

MONSANTO



**Application to Food Standards Australia New Zealand
for the Inclusion of
MON 87427 Maize with Tissue-Selective Glyphosate Tolerance
Facilitating the Production of Hybrid Maize Seed
in Standard 1.5.2 - Food Derived from Gene
Technology**

Submitted by:

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ABBREVIATIONS AND DEFINITIONS¹

| | |
|------------------|---|
| AD_2010 | Allergen and gliadin protein sequence database (Release date January 22, 2010) |
| ADF | acid detergent fiber |
| ae/A | acid equivalent per acre |
| AMPA | aminomethylphosphonic acid |
| 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 <i>Agrobacterium</i> sp. strain CP4 |
| <i>cp4 epsps</i> | Codon-optimized coding sequence of the <i>aroA</i> gene from the <i>Agrobacterium</i> sp. strain CP4 encoding the CP4 EPSPS protein |
| CTP2 | chloroplast transit peptide |
| dwt | Dry weight |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| 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 |
| FSANZ | Food Standard Australia and New Zealand |
| 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 |
| IgE | Immunoglobulin E |
| kDa | KiloDalton |
| LOQ | Limit of quantitation |
| MALDI-TOF MS | Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry |
| MRL | Maximum Residue Limit |
| MW | Molecular Weight |
| MWCO | Molecular Weight Cutoff |
| NDF | neutral detergent fiber |
| 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 |
| sp. | Species |
| T-DNA | Transfer DNA |
| TDF | Total dietary fiber |
| TOX_2010 | Toxin protein sequence database (Release date January 22, 2010) |
| v/v | Volume to Volume ratio |
| w/v | Weight to Volume ratio |

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.

Part 1 GENERAL INFORMATION

Applicant Details

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| (f) Nature of applicant's business | Technology Provider to the Agricultural and Food Industries |
| (g) Details of other individuals, companies or organisations associated with the application | Not applicable |

Purpose of the Application

This application is submitted to Food Standards Australia New Zealand by Monsanto Australia Limited and is not made on behalf of any other party.

The purpose of this submission is to make an application to vary **Standard 1.5.2 – Food Produced Using Gene Technology** to seek the addition of tissue-selective glyphosate tolerant maize MON 87427 and products containing maize MON 87427 (hereafter referred to as MON 87427) to the Table to Clause 2 (see below).

| Food derived from gene technology | Special requirements |
|--|----------------------|
| Food derived from tissue-selective glyphosate tolerant maize MON 87427 | None |

Relevant Overseas Approvals

Monsanto has submitted a food and feed safety and nutritional assessment summary for MON 87427 to the United States Food and Drug Administration (FDA) in December 2010. Monsanto has also 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.

It has been also submitted to Canadian Food Inspection Agency (CFIA) and Health Canada (HC) in January 2011; Korea Food and Drug Administration (KFDA) and Rural Development Administration (RDA) in June 2011; Japan's Ministry of Environment in May 2011 and Ministry of Health, Labour, and Welfare (MHLW) in June 2011; Taiwan Food and Drug Administration (TFDA) in June 2011; and Philippines Bureau of Plant Industry (BPI) in July 2011.

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 Mexico and EU 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.

Justification for the Application

The need and/or advantages for the proposed change

Maize (*Zea mays* L.) is the largest crop grown in the U.S. in terms of acreage planted and net value. 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.

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.

Assessment Procedure

Monsanto Australia is submitting this application in anticipation that it will fall within the general procedure category.

Part 2 **SPECIFIC DATA REQUIREMENTS FOR SAFETY ASSESSMENT**

A1. Technical information on the GM food

A1(a) A description of the new GM organism

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 will be combined with other glyphosate-tolerant maize using traditional breeding techniques.

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 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).

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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. 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.

A1(b) Name, number or other identifier of each new line or strain

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.

A1(c) The name the food will be marketed under (if known)

Maize containing the transformation event MON 87427 will be produced in North America. There are currently no plans to produce this product in Australia or New Zealand. A commercial trade name for the product has not been determined at the time of this submission and will be available prior to commercial launch of the product.

A1(d) The types of products likely to include the food or food ingredient

Maize is widely used for a variety of food and feed purposes, and it is intended that MON 87427 will be utilized in the same manner and for the same uses as conventional maize. Maize grain and its processed products are consumed in a multitude of human food and animal feed products. Maize forage (as silage) is extensively consumed as an animal feed by ruminants.

A2 Description of donor and host organisms**A2(a) Description of all donor organism(s)****A2(a)(i) Common and scientific names and taxonomic classification**

The donor organism, *Agrobacterium* sp. strain CP4, is related to microbes commonly present in the soil and in the rhizosphere of plants, and was isolated based on its tolerance to glyphosate brought about by the production of a naturally glyphosate-tolerant EPSPS protein (Padgett 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*

A2(a)(ii) Information on pathogenicity, toxicity, allergenicity

Agrobacterium sp. strain 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/WHO, 2001). Furthermore, *Agrobacterium* sp. strain CP4 has been previously reviewed as a part of the safety assessment of the donor organism during past Monsanto applications that have been approved by FSANZ including 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.

A2(a)(iii) History of use of the organism in food supply or human exposure

As described above, *Agrobacterium* sp. strain CP4 is related to microbes commonly present in the soil and in the rhizosphere of plants.

A2(b) Description of the host organism**A2(b)(i) Phenotypic information**

Maize (*Zea mays* L.) is a member of the tribe Maydae, which is included in the subfamily Panicoideae of the grass family Gramineae.

Family - Gramineae
 Subfamily - Panicoideae
 Tribe - Maydae
 Western Hemisphere:
 Genus - *Zea*
 A. Subgenus - *Luxuriantes*

1. *Zea luxurians* (2n = 20)
 2. *Zea perennis* (2n = 40)
 3. *Zea diploperennis* (2n = 20)
- B. Subgenus - *Zea*
1. *Zea mays* (2n = 20)
Subspecies
 1. *Z. mays parviglumis* (2n = 20)
 2. *Z. mays huehuetenangensis* (2n = 20)
 3. *Z. mays mexicana* (Schrad.) (2n = 20)

The genera included in the tribe Maydaceae include *Zea* and *Tripsacum* in the Western Hemisphere, and *Coix*, *Polytoca*, *Chionachne*, *Schlerachne*, and *Trilobachne* in Asia. Although some researchers have implicated the Asian genera in the origin of maize, the evidence for them is not as extensive and convincing as for the genera located in the Western Hemisphere.

The genus *Zea* includes two sub-genera: *Luxuriantes* and *Zea*. Maize (*Zea mays* L.) is a separate species within the subgenus *Zea*, along with three subspecies. All species within the genus *Zea*, except maize, are different species of teosinte. Until recently, the teosinte species were included in the genus *Euchlaena* rather than the genus *Zea*.

A2(b)(ii) How the organism is propagated for food use

Maize is wind-pollinated, and the distances that viable pollen can travel depend on prevailing wind patterns, humidity, and temperature. Pollen is shed from the tassel and is viable for approximately 10 to 30 minutes as it is rapidly desiccated in the air (Kiesselbach, 1980). Maize plants shed pollen for up to 14 days.

The reproductive phase begins when one or two auxiliary buds, present in the leaf axils, develop and form the pistillate inflorescence of female flower. The auxiliary bud starts the transformation to form a long 'cob' on which the flowers will be borne. From each flower a style begins to elongate towards the tip of the cob in preparation for fertilization. These styles form long threads, known as silks. The base of the silk is unique, as it elongates continuously until fertilization occurs. Styles may reach a length of 30 centimetres, the longest known in the plant kingdom. Individual maize kernels, or fruit, are unique in that mature seed is not covered by floral bracts (glumes, lemmas, and paleas) as in most other grasses, but rather the entire structure is enclosed and protected by large modified leaf bracts, collectively referred to as the ear. The mature female flowers will remain ready for fertilization for up to two weeks, at which point if fertilization has not occurred, the nucleus will de-organize and fertilization will no longer be possible.

The pollen of maize, a protandrous plant, matures before the female flower is receptive. This may have been an ancient mechanism to ensure cross-pollination, but is no longer considered conducive to modern agricultural practices. However, decades of conventional selection and improvement have produced many maize varieties with similar maturities for both male and female flowers, to ensure seed set for agricultural purposes.

Under natural conditions, maize reproduces only by seed production. Pollination occurs with the transfer of pollen from the tassels to the silks of the ear. About 95% of the ovules are cross-pollinated and about 5% are self-pollinated, although plants are completely self-compatible. Maize, as a thoroughly domesticated plant, has lost all ability to disseminate its seeds and relies entirely on the aid of man for its distribution.

A2(b)(iii) What part of the organism is used for food

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).

The majority of the maize used for food 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 breadings; 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, ethanol (Watson, 1988), and distilled beverages (Rooney and Serna-Saldivar, 1987).

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).

A2(b)(iv) Whether special processing is required to render food safe to eat

Maize kernel fractions contain 82% endosperm, 12% germ, 5% bran, and 1% tip cap (Earle et al., 1946). Maize is widely used for a variety of food and feed purposes; maize grain and its processed products are consumed in several human food and animal feed products. The major maize processing methods include wet milling and dry milling processes that are used to separate the different fractions of the maize kernel. Several food and livestock feed products are derived from the direct products and by-products of these milling processes.

Wet Milling:

For the wet milling process, maize kernels are thoroughly cleaned and then transferred into a mixture of hot water (49° to 54°C) and 0.2% sulfur dioxide to soften the pericarp (the outer layer covering the seed). The softened kernels are then cracked and passed through a floatation process that separates the germ portion from the endosperm. The germ portion of the kernel is the main source of oil derived from maize. The germ portion is pressed to separate the oil from the germ fiber. Maize oil derived as a result of this process is used in margarine, cooking oil, baking, and frying. The endosperm portion is milled and passed through screens to remove the hulls. The finely milled endosperm portion is then passed through centrifugation to separate starch from gluten. The gluten is dried and used as maize gluten meal for livestock feed. Maize starch is the primary product of wet milling process. Approximately 40% of the starch obtained through the wet milling processing is consumed directly as food or used for industrial purposes, while the remaining 60% is used in the production of sweeteners.

Dry Milling:

The dry milling process usually refers to three processing methods: 1) stone grinding, 2) dry-grinding for producing commercially used ethanol and 3) tempering degerminating (TD) system. Of these three methods, tempering degerminating system is the most widely used for food processing. Cleaned maize kernels are “tempered” with water or steam to condition the maize kernels for the dry milling process. After tempering, the maize kernels are passed through the degerminator for a clean separation of germ from the rest of the kernel. The products of the tempering degerminating process include 12% flaking grits (used in the production of corn flakes), 15% coarse grits (these are often cooked and eaten), 23% fine grits

(used by snack and brewing industries), 6% meal, 4% flour, 1% oil, and 35% livestock feed.

In addition to livestock feed generated as a by product of wet and dry milling processes, the entire maize plant can also be harvested and used as livestock feed, i.e. whole plant maize silage. To produce whole plant maize silage, the whole plant 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.

A2(b)(v) The significance to the diet in Australia and NZ of the host organism

Worldwide maize grain production averaged over 780 million metric tons (MMT) per year from 2006 to 2010 (USDA-FAS, 2011), and it was approximately 350,000 metric tons in Australia in 2010.

Maize has been a staple of the human diet for centuries, and its processed fractions are consumed in a multitude of food. Estimates of maize consumption are available from the WHO Global Environmental Monitoring System - Food Contamination Monitoring and Assessment Programme (GEMS/Food) (www.who.int/foodsafety/chem/gems). The GEMS/Food programme has developed 13 Cluster Diets which are considered to be representative of the major food consumption patterns exhibited by regional and cultural groups around the world. Australia is included in Cluster M, along with United States and Canada and several other countries.

A3 The nature of the genetic modification

A3(a) Method used to transform host organism

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 R₀ plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R₀ 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 R₀ 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 1.

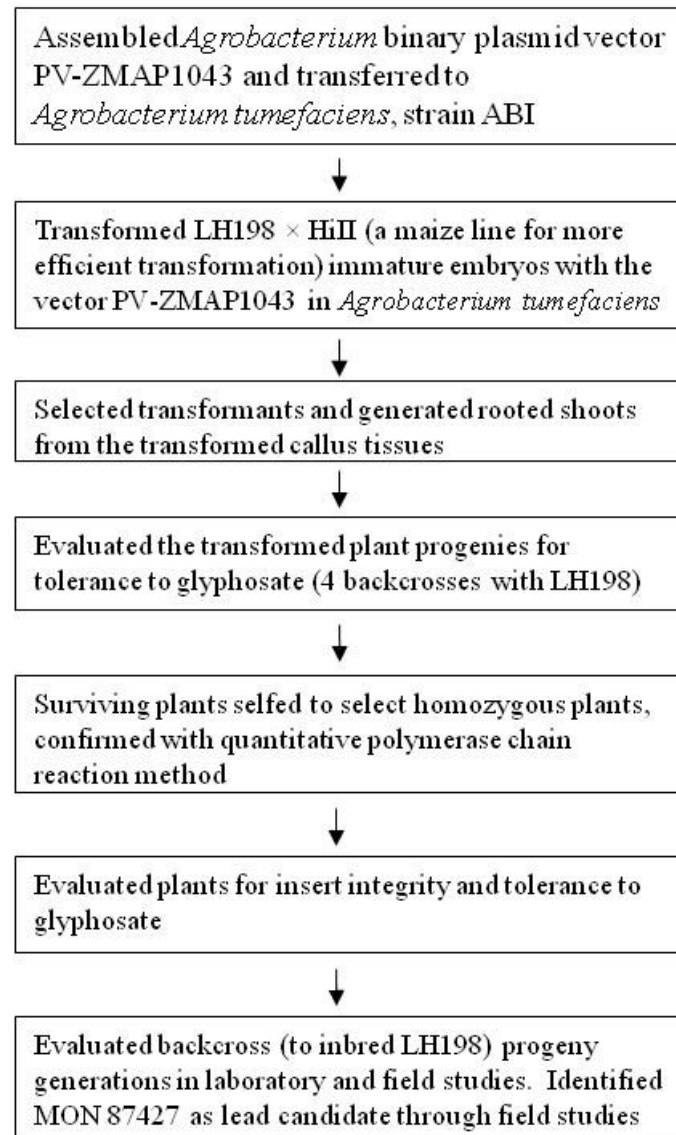


Figure 1. Schematic of the Development of MON 87427

A3(b) Intermediate hosts (eg. bacteria)

A disarmed strain of *Agrobacterium tumefaciens* was the intermediate host used to transfer the plasmid PV-ZMAP1043 into maize cells. PV-ZMAP1043 contains one T-DNA, containing the full *cp4 epsps* expression cassette. Following transformation, self-pollination breeding and segregation methods were used to produce MON 87427.

