Application to Food Standards Australia New Zealand for the Inclusion of Glyphosate-Tolerant Canola MON 88302 in Standard 1.5.2 - Food Derived from Gene Technology

Submitted by:

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UNPUBLISHED REPORTS BEING SUBMITTED

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[redacted]. 2010. Bioinformatics Evaluation of DNA Sequences Flanking the 5′ and 3′ Junctions of Inserted DNA in MON 88302: Assessment of Putative Polypeptides. **MSL0023088.** Monsanto Company. **(Volume 1)**

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[redacted] 2010. Compositional Analyses of Canola Seed Collected from MON 88302 Grown in the United States and Canada during the 2009 Growing Season. **MSL0022806**. Monsanto Company. **(Volume 7 & 8)**

[redacted]. 2011. Analysis of Tannins in Canola Seed Collected from Glyphosate-Treated MON 88302 Grown in the United States and Canada during the 2009 Growing Season. **RAR-2011-0237**. Monsanto Company. **(Volume 9)**

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ABBREVIATIONS AND DEFINITIONS[1]

~	Approximately
a.e.	Acid Equivalent
AA	Amino Acid
ADF	Acid Detergent Fiber
AMPA	Aminomethylphosphonic acid
APHIS	Animal and Plant Health Inspection Service
Ave	Average
BBCH Scale	Bayer, BASF, Ciba-Geigy and Hoechst Cereal Grain Growth Scale
BLOCKS	A database of amino acid motifs found in protein families
BLOSUM	Blocks Substitution Matrix, used to score similarities between pairs of distantly related protein or nucleotide sequences
BSA	Bovine Serum Albumin
bw	Body Weight
C8-C24	8-24 Carbon-Chain Fatty Acids
CFIA	Canadian Food Inspection Agency
CFR	Code of Federal Regulations
CI	Confidence Interval
cp4 epsps	Codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase protein from the *Agrobacterium* sp. strain CP4
CPI	Canola Protein Isolate
CSFII	Continuing Surveys of Food Intakes by Individuals
DDI	Daily Dietary Intake
DEEM-FCID	Dietary Exposure Evaluation Model-Food Commodity Intake Database
DTT	Dithiothreitol
dw	Dry Weight
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EPA	Environmental Protection Agency
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase enzyme
FA	Fatty Acid
FAO/WHO	Food and Agriculture Organization of the United Nations/World Health Organization
FSANZ	Food Standard Australia and New Zealand
fw	Fresh Weight
g	g-force
GLP	Good Laboratory Practice
GRAS	Generally Recognized As Safe
HEPES	N-[2-(hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)]

[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.

ILSI	International Life Sciences Institute
kDa	Kilodalton
LB	Laemmli buffer
LOD	Limit of Detection
LOQ	Limit of Quantitation
MMT	Million metric tons
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry
MOE	Margin of Exposure
MRL	Maximum Residue Limit
MW	Molecular Weight
MWCO	Molecular Weight Cutoff
NDF	Neutral Detergent Fiber
NFDM	Non-Fat Dry Milk
NIST	National Institute of Standards and Technology
NOAEL	No Observable Adverse Effect Level
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame
OSL	Over Season Leaf
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline containing 0.05% (v/v) Tween-20
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate
P_i	Inorganic phosphate
ppm	Parts Per Million
PRESS	Predicted Residual Sum Of Squares
PVDF	Polyvinylidene Difluoride
RBD	Refined, Bleached, and Deodorized
S3P	Shikimate-3-phosphate
Sarkosyl	*N*-lauroylsarcosine, sodium salt
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SE	Standard Error
SGF	Simulated Gastric Fluid
sp.	Species
TDF	Total Dietary Fiber
T-DNA	Transfer DNA
Tm	Melting temperature
TRR	Total Radioactive Residues

Part 1 GENERAL INFORMATION

Applicant Details

(a) Applicant's name/s	Michael Leader
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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 glyphosate-tolerant canola MON 88302 and products containing canola MON 88302 (hereafter referred to as MON 88302) to the Schedule to the Standard (see below).

Food derived from gene technology	Special requirements
Food derived from glyphosate-tolerant canola MON 88302	None

Relevant Overseas Approvals and International Standards

In 2011, Monsanto submitted a food and feed safety and nutritional assessment summary for MON 88302 to the United States Food and Drug Administration (FDA). Monsanto also requested a Determination of Nonregulated Status for MON 88302, including all progenies derived from crosses between MON 88302 and conventional canola or other canola lines previously deregulated in the United States, from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA).

Monsanto has submitted dossiers in 2011 to the Canadian Food Inspection Agency (CFIA) and Health Canada (HC); the Korean Food and Drug Administration (KFDA) and Rural Development Administration (RDA); Japan's Ministry of Health, Labour, and Welfare (MHLW) and Ministry Forestry and Fisheries; and to the European Food Safety Authority. Submissions have also been made in the Philippines and Singapore.

MON 88302 regulatory submissions will be made to countries that import significant canola or food and feed products derived from U.S., Canadian and Australian canola and that have functional regulatory review processes in place. These governmental regulatory agencies include, but are not limited to, Mexico and China, as well as to regulatory authorities in other canola importing countries with functioning regulatory systems. As appropriate, notifications will be made to countries that import significant quantities of U.S., Canadian and Australian canola and canola products and that do not have a formal regulatory review process for biotechnology-derived crops.

Monsanto makes all efforts to ensure that safety assessments are aligned, as closely as possible, with relevant international standards such as the Codex Alimentarius Commission's *Principles for the Risk Analysis of Foods Derived from Modern Biotechnology* and supporting *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants*.

Justification for the Application

-The need and/or advantages for the proposed change

Canola is grown principally for its oil which is extracted from the seed, and has both food and industrial applications. Processing canola seed yields approximately 40% oil and 60% meal (Colton and Sykes, 1992). Canola oil can be derived from any one of three species: *Brassica napus, Brassica rapa,* and *Brassica juncea* (OGTR, 2008; U.S. FDA, 1988; U.S. FDA, 2000). The host plant of MON 88302, *Brassica napus* L., is a versatile crop that provides both food and feed to the global economy and whose biology is well understood and documented.

Monsanto Company has developed a second-generation glyphosate-tolerant canola product, MON 88302, designed to provide growers with improved weed control through tolerance to higher rates of glyphosate and greater flexibility for glyphosate herbicide application. Weed competition can be a major limiting factor in canola production leading to significant yield reductions (CCC, 2006). Certain weeds, such as Canada thistle, and in Australia, silver grass, wild radish and turnip are known to be particularly important to control in Australian canola production (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/riskassessments-1). Glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaf weeds (NDSU, 2005; Padgette et al., 1996). With an increased window of application and higher spray rates, MON88302 will provide superior weed control by providing growers with the opportunity to ensure weeds that may impact yields are removed at the optimal time.

Assessment Procedure

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

Cost-Benefit Effect

If the draft variation to permit the sale and use of food derived from MON 88302 is approved possible affected parties may include consumers, industry sectors and government. The consumers who may be affected are those particularly concerned about the use of biotechnology. Industry sectors affected may be food importers and exporters, distributors, processors and manufacturers. Lastly, government enforcement agencies may be affected.

A cost/benefit analysis quantified in monetary terms is difficult to determine. In fact, most of the impacts that need to be considered cannot be assigned a dollar value. Criteria would need to be deliberately limited to those involving broad areas such as trade, consumer information and compliance. If the draft variation is approved:

Consumers:

- There would be benefits in the broader availability of canola products as there would be no restriction on foods containing canola MON 88302.
- There is unlikely to be any significant increase in the prices of foods if manufacturers are able to use comingled canola products.
- Consumers wishing to do so will be able to avoid GM canola products as a result of labeling requirements and marketing activities.

Government:

- Benefit that if canola MON 88302 was detected in food products, approval would ensure compliance of those products with the Code. This would ensure no potential for trade disruption on regulatory grounds.
- Approval of canola MON 88302 would ensure no potential conflict with WTO responsibilities.

In the case of approved GM foods, monitoring is required to ensure compliance with the labeling requirements, and in the case of GM foods that have not been approved, monitoring is required to ensure they are not illegally entering the food supply. The costs of monitoring are thus expected to be comparable, whether a GM food is approved or not.

Industry:

- Sellers of processed foods containing canola derivatives would benefit as foods derived from canola MON 88302 would be compliant with the Code, allowing broader market access and increased choice in raw materials. Retailers may be able to offer a broader range of canola products or imported foods manufactured using canola derivatives.
- Possible cost to food industry as some food ingredients derived from canola MON 88302 would be required to be labelled.

In addition, if the draft variation to permit the sale and use of food derived from MON 88302 was rejected it would result in the requirement for segregation of any products containing canola MON 88302 from those containing approved canola, which would be likely to increase the costs of imported canola-derived foods.

It is important to note that if the draft variation is approved canola MON 88302 will not have a mandatory introduction. The consumer will always have the right to choose not to use/consume this product.

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 a second-generation glyphosate-tolerant canola product, MON 88302, designed to provide growers with improved weed control through tolerance to higher rates of glyphosate and greater flexibility for glyphosate herbicide application. MON 88302 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. The CP4 EPSPS protein confers tolerance to the herbicide glyphosate, the active ingredient in the family of Roundup agricultural herbicides.

MON 88302 utilizes a *FMV/Tsf1* chimeric promoter sequence to drive CP4 EPSPS expression in different plant tissues including pollen. By virtue of CP4 EPSPS expression in pollen, MON 88302 provides tolerance to glyphosate during the sensitive reproductive stages of growth, and enables the application of glyphosate at higher rates up to first flower with no detectable impact to male fertility.

Weed competition can be a major limiting factor in canola production leading to significant yield reductions. For example, studies have demonstrated that only ten Canada thistle plants per square meter have resulted in 10% yield loss while forty plants per square meter have resulted in over 50% yield loss (CCC, 2006). While glyphosate is highly effective against the majority of annual and perennial grasses and broad-leaf weeds (NDSU, 2005; Padgette et al., 1996), the higher glyphosate rates and extended timing for applications possible with MON 88302 will enable better control of difficult to manage weeds. Use of MON 88302 will provide (1) an opportunity to control weeds if glyphosate application is delayed due to weather or equipment failure; (2) an increased ability to apply glyphosate according to the weed development stage instead of the canola developmental stage; (3) enhanced protection of canola plants at more advanced development stages at the time of glyphosate application and (4) better control of weeds such as silver grass, wild radish, turnip, Canada thistle, dandelion, common lambsquarters, kochia, smartweed and wild buckwheat. Use of MON 88302 will provide growers with the opportunity to ensure weeds that may impact yields are removed at the optimal time.

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 88302 has been assigned the unique identifier MON-883Ø2-9.

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

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

MON 88302 will be utilized in the same manner and for the same uses as conventional canola because MON 88302 is not materially different from conventional canola other than the

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introduction of the herbicide tolerance trait and can be processed into a wide variety of food products as described in section A2(b)(iii).

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

Kingdom : Bacteria
Phylum : Proteobacteria
Class : Alphaproteobacteria
Order : Rhizobiales
Family : Rhizobiaceae
Genus : *Agrobacterium*

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

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 commonly known for human or animal pathogenicity or allergenicity. According to a report of a joint FAO/WHO Expert Consultation (FAO/WHO, 2001), there is no known population of individuals sensitized to bacterial proteins. 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 (Application number A338), Roundup Ready 2 Yield soybean (A592), Roundup Ready corn 2 (A416), Roundup Ready canola (A363), Roundup Ready sugar beet (A525), Roundup Ready cotton (A355), Roundup Ready Flex cotton (A553), and Roundup Ready alfalfa (A575).

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

The host organism, *Brassica napus* (*B. napus*) is a member of the family Brassicaceae, previously known as Crucifereae (OECD, 2011).

Family – Brassicaceae (previously known as Crucifereae)
Tribe – Brassiceae
Genus – *Brassica* L.
Species – *Brassica napus* L.
Subspecies
Brassica napus oleifera (common name: spring/summer oilseed rape, Canola)
Brassica napus f. *biennis* (common name: winter oilseed rape, winter Canola)

There are numerous terms used to describe oil-producing *B. napus* varieties including oilseed rape, rapeseed, rape, low erucic acid rapeseed and canola. In this application, *B napus* will be referred to as oilseed rape and the term canola will be used to denote *B. napus* varieties that produce low (< 2%) erucic acid oil and have levels of glucosinolates below the accepted standard of 30 µmoles/g in meal (OECD, 2001).

The *B. napus* canola variety used as the recipient for the DNA insertion to create MON 88302 was Ebony, a non-transgenic conventional spring canola variety registered with the Canadian Food Inspection Agency in 1994 by Monsanto Company (CFIA, 1994). Ebony originated from a cross of varieties (Bienvenu × Alto) × Cesar. Selection criteria for the non-transgenic variety included yield, oil and protein content, and tolerance to the fungus *Leptosphaeria maculans*, commonly known as blackleg (CFIA, 2010). Ebony was used to produce the glyphosate-tolerant canola MON 88302 because it responds well to *Agrobacterium*-mediated transformation and tissue regeneration.

