4.37 - 9.31)
Total Fat	1.57 (0.27) (1.09 - 1.85)	1.32 (0.27) (0.58 - 2.20)	0.25 (0.39) (-1.11 - 1.18)	-0.64, 1.14	0.535	0.44, 3.33 (0.78 - 3.16)
Fiber (% dw)						
Acid Detergent Fiber	27.86 (1.16) (26.42 - 29.00)	26.59 (1.16) (24.57 - 27.71)	1.27 (1.61) (-1.28 - 3.58)	-2.45, 4.99	0.452	14.84, 38.51 (21.33 - 35.92)

Table H-14 (continued). Statistical Summary of Site ILWY Forage Nutrient Content for MON 87427 vs. the Conventional Control

Analytical Component (Units)[1]	MON 87427[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 87427 minus Control) Mean (S.E.) (Range)	95% CI Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	40.98 (2.55) (36.14 - 43.70)	40.76 (2.55) (36.53 - 43.43)	0.22 (2.54) (-0.39 - 1.36)	-5.64, 6.08	0.933	25.12, 54.99 (29.68 - 60.16)
Mineral						
Calcium (% dw)	0.21 (0.013) (0.19 - 0.22)	0.22 (0.013) (0.18 - 0.25)	-0.011 (0.018) (-0.063 - 0.036)	-0.054, 0.032	0.568	0.075, 0.29 (0.10 - 0.24)
Phosphorus (% dw)	0.21 (0.017) (0.20 - 0.21)	0.23 (0.017) (0.19 - 0.27)	-0.019 (0.019) (-0.074 - 0.023)	-0.062, 0.025	0.345	0.063, 0.37 (0.16 - 0.31)

[1]dw = dry weight; fw = fresh weight.
[2] MON 87427 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the near isogenic, conventional control.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

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