A3(c)(i) Gene construct including size, source and function of all elements**Plasmid Vector PV-ZMAP1043**

PV-ZMAP1043 was used in the transformation of maize to produce MON 87427 and is shown in Figure 3, and the elements included in this vector are described in Table 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 1.

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 2) (Padgett 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; Padgett 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; Padgett et al., 1996).

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).

T-DNA Borders

PV-ZMAP1043 contains Right Border and Left Border regions (Figure 3 and Table 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.

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 Tn7 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 A3(d)(i)).

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

| Genetic Element | Location in Plasmid Vector | Function (Reference) |
|---|-----------------------------------|--|
| T-DNA | | |
| B¹-Left Border Region | 1-442 | DNA region from <i>Agrobacterium tumefaciens</i> 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 |
| P²-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 |
| I³-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 |
| TS⁴-CTP2 | 1954-2181 | Targeting sequence from the <i>ShkG</i> gene encoding the chloroplast transit peptide region of <i>Arabidopsis thaliana</i> EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast |
| CS⁵-cp4 epsps | 2182-3549 | Codon-optimized coding sequence of the <i>aroA</i> gene from the <i>Agrobacterium</i> sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgett et al., 1996) |
| Intervening Sequence | 3550-3555 | Sequences used in DNA cloning |
| T⁶-nos | 3556-3808 | 3' nontranslated region of the <i>nopaline synthase (nos)</i> gene from <i>Agrobacterium tumefaciens</i> 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 <i>Agrobacterium tumefaciens</i> containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982) |

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

| Vector Backbone | | |
|----------------------------------|-----------|---|
| Intervening Sequence | 4193-4328 | Sequences used in DNA cloning |
| <i>aadA</i> | 4329-5217 | Bacterial promoter, coding sequence, and 3' untranslated region for an aminoglycoside-modifying enzyme, 3''(9)-O-nucleotidyltransferase from the transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance |
| Intervening Sequence | 5218-5747 | Sequences used in DNA cloning |
| OR⁷-ori-pBR322 | 5748-6336 | Origin of replication from pBR322 for maintenance of plasmid in <i>E. coli</i> (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 <i>E. coli</i> (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 <i>Agrobacterium</i> (Stalker et al., 1981) |
| Intervening Sequence | 8861-8946 | Sequences used in DNA cloning |