Ebony was used as the conventional canola comparator (referred to in this application as the conventional control) in the safety assessment of MON 88302. MON 88302 and the conventional control have similar genetic backgrounds with the exception of the *cp4 epsps* expression cassette. In addition, commercial conventional canola varieties (referred to in this application as commercial reference varieties) were used to establish ranges of natural variability or responses representative of commercial canola varieties. The commercial reference varieties used at each location were selected based on their availability and agronomic fit for the geographic region.

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

Brassica napus is predominantly self-pollinating although interplant (plants are touching one another) outcrossing rates range from 12% to 55% with a mean of 30% (Beckie et al., 2003). Pollen of *B. napus* is heavy and sticky (OECD, 1997) and pollen movement is primarily by insects, such as honey bees (Thompson et al., 1999) although wind is also responsible for some pollen movement. Most (98.8%) of pollen travels less than twelve meters from its source (Scheffler et al., 1993) although dispersal due to pollinators may occur over greater distances at low frequency (Thompson et al., 1999). In general, the percentage of pollen flow and potential for outcrossing diminishes with increasing distance from the source.

Brassica napus produces a large amount of pollen (OGTR, 2008) which can remain viable for four to five days under field conditions (Rantio-Lehtimäki, 1995). Seed ripening begins when the petal on the last formed flower on the main stem falls. Seed fill is complete approximately 35 to 45 days after flower initiation (NDSU, 2005). In Australia, canola matures 5-7 months after sowing, depending on latitude, rainfall, temperature and sowing date. Canola is typically swathed prior to full seed maturity in order to prevent pod shattering and to allow uniform drying of the grain to ideal moisture content. The grain is collected 7-10 days after swathing when the moisture is at a maximum of 8.5% (OGTR, 2008).

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

Canola is grown principally for its oil which is extracted from the seed, and has both food and industrial applications. Processing canola seed yields approximately 40% oil and 60% meal (Colton and Sykes, 1992). Canola oil is high quality oil that is used in a variety of foods including frying and baking oils, salad oils, margarines and shortenings, and is the most valuable component of canola seed. It is the world's third largest source of vegetable oil with 15% of world vegetable oil consumption after soybean oil at 28% and palm oil at 32% (ASA, 2010; USDA-ERS, 2010). Canola oil contains a low level (<10% of total fatty acids), of saturated fatty acids; a high level (approximately 60%) of the monounsaturated fatty acid, oleic acid, a moderate level (approximately 20%) of linoleic acid, and an appreciable amount (approximately 10%) of alpha-linolenic acid (CCC, 2010). Dietary guidance calls for limiting saturated fats in the diet in favor of monounsaturated and polyunsaturated fats. Canola oil helps achieve this guidance by replacing saturated fats with unsaturated fats. Furthermore, canola oil provides alpha-linolenic acid, which is essential to human health and must be supplied in the diet. Canola oil has well established heart health benefits and the U.S. FDA has issued a qualified health claim based on its ability to reduce the risk of coronary heart disease (U.S. FDA, 2006).

Another product derived from canola, canola meal is important for animal. It is used in poultry, pig, beef and dairy cattle feeds, and can also be used in aquaculture diets for salmon, catfish and trout (CCC, 2009). Canola meal contains approximately 40% protein with a good balance of essential amino acids, and approximately 13% crude fiber (Bell, 1995).

The demand for canola has risen sharply, particularly in canola oil, margarine and other canola based products. Canola is the leading oilseed crop in Australia and is a growing export industry. These canola-based products are routinely used in food and have a history of safe use.

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

Canola seed is processed into two major products with typical percent yields of (by weight): oil (40%) and meal (60%).

Preparation

In the first step of processing, canola seeds are mechanically cleaned to remove any leaves, twigs, etc. After cleaning, particularly in colder climates, the seed is pre-conditioned or tempered by heating the seeds to approximately 35ºC to prevent shattering which may occur when seed is taken directly from cold storage and processed. Due to the small seed size and high oil content which contributes to the difficulty in economically removing the protective hull which surrounds the rapeseed, the hull is usually left on the seed in large commercial operations. In some instances the hull may be removed to produce meal for special markets

i.e. canola meal with reduced fiber and increased protein. Cleaned canola seeds are flaked by a rolling process in preparation for oil extraction.

Extraction

For the first step in oil extraction, canola flakes are placed in a cooker or conditioner where they are "cooked" and the moisture reduced to a low level. Heating reduces the viscosity of the oil, and inactivates enzymes including the enzyme myrosinase found in canola seed. Myrosinase, if not deactivated, can break down glucosinolates to produce isothiocyanates and nitriles which are harmful when fed to animals. After heating, it is a common practice for canola seeds to undergo mechanical extraction to produce a cake with an oil content of less than 20%, followed by solvent extraction using hexane to remove the bulk of the remaining oil.

Further Processing

This crude oil undergoes further processing which may include: a) water or acid "degumming" to remove phospholipids; b) physical or acid refining to remove free fatty acids; c) bleaching to remove pigments and oxidation products present in the oil; d) winterization to remove some saturates that crystallize out at lower temperatures that make the oil appear cloudy; e) hydrogenation to increase oxidative stability and increasing melting points of triglycerides; f) interesterification to prevent phase separation in fats; and g) deodorization to remove undesirable odors and off flavors. Canola seed oil is premium quality oil that is used in a variety of foods including frying oils, salad oils, margarines and shortenings and is the most valuable component of canola seed.

After solvent extraction, the flakes are treated in a desolventiser-toaster to remove the residual hexane as well as to dry (toast) the flakes. The toasted flakes are then cooled and ground to produce meal. Meal produced by solvent processes usually contains 2-4% residual fat and is used principally as high-protein feed for livestock.

The processing of MON 88302 is not expected to be any different from that of conventional canola. As summarized in section B6 below, detailed compositional analyses of key components of MON 88302 have been performed and have demonstrated that MON 88302 is compositionally equivalent to conventional canola. Additionally, the mode of action of the CP4 EPSPS protein, as described in section B2(a), is well understood, and there is no reason to expect interactions of this protein with important nutrients or endogenous toxicants that may be present in canola. Therefore, when MON 88302 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 canola.

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

The European Union, Canada, and China are the largest producers of oilseed rape with 19.0, 12.64, and 12.10 million metric tons, respectively, in 2008/2009 (USDA Foreign Agricultural Service (FAS *Annual Report on World Agricultural Production,* www.fas.usda.gov).

In Australia, canola is an established crop in the medium and high rainfall (400 mm and above) areas of southern Australia, which represents the winter production cereal belt. However the development of early maturing varieties is expanding growing areas of canola into the low rainfall areas of the wheat belt. As the world's second largest exporter of canola seed, Australia's exports consistently exceed one million tonnes. From a minor crop in the late 1980s, canola is now Australia's third largest broad-acre crop (after wheat and barley). In 2010 there was 1,650,000 hectares of canola planted in Australia, resulting in a

harvest of 2,115,000 tonnes (Australian Oilseeds Federation, http://www.australianoilseeds.com/) Canola oil has become an established component in Australian diets and its use is continuing to increase with greater recognition of its healthy properties.

Estimates of canola 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 88302 was developed through *Agrobacterium*-mediated transformation of canola hypocotyls, based on the method described by Radke et al. (1992), utilizing PV-BNHT2672 (Figure 3). In summary, hypocotyl segments were excised from dark grown seedlings of germinated Ebony seed. After co-culturing with the *Agrobacterium* carrying the vector, the hypocotyl segments were placed on medium for callus growth containing carbenicillin, ticarcillin disodium and clavulanate potassium to inhibit the growth of excess *Agrobacterium*. The hypocotyls were then placed in selection media containing glyphosate to inhibit the growth of untransformed cells and plant growth regulators conducive to shoot regeneration. Rooted R_0 plants with normal phenotypic characteristics were selected and transferred to soil for growth and further assessment.

The R_0 plants generated through the *Agrobacterium*-mediated transformation were transferred to soil for growth and then selfed to produce R_1 seed. R_0 and R_1 plants were evaluated for tolerance to glyphosate and screened for the presence of the T-DNA (*cp4 epsps* expression cassette) and absence of plasmid vector backbone (*Ori V*). Subsequently, the *cp4 epsps* homozygous R_1 plant was self-pollinated to give rise to R_2 plants. Homozygous R_2 plants containing only a single T-DNA insertion were identified by a combination of analytical techniques including glyphosate spray, polymerase chain reaction (PCR), and Southern blot analysis, resulting in production of glyphosate-tolerant canola MON 88302. MON 88302 was selected as the lead event based on superior phenotypic characteristics and its comprehensive molecular profile. Regulatory studies on MON 88302 were initiated to further characterize the genetic insertion and the expressed protein, and to establish the food, feed, and environmental safety relative to conventional canola. The major steps involved in the development of MON 88302 are depicted in Figure 1.

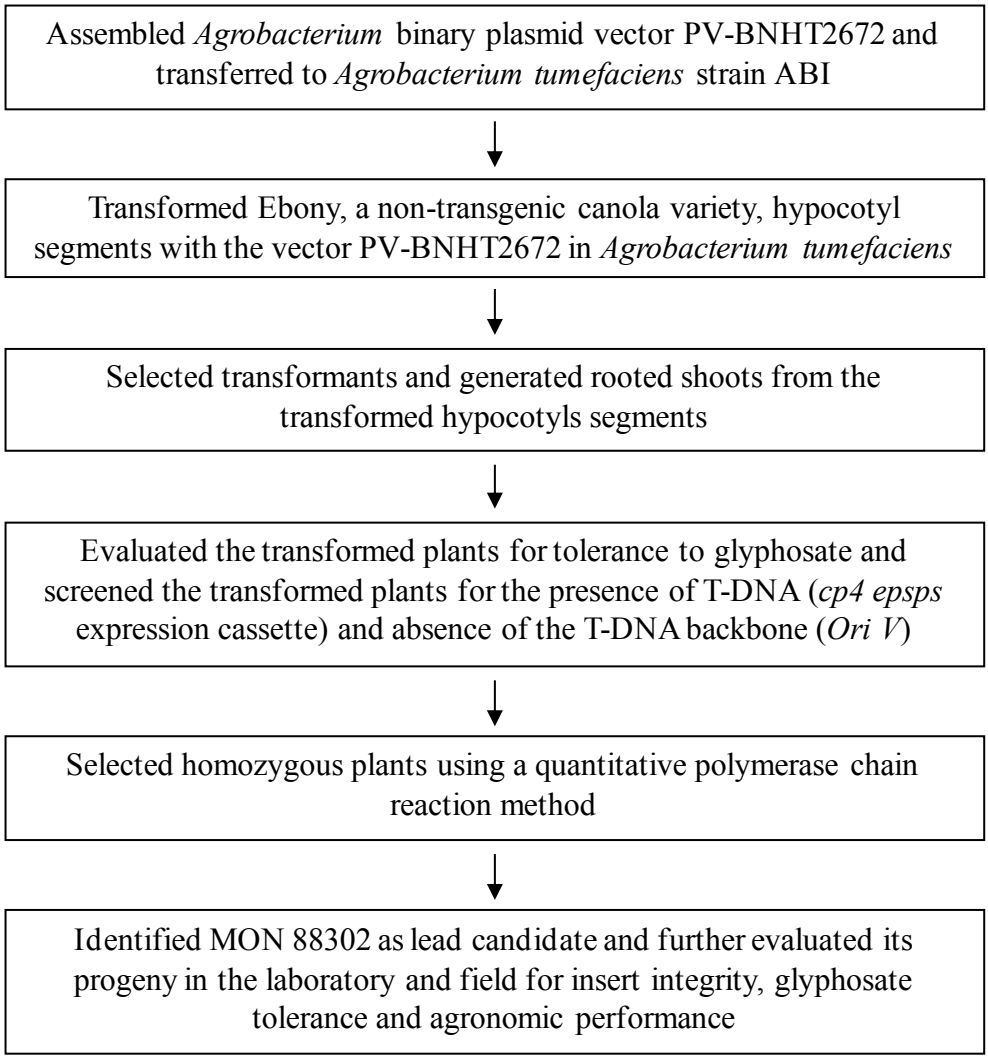

Figure 1. Schematic of the Development of MON 88302

A3(b) Intermediate hosts (eg. bacteria)

A disarmed strain of *Agrobacterium tumefaciens* was the intermediate host used to transfer the plasmid PV-BNHT2672 into canola cells to produce MON 88302. PV-BNHT2672 contains one T-DNA, containing the full *cp4 epsps* expression cassette. Following transformation, self-pollination breeding and segregation methods were used to produce MON 88302.

A3(c)(i) Gene construct including size, source and function of all elements

Plasmid Vector PV-BNHT2672

PV-BNHT2672 was used in the transformation of canola to produce MON 88302 and is shown in Figure 3. The elements included in this plasmid vector are described in Table 1. PV-BNHT2672 is approximately 9.7 kb and contains one T-DNA that is delineated by Left Border and Right Border regions. The T-DNA contains the *cp4 epsps* coding sequence under the control of the *FMV/Tsf1* chimeric promoter, the *Tsf1* leader and intron sequences, and the *E9* 3′ untranslated region. The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987).