¹ B, Border² P, Promoter³ I, Intron⁴ TS, Targeting Sequence⁵ CS, Coding Sequence⁶ T, Transcription Termination Sequence⁷ OR, Origin of Replication

```

1   MAQVSRICNG VQNPSLISNL SKSSQKKSPL 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 EPIMTRDHT E KMLQGFGANL TVETDADGVR
301 TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR
351 TGLILTLQEM GADIEVINPR LAGGEDVADL RVRSSSTLKG TVPEDRAPSM
401 IDEYPILAVA AAFAEGATVM NGLEELRVKE SDRLSAVANG LKLNGVDCDE
451 GETSLVVRGR PDGKGLGNAS GAAVATHLDH RIAMSFLVMG LVSENPVTVD
501 DATMIATSFP EFMDLMAGLG AKIELSDTKA A

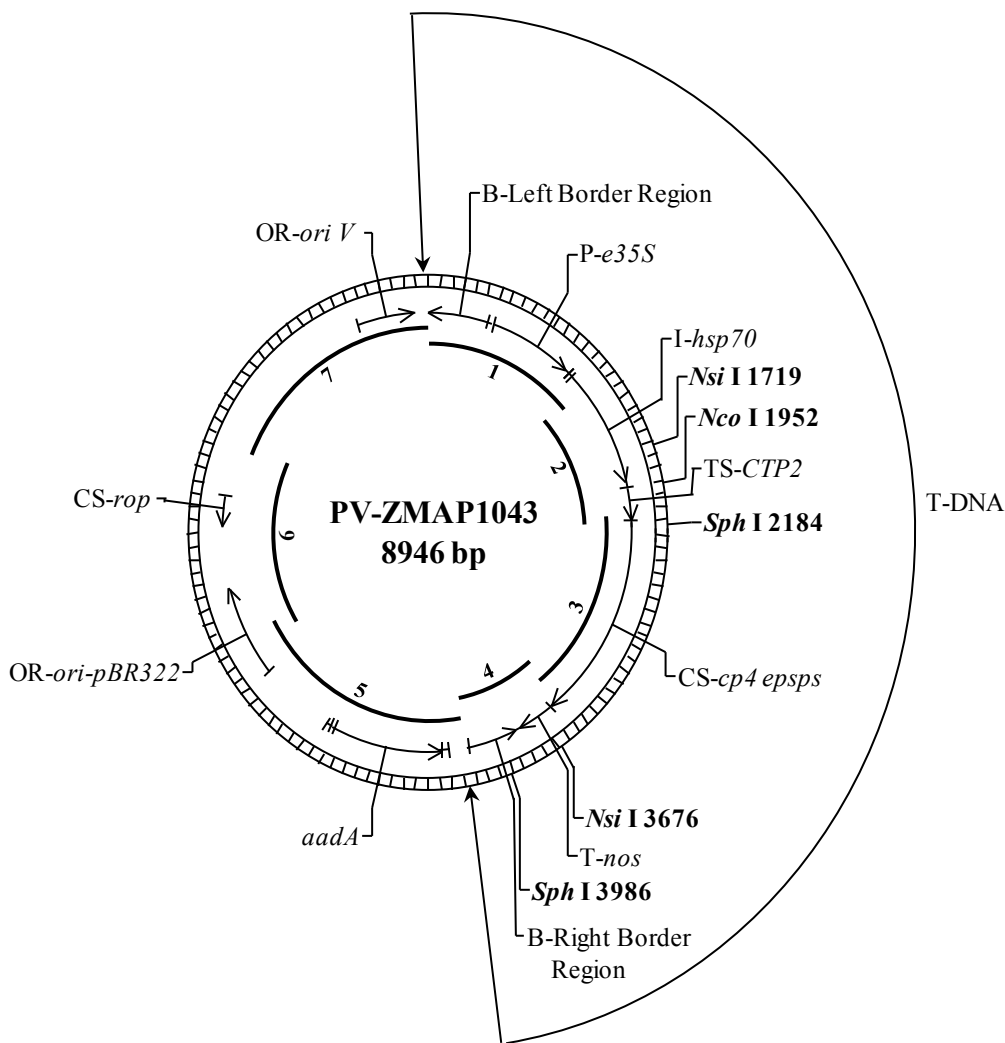
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Figure 2. Deduced Amino Acid Sequence of the MON 87427 CP4 EPSPS Precursor Protein

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.

A3(c)(ii) Detailed map of the location and orientation of all genetic elements

PV-ZMAP1043 was used in the transformation of maize to produce MON 87427 and is shown in Figure 3, and the elements included in this vector are described in Table 1.



| 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 3. 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.

A3(d)(i) Molecular characterisation including identification of GM elements

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 (Figure 5 - Figure 7).

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 13) 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 4).

The Southern blot analyses confirmed that the T-DNA reported in Figure 4 represents the only detectable insert in MON 87427. Figure 4 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 3. 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 2. The genetic elements integrated in MON 87427 are summarized in Table 3. The generations used in the generational stability analysis are depicted in the breeding history shown in Figure 13.

The generations used in the assessment are depicted in the breeding history shown in Figure 13. The maize germplasm that was utilized as the recipient of the transgene to create MON 87427 was LH198 x HiII. LH198 was used as the recurrent parent through four backcrossing generations. Molecular characterization was conducted with the MON 87427 test material generation LH198 BC3F4 (Figure 13) that was used to initiate commercial breeding efforts.

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 (section A3), safety assessment of the expressed protein (sections B2, B3, and B4), and composition analysis (section B5). Molecular characterization was conducted with the MON 87427 test material generation LH198 BC3F4

(Figure 13) 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 13).

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 B5), and protein safety assessment (sections B2, B3, and B4). 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.

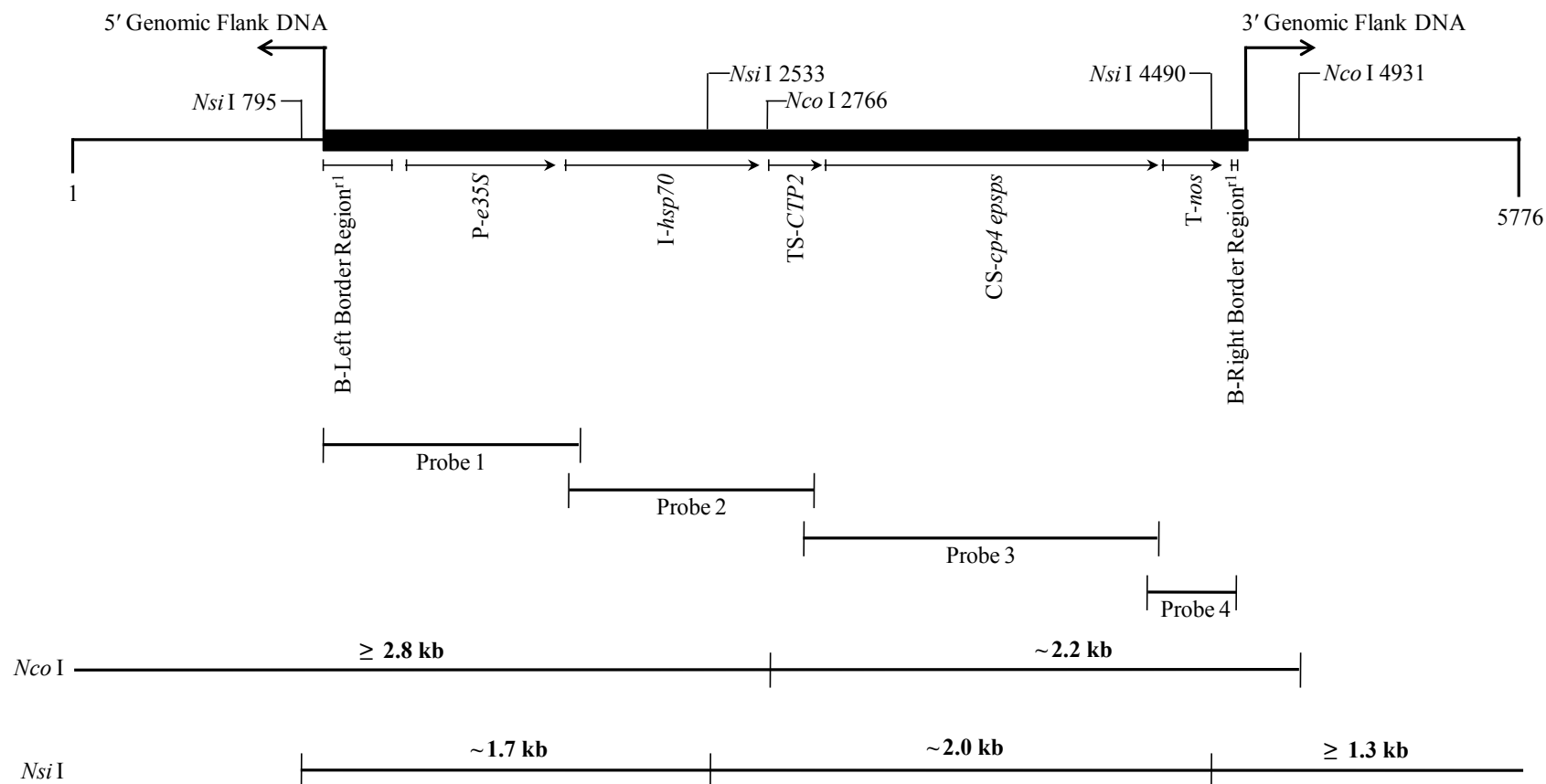


Figure 4. Schematic Representation of the Insert and Genomic Flanking Sequences in MON 87427

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 3.

Table 2. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in MON 87427 Analyses

| Southern Blot Figure | | 5 | 6 | 7 | 8 | 14 |
|------------------------------------|------------------|---|---------------------|--------------------|-------------------------------|--------------------------------|
| Probes Used | | 1, 4 | 2 | 3 | 5, 6, 7 | 1, 4 |
| Probing Target | Digestion Enzyme | Expected Band Sizes on Each Southern Blot | | | | |
| PV-ZMAP1043 | <i>Sph</i> 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 | <i>Nco</i> I | ≥ 2.8 kb ~2.2 kb | ≥ 2.8 kb ~2.2 kb | ~2.2 kb | No band | -- ² |
| | <i>Nsi</i> 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 3. 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 <i>Agrobacterium tumefaciens</i> 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 - <i>e35S</i> | 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 - <i>hsp70</i> | 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 - <i>CTP2</i> | 2767-2994 | Targeting sequence from the <i>ShkG</i> gene encoding the chloroplast transit peptide region of <i>Arabidopsis thaliana</i> EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast |
| CS ⁵ - <i>cp4 epsps</i> | 2995-4362 | Codon-optimized coding sequence of the <i>aroA</i> gene from the <i>Agrobacterium</i> sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgett et al., 1996) |
| Intervening Sequence | 4363-4368 | Sequences used in DNA cloning |
| T ⁶ - <i>nos</i> | 4369-4621 | 3' nontranslated region of the <i>nopaline synthase (nos)</i> gene from <i>Agrobacterium tumefaciens</i> 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 <i>Agrobacterium tumefaciens</i> 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, Border² P, Promoter³ TS, Targeting Sequence⁴ I, Intron⁵ CS, Coding Sequence⁶ 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

A3(d)(ii) Determination of number of insertion sites, and copy number

A3(d)(ii)(1) 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 3). Each restriction digest is expected to produce a specific banding pattern on the Southern blots (Table 2) 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 4). 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 4 and Table 2). 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 4). 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 4 and Table 2). 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 A3(c)(i)). 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 3 and Table 2). The results of these analyses are shown in Figure 5 through Figure 7.

Probes 1 and 4

Conventional control genomic DNA digested with *Nco* I (Figure 5, lane 1 and lane 8) and hybridized with Probe 1 and Probe 4 (Figure 3) produced endogenous hybridization bands of ~6.1 kb and ~4.1 kb. Conventional control genomic DNA digested with *Nsi* I (Figure 5, lane 3 and lane 10) and hybridized with Probe 1 and Probe 4 (Figure 3) 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 3). 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 *Nci* I (Figure 5, lane 7) produced the two expected bands at ~7.1 kb and ~1.8 kb (Figure 3 and Table 2) in addition to the endogenous hybridization bands listed above. Probe templates generated from PV-ZMAP1043 (Figure 3) were mixed with conventional control genomic DNA pre-digested with the restriction enzyme *Nco* I (Figure 5, lane 5 and lane 6) produced the expected bands at ~1.2 kb and ~0.7 kb (Figure 3 and Table 2) 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 3) produced two bands in addition to the endogenous hybridization bands (Figure 5, 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 4). 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 4).

MON 87427 genomic DNA digested with *Nsi* I (Figure 5, lane 4 and lane 11) and hybridized with Probe 1 and Probe 4 produced three bands (Table 2) 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 4). 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 4). 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 4).

No additional bands were detected using Probe 1 and Probe 4 other than those listed above. Based on the results presented in Figure 5, it was concluded that T-DNA sequences covered by Probe 1 and Probe 4 reside at a single integration locus in MON 87427.

Probe 2

Conventional control genomic DNA digested with *Nco* I (Figure 6, lane 1 and lane 8) and hybridized with Probe 2 (Figure 3) produced an endogenous hybridization band of ~4.1 kb. Conventional control genomic DNA digested with *Nsi* I (Figure 6, lane 3 and lane 10) and hybridized with Probe 2 (Figure 3) 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 3). 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 6, lane 7) produced the expected band of ~7.1 kb (Figure 3 and Table 2) in addition to the endogenous hybridization bands listed above. Probe template generated from PV-ZMAP1043 (Figure 3)

was mixed with conventional control genomic DNA predigested with the restriction enzyme *Nco* I (Figure 6, lane 5 and lane 6) produced the expected band of ~1.0 kb (Figure 3 and Table 2) 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 6, 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 4). 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 4).

MON 87427 genomic DNA digested with *Nsi* I (Figure 6, 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 4). 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 4).

No additional bands were detected using Probe 2 other than those listed above. Based on the results presented in Figure 6, it was concluded that the T-DNA sequences covered by Probe 2 reside at a single integration locus in MON 87427.

Probe 3

Conventional control genomic DNA digested with the restriction enzyme *Nco* I (Figure 7, lane 1 and lane 8) or *Nsi* I (Figure 7, lane 3 and lane 10) and hybridized with Probe 3 (Figure 3) 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 7, lane 7) produced one band at ~1.8 kb (Figure 3 and Table 2). 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 3) was mixed with conventional control DNA predigested with the restriction enzyme *Nco* I (Figure 7, lane 5 and lane 6) produced the expected band at ~1.5 kb (Figure 3 and Table 2). 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 3) produced one band (Figure 7, 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 4).

MON 87427 genomic DNA digested with the restriction enzyme *Nsi* I and hybridized with Probe 3 (Figure 3) produced one band (Figure 7, 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 4).

No additional bands were detected using Probe 3 other than those listed above. Based on the results presented in Figure 7, it was concluded that the sequence covered by Probe 3 resides at a single integration locus in MON 87427.

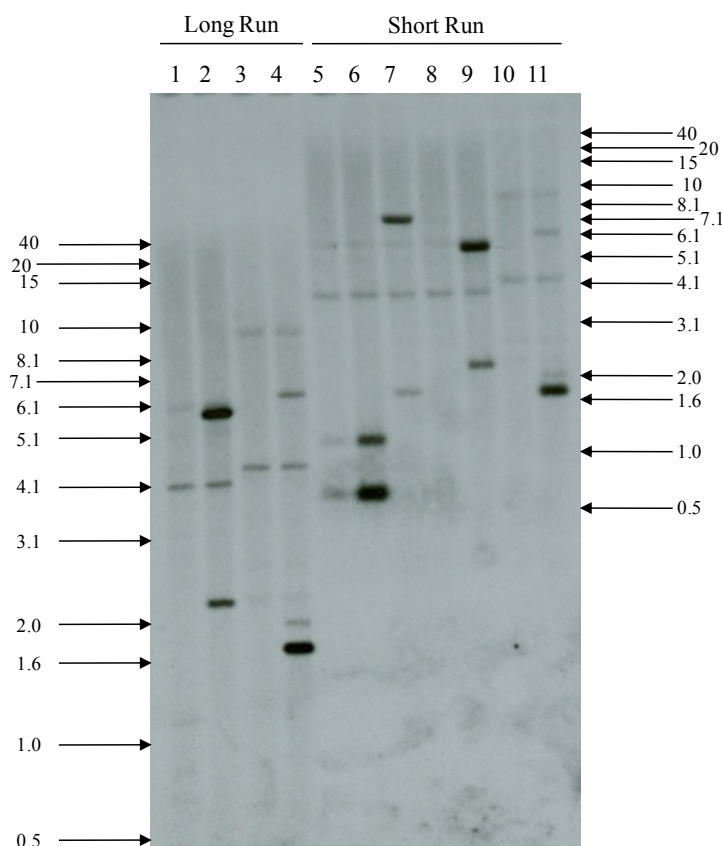


Figure 5. Southern Blot Analysis to Determine Insert and Copy Number of the T-DNA in MON 87427: Probe 1 and Probe 4

The blot was hybridized with two ^{32}P -labeled probes that spanned portions of the T-DNA sequence (Figure 3, 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 (<i>Nco</i> I) |
| 2 | MON 87427 (<i>Nco</i> I) |
| 3 | Conventional control (<i>Nsi</i> I) |
| 4 | MON 87427 (<i>Nsi</i> I) |
| 5 | Conventional control (<i>Nco</i> I) spiked with Probe 1 and Probe 4 [~0.1 genome equivalent] |
| 6 | Conventional control (<i>Nco</i> I) spiked with Probe 1 and Probe 4 [~1.0 genome equivalent] |
| 7 | Conventional control (<i>Nco</i> I) spiked with PV-ZMAP1043 (<i>Sph</i> I) [~1.0 genome equivalent] |
| 8 | Conventional control (<i>Nco</i> I) |
| 9 | MON 87427 (<i>Nco</i> I) |
| 10 | Conventional control (<i>Nsi</i> I) |
| 11 | MON 87427 (<i>Nsi</i> 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.

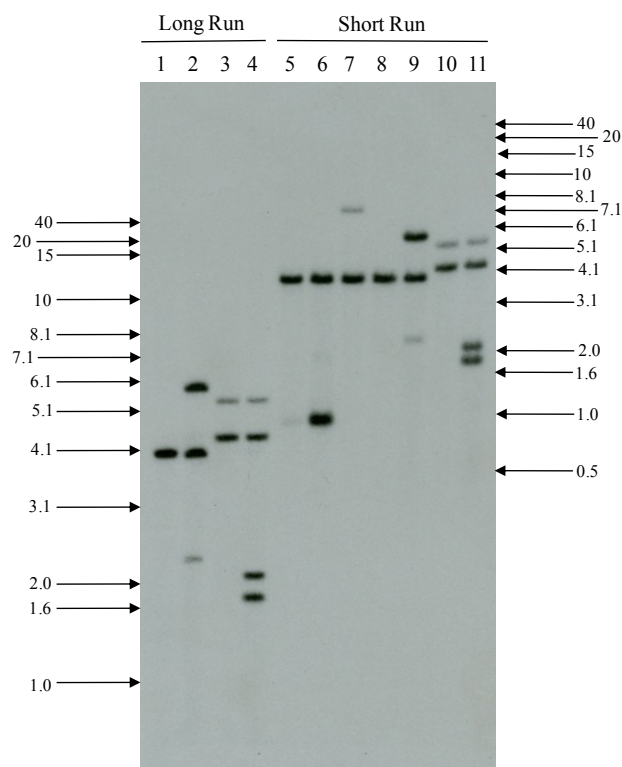


Figure 6. Southern Blot Analysis to Determine Insert and Copy Number of the T-DNA in MON 87427: Probe 2

The blot was hybridized with one ^{32}P -labeled probe that spanned a portion of the T-DNA sequence (Figure 3, 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.

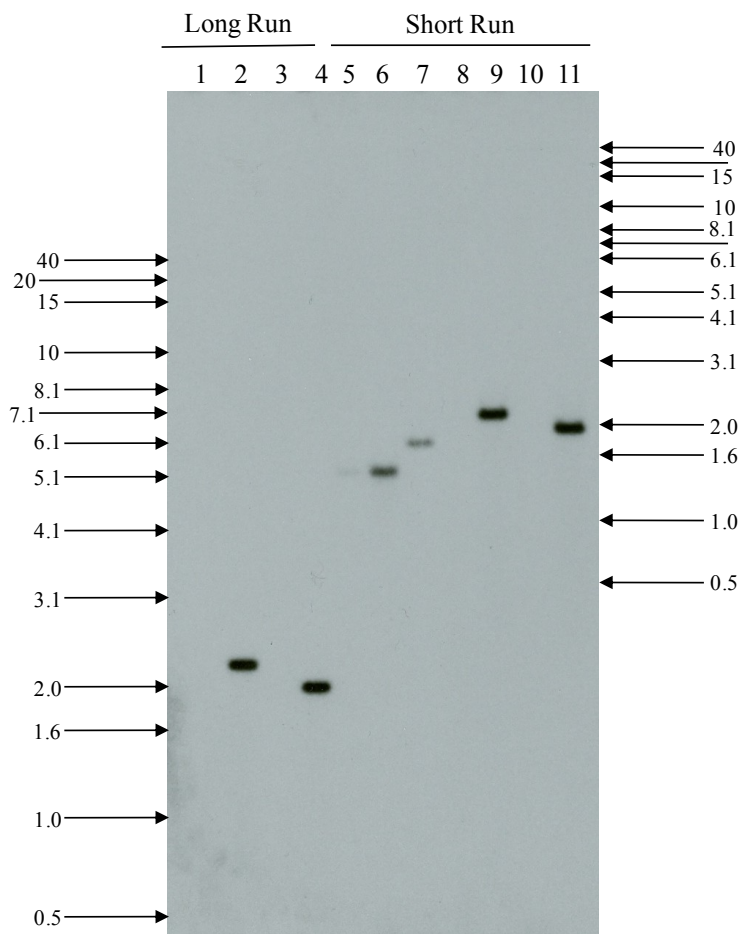


Figure 7. Southern Blot Analysis to Determine Insert and Copy Number of the T-DNA in MON 87427: Probe 3

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

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

A3(d)(ii)(2) 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 3, 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 3, 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 8.

Plasmid Vector Backbone Probes 5, 6, 7