The backbone region of PV-BNHT2672, located outside of the T-DNA, contains two origins of replication for maintenance of plasmid vector in bacteria (*ori V* and *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 *Escherichia coli (E. coli)* (*rop*). A description of the genetic elements and their prefixes (e.g., B-, P-, L-, I-, TS-, CS-, T-, and OR-) in PV-BNHT2672 is provided in Table 1.

The *cp4 epsps* expression cassette used in MON 88302 canola is similar to that used in other crops such as Roundup Ready Flex cotton and Roundup Ready 2 Yield soybean.

The *cp4 epsps* Coding Sequence and CP4 EPSPS Protein

The *cp4 epsps* expression cassette, or T-DNA in this application, encodes a 47.6 kDa CP4 EPSPS protein consisting of a single polypeptide of 455 amino acids (Figure 2) (Padgette et al., 1996). The *cp4 epsps* coding sequence is the codon optimized coding sequence of the *aroA* gene from *Agrobacterium* sp. strain CP4 encoding CP4 EPSPS (Barry et al., 2001; Padgette et al., 1996). The CP4 EPSPS protein is similar and functionally identical to endogenous plant EPSPS enzymes, but has a much reduced affinity for glyphosate, the active ingredient in Roundup agricultural herbicides, relative to endogenous plant EPSPS (Barry et al., 2001; Padgette et al., 1996). The amino acid sequence of the mature CP4 EPSPS protein produced in MON 88302 canola is identical to that in Roundup Ready canola, Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready Flex®[2] cotton, Roundup Ready sugar beet and Roundup Ready alfalfa.

Regulatory Sequences

The *cp4 epsps* coding sequence in MON 88302 is under the regulation of the *FMV/Tsf1* chimeric promoter, the *Tsf1* leader and intron sequences, and the *E9* 3′ untranslated region. The *FMV/Tsf1* chimeric promoter, which directs transcription in plant cells, contains enhancer sequences from the promoter of the figwort mosaic virus 35S RNA (Richins et al.,

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1987) combined with the promoter from the *Tsf1* gene of *Arabidopsis thaliana* that encodes elongation factor EF-1α (Axelos et al., 1989). The *Tsf1* leader sequence is the 5' untranslated region from the *Tsf1* gene of *Arabidopsis thaliana* (Axelos et al., 1989). The *E9* 3' untranslated region is the 3' untranslated region of the pea (*Pisum sativum*) ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS2*) *E9* gene (Coruzzi et al., 1984) and is present to direct polyadenylation of the *cp4 epsps* transcript. The chloroplast transit peptide CTP2 directs transport of the CP4 EPSPS protein to the chloroplast and is derived from *CTP2* target sequence of the *Arabidopsis thaliana shkG* gene (Herrmann, 1995; Klee et al., 1987).

T-DNA Border Regions

PV-BNHT2672 contains Right Border and Left Border regions (Figure 3 and Table 1) that were derived from *Agrobacterium tumefaciens* plasmids. The border regions each contain a 24-25 bp nick site that is the site of DNA exchange during transformation (Barker et al., 1983; Depicker et al., 1982; Zambryski et al., 1982). The border regions separate the T-DNA from the plasmid backbone region and are involved in the efficient transfer T-DNA into the canola genome.

Genetic Elements Outside the T-DNA Border Regions

Genetic elements that exist outside of the T-DNA border regions are those that are essential for the maintenance or selection of PV-BNHT2672 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* encodes the repressor of primer (ROP) protein which 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 canola genome. The absence of detectable backbone sequence in MON 88302 has been confirmed by Southern blot analyses (see section A3(d)(i)).

Table 1. Summary of Genetic Elements in PV-BNHT2672

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA		
B[1]-Right Border Region	1-357	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening Sequence	358-427	Sequence used in DNA cloning
P[2]-*FMV/Tsf1*	428-1467	Chimeric promoter consisting of the promoter of the *Tsf1* gene from the *Arabidopsis thaliana* encoding elongation factor EF-1α (Axelos et al., 1989) and enhancer sequences from the 35S promoter from the figwort mosaic virus (Richins et al., 1987)
L[3]-*Tsf1*	1468-1513	5' untranslated leader (exon 1) from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1α (Axelos et al., 1989)
I[4]-*Tsf1*	1514-2135	Intron from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1α (Axelos et al., 1989)
Intervening Sequence	2136-2144	Sequence used in DNA cloning
TS[5]-*CTP2*	2145-2372	Targeting sequence from the *shkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS[6]-*cp4 epsps*	2373-3740	Codon optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgette et al., 1996)
Intervening Sequence	3741-3782	Sequence used in DNA cloning
T[7]-*E9*	3783-4425	3' untranslated sequence from the *rbcS2* gene of *Pisum sativum* (pea) encoding the Rubisco small subunit (Coruzzi et al., 1984)
Intervening Sequence	4426-4468	Sequence used in DNA cloning
B-Left Border Region	4469-4910	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983; Zambryski et al., 1982)

Table 1. Summary of Genetic Elements in PV-BNHT2672 (continued)

Genetic Element	Location in Plasmid	Function (Reference)
Vector Backbone		
Intervening Sequence	4911-4996	Sequence used in DNA cloning
OR[8]- *oriV*	4997-5393	Origin of replication from the broad host range plasmid RK2 for maintenance of plasmid in *Agrobacterium* (Stalker et al., 1981)
Intervening Sequence	5394-6901	Sequence used in DNA cloning
CS-*rop*	6902-7093	Coding sequence for repressor of primer protein for maintenance of plasmid copy number in *E. coli* (Giza and Huang, 1989)
Intervening Sequence	7094-7520	Sequence used in DNA cloning
OR-*ori-pBR322*	7521-8109	Origin of replication from pBR322 for maintenance of plasmid in *E. coli* (Sutcliffe, 1979)
Intervening Sequence	8110-8639	Sequence used in DNA cloning
aadA	8640-9528	Bacterial promoter, coding sequence, and 3′ untranslated region for an aminoglycoside-modifying enzyme, 3"(9)-*O*-nucleotidyl-transferase from the transposon Tn7 (Fling et al., 1985) that confers spectinomycin and streptomycin resistance
Intervening Sequence	9529-9664	Sequence used in DNA cloning

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

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  1   MAQVSRICNG VQNPSLISNL SKSSQRKSPL SVSLKTQQHP RAYPISSSWG
 51   LKKSGMTLIG SELRPLKVMS SVSTACMLHG ASSRPATARK SSGLSGTVRI
101   PGDKSISHRS FMFGGLASGE TRITGLLEGE DVINTGKAMQ AMGARIRKEG
151   DTWIIDGVGN GGLLAPEAPL DFGNAATGCR LTMGLVGVYD FDSTFIGDAS
201   LTKRPMGRVL NPLREMGVQV KSEDGDRLPV TLRGPKTPTP ITYRVPMASA
251   QVKSAVLLAG LNTPGITTVI EPIMTRDHTE KMLQGFGANL TVETDADGVR
301   TIRLEGRGKL TGQVIDVPGD PSSTAFPLVA ALLVPGSDVT ILNVLMNPTR
351   TGLILTLQEM GADIEVINPR LAGGEDVADL RVRSSTLKGV TVPEDRAPSM
401   IDEYPILAVA AAFAEGATVM NGLEELRVKE SDRLSAVANG LKLNGVDCDE
451   GETSLVVRGR PDGKGLGNAS GAAVATHLDH RIAMSFLVMG LVSENPVTVD
501   DATMIATSFP EFMDLMAGLG AKIELSDTKA A
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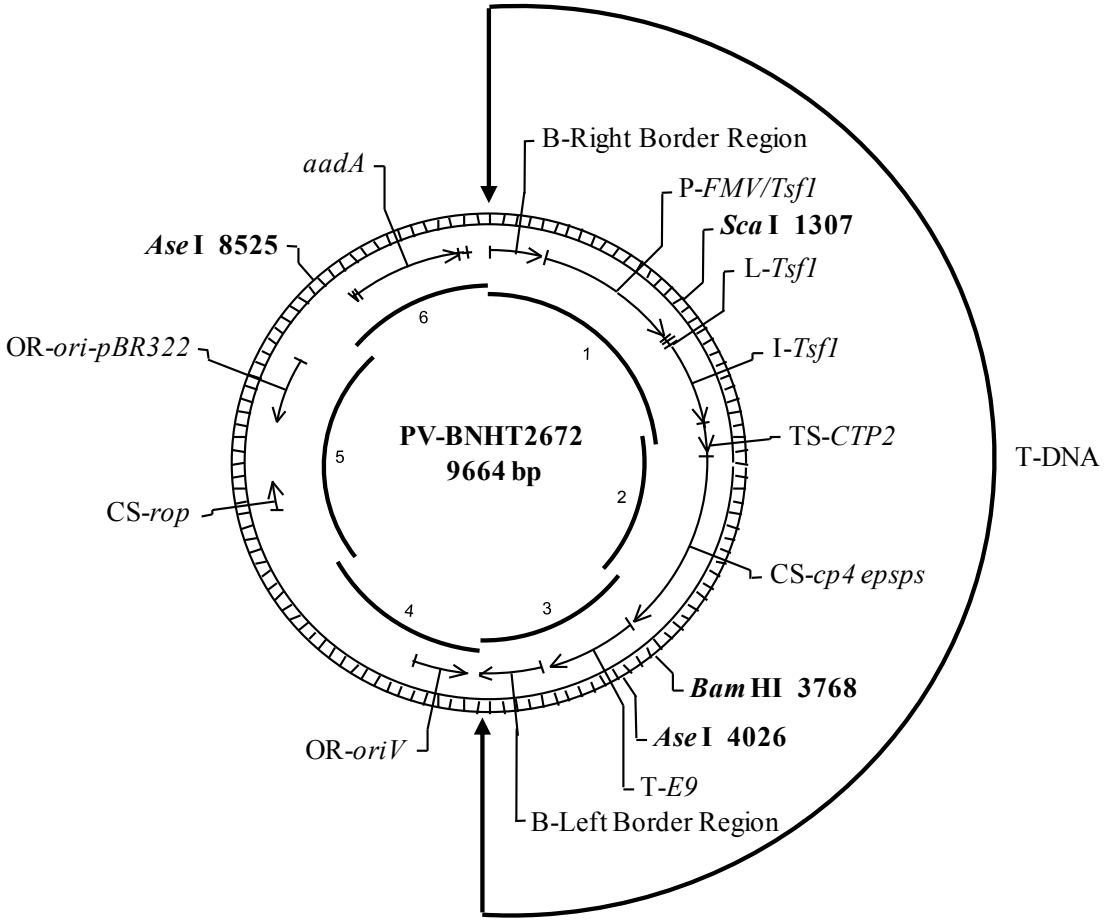
Figure 2. Deduced Amino Acid Sequence of the MON 88302 CP4 EPSPS Precursor Protein

The amino acid sequence of the CP4 EPSPS precursor protein was deduced from the full-length coding nucleotide sequence present in PV-BNHT2672. The 76 amino acid CTP2, the transit peptide of the *Arabidopsis thaliana* EPSPS protein, 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

The vector PV-BNHT2672 that was used in the transformation of canola to produce MON 88302 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	2287	~2.3
2	T-DNA Probe 2	2231	3618	~1.4
3	T-DNA Probe 3	3562	4910	~1.3
4	Backbone Probe 4	4911	6564	~1.7
5	Backbone Probe 5	6512	8383	~1.9
6	Backbone Probe 6	8329	9664	~1.3

Figure 3. Circular Map of PV-BNHT2672 Showing Probes 1-6

A circular map of PV-BNHT2672 used to develop MON 88302 is shown. Genetic elements and restriction sites (in bold) used in Southern analyses (with positions relative to the first base pair 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 and listed in the table. PV-BNHT2672 contains a single T-DNA.

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

Characterization of the DNA insert in MON 88302 was conducted by Southern blot, PCR and DNA sequence analyses (study report MSL0022523). The results of this characterization demonstrate that MON 88302 contains a single copy of the *cp4 epsps* expression cassette, i.e., the 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 canola genome for the presence of T-DNA and the absence of the plasmid backbone sequences derived from PV-BNHT2672, and demonstrated that only a single copy of the T-DNA was inserted at a single site; 2) DNA sequence analyses determined the exact sequence of the inserted DNA and the DNA sequences flanking the 5' and 3' ends of the insert, and allowed a comparison to the T-DNA sequence in the plasmid vector to confirm that only the expected sequences were integrated; and 3) DNA sequences flanking the 5' and 3' ends of the insert were compared to the sequence of the insertion site in conventional canola to identify any rearrangements that 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 canola genome and that no plasmid vector backbone sequences are present in MON 88302.

Southern blot analyses were used to determine the copy number 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 be identified. The entire canola genome was assayed with probes that spanned the complete plasmid vector to detect the presence of the insert as well as confirm the absence of any plasmid vector backbone sequences. This was accomplished by using probes that were not more than 2.5 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 copies per genome equivalent. Two sets of restriction enzymes were specifically chosen to fully characterize the T-DNA and detect any potential fragments of the T-DNA and backbone sequences. The restriction enzyme sets were chosen such that each enzyme set cleaves once within the inserted T-DNA and at least once within the known DNA flanking the 5' or 3' end of the insert. As a consequence, at least one segment containing a portion of the insert with the adjacent 5' flanking DNA generated by one set of the enzyme(s) is of a predictable size and overlaps with another predictable size segment containing a portion of the insert with the adjacent 3' flanking DNA generated by another set of the enzyme(s). This two-set-enzyme design ensures that the entire insert is identified in a predictable hybridization pattern. This strategy also maximizes the possibility of detecting an insertion elsewhere in the genome that could be overlooked if that fragment co-migrated on the gel with an expected fragment.

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 an 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 for retaining the small molecular weight DNA on the gel. 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 through Figure 9). Southern blot analyses determined that a single copy of the T-DNA was inserted at a single locus of the canola genome, and no additional genetic elements, including backbone sequences, from PV-BNHT2672 were detected in MON 88302.

PCR and DNA sequence analyses complement the Southern analyses. PCR and DNA sequence analyses performed on MON 88302 determined the complete DNA sequence of the insert and flanking genomic DNA sequences in MON 88302, confirmed the predicted organization of the genetic elements within the insert, and determined the sequences flanking the insert. In addition, DNA sequence analyses confirmed that each genetic element in the insert is intact and the sequence of the insert is identical to the corresponding sequence in PV-BNHT2672. Furthermore, genomic organization at the MON 88302 insertion site was determined by comparing the 5' and 3' flanking sequences of the insert to the sequence of the insertion site in conventional canola.

The stability of the T-DNA present in MON 88302 across multiple generations was demonstrated by Southern blot fingerprint analysis. Genomic DNA from multiple generations of MON 88302 (Figure 14) was digested with one of the enzyme sets used for the insert and copy number analyses and was hybridized with two probes that detect restriction segments that encompass the entire insert. This fingerprint strategy consists of two insert segments each containing its adjacent genomic DNA that assesses not only the stability of the insert, but also the stability of the DNA directly adjacent to the insert.

Segregation analysis was conducted to determine the inheritance and stability of the T-DNA insert in MON 88302. Results from this analysis demonstrated the inheritance and stability of the insert was as expected across multiple generations (Figure 13 and Table 4), which corroborates the molecular insert stability analysis and establishes the genetic behavior of the T-DNA at a single chromosomal locus.

The Southern blot analyses confirmed that the T-DNA reported in Figure 4 represents the only detectable insert in MON 88302. A circular map of PV-BNHT2672 annotated with the probes used in the Southern blot analysis is presented in Figure 3 and the genetic elements within the MON 88302 insert are summarized in Table 3. A linear map depicting restriction sites within the insert as well as within the DNA immediately flanking the insert in MON 88302 is shown in Figure 4. Based on the plasmid map and the linear map of the insert, a table summarizing the expected DNA segments for Southern analyses is presented in Table 2. The results from the Southern blot analyses are presented in Figure 5 through Figure 9. PCR amplification of the MON 88302 insert and the insertion site in conventional control (Ebony) for DNA sequence analysis are shown in Figure 10 and Figure 11, respectively. The generations used in the generational stability analysis are depicted in the breeding history shown in Figure 14 and the results from the generational stability analysis are presented in Figure 15. The breeding path for generating the segregation data is shown in Figure 13 and the results for the segregation analysis are presented in Table 4.

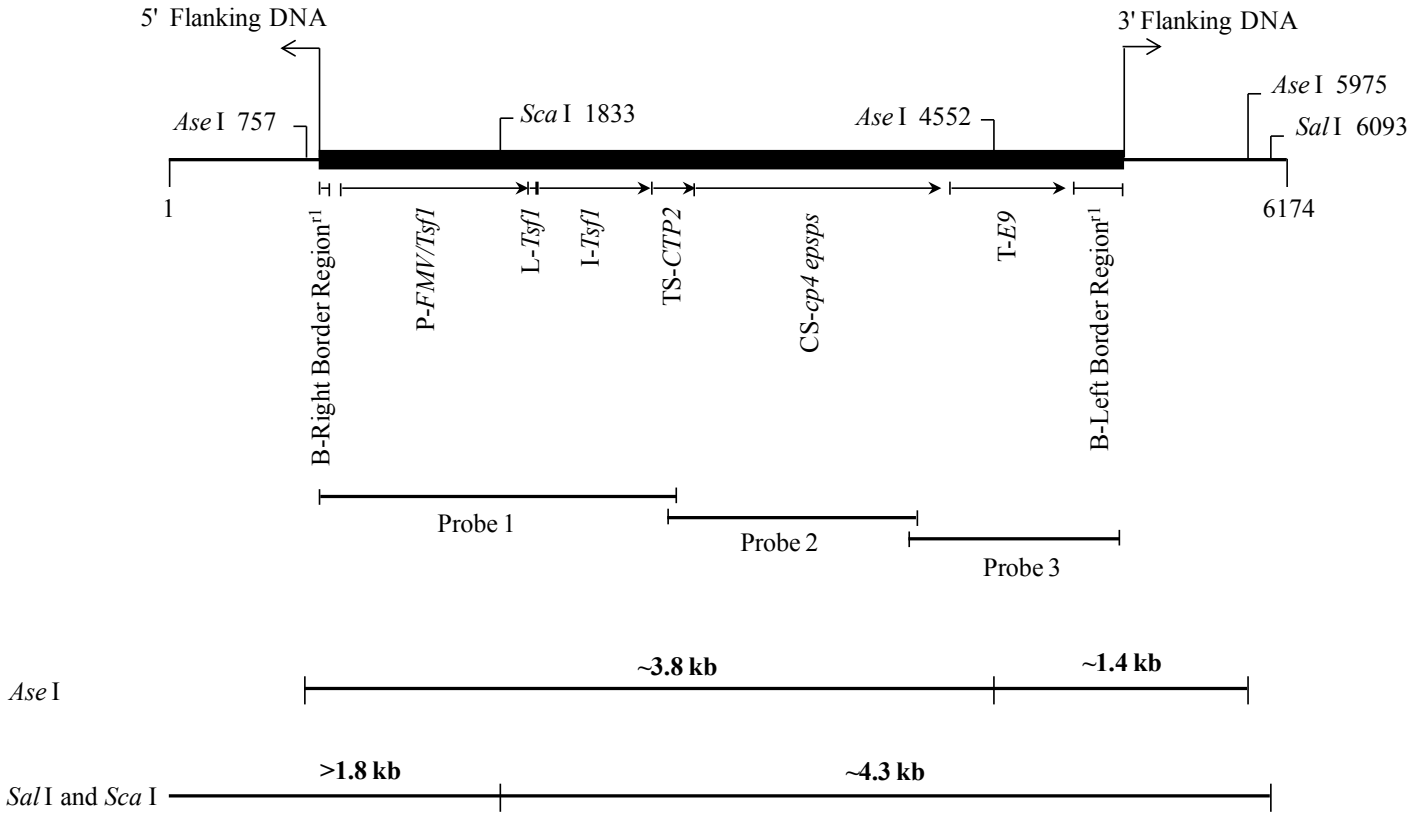


Figure 4. Schematic Representation of the Insert and Flanking DNA in MON 88302

A linear map of the insert and DNA flanking the insert in MON 88302 is shown. Right-angled arrows indicate the ends of the integrated T-DNA and the beginning of the flanking DNA. Identified on the linear map are genetic elements within the insert, as well as the sites of the restriction enzymes used in the Southern analyses with positions relative to the first base pair of the DNA sequence represented in this map. The relative sizes and locations of the T-DNA probes and the expected sizes of restriction fragments are indicated in the lower portion of the scheme. This schematic diagram is not drawn to scale. Locations of genetic elements and T-DNA probes are approximate. Probes are also shown in Figure 3.

Table 2. Summary Chart of the Expected DNA Segments Based on Hybridizing Probes and Restriction Enzymes Used in MON 88302 Analysis

Southern Blot Analysis		**T-DNA**		**Backbone**			**Insert Stability**
Figure Number		Figure 5	Figure 6	Figure 7	Figure 8	Figure 9	Figure 15
Probe Used		1, 3	2	4	5	6	1, 3
Probing Target	**Digestion Enzyme**	**Expected Band Sizes on Each Southern Blot**					
Plasmid PV-BNHT2672	*Bam* HI and *Sca* I	~2.5 kb ~7.2 kb	~2.5 kb	~7.2 kb	~7.2 kb	~7.2 kb	~2.5 kb ~7.2 kb
Probe Template Spikes[1]	N/A	~2.3 kb ~1.3 kb	~~[2]	~~[2]	~~[2]	~~[2]	~2.3 kb ~1.3 kb
MON 88302	*Ase* I	~3.8 kb ~1.4 kb	~3.8 kb	No band	No band	No band	~3.8 kb ~1.4 kb
	Sal I and *Sca* I	>1.8 kb ~4.3 kb	~4.3 kb	No band	No band	No band	--[3]

1 probe template spikes were used as positive hybridization controls in Southern blot analyses when multiple probes were hybridized to the Southern blot simultaneously.
2 '~~' indicates that probe template spikes were not used.
3 '--' indicates that the combination of the restriction enzymes was not used in the analysis.

Table 3. Summary of Genetic Elements in MON 88302

Genetic Element	Location in Sequence	Function (Reference)
5' Flanking Sequence	1-839	DNA sequence adjacent to the 5' end of the insertion site
B[1]-Right Border Region [r1]	840-882	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening Sequence	883-952	Sequence used in DNA cloning
P[2]-*FMV/Tsf1*	953-1992	Chimeric promoter consisting of the promoter of the *Tsf1* gene from the *Arabidopsis thaliana* encoding elongation factor EF-1α (Axelos et al., 1989) and enhancer sequences from the 35S promoter from the figwort mosaic virus (Richins et al., 1987)
L[3]-*Tsf1*	1993-2038	5' untranslated leader (exon 1) from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1 α (Axelos et al., 1989)
I[4]-*Tsf1*	2039-2660	Intron from the *Arabidopsis thaliana Tsf1* gene encoding elongation factor EF-1α (Axelos et al., 1989)
Intervening Sequence	2661-2669	Sequence used in DNA cloning
TS[5]-*CTP2*	2670-2897	Targeting sequence from the *shkG* gene encoding the chloroplast transit peptide region of *Arabidopsis thaliana* EPSPS (Herrmann, 1995; Klee et al., 1987) that directs transport of the CP4 EPSPS protein to the chloroplast
CS[6]-*cp4 epsps*	2898-4265	Codon optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein (Barry et al., 2001; Padgette et al., 1996)
Intervening Sequence	4266-4307	Sequence used in DNA cloning
T[7]-*E9*	4308-4950	3' untranslated sequence from the *rbcS2* gene of *Pisum sativum* encoding the Rubisco small subunit (Coruzzi et al., 1984)
Intervening Sequence	4951-4993	Sequence used in DNA cloning
B-Left Border Region [r1]	4994-5267	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Barker et al., 1983; Zambryski et al., 1982)
3' Flanking Sequence	5268-6174	DNA sequence adjacent to the 3' end of the insertion site

[1] B, Border
[2] P, Promoter
[3] L, Leader
[4] I, Intron
[5] TS, Targeting Sequence
[6] CS, Coding Sequence
[7] T, Transcription Termination Sequence
[r1] Superscripts in Left and Right Border Regions indicate that the sequences in MON 88302 were truncated compared to the sequences in PV-BNHT2672.

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 88302

The numbers of copies and insertion sites of the T-DNA sequences in the canola genome were evaluated by digesting MON 88302 and conventional control genomic DNA samples with the restriction enzyme *Ase* I or the combination of restriction enzymes *Sal* I and *Sca* 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). Any additional copies and/or integration sites would be detected as additional bands on the blots.

The restriction enzyme *Ase* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 5' and 3' ends of the insert (Figure 4). Therefore, if T-DNA sequences were present as a single copy at a single integration site in MON 88302, the digestion with *Ase* I was expected to generate two border segments with expected sizes of ~3.8 kb and ~1.4 kb (Figure 4 and Table 2). The combination of restriction enzymes *Sal* I and *Sca* I cleaves once within the inserted T-DNA and within the known genomic DNA flanking the 3' end of the insert (Figure 4). If T-DNA sequences were present as a single copy at a single integration site in MON 88302, the digestion with *Sal* I and *Sca* I was expected to generate two border segments with expected sizes of >1.8 kb and ~4.3 kb (Figure 4 and Table 2).

The Southern blots were hybridized with T-DNA probes that collectively span the entire inserted DNA sequence (Figure 3 and Figure 4, Probe 1, Probe 2, and Probe 3). Conventional control genomic DNA digested with the restriction enzyme *Ase* I and spiked with either probe templates and/or digested PV-BNHT2672 DNA served as positive hybridization controls. The positive hybridization control was spiked at approximately 0.1 and 1 genome equivalents to demonstrate sufficient sensitivity of the Southern blot. Conventional control genomic DNA digested with the appropriate restriction enzymes was used as a negative control. The results of these analyses are shown in Figure 5 and Figure 6.