Conventional control genomic DNA digested with *Nco* I (Figure 8, lane 1 and lane 10) or *Nsi* I (Figure 8, lane 3 and lane 12) and hybridized simultaneously with overlapping probes spanning the plasmid vector backbone of PV-ZMAP1043 (Figure 3, 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 8, lane 9) produced one expected band of ~7.1 kb (Figure 3 and Table 2). Probe templates generated from PV-ZMAP1043 (Figure 3, Probe 5 and Probe 6) were mixed with conventional control DNA predigested with the restriction enzyme *Nco* I (Figure 8, lane 5 and lane 6) produced two expected bands at ~1.8 kb and ~1.5 kb, respectively (Figure 3 and Table 2). Probe template generated from PV-ZMAP1043 (Figure 3, Probe 7) was mixed with conventional control DNA predigested with the restriction enzyme *Nco* I (Figure 8, 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 8, lane 2 and lane 11) or *Nsi* I (Figure 8, 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 8, it was concluded that MON 87427 contains no detectable backbone sequences from PV-ZMAP1043.

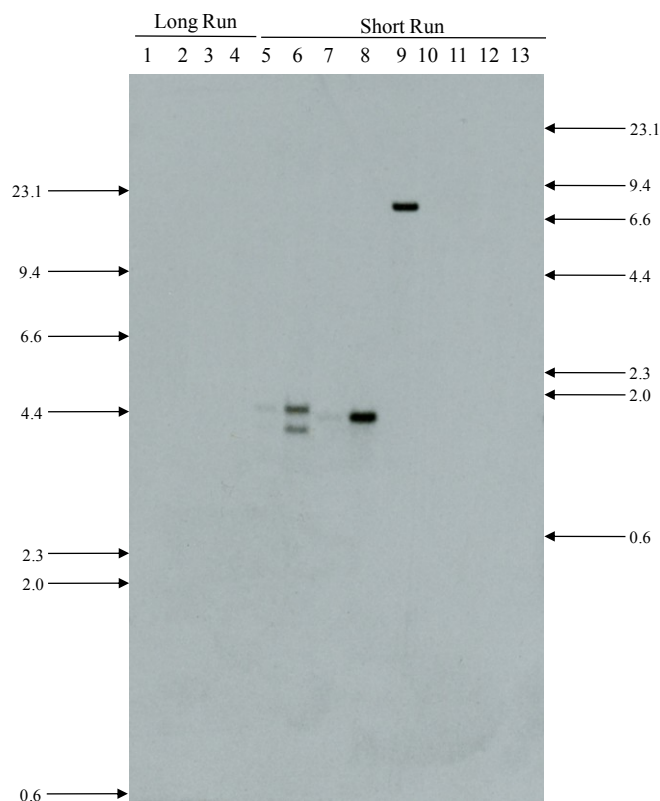


Figure 8. Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector PV-ZMAP1043 Backbone Sequences in MON 87427: Probes 5, 6, and 7

The blot was hybridized with three ^{32}P -labeled probes that spanned portions of the T-DNA sequence (Figure 3, Probe 5, Probe 6, and Probe 7). Each lane contains $\sim 10 \mu\text{g}$ 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 $\lambda\text{DNA}/\text{Hind III}$ Fragments (Invitrogen) on the ethidium bromide stained gel.

A3(d)(iii) Full DNA sequence, including junction regions, or bioinformatics

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 9). 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 1(CBI).

A3(d)(iv) Map of the organisation of the inserted DNA (each 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 10). 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).

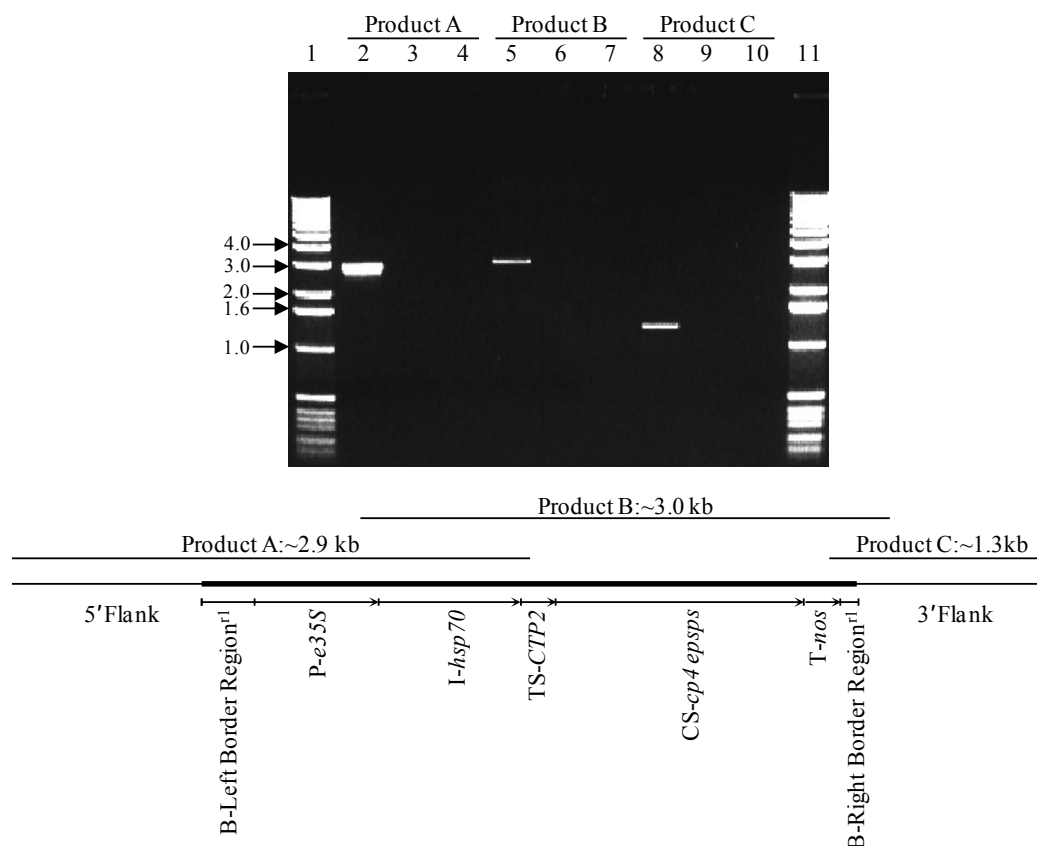


Figure 9. Overlapping PCR Analysis Across the Insert in MON 87427

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.

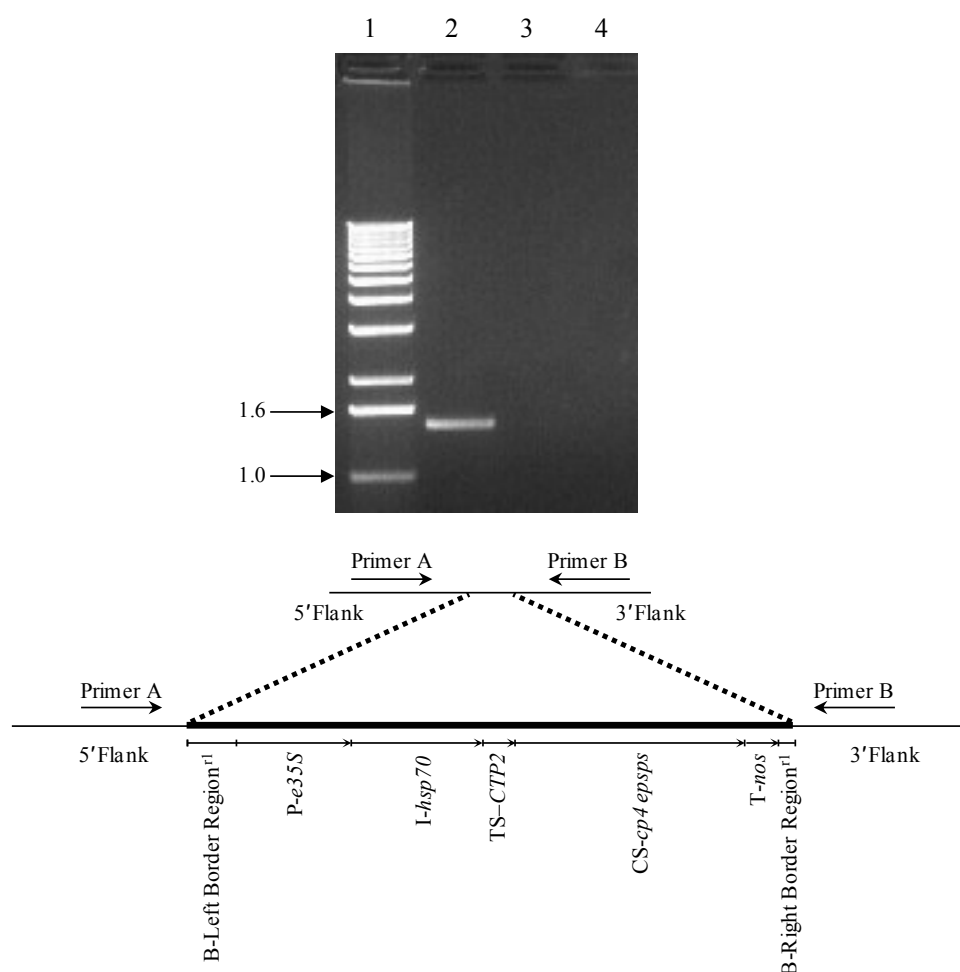


Figure 10. PCR Amplification of the MON 87427 Insertion Site in Conventional Maize

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.

A3(d)(v) Identification and characterisation of unexpected ORFs

The 2003 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2003) 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 (section B3(a) and B4(b)) 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 11. 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.

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 11).

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, 2003) 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 8.2×10^{-6} . 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 1×10^{-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-enolpyruvylshikimate-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.

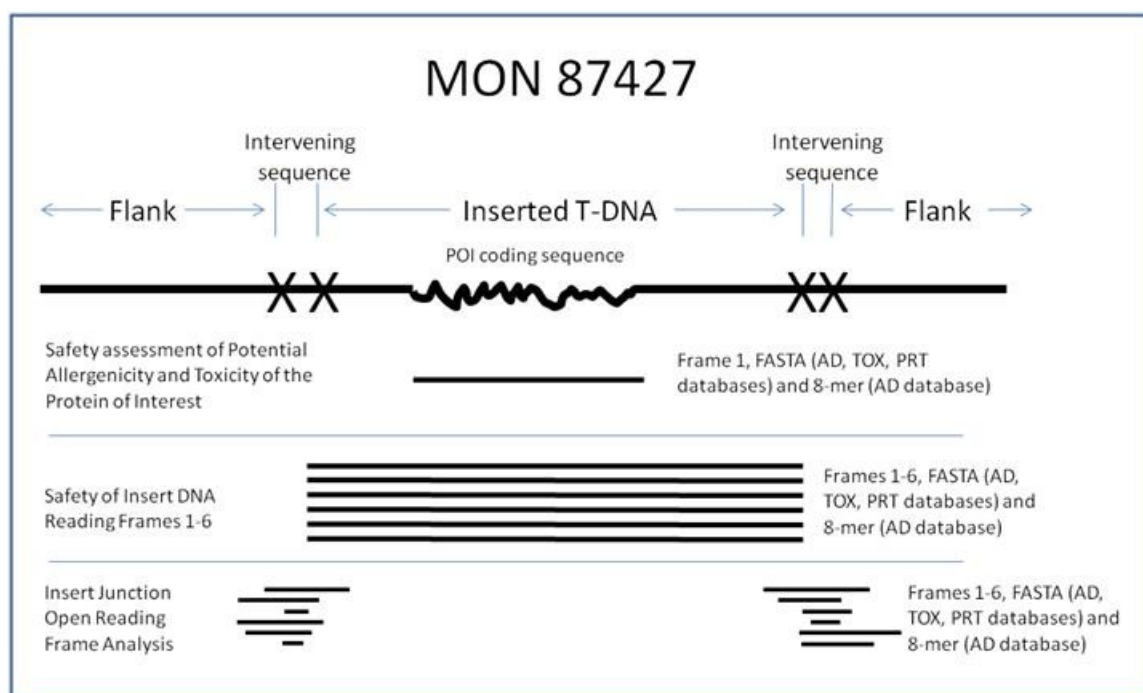
Insert Junction Open Reading Frame Bioinformatics Analysis

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 11). 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 11) 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, 2003) 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.



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

Figure 11. Schematic Summary of MON 87427 Bioinformatic Analyses

A3(e) Family tree or breeding map

Please refer to section A3(f)(i).

A3(f)(i) Pattern of inheritance of insert and no. of generations monitored

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 12. The transformed R₀ 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 4. 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 4. Segregation of the Tissue-selective Glyphosate Tolerance Trait During the Development of MON 87427

| Generation¹ | Number of plants² | Observed Positives | Observed Negatives | Expected Positives | Expected Negatives | χ^2 | Probability |
|-------------------------------|-------------------------------------|---------------------------|---------------------------|---------------------------|---------------------------|----------------------------|--------------------|
| BC1F1 | 238 | 109 | 129 | 119 | 119 | 1.6807 | 0.1948 |
| BC2F1 | 290 | 145 | 145 | 145 | 145 | 0 | 1.0000 |
| BC2F2 | 1107 | 820 | 287 | 830 | 277 | 0.5062 | 0.4768 |

¹The BC1F1 and BC2F2 generations listed here are those from the trait integration breeding pathway as shown in Figure 12.

²The plants were evaluated for the presence or absence of the glyphosate tolerance phenotype.

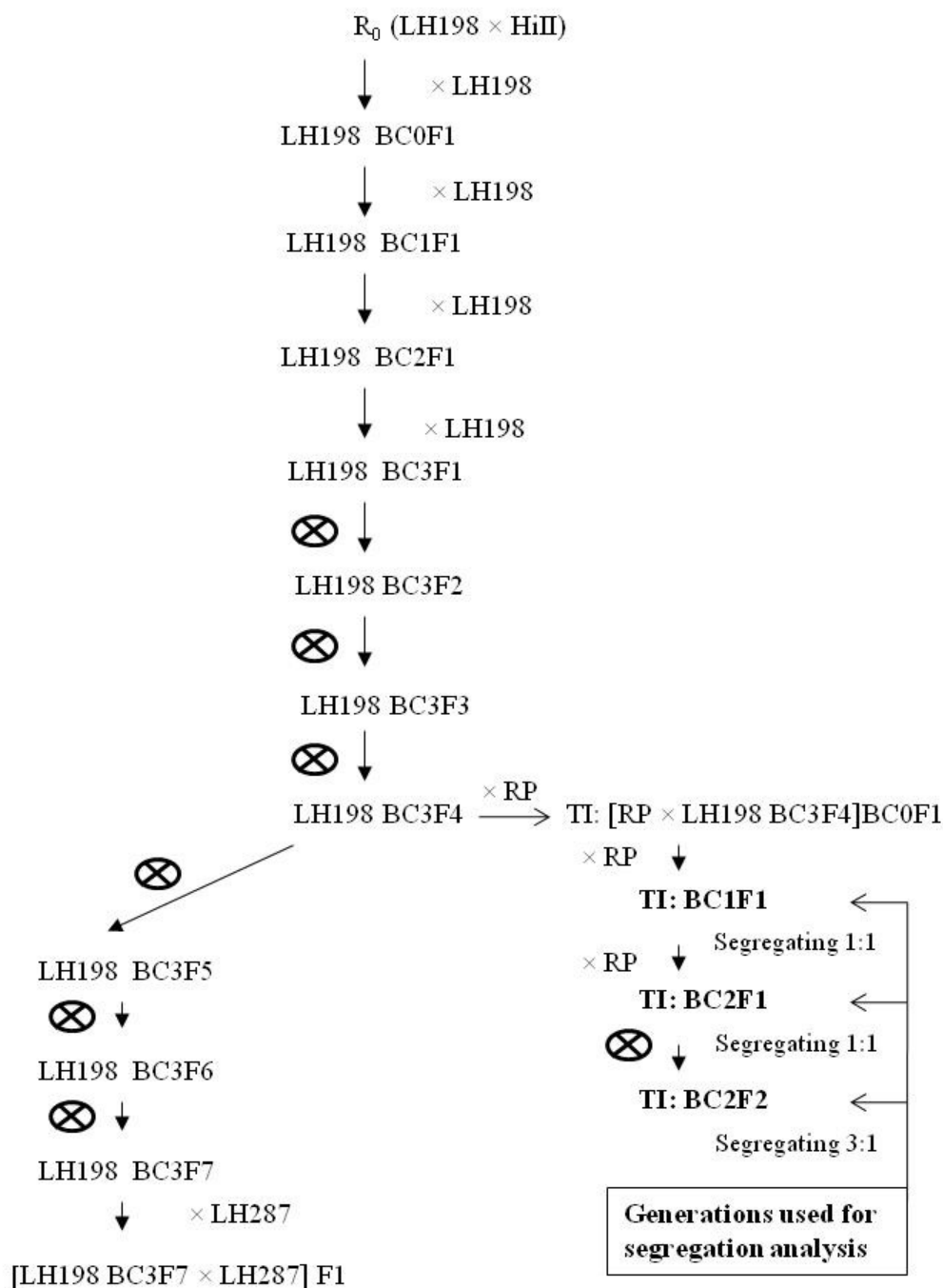


Figure 12. Breeding Path for Generating Segregation Data for MON 87427

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. \otimes designates self-pollination. BC# is the backcross generation, and TI corresponds to trait integration for commercial seed development.

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 13, and the specific generations tested are indicated in the legend of Figure 14. The LH198 BC3F4 generation was used for the molecular characterization analyses shown in Figure 5 through Figure 8. 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 4) and hybridized with Probe 1 and Probe 4 (Figure 3). 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 A3(d)(i). The results are shown in Figure 14.

Probes 1 and 4