T-DNA Probes 1 and 3

Conventional control genomic DNA digested with *Ase* I (Figure 5, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure 5, Lane 3 and Lane 7) and simultaneously hybridized with Probe 1 and Probe 3 (Figure 3 and Figure 4) produced no detectable hybridization bands as expected for the negative control. Conventional control genomic DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure 3), produced two bands at ~7.2 kb and ~2.5 kb (Figure 5, Lane 10), as expected. Conventional control genomic DNA digested with *Ase* I and spiked with probe templates of Probe 1 and Probe 3 (Figure 3) produced the expected bands at ~2.3 kb and ~1.3 kb (Figure 5, Lane 11 and Lane 12). Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 88302 DNA digested with *Ase* I and simultaneously hybridized with Probe 1 and Probe 3 (Figure 3 and Figure 4) produced the expected bands at ~3.8 kb and ~1.4 kb (Figure 5, Lane 2 and Lane 6). MON 88302 DNA digested with the combination of restriction enzymes *Sal* I and *Sca* I and hybridized with Probe 1 and Probe 3 (Figure 3 and Figure 4) produced two bands at ~2.7 kb and ~4.3 kb (Figure 5, Lane 4 and Lane 8), which is consistent with the expected >1.8 kb and ~4.3 kb bands (Figure 4 and Table 2), respectively.

The results presented in Figure 5 indicate that the sequences covered by Probe 1 and Probe 3 reside at a single detectable locus of integration in MON 88302.

T-DNA Probe 2

Conventional control DNA digested with *Ase* I (Figure 6, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure 6, Lane 3 and Lane 7) and hybridized with Probe 2 (Figure 3 and Figure 4) produced no detectable hybridization bands as expected for the negative control. Conventional control genomic DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure 3), produced a unique band at ~2.5 kb (Figure 6, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I and hybridized with Probe 2 (Figure 3 and Figure 4) produced the expected band at ~3.8 kb (Figure 6, Lane 2 and Lane 6). MON 88302 DNA digested with the combination of restriction enzymes *Sal* I and *Sca* I and hybridized with Probe 2 (Figure 3 and Figure 4) produced the expected band at ~4.3 kb (Figure 6, Lane 4 and Lane 8, Figure 4, and Table 2).

The results presented in Figure 6 indicate that the sequence covered by Probe 2 resides at a single detectable locus of integration in MON 88302.

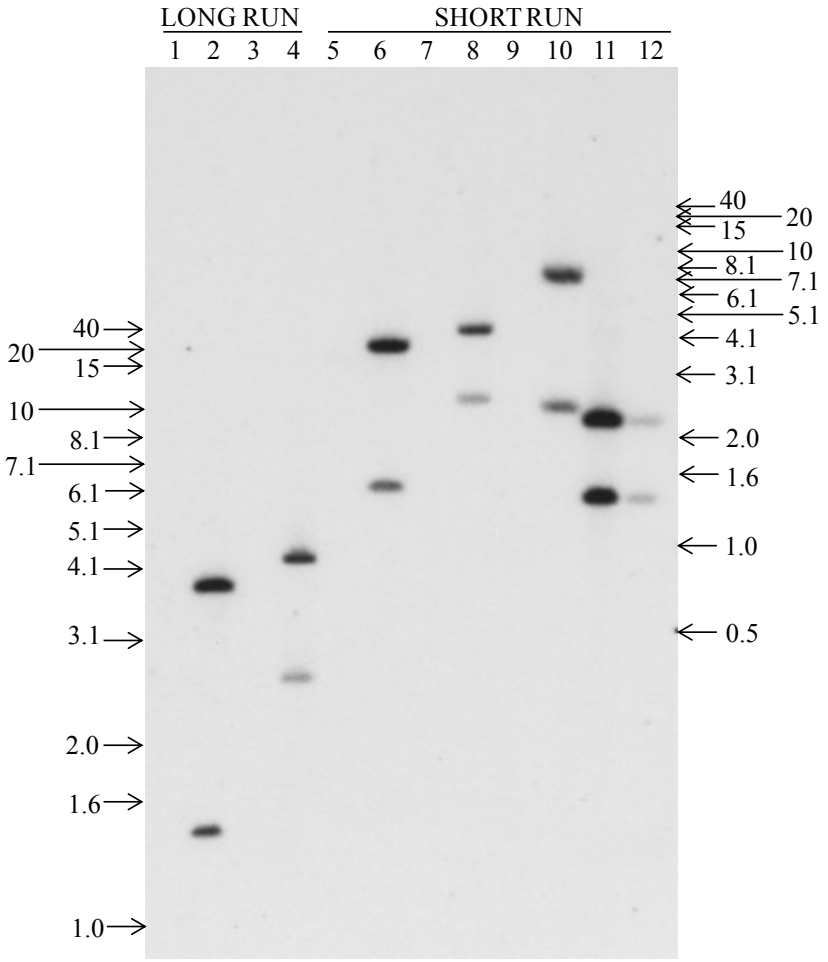


Figure 5. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88302: Probes 1 and 3

The blot was simultaneously hybridized with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure 3, Probe 1 and Probe 3). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional control (*Ase* I)
2	MON 88302 (*Ase* I)
3	Conventional control (*Sal* I/*Sca* I)
4	MON 88302 (*Sal* I/*Sca* I)
5	Conventional control (*Ase* I)
6	MON 88302 (*Ase* I)
7	Conventional control (*Sal* I/*Sca* I)
8	MON 88302 (*Sal* I/*Sca* I)
9	Blank
10	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent]
11	Conventional control (*Ase* I) spiked with Probe 1 and Probe 3 [~1 genome equivalent]
12	Conventional control (*Ase* I) spiked with Probe 1 and Probe 3 [~0.1 genome equivalent]

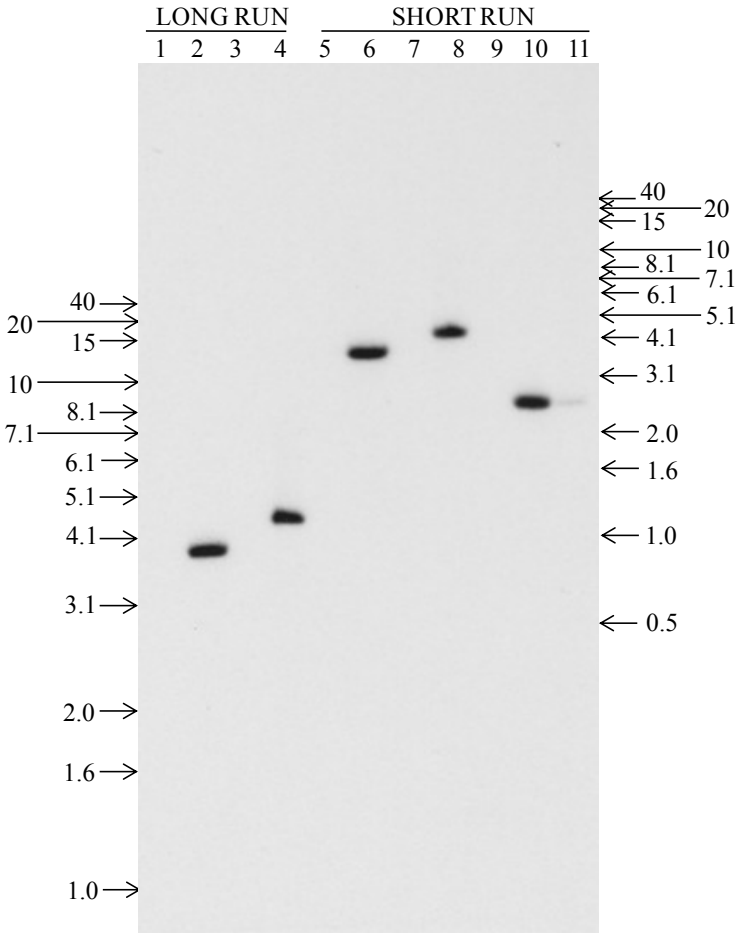


Figure 6. Southern Blot Analysis to Determine Insert and Copy Number of T-DNA in MON 88302: Probe 2

The blot was hybridized with a 32P-labeled probe that spans a portion of the T-DNA sequence (Figure 3, Probe 2). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional control (*Ase* I)
2	MON 88302 (*Ase* I)
3	Conventional control (*Sal* I/*Sca* I)
4	MON 88302 (*Sal* I/*Sca* I)
5	Conventional control (*Ase* I)
6	MON 88302 (*Ase* I)
7	Conventional control (*Sal* I/*Sca* I)
8	MON 88302 (*Sal* I/*Sca* I)
9	Blank
10	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent]
11	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent]

A3(d)(ii)(2) Southern Blot Analysis to Determine the Presence or Absence of Plasmid Vector PV-BNHT2672 Backbone Sequences in MON 88302

To determine the presence or absence of the PV-BNHT2672 backbone sequences, MON 88302 and conventional control genomic DNA were digested with the restriction enzyme *Ase* I or the combination of restriction enzymes *Sal* I and *Sca* I, and hybridized with one of the three backbone probes that collectively span the entire backbone sequences (Figure 3, Probe 4, Probe 5, and Probe 6). If backbone sequences are present in MON 88302, then probing with backbone probes should result in hybridizing bands. Conventional control genomic DNA digested with the restriction enzyme *Ase* I and spiked with digested PV-BNHT2672 DNA served as positive hybridization controls. The positive hybridization control was spiked at approximately 0.1 and 1 genome equivalents to demonstrate sufficient sensitivity of the Southern blot. Conventional control genomic DNA digested with the appropriate restriction enzymes was used as a negative control. The results of these analyses are shown in Figure 7, Figure 8, and Figure 9.

Backbone Probe 4

Conventional control DNA digested with *Ase* I (Figure 7, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure 7, Lane 3 and Lane 7) and hybridized with Probe 4 (Figure 3) produced no detectable hybridization bands as expected for the negative control. Conventional control DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure 3), produced a unique band at ~7.2 kb (Figure 7, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I (Figure 7, Lane 2 and Lane 6) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure 7, Lane 4 and Lane 8) and hybridized with Probe 4 produced no detectable bands.

The results presented in Figure 7 indicate that MON 88302 contains no detectable backbone sequences covered by Probe 4.

Backbone Probe 5

Conventional control DNA digested with *Ase* I (Figure 8, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure 8, Lane 3 and Lane 7) and hybridized with Probe 5 (Figure 3) produced no detectable hybridization bands as expected for the negative control. Conventional control DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure 3), produced a unique band at ~7.2 kb (Figure 8, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I (Figure 8, Lane 2 and Lane 6) or the combination of restriction enzymes *Sal* I and Sca I (Figure 8, Lane 4 and Lane 8) and hybridized with Probe 5 produced no detectable bands.

The results presented in Figure 8 indicate that MON 88302 contains no detectable backbone sequences covered by Probe 5.

Backbone Probe 6

Conventional control DNA digested with *Ase* I (Figure 9, Lane 1 and Lane 5) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure 9, Lane 3 and Lane 7) and hybridized with Probe 6 (Figure 3) produced no detectable hybridization bands as expected for the negative control. Conventional control DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure 3), produced a unique band at ~7.2 kb (Figure 9, Lane 10 and Lane 11), as expected. Detection of the positive controls indicates that the probe hybridized to its target sequence.

MON 88302 DNA digested with *Ase* I (Figure 9, Lane 2 and Lane 6) or the combination of restriction enzymes *Sal* I and *Sca* I (Figure 9, Lane 4 and Lane 8) and hybridized with Probe 6 produced no detectable bands.

The results presented in Figure 9 indicate that MON 88302 contains no detectable backbone sequences covered by Probe 6.

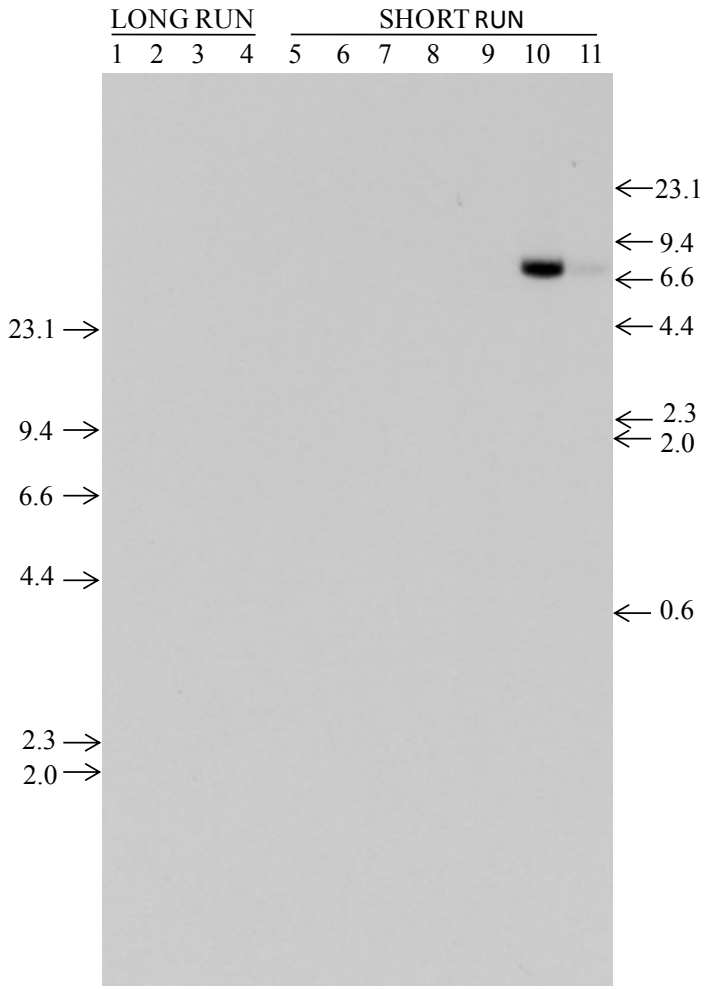


Figure 7. Southern Blot Analysis to Determine the Presence or Absence of the PV-BNHT2672 Backbone Sequences in MON 88302: Probe 4

The blot was hybridized with a ^{32}P-labeled probe that spans a portion of the plasmid vector backbone sequences (Figure 3, Probe 4). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from λ DNA/*Hind* III Fragments on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional control (*Ase* I)
2	MON 88302 (*Ase* I)
3	Conventional control (*Sal* I/*Sca* I)
4	MON 88302 (*Sal* I/*Sca* I)
5	Conventional control (*Ase* I)
6	MON 88302 (*Ase* I)
7	Conventional control (*Sal* I/*Sca* I)
8	MON 88302 (*Sal* I/*Sca* I)
9	Blank
10	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent]
11	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent]

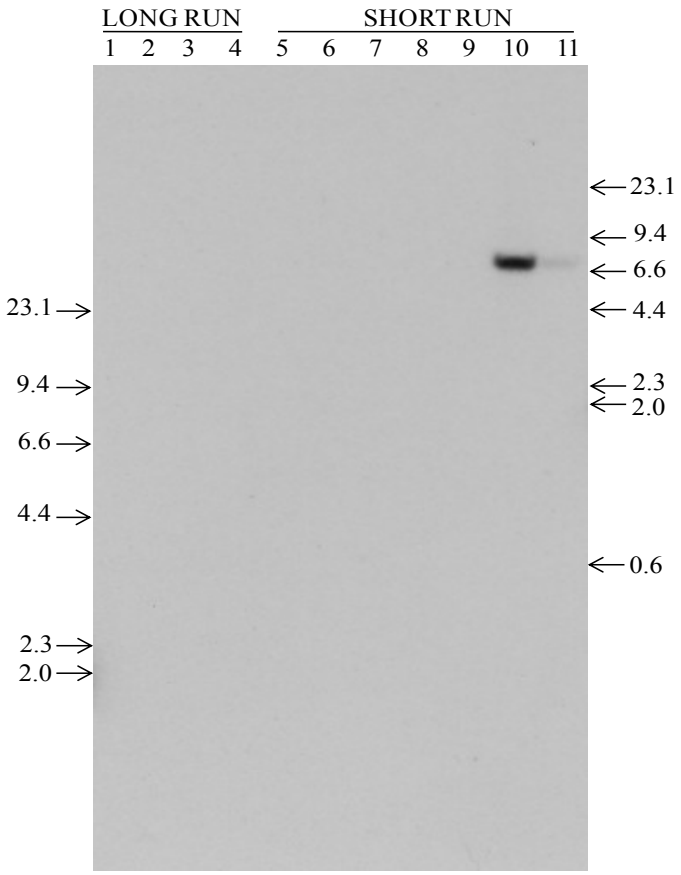


Figure 8. Southern Blot Analysis to Determine the Presence or Absence of the PV-BNHT2672 Backbone Sequences in MON 88302: Probe 5

The blot was hybridized with a ^{32}P-labeled probe that spans a portion of the plasmid vector backbone sequences (Figure 3, Probe 5). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from λ DNA/*Hind* III Fragments ethidium bromide stained gel. Fragments on the Lane designations are as follows:

Lane	
1	Conventional control (*Ase* I)
2	MON 88302 (*Ase* I)
3	Conventional control (*Sal* I/*Sca* I)
4	MON 88302 (*Sal* I/*Sca* I)
5	Conventional control (*Ase* I)
6	MON 88302 (*Ase* I)
7	Conventional control (*Sal* I/*Sca* I)
8	MON 88302 (*Sal* I/*Sca* I)
9	Blank
10	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent]
11	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent]

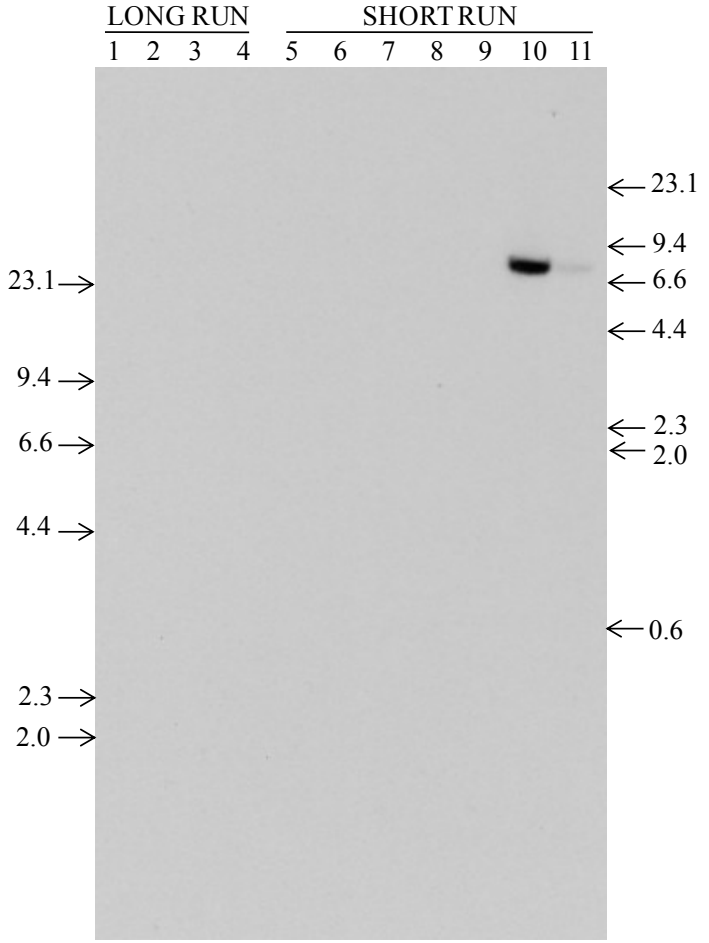


Figure 9. Southern Blot Analysis to Determine the Presence or Absence of the PV-BNHT2672 Backbone Sequences in MON 88302: Probe 6

The blot was hybridized with a ^{32}P-labeled probe that spans a portion of the plasmid vector backbone sequences (Figure 3, Probe 6). Each lane contains approximately 10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from λ DNA/*Hind* III Fragments on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional control (*Ase* I)
2	MON 88302 (*Ase* I)
3	Conventional control (*Sal* I/*Sca* I)
4	MON 88302 (*Sal* I/*Sca* I)
5	Conventional control (*Ase* I)
6	MON 88302 (*Ase* I)
7	Conventional control (*Sal* I/*Sca* I)
8	MON 88302 (*Sal* I/*Sca* I)
9	Blank
10	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent]
11	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~0.1 genome equivalent]

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

The organization and sequence of the elements within the MON 88302 insert was confirmed by DNA sequence analysis. PCR primers were designed with the intent to amplify two overlapping DNA amplicons that span the entire length of the insert and the associated DNA flanking the 5' and 3' ends of the insert (Figure 10). The amplified PCR products were subjected to DNA sequence analyses. This analysis determined that the DNA sequence of the MON 88302 insert is 4428 bp long (Table 3) and is identical to the corresponding T-DNA sequence of PV-BNHT2672 as described in Table 1.

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

PCR and sequence analyses were performed on genomic DNA extracted from MON 88302 and the conventional control to examine the MON 88302 insertion site. The PCR was performed with a forward primer specific to the genomic DNA sequence flanking the 5' end of the insert paired with a reverse primer specific to the genomic DNA sequence flanking the 3' end of the insert (Figure 11). The amplified PCR product from the conventional control was subjected to DNA sequence analysis. Alignments between the conventional control sequence obtained from this analysis and the sequences immediately flanking the 5' and 3' end of the MON 88302 insert were separately performed to determine the integrity and genomic organization of the insertion site in MON 88302. From these alignment analyses, a 9 base pair insertion immediately adjacent to the 3' end of the MON 88302 insert and a 29 base pair deletion from the conventional genomic DNA were identified. 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). A single nucleotide difference between the conventional control sequence and the genomic DNA sequence flanking the 3' end of the MON 88302 insert was also identified. The difference was most likely caused by a single nucleotide polymorphism (SNP) segregating in the canola population (Trick et al., 2009).

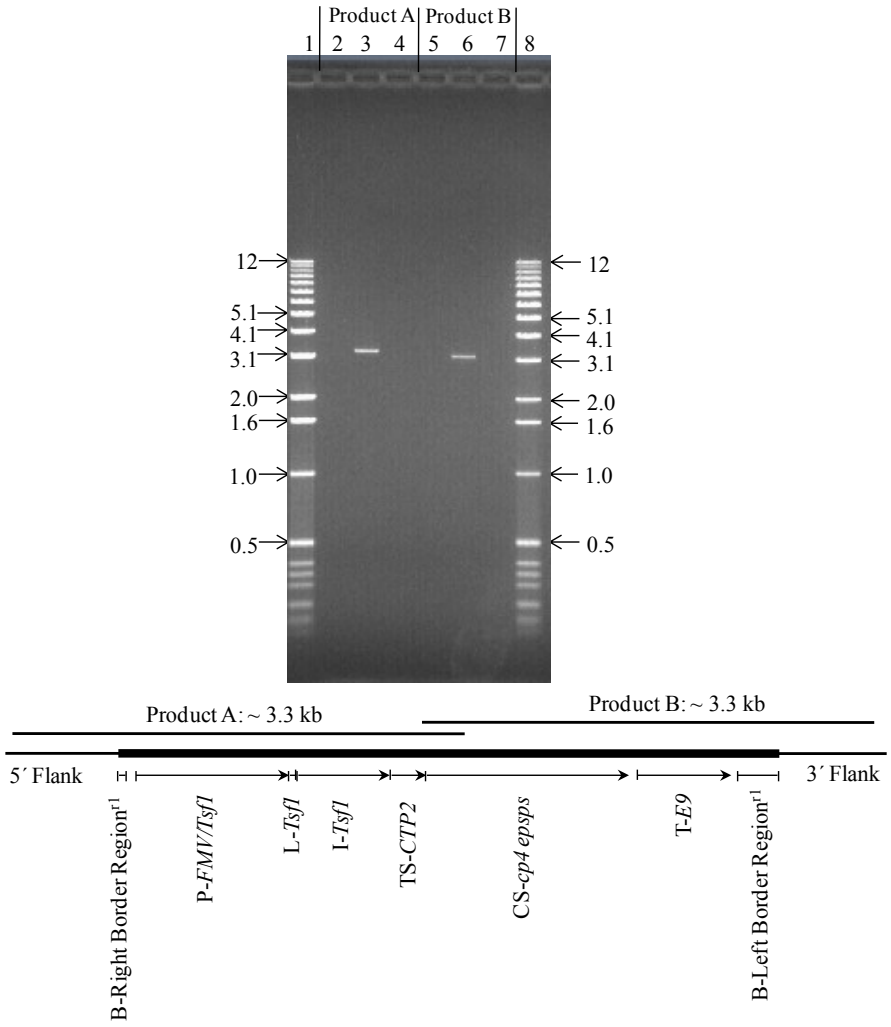


Figure 10. Overlapping PCR Analysis across the Insert in MON 88302
PCR was performed on both conventional control genomic DNA and MON 88302 genomic DNA using two pairs of primers to generate overlapping PCR fragments from MON 88302 for sequence analysis. Five microliters of each of the PCR reactions was loaded on the gel. The expected product size for each amplicon is provided in the illustration of the insert in MON 88302 that appears at the bottom of the figure. Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from 1 Kb DNA Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	1 Kb DNA Ladder
2	Conventional control
3	MON 88302
4	No template DNA control
5	Conventional control
6	MON 88302
7	No template DNA control
8	1 Kb DNA Ladder