Conventional control genomic DNA digested with *Nsi* I (Figure 14) and hybridized with Probe 1 and Probe 4 (Figure 4) 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 14, 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 14, 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 14, 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 *Nco* I (Figure 14, lane 3) produced the two expected bands at ~7.1 kb and ~1.8 kb (Figure 3 and Table 2) in addition to the endogenous hybridization bands. Probe templates generated from PV-ZMAP1043 (Figure 4) were mixed with conventional control DNA predigested with the restriction enzyme *Nsi* I (Figure 14, lane 1 and lane 2) produced the expected bands at ~1.2 kb and ~0.7 kb (Figure 4 and Table 2) 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 14, lane 5, lane 6, lane 7, lane 8, and lane 10) produced three bands (Table 2) 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 4). The ~2.0 kb band contains an internal portion of the inserted T-DNA (Figure 4). 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 4). 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 14, lane 5, lane 7, lane 8, and lane 10) is consistent with that from the fully characterized generation MON 87427 LH198 BC3F4 (Figure 5, lane 4 and lane 11; Figure 14, 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.

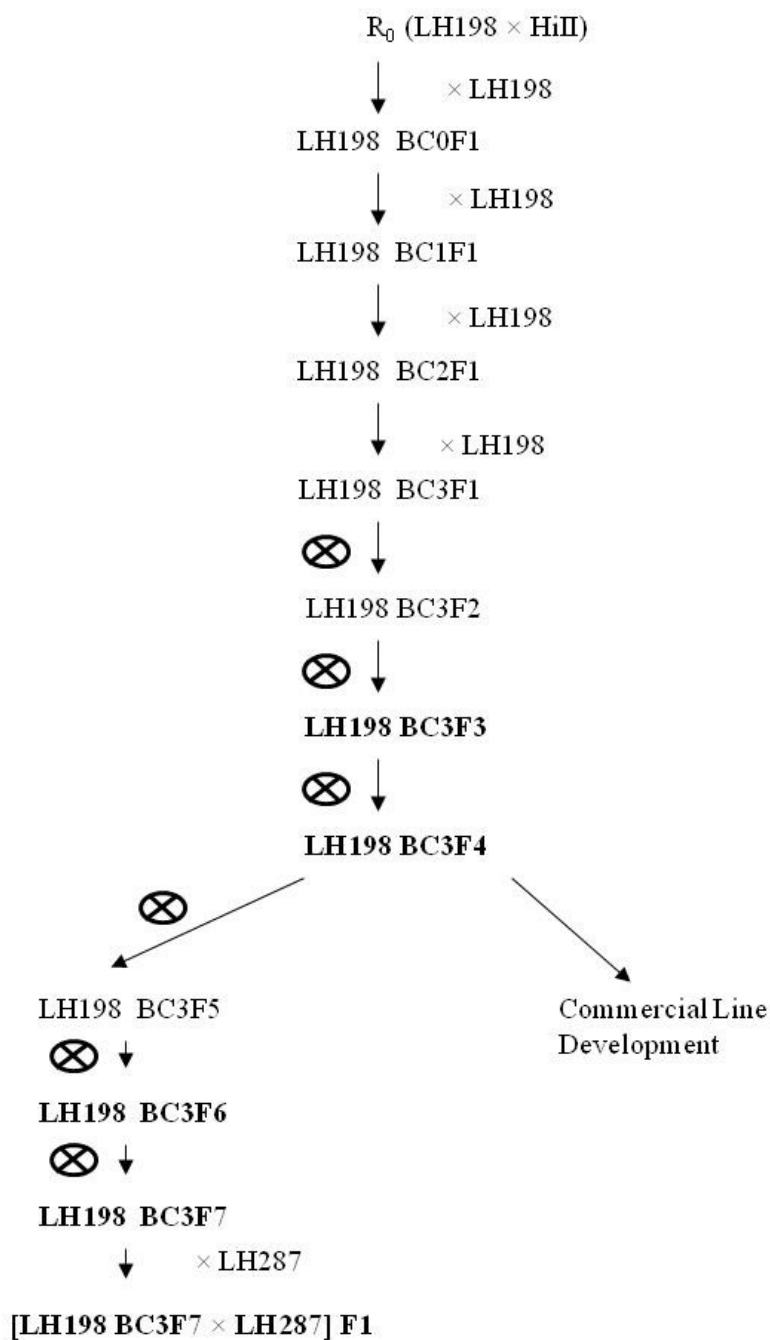


Figure 13. Breeding History of MON 87427

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.

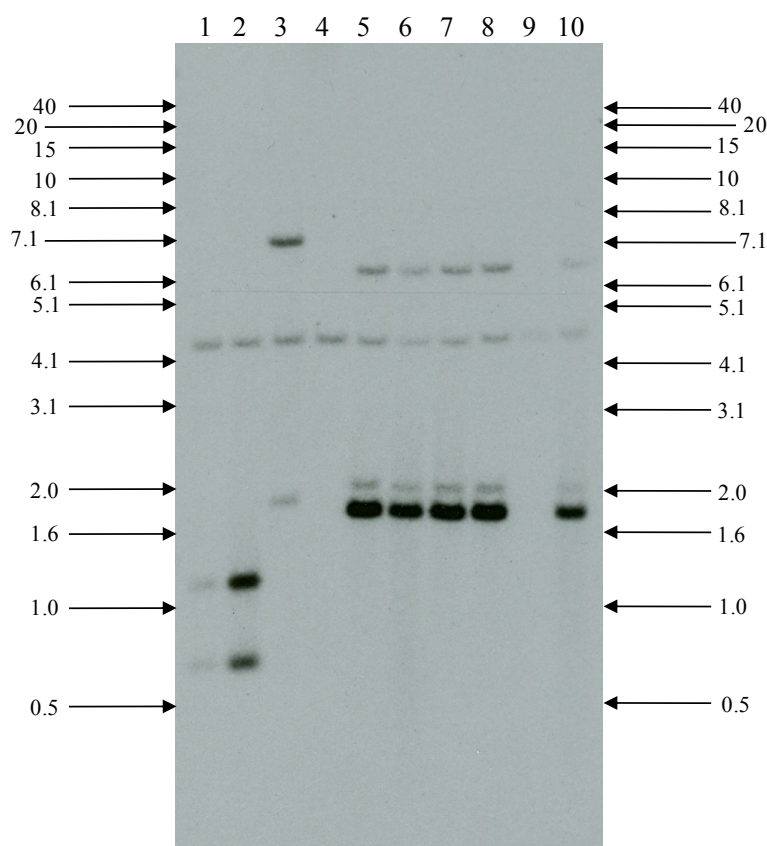


Figure 14. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 87427: Probes 1 and 4

The blot was hybridized with two ^{32}P -labeled probes that spanned portions of the T-DNA sequence (Figure 3, 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 \times HiII (*Nsi* I) spiked with Probe 1 and Probe 4 [~0.1 genome equivalent]
- 2 Conventional control LH198 \times HiII (*Nsi* I) spiked with Probe 1 and Probe 4 [~1.0 genome equivalent]
- 3 Conventional control LH198 \times HiII (*Nsi* I) spiked with PV-ZMAP1043 (*Sph* I) [~1.0 genome equivalent]
- 4 Conventional control LH198 \times 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 \times LH287 (*Nsi* I)
- 10 MON 87427([LH198 BC3F7 \times 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.

A3(f)(ii) Pattern of expression of phenotype over several generations

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 15). 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 15, lanes 4-8) to the CP4 EPSPS protein reference standard (Figure 15, lane 3). As shown in Figure 15, 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 9).

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 15, lane 10).

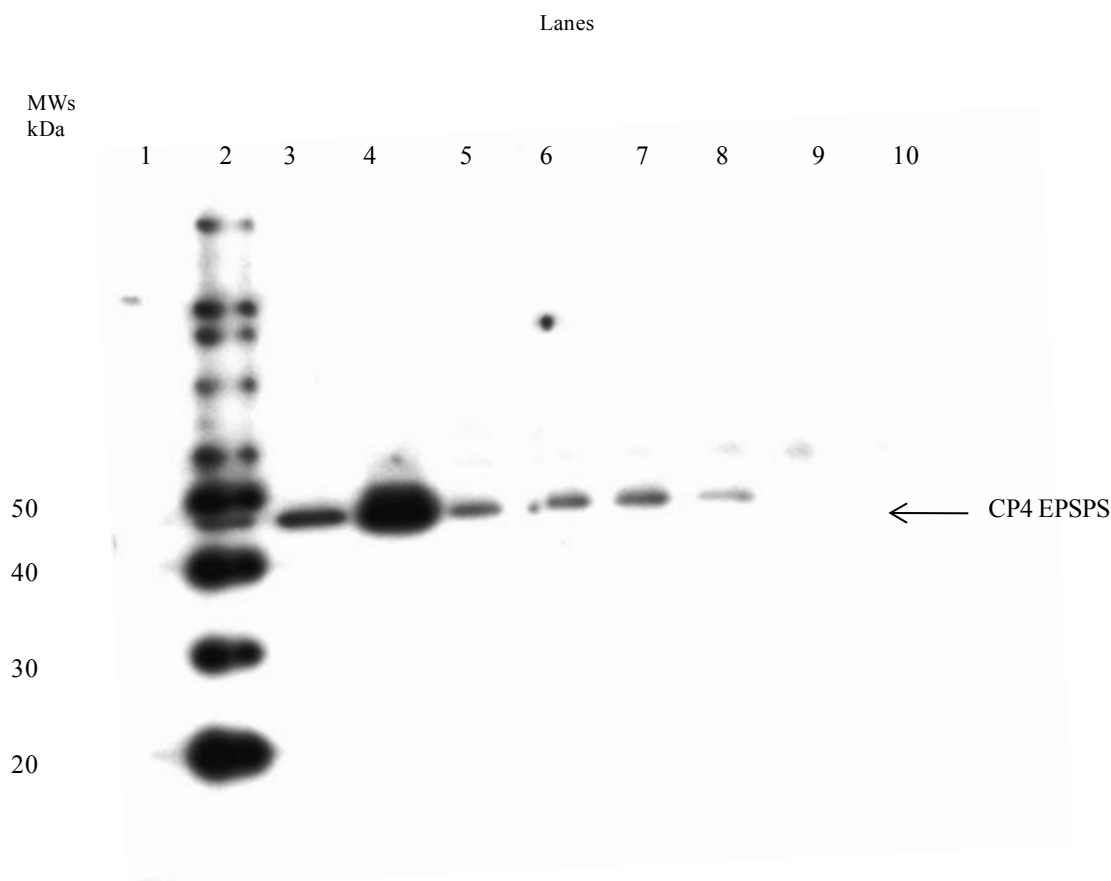


Figure 15. Presence of CP4 EPSPS Protein in Multiple Generations of MON 87427

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.

| <u>Lane</u> | <u>Description</u> | <u>Amount Loaded on Gel</u> |
|-------------|---|-----------------------------|
| 1 | Blank | NA |
| 2 | Magic Marker Molecular Weight Marker | 0.5 µl |
| 3 | <i>E. coli</i> -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 |

A4. Analytical method for detection

Information suitable for detection of novel DNA or novel protein in GM food

The DNA sequence of the insert and adjacent Genomic DNA in MON87427 has been provided to FSANZ in the Confidential Attachment.

B1 Antibiotic Resistance Marker Genes

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 this application demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 87427.

B1(a) Clinical importance of antibiotic that GM is resistant to (if any)

Not applicable

B1(b) Presence in food of antibiotic resistance protein (if any)

Not applicable

B1(c) Safety of antibiotic protein

Not applicable

B1(d) If GM organism is micro-organism, is it viable in final food?

Not applicable

B2 Characterisation of novel proteins or other novel substances**B2(a) Biochemical function and phenotypic effects of novel protein(s)**

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; Schönbrunn et al., 2001; Steinrücken 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 (Steinrücken 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 (Padgett 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 (Padgett 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 (Steinrücken 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

(Padgett 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.

B2(b) Identification of novel substances (e.g. metabolites), levels and site

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.

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.

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 16) 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 Meinel, 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 16. 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.

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 5). The identified masses were used to assemble a mass fingerprint map of the entire CP4 EPSPS protein (Figure 17). 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 5. Summary of the Tryptic Masses Identified for the MON 87427-produced CP4 EPSPS Protein Using MALDI-TOF MS

| Matrix | | | | | | Expected Mass ¹ | Diff. ² | AA Position ³ | Fragment |
|-----------------|-----------------|--------------|-----------|----------------|----------------|----------------------------|--------------------|--------------------------|---|
| α -Cyano | α -Cyano | DHB | DHB | Sinapinic acid | Sinapinic acid | | | | |
| 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 | TPTPTTYR |
| 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 | SFMFGGLASGETR |
| | | 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 | SAVLLAGLNTPGTTTVEIPIMTR |
| | | | | 2449.44 | | 2450.23 | 0.79 | 24-46 | IPGDKSISHRSFPMFGGLASGETR |
| | | | | 2449.44 | | 2450.22 | 0.78 | 105-127 | LTMGLGVYDFDSTFIGDASLTK |
| 3250.78(AVE) | | 3251.23(AVE) | | 3250.80(AVE) | 3252.37(AVE) | 3251.75 | 0.97 | 321-351 | APSMIDEYPILAVAAFAEGATVMNGLEELR |
| | | 4190.17(AVE) | | 4190.98(AVE) | 4190.14(AVE) | 4180.89 | 0.72 | 234-274 | LTGQVIDVPGDPSSTAFPLVAALLVPGSDVTILNVLMPNTR |

¹Only experimental masses that matched expected masses are listed in the table.

²The 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.

³AA position refers to amino acid residues within the predicted CP4 EPSPS sequence as depicted in Figure 17.
AVE = mass average.

```

001  MLHGASSRPA TAR[KSSGLSG TVRIPGDKSI SHRSFMFGGL ASGETRITGL]
051  [LEGEDVINTG KAMQAMGAR]I RKEGDTWIID GVGNGGLLAP EAPLDFGNAA
101  TGCR[LTMGLV GYDFDSTFI GDASLTKRPM GRVLNPLR]EM GVQVK[SEDGD]
151  [RLPVTLR]GPK [TPTPITYR]VP MASAQVK[SAV LLAGLNTPGI TTVIEPIMTR]
201  [DHTEKMLQGF GANLTVETDA DGVR]TIRLEG RGK[LTGQVID VPGDPSSTAF]
251  [PLVAALLVPG SDVTILNVLM NPTRTGLILT LQEMGADIEV INPRLAGGED]
301  [VADLR]VRSST LK[GVTVPEDR APSMIDEYPI LAVAAAFAG ATVMNGLEEL]
351  [RVK]ESDRLSA VANGKLKNGV DCDEGETSLV VRGRPDGKGL GNASGAAVAT
401  [HLDHR]IAMSF LVMGLVSENP VTVDDATMIA TSFPEFMDLM AGLGAKIELS
451  DTKAA

```

Figure 17. 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.

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 ($\pm 0.4\%$) of the MALDI-TOF MS instrument. This analysis confirmed the identity of the MON 87427-produced CP4 EPSPS protein.

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 18). 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 6). This observed difference was within the preset acceptance criteria for immunoreactivity ($\pm 35\%$). 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.

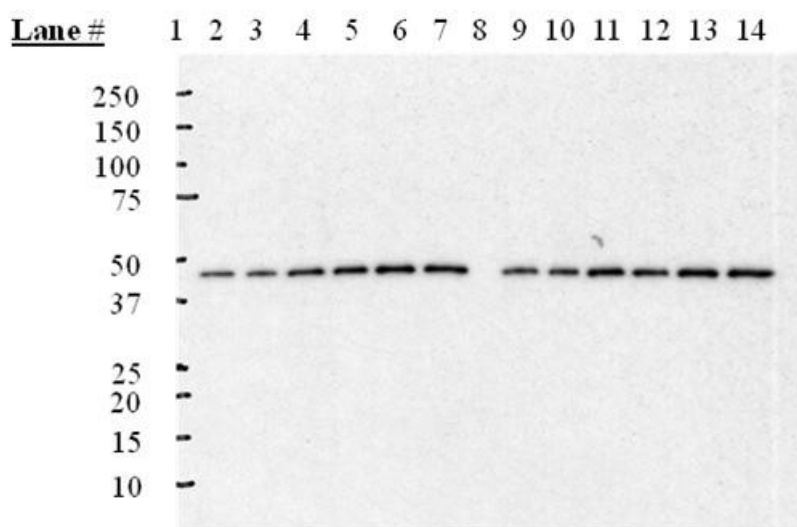


Figure 18. Western blot Analysis of MON 87427- and *E. coli* -produced CP4 EPSPS Protein

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 | <i>E. coli</i> -produced CP4 EPSPS protein | 1 |
| 3 | <i>E. coli</i> -produced CP4 EPSPS protein | 1 |
| 4 | <i>E. coli</i> -produced CP4 EPSPS protein | 2 |
| 5 | <i>E. coli</i> -produced CP4 EPSPS protein | 2 |
| 6 | <i>E. coli</i> -produced CP4 EPSPS protein | 3 |
| 7 | <i>E. coli</i> -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 6. 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 | 9.6 |
| 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 | |
| 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 | | | |

¹Average Contour Quantity = $\sum(\text{Contour Quantity})/2$; contour quantity is average pixel density \times band area

²Percent Difference(%) = $(| \text{Average Contour Quantity plant} - \text{Average Contour Quantity } E. coli |) / (\text{Average Density plant}) \times 100\%$

³Average difference (%) = $\sum [\% \text{ difference}] / 3$

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 19). The MON 87427-produced CP4 EPSPS protein (Figure 19, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS protein standard (Figure 19, lane 2) and had an apparent molecular weight of 44.1 kDa (Table 7). 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 7). Because the experimentally determined difference in apparent molecular weights met the preset acceptance criteria ($\pm 10\%$) 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 19, lanes 3 to 8). The average purity was determined to be 96%.

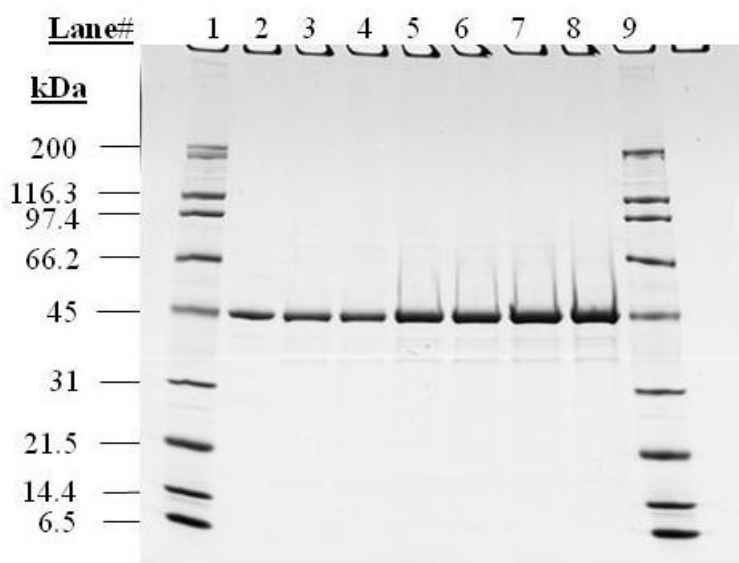


Figure 19. Molecular Weight and Purity Analysis of the MON 87427-produced CP4 EPSPS Protein

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 | <i>E. coli</i> -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 7. 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 <i>E. coli</i>-Produced CP4 EPSPS Protein | % Difference from <i>E. coli</i>-Produced CP4 EPSPS Protein¹ |
|---|--|--|
| 44.1 kDa | 43.8 kDa | 0.7% |

¹Percent difference =

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

CP4 EPSPS Glycosylation Equivalence

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple monosaccharides. In contrast, glycosylation in prokaryotes is uncommon. In *E. coli*, the organism used to produce the reference protein used in this study, only a few specific proteins have been confirmed to be glycosylated (Sherlock et al., 2006).

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 20. The positive controls were clearly detected at the expected molecular weight and the bands increased with increasing protein concentration (Figure 20 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 20, 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 20, Panel B). Both the MON 87427- and *E. coli*-produced CP4 EPSPS proteins were detected on the membrane (Figure 20, 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.

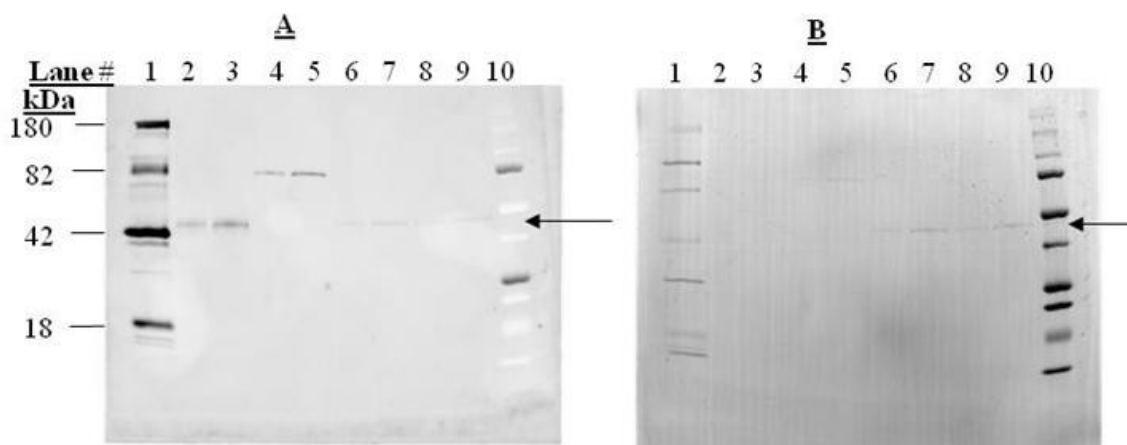


Figure 20. Glycosylation Analysis of the MON 87427 CP4 EPSPS Protein.

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 | <i>E. coli</i> -produced CP4 EPSPS protein(negative control) | 30 |
| 9 | <i>E. coli</i> -produced CP4 EPSPS protein(negative control) | 60 |
| 10 | Precision Plus Protein TM Standards Dual color | - |

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 μ mole 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 2-fold (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 8. 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 8), 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 8. CP4 EPSPS Functional Assay

| MON 87427-produced CP4 EPSPS PROTEIN¹ (U/mg) | <i>E. coli</i>-produced CP4 EPSPS PROTEIN¹ (U/mg) | Previously set acceptance limits² (U/mg) |
|--|---|--|
| 8.67 \pm 0.23 | 5.41 \pm 0.37 | 2.71-10.82 |

¹Value refers to mean and standard deviation calculated based on n = 6 which includes three replicate assays spectrophotometrically analyzed at 660 nm in duplicate.

²Within 2-fold (50% difference) of the *E. coli*-produced CP4 EPSPS protein specific activity (5.41 \div 2 U/mg to 5.41 \times 2 U/mg).

B2(c) Site of expression of all novel substances and levels

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 9. 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 9).

The CP4 EPSPS protein expression data from MON 87427 is consistent with the MON 87427 product concept. As discussed in section A1(a), 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 9). 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 9. 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 9. (continued) Summary of CP4 EPSPS Protein Levels in Maize Tissues from MON 87427 Grown in 2008 U.S. Field Trials

| Tissue Type¹ | Development Stage² | Days after planting (DAP) | Mean (SD) Range (µg/g fwt)³ | Mean (SD) Range (µg/g dwt)⁴ | LOD/LOQ⁵ (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 |

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

²The maize development stage each tissue was collected.

³Protein 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.

⁴Protein 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.

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

⁶CP4 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.

B2(d) Post-translational modifications to the novel protein(s)

Please refer to section B2(b).

B2(e) Evidence of silencing, if silencing is the method of modification

Not applicable

B2(f) History of human consumption of novel substances or similarity to substances previously consumed in food

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; Schönbrunn et al., 2001; Steinrücken 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 (Padgett 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 (Padgett et al., 1996).

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by FSANZ. 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 B2(b)) used in several of the safety studies. The safety of CP4 EPSPS proteins present in numerous Roundup Ready crops has been extensively assessed (Harrison et al., 1996). 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.

B3 Assessment of Potential Toxicity

The history of safe use of the introduced protein (section B2(f)) is one important consideration in the assessment for potential toxicity.

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 (B3(a)); 2) if the protein is rapidly digested in mammalian gastrointestinal systems (B3(b)); 3) if the protein is stable to heat treatment (B3(b)); 4) if the protein exerts any acute toxic effects in mammals (B3(c)). The CP4 EPSPS protein in MON 87427 has been assessed for its potential toxicity based on these criteria, and was determined to pose no significant toxicological risk.

B3(a) Bioinformatic comparison (aa) of novel protein(s) to 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 (Caetano-Anollés et al., 2009; Illergård et al., 2009). 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 (Silvanovich et al., 2009). 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.

B3(b) Stability to heat or processing and/or degradation in gastric model

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 75°C (section B4(c).2). 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 B4(c).1). 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.

B3(c) Acute or short-term oral toxicity on novel protein(s)

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 (few-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.

B4 Assessment of Potential Allergenicity

The history of safe use of the introduced protein (section B2(f)) is one important consideration in the assessment for potential allergenicity.

Additionally, according to guidelines adopted by the Codex Alimentarius Commission (2003) 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, 2003). A protein is not likely to be associated with allergenicity if: 1) the protein is from a nonallergenic source (section B4(a)); 2) the protein does not share structural similarities based on the amino acid sequence to known allergens (section B4(b)); 3) the protein is rapidly digested in mammalian gastrointestinal systems (section B4(c)(i)); 4) the protein is not stable to heat treatment (section B4(c)(ii); and 5) the protein represents only a very small portion of the total plant protein (section B4(e)). The CP4 EPSPS protein in MON 87427 has been assessed for its potential allergenicity according to these safety assessment guidelines.

B4(a) Source of introduced protein

As described in Sections A2(a)(i) and A2(a)(ii), 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 (Padgett 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/WHO, 2001).

B4(b) Bioinformatic comparison (aa) of novel protein(s) to allergens

The Codex guidelines for the evaluation of the allergenicity potential of introduced proteins (Codex Alimentarius, 2003) 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, 2003; 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 (Caetano-Anollés et al., 2009; Illergård et al., 2009). 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 (Silvanovich et al., 2009). 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.

B4(c) Structural properties, including digestion by pepsin, heat treatment

B4(c)(i) 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 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, when digested in simulated intestinal fluid (SIF), the half life of CP4 EPSPS protein

was less than 10 minutes (Harrison et al., 1996). 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. 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 B2(b)), 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 21). Western blot analysis confirmed that greater than 95% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 seconds (Figure 22). 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.

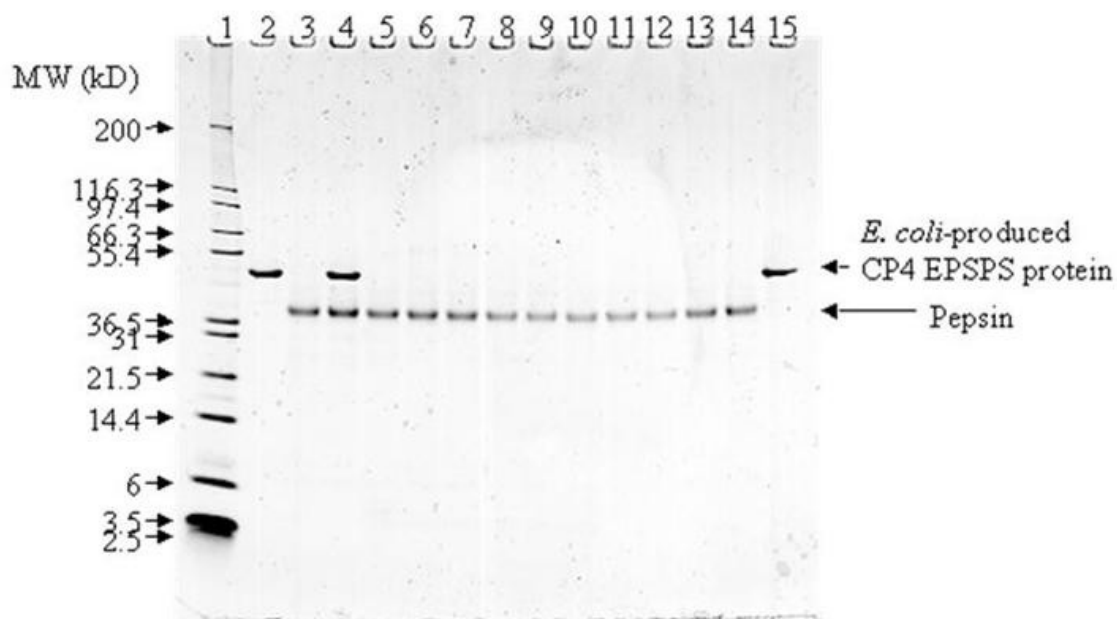


Figure 21. Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid

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 |

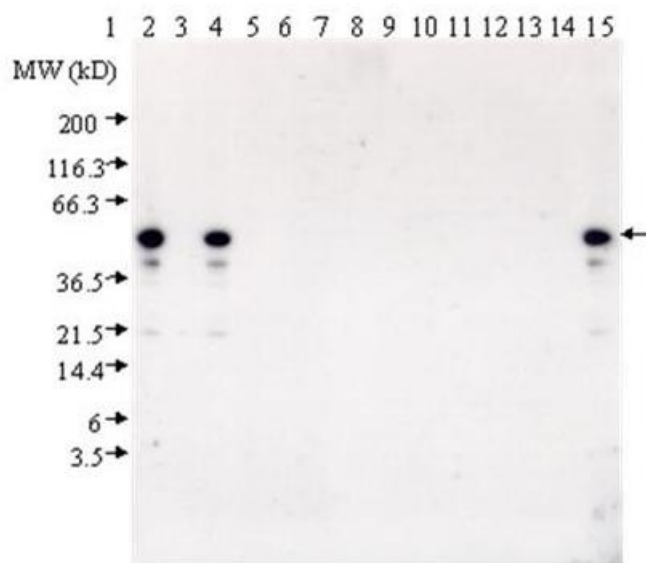


Figure 22. Western Blot Analysis of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid

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 |

B4(c)(ii) 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 *E. coli*-produced CP4 EPSPS protein was evaluated using purified protein. CP4 EPSPS protein was heated to 25, 37, 55, 75, and 95 °C for either 15 min or 30 min. Heat-treated samples and an unheated control sample of CP4 EPSPS protein 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 for 15 min and 30 min is presented in Table 10 and Table 11, respectively. After treatment at temperatures of 75 °C and higher CP4 EPSPS functional activity was below the limit of detection. There was no effect on band intensity, as measured by SDS-PAGE, of heat-treated samples after incubation for 15 or 30 minutes at all temperatures tested (Figure 23 and Figure 24, respectively). These data demonstrate that CP4 EPSPS behaves with a predictable tendency toward enzyme denaturation at elevated temperatures.

Table 10. Activity of CP4 EPSPS after 15 Minutes at Elevated Temperatures

| Temperature | Functional Activity CP4 EPSPS (U/mg) (Mean ¹ ± SD ²) | Relative activity ³ |
|-------------------------|---|--------------------------------|
| Unheated Control (0 °C) | 6.03 ± 0.29 | 100% |
| 25 °C | 4.88 ± 0.24 | 81% |
| 37 °C | 5.08 ± 0.33 | 84% |
| 55 °C | 4.22 ± 0.12 | 70% |
| 75 °C | < LOD ⁴ | < 3% ⁵ |
| 95 °C | < LOD ⁴ | < 3% ⁵ |

¹ Mean specific activity determined from n = 3.

² SD = standard deviation

³ CP4 EPSPS activity of unheated control was assigned 100 %.

⁴ LOD is defined as the value that is three standard deviations above the mean of the assay blank.

⁵ Calculated from the LOD of the CP4 EPSPS activity assay.

Table 11. Activity of CP4 EPSPS after 30 Minutes at Elevated Temperatures

| Temperature | Functional Activity CP4 EPSPS (U/mg) (Mean ¹ ± SD ²) | Relative activity ³ |
|-------------------------|---|--------------------------------|
| Unheated Control (0 °C) | 2.8 ± 0.26 | 100% |
| 25 °C | 3.1 ± 0.23 | 110% |
| 37 °C | 2.5 ± 0.05 | 88% |
| 55 °C | 0.70 ± 0.09 | 25% |
| 75 °C | < LOD ⁴ | < 8% ⁵ |
| 95 °C | < LOD ⁴ | < 8% ⁵ |

¹ Mean specific activity determined from n = 3.

² SD = standard deviation

³ CP4 EPSPS activity of unheated control was assigned 100 %.

⁴ LOD is defined as the value that is three standard deviations above the mean of the assay blank.

⁵ Calculated from the LOD of the CP4 EPSPS activity assay.

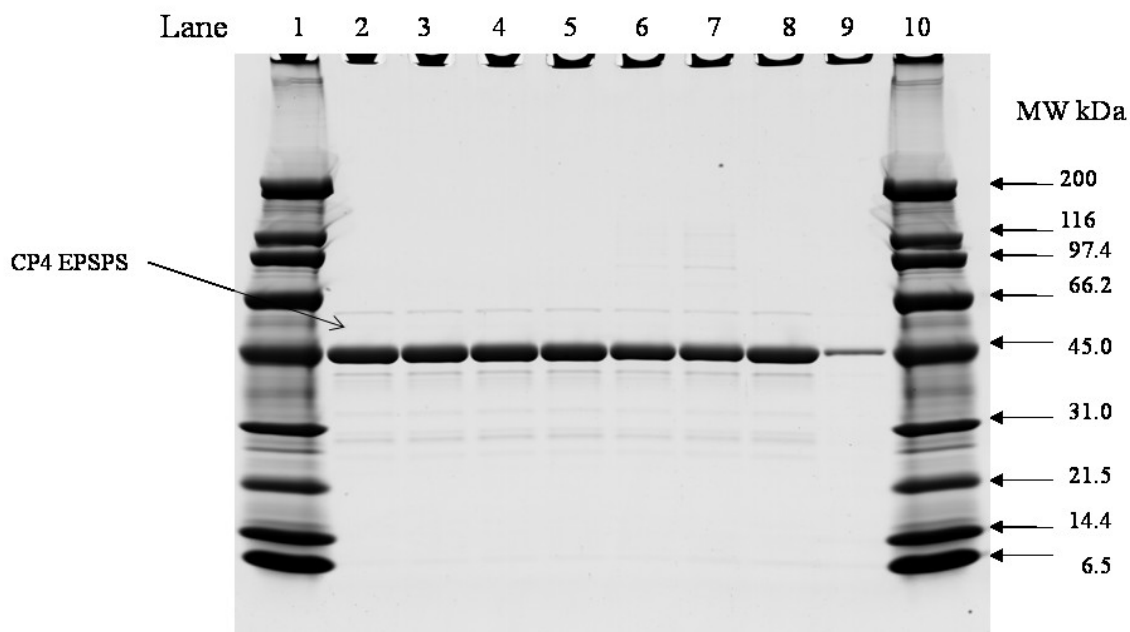


Figure 23. SDS-PAGE of CP4 EPSPS Following Heat Treatment for 15 Minutes

Heated-treated samples of CP4 EPSPS (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 | Description | Amount (µg) |
|------|---|-------------|
| 1 | Broad Range Molecular Weight Markers | 4.5 |
| 2 | CP4 EPSPS Temperature Unheated Control (0 °C) | 3.2 |
| 3 | CP4 EPSPS 25 °C | 3.2 |
| 4 | CP4 EPSPS 37 °C | 3.2 |
| 5 | CP4 EPSPS 55 °C | 3.2 |
| 6 | CP4 EPSPS 75 °C | 3.2 |
| 7 | CP4 EPSPS 95 °C | 3.2 |
| 8 | CP4 EPSPS Reference | 3.2 |
| 9 | CP4 EPSPS Reference | 0.32 |
| 10 | Broad Range Molecular Weight Markers | 4.5 |

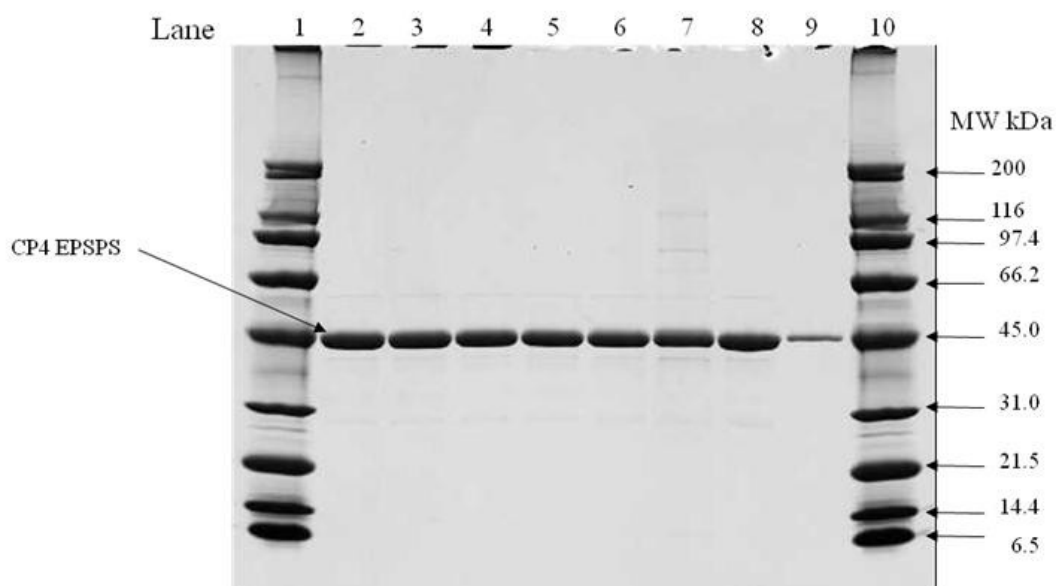


Figure 24. SDS-PAGE of CP4 EPSPS Following Heat Treatment for 30 Minutes

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 | Description | Amount (µg) |
|------|--------------------------------------|-------------|
| 1 | Broad Range Molecular Weight Markers | 4.5 |
| 2 | CP4 EPSPS 25 °C | 3.2 |
| 3 | CP4 EPSPS 37 °C | 3.2 |
| 4 | CP4 EPSPS 55 °C | 3.2 |
| 5 | CP4 EPSPS 75 °C | 3.2 |
| 6 | CP4 EPSPS 95 °C | 3.2 |
| 7 | CP4 EPSPS Unheated Control (0 °C) | 3.2 |
| 8 | CP4 EPSPS Reference | 3.2 |
| 9 | CP4 EPSPS Reference | 0.32 |
| 10 | Broad Range Molecular Weight Markers | 4.5 |

B4(d) Specific serum screening if protein from allergenic source

Not applicable. CP4 EPSPS protein is derived from *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/WHO, 2001). In addition, 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.