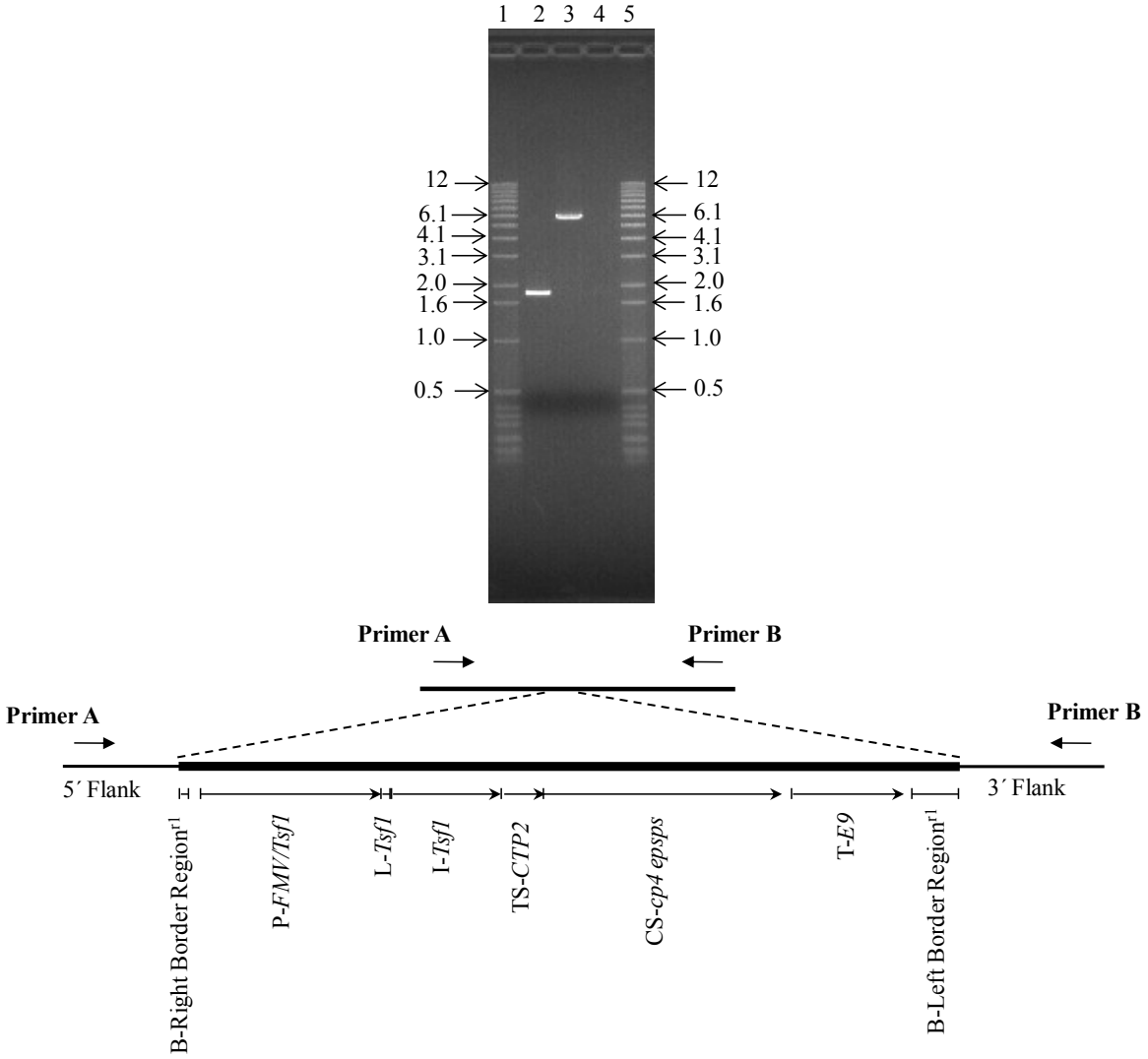


Figure 11. PCR Amplification of the MON 88302 Insertion Site in Conventional Control

PCR was performed on both conventional control genomic DNA and MON 88302 genomic DNA, using Primer A specific to the 5' flanking sequence and Primer B specific to the 3' flanking sequence of the insert in MON 88302, to generate DNA fragments for sequence analysis. The insertion site in conventional control (top) and MON 88302 (bottom) are illustrated at the bottom of the figure. Five microliters of each of the PCR reactions were loaded on the gel. Arrows on the agarose gel photograph denote the size of the DNA, in kilobase pairs, obtained from 1Kb DNA Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	1 Kb DNA Ladder
2	Conventional control
3	MON 88302
4	No template DNA control
5	1 Kb DNA Ladder

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

The 2009 Codex Alimentarius Commission guidelines for the safety assessment of food derived from biotechnology crops (Codex Alimentarius, 2009) 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 bioinformatic analyses demonstrate that any putative polypeptides in MON 88302 are unlikely to exhibit allergenic, toxic or otherwise biologically adverse properties.

In addition to the bioinformatic analysis conducted on MON 88302-produced CP4 EPSPS protein (see sections B3(a) and B4(b)) bioinformatic analyses were also performed on the MON 88302 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 88302 insert DNA (Table 3), as well as ORFs present in the 5' and 3' inserted DNA-5' and 3' flanking sequence junctions (MSL0023088). These various bioinformatic evaluations are depicted in Figure 12. 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 protein databases using bioinformatic tools. Similarly, the entire MON 88302 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 88302-produced CP4 EPSPS protein 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 88302-produced CP4 EPSPS protein 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 88302 relatedness to known toxins and 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 88302 (Figure 12).

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, 2009) 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. 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.

The results of the search comparisons showed that no relevant structural similarity to known allergens and toxins were observed for any of the putative polypeptides when compared to proteins in the allergen (AD_2010) or toxin (TOX_2010) databases. 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 frames 1 to 5 yielded alignments with *E*-scores less than or equal to a 1×10^{-5} threshold. Translation of frame 1 yielded numerous alignments with *E*-scores less than or equal to 1×10^{-5} when used to search the PRT_2010 database. The top alignment yielding the most significant *E*-score positively identified CP4 EPSPS in the MON 88302 T-DNA insert. Translation of frame 2 yielded two alignments with *E*-scores less than or equal to 1×10^{-5}. The top alignment yielding the most significant *E*-score was with an unknown protein product derived from Figwort Mosaic Virus. This result is not unexpected as the translated DNA sequence yielding this alignment was derived from the promoter for CP4 EPSPS which is partially derived from Figwort Mosaic Virus. While this alignment reflects conserved structure, there is no indication that it reflects the potential for adverse biological activity.

Translation of frames 3 and 5 each yielded two alignments with *E*-scores less than or equal to 1×10^{-5}. Inspection of the alignments for both the frame 3 and 5 translations revealed that the query sequences were punctuated with numerous stop codons and required numerous gaps to optimize the alignment. As a result, it is unlikely these alignments reflect conserved structure. Translation of frame 4 yielded an alignment displaying an *E*-score of 4×10^{-7} with an unknown amino acid sequence found in a patent submission. The alignment which displayed only 27.7% identity in a 173 amino acid overlap did not provide any indication of the potential for adverse effects human or animal health if it were to be produced. Taken together, these data demonstrate the lack of relevant similarities between known allergens and toxins for putative peptides derived from all six reading frames from the inserted DNA sequence of MON 88302.

As a result, in the unlikely event that any translation products other than MON 88302-produced CP4 EPSPS protein were derived from reading frames 1 to 6, then such putative polypeptides would not be expected to be cross-reactive allergens, toxins, or display adverse biological activity.

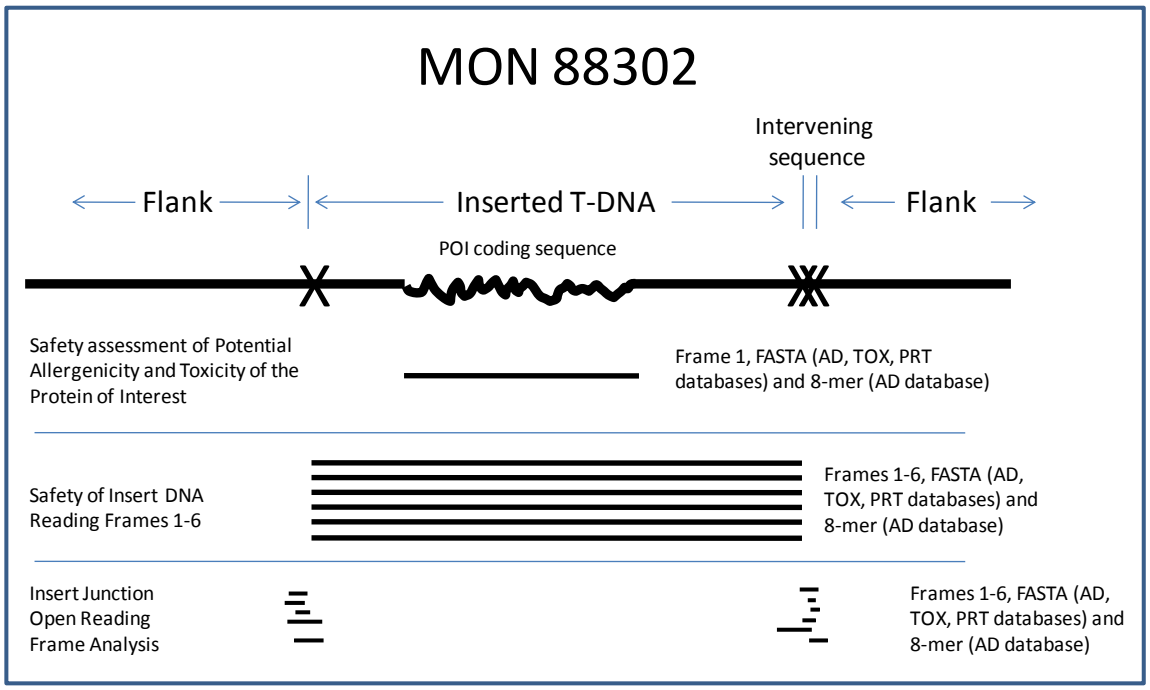
Insert Junction Open Reading Frame Bioinformatics Analysis

Analyses of putative polypeptides encoded by DNA spanning the 5' and 3' genomic junctions of the MON 88302 inserted DNA were performed using a bioinformatic comparison strategy. 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' genomic DNA-T-DNA and the 3' genomic DNA-intervening DNA and/or intervening DNA-T-DNA junctions, (Figure 12) 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 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, 2009) (thresholds for FASTA searches of the AD_2010 database), and the *E*-score. Alignments having an *E*-score less than 1×10^{-5} are deemed significant because they may reflect shared structure and function among sequences. 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.

No biologically relevant structural similarity to known allergens and 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 any translation products were derived from DNA spanning the 5' or 3' genomic DNA insert DNA junctions of MON 88302, then such putative polypeptides would not be 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 12. Schematic Summary of MON 88302 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 88302

During development of MON 88302, segregation data were recorded to assess the inheritance and stability of the coding sequence present in MON 88302. Chi-square (χ^2) analysis was performed over several generations to confirm the segregation and stability of the MON 88302 insert. The χ^2 analysis is based on testing the observed segregation ratio to the expected segregation ratio according to Mendelian principles.

The MON 88302 breeding path for generating segregation data is described in Figure 13. The transformed R_0 plant was self-pollinated to generate R_1 seed. From the R_1 segregating population, an individual plant homozygous for the *cp4 epsps* coding sequence (subsequently designated MON 88302) was identified via TaqMan PCR copy number assay and Southern blot copy number analysis. The *cp4 epsps* homozygous R_1 plant was self-pollinated to give rise to R_2 plants that were self-pollinated to produce R_3 seed. At each generation, the homozygous plants were tested for the expected segregation pattern of 1:0 (positive: negative) for the *cp4 epsps* gene using a glyphosate spray test and/or TaqMan PCR assay.

An individual *cp4 epsps* positive R_3 plant, which was confirmed by Endpoint TaqMan PCR assay, was crossed to a Monsanto proprietary canola inbred, which does not contain the MON 88302 insert, via traditional breeding techniques to produce hemizygous F_1 seed. The resulting F_1 plant was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR, and was then self-pollinated to produce F_2 seed. A *cp4 epsps* hemizygous F_2 plant from the F_2 population was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR and was then self-pollinated to produce the F_3 population. A *cp4 epsps* hemizygous F_3 plant from the F_3 population was shown to contain a single copy of the *cp4 epsps* gene by real-time TaqMan PCR and was self-pollinated to produce the F_4 population. The copy number of the *cp4 epsps* gene in the F_2, F_3, and F_4 populations was then assessed using a real-time TaqMan PCR assay.

A χ^2 analysis was performed on each of the F_2, F_3, and F_4 populations using the statistical program R (Version 2.10.1) to compare the observed segregation ratio of *cp4 epsps* coding sequence to the expected ratio according to Mendelian principles of inheritance. 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% ($\alpha = 0.05$).

The results of the χ^2 analysis of the MON 88302 segregating progeny are presented in Table 4. The χ^2 value in the F_2, F_3, and F_4 populations indicated no statistically significant difference between the observed and expected 1:2:1 segregation ratio (homozygous positive: hemizygous: homozygous negative) of *cp4 epsps* coding sequence. These results support the conclusion that the *cp4 epsps* expression cassette in MON 88302 resides at a single locus within the canola genome and is inherited according to Mendelian

principles of inheritance. These results are also consistent with the molecular characterization data indicating that MON 88302 contains a single, intact copy of the *cp4 epsps* expression cassette inserted at a single locus in the canola genome.