B4(e) 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 9) 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 100,500µg/g). The percentage of CP4 EPSPS in MON 87427 harvested grain is calculated as follows:

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

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

B5 Toxicity of novel herbicide metabolites in GM herbicide-tolerant plants**Identification of novel residues and calculation of residue levels**

Glyphosate metabolism studies have been conducted in a variety of conventional crops and in glyphosate-tolerant crops with the *cp4 epsps* gene (FAO, 2005). The metabolic pathway of glyphosate and the nature of the metabolites are the same for conventional and glyphosate-tolerant crops sprayed with glyphosate - only the relative distribution varies depending on the extent of glyphosate conversion to aminomethylphosphonic acid (AMPA), the major degradate found in plants (FAO, 2005). AMPA can be further modified via conjugation with naturally occurring small molecular weight organic compounds to produce trace level components (FAO, 2005).

Previous investigations of the metabolism and distribution of glyphosate following root uptake from the soil in conventional crops like soybeans, cotton, wheat, and corn have been conducted. Analyses of concentrated aqueous extracts of plant tissues showed that glyphosate was partially metabolized to AMPA. Glyphosate was the major ¹⁴C-containing compound in the aqueous extracts in all samples except corn forage. In corn forage, comparable amounts of glyphosate and AMPA were found. The major ¹⁴C-containing metabolite found in all extracts of plant samples was AMPA. In all cases, AMPA accounts for less than 28% of the radioactive residues, and typically is less than 10%. With the exception of AMPA, no other metabolites of glyphosate were present at greater than 2% of the total radioactive residues. The results from investigations with crops exposed to ¹⁴C-glyphosate via hydroponic solution also indicate that ¹⁴C-glyphosate, presumably via the intermediacy of

AMPA, is degraded to carbon dioxide and other one-carbon fragments that are expired or incorporated in natural plant metabolic processes (FAO, 2005).

Metabolism studies have also been completed in glyphosate-tolerant soybeans, sugar beet, and cotton crops that contain the *cp4 epsps* gene. In glyphosate-tolerant soybeans, glyphosate is metabolized substantially to AMPA, which can be further conjugated with natural plant constituents to give trace level metabolites, or degraded to one carbon fragments that are incorporated into natural products (FAO, 2005). None of the trace level metabolites account for greater than 2% of the total radioactive residues (TRR) in any soybean raw agricultural commodity. Glyphosate plus AMPA account for at least 66% of the total radioactive residues in forage, hay, and grain of soybean. In glyphosate-tolerant cotton, glyphosate is partially metabolized to AMPA (FAO, 2005). Glyphosate and AMPA account, respectively, for 91 - 95% and 0.7 - 1.6% of the TRR in forage. The metabolism of ¹⁴C-glyphosate in glyphosate-tolerant sugar beet was very similar to soybeans and cotton (FAO, 2005). The results of the study show that glyphosate is partially metabolized to AMPA and low levels of AMPA conjugates in sugar beet. Glyphosate and AMPA together account for at least 99% and 81% of the total radioactive residues in roots and tops, respectively. AMPA is further converted to a limited degree to produce low levels of simple conjugates. In addition to conjugation, the results indicated that glyphosate and AMPA are further degraded to one-carbon fragments that become broadly incorporated into a wide variety of natural products and plant constituents.

The results of these studies demonstrate that the metabolic fate of glyphosate in glyphosate-tolerant plants is the same as in conventional plants. The addition of the *cp4 epsps* gene into the plant should not affect the route of glyphosate metabolism.

A glyphosate residue study was conducted on maize hybrids that contained MON 87427 and the Roundup Ready[®] maize events Roundup Ready Corn 2 (NK603) or YieldGard VT Rootworm / Roundup Ready Corn 2 (MON 88017). These hybrids were selected because MON 87427 will only be used for the production of viable commercial hybrid corn seed through the pollination of a MON 87427 female parent inbred with a male parent inbred that has a commercially desirable and approved trait with vegetative and reproductive tolerance to glyphosate. MON 87427 will be combined with other glyphosate-tolerant maize using traditional breeding techniques and will not be released commercially as a single trait. The labeled glyphosate application rates for weed control will be the same in the combined trait products and the commercially desirable male parent inbreds that are used in the production of commercial hybrid corn seed.

Data were obtained on the residue levels of glyphosate and AMPA in maize grain grown from maize hybrids containing both MON 87427 and NK603 or MON 88017, following applications of the glyphosate-based herbicide, Roundup WeatherMAX[®] (Mueth, 2010). The combination of applications represent the maximum pre-emergence and in-crop applications allowed on maize hybrids containing Roundup Ready 2 Technology (NK603 or MON 88017), and the total combined in-season rate of 6.9 kg glyphosate acid per hectare is consistent with the current U.S. label. Glyphosate and AMPA residues ranged from 0.01 - 0.13 ppm and 0.01 - 0.03 ppm in MON 87427 × NK603 grain, respectively, and from 0.01 - 0.20 ppm and 0.01 - 0.04 ppm in MON 87427 × MON 88017 grain, respectively. However, the median levels of glyphosate and AMPA in MON 87427 × NK603 grain are 0.04 ppm and 0.01 ppm, respectively, and are 0.06 ppm and 0.02 ppm in MON 87427 × MON 88017 grain, respectively. These results are below the tolerance or Maximum Residue Limit (MRL) established for glyphosate in maize grain by numerous regulatory authorities. These MRLs include 5 ppm established by the United States EPA (2000) and CODEX (Codex

Alimentarius, 2010). Monsanto Australia Limited is therefore separately requesting that FSANZ consider amending Standard 1.4.2 – Maximum Residue Limits to raise the MRL tolerance for glyphosate on maize food imports to that which is set out in the Code of Federal Regulations of the United States of America.

B6 Compositional Assessment

Several Roundup Ready crops that produce the CP4 EPSPS protein have been reviewed by FSANZ. 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 FSANZ 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 biotechnology-derived crop to that of conventional counterparts (Codex Alimentarius, 2003). 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 biotechnology-derived 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.

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), 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. 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 ($\alpha=0.05$). 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 ($\alpha=0.05$) 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.

B6(a) Levels of key nutrients, toxicants and anti-nutrients

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), B₁, B₂, B₆, 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 (Table 12 and Table 13).

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 (Table 12 and Table 13).
- 3) Assessment of reproducibility for the combined-site significant differences at the three individual sites demonstrated significant differences ($\alpha=0.05$) 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, 2006²); (Table 17).

The six combined-site significant differences ($\alpha=0.05$) 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

² ILSI, 2006. Crop Composition Database Version 3.0. <http://www.cropcomposition.org/>

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) (Table 13). 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, 2006) highlight the extensive natural variability in compositional component levels in maize, as presented in Table 17. 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 17). 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.

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 ($\alpha=0.05$) between MON 87427 and conventional control (Table 12 and Table 14) 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 (Table 12 and Table 14)
- 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, 2006).

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.

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 ($\alpha=0.05$) 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.

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 ($\alpha=0.05$) 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 12. Summary of Differences ($\alpha=0.05$) 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) | | MON 87427 Range | Commercial Tolerance Interval ⁵ |
|--|---|------------------------------|--|---------------------------|--------------------|---|
| | | | Mean Difference (% of Control) | Significance (p-Value) | | |
| 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 12 (continued). Summary of Differences ($\alpha=0.05$) 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) | | MON 87427 Range | Commercial Tolerance Interval ⁵ |
|--|---|------------------------------|--|---------------------------|--------------------|---|
| | | | Mean Difference (% of Control) | Significance (p-Value) | | |
| 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 12 (continued). Summary of Differences ($\alpha=0.05$) 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) | | MON 87427 Range | Commercial Tolerance Interval ⁵ |
|---|---|------------------------------|--|---------------------------|--------------------|---|
| | | | Mean Difference (% of Control) | Significance (p-Value) | | |
| 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 12 (continued). Summary of Differences ($\alpha=0.05$) 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) | | MON 87427 Range | Commercial Tolerance Interval ⁵ |
|---|---|------------------------------|--|---------------------------|--------------------|---|
| | | | Mean Difference (% of Control) | Significance (p-Value) | | |
| 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 12 (continued). Summary of Differences ($\alpha=0.05$) 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) | | MON 87427 Range | Commercial Tolerance Interval ⁵ |
|---|---|------------------------------|--|---------------------------|--------------------|---|
| | | | Mean Difference (% of Control) | Significance (p-Value) | | |
| 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 12 (continued). Summary of Differences ($\alpha=0.05$) 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) | | MON 87427 Range | Commercial Tolerance Interval ⁵ |
|---|---|------------------------------|--|---------------------------|--------------------|---|
| | | | Mean Difference (% of Control) | Significance (p-Value) | | |
| 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 |

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

Table 13. 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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 13 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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) |

¹dw = dry weight; fw = fresh weight; FA = fatty acid.

² MON 87427 treated with glyphosate.

³Mean (S.E.) = least-square mean (standard error); CI = confidence interval.

⁴Control refers to the near isogenic, conventional control.

⁵With 95% confidence, interval contains 99% of the values expressed in the population of commercial references. Negative limits were set to zero.

Table 14. 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) | | | Commercial Tolerance Interval (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|---|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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) |

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

Table 15. 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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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) |

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

Table 16. 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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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 16 (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) | | | Commercial Tolerance Interval ⁵ (Range) |
|---|---|--|--------------------------------------|------------------------|---------------------------|--|
| | | | Mean (S.E.) (Range) | 95% CI Lower, Upper | Significance (p-Value) | |
| 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) |

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

Table 17. Literature and ILSI Ranges for Components in Maize Forage and Grain

| Grain Tissue Components¹ | Literature Range² | ILSI Range³ |
|--|---|-------------------------------|
| 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 17 (continued). Literature and ILSI Ranges for Components in Maize Forage and Grain

| Grain Tissue Components¹ | Literature Range² | ILSI Range³ |
|---|--|-------------------------------|
| 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 Components¹ | Literature Range² | ILSI Range³ |
| 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 |

¹dw=dry weight; fw=fresh weight, FA = fatty acids.²Literature range references: a(Harrigan et al., 2009)[US 2006], b(Harrigan et al., 2009)[Chile 2006/2007].³ILSI range is from ILSI Crop Composition Database Version 3.0. International Life Science Institute, Washington, D.C. <http://www.cropcomposition.org/> (ILSI, 2006).

B6(b) Levels of other GM-influenced constituents

As described in section B6(a), detailed compositional analyses of key components of MON 87427 have been performed and have demonstrated that MON 87427 is compositionally equivalent to conventional maize. The processing of MON 87427 is not expected to be any different from that of conventional maize. Additionally, the mode of action of CP4 EPSPS protein, as described in section B2(a), 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.

B6(c) Levels of naturally-occurring allergenic proteins

Not applicable

C Nutritional Impact**C1 Data on nutritional impact of compositional changes**

There is no compositional change shown in MON 87427 as described in section B5(a).

C2 Data from an animal feeding study, if available

The data and information presented in this submission demonstrate that the food and feed derived from MON 87427 are as safe and nutritious as those derived from commercially-available, conventional maize for which there is an established history of safe consumption. Therefore, animal feeding studies do not add value to the safety of MON 87427.

Part 3 STATUTORY DECLARATION – AUSTRALIA

I, Amanda Forster, declare that the information provided in this application fully sets out the matters required and that the same are true to the best of my knowledge and belief, and that no information has been withheld that might prejudice this application.

Signature: _____

Declared before me

This day of 2011.

Part 4 REFERENCES

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