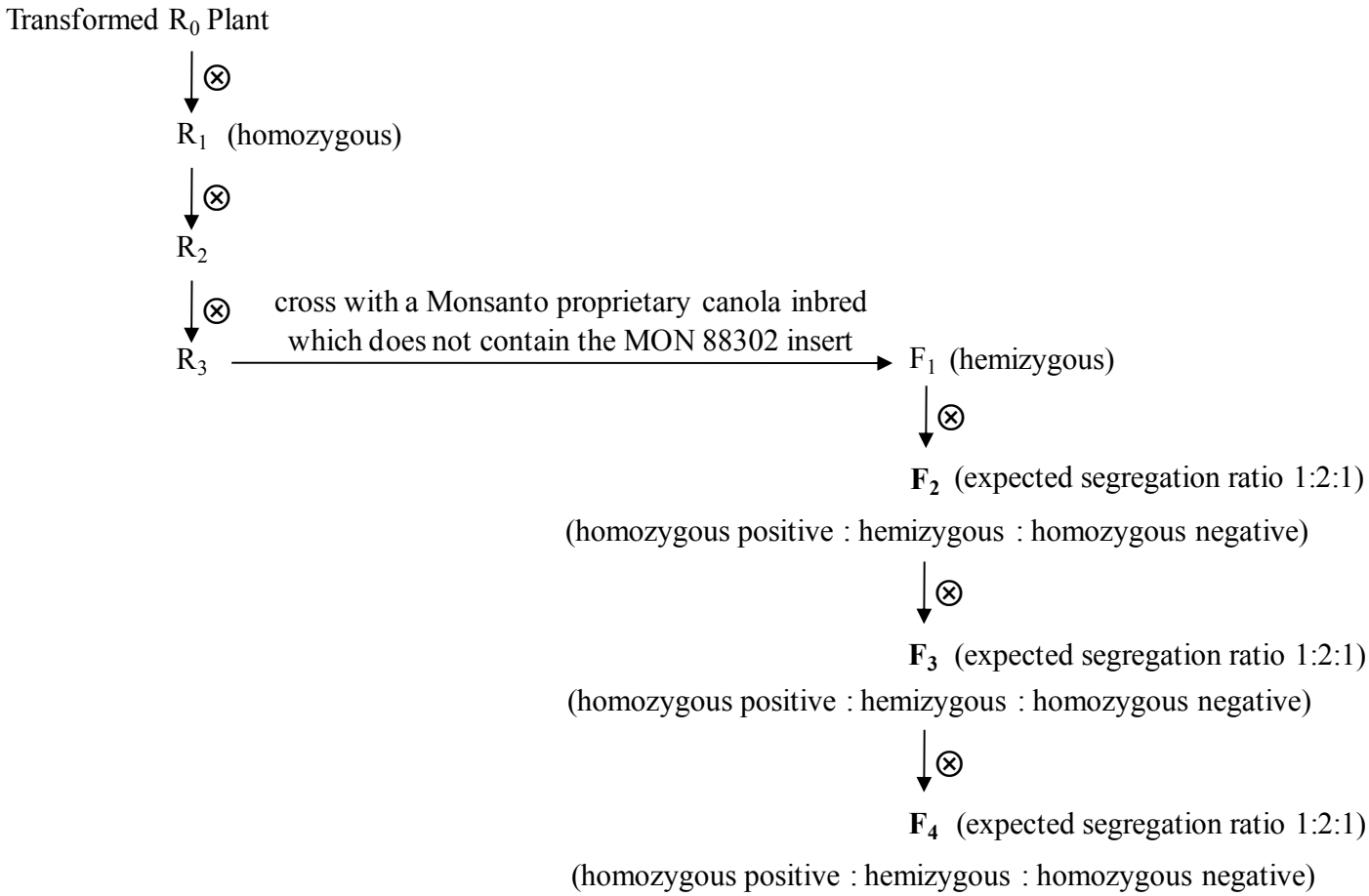


Figure 13. Breeding Path for Generating Segregation Data for MON 88302

An individual hemizygous plant from each of the F_1, F_2, and F_3 populations was self-pollinated to produce the population of the next generation. Chi-square analyses were conducted on segregation data from the F_2, F_3, and F_4 populations.

Table 4. Segregation of the *cp4 epsps* Gene During the Development of MON 88302

					1:2:1 Segregation				
Generation	**Total Plants***	**Observed # Plants Homozygous Positive**	**Observed # Plants Hemizygous**	**Observed # Plants Homozygous Negative**	**Expected # Plants Homozygous Positive**	**Expected # Plants Hemizygous**	**Expected # Plants Homozygous Negative**	**χ^2**	**Probability**
F_2	220	51	122	47	55.00	110.00	55.00	2.76	0.2511
F_3	166	39	94	33	41.50	83.00	41.50	3.35	0.1874
F_4	198	53	97	48	49.50	99.00	49.50	0.33	0.8465

*Plants were evaluated for the copy number of the *cp4 epsps* gene using a real-time TaqMan PCR assay.

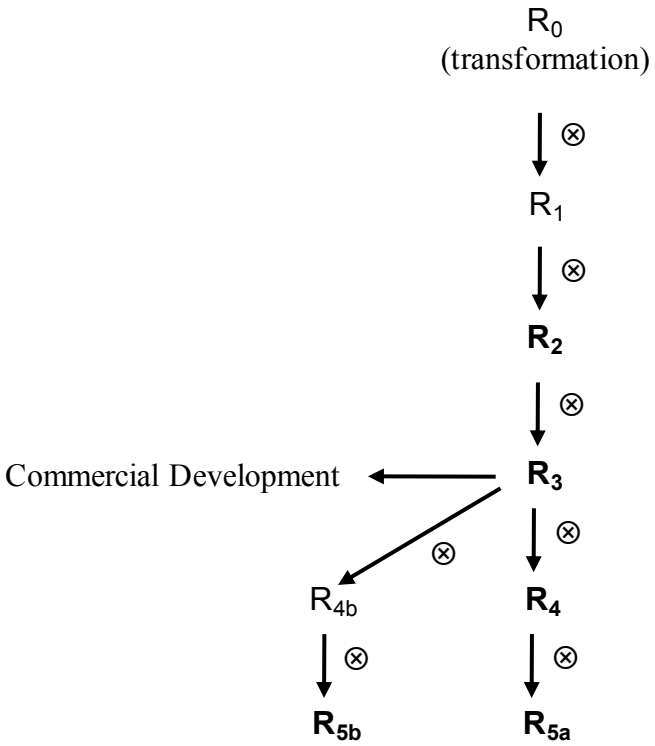
Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88302

In order to demonstrate the stability of the insert in MON 88302, Southern blot analysis was performed using genomic DNA extracted from leaf tissues from four breeding generations of MON 88302. For reference, the breeding history of MON 88302 is presented in Figure 14. The specific generations tested are indicated in the legend of Figure 14. The R_3 generation was used for the molecular characterization analyses shown in Figure 5 through Figure 9. To analyze insert stability, four samples from three additional generations of MON 88302 were evaluated by Southern blot analysis and compared to the R_3 generation. Genomic DNA, isolated from each of the selected generations of MON 88302, was digested with the restriction enzyme *Ase* I and simultaneously hybridized with Probe 1 and Probe 3 (Figure 3 and Figure 4), which was designed to detect both fragments generated by the *Ase* I digest. Any instability associated with the insert would be detected as extra bands within the fingerprint on the Southern blot. The Southern blot has the same controls as described in section A3(d)(i).

T-DNA Probes 1 and 3

Conventional control genomic DNA digested with restriction enzyme *Ase* I and simultaneously hybridized with Probe 1 and Probe 3 (Figure 3 and Figure 4) produced no hybridization signals (Figure 15, Lane 1) as expected for the negative control. Conventional control genomic DNA digested with *Ase* I and spiked with the PV-BNHT2672 DNA, previously digested with the combination of restriction enzymes *Bam* HI and *Sca* I (Figure 3 and Table 2), produced the expected bands at ~2.5 kb and ~7.2 kb (Figure 15, Lane 8). Conventional control genomic DNA digested with *Ase* I and spiked with probe templates of Probe 1 and Probe 3 produced the expected bands at ~2.3 kb and ~1.3 kb (Figure 15, Lane 9 and Lane 10). Detection of the positive controls indicates that the probes hybridized to their target sequences.

MON 88302 genomic DNA digested with *Ase* I and hybridized with Probe 1 and Probe 3 (Figure 3 and Figure 4) is expected to produce a Southern fingerprint with two bands at ~3.8 kb and ~1.4 kb (Figure 4 and Table 2). Southern fingerprints produced from multiple generations (Figure 15, Lane 2, Lane 4, Lane 5, and Lane 6), of MON 88302 are consistent with the one produced from the fully characterized generation R_3 (Figure 5, Lane 2 and Lane 6, and Figure 15, Lane 3), indicating that MON 88302 contains one copy of the T-DNA insert that is stable across multiple generations.



R_0- Originally transformed plant ; ⊗-self pollination

Figure 14. Breeding History of MON 88302

R_0 corresponds to the transformed canola plant. All generations were self pollinated. ⊗ designates self-pollination. The R_3 generation was used for the molecular characterization and commercial development of MON 88302. The R_2, R_3, R_4, R_{5a}, and R_{5b} (bolded in the breeding tree) generations of MON 88302 were used for analyzing the stability of the insert across generations. R_{5b} was propagated independently of R_{5a} beginning with the R_3 generation.

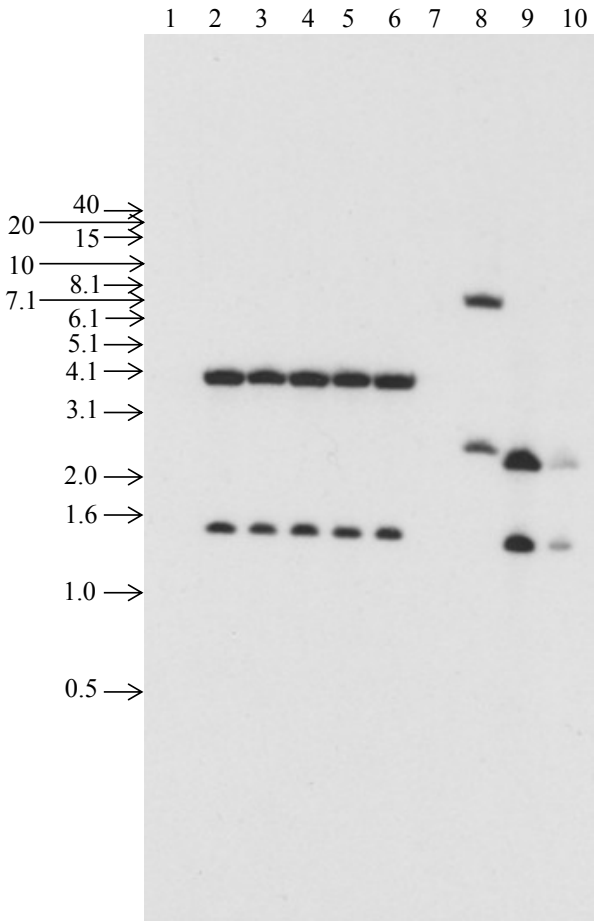


Figure 15. Southern Blot Analysis to Examine Insert Stability in Multiple Generations of MON 88302: Probes 1 and 3

The blot was simultaneously hybridized with two ^{32}P-labeled probes that span a portion of the T-DNA sequence (Figure 3, Probe 1 and Probe 3). Each lane contains ~10 µg of digested genomic DNA. Arrows denote the size of the DNA, in kilobase pairs, obtained from 1Kb DNA Extension Ladder on the ethidium bromide stained gel. Lane designations are as follows:

Lane	
1	Conventional control (*Ase* I)
2	R_2 generation of MON 88302 (*Ase* I)
3	R_3 generation of MON 88302 (*Ase* I)
4	R_4 generation of MON 88302 (*Ase* I)
5	R_{5a} generation of MON 88302 (*Ase* I)
6	R_{5b} generation of MON 88302 (*Ase* I)
7	Blank
8	Conventional control (*Ase* I) spiked with PV-BNHT2672 (*Bam* HI/*Sca* I) [~1 genome equivalent]
9	Conventional control (*Ase* I) spiked with probe templates Probe 1 and Probe 3 [~1 genome equivalent]
10	Conventional control (*Ase* I) spiked with probe templates Probe 1 and Probe 3 [~0.1 genome equivalent]

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

In order to confirm the presence of the CP4 EPSPS protein in MON 88302 across multiple generations, western blot analysis of MON 88302 was conducted on leaf tissue collected from generations R_2, R_3, R_4, R_{5a}, and R_{5b} (Figure 14) of MON 88302, and on leaf tissue of the conventional control (Ebony). The presence of the CP4 EPSPS protein in harvested leaf tissue of the R_2, R_3, R_4, R_{5a}, and R_{5b} generations of MON 88302 was demonstrated (Figure 16). An *E. coli*-produced CP4 EPSPS standard (2 ng) was used as a reference for the identification of the CP4 EPSPS protein. The presence of CP4 EPSPS protein in MON 88302 leaf tissue samples was determined by visual comparison of the bands produced in the multiple breeding generations (Figure 16, lanes 4 through 8) to the CP4 EPSPS reference standard (Figure 16, lane 2). As shown in Figure 16, CP4 EPSPS protein was present in multiple generations of MON 88302 tissue samples and migrated with a 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 extract (Figure 16, lane 3). Two additional faint bands were observed at approximately 30 kDa and 60 kDa in the MON 88302 samples (Figure 16, lanes 4 through 8). These bands are likely the result of non-specific binding of either the primary or secondary antibody to endogenous canola leaf proteins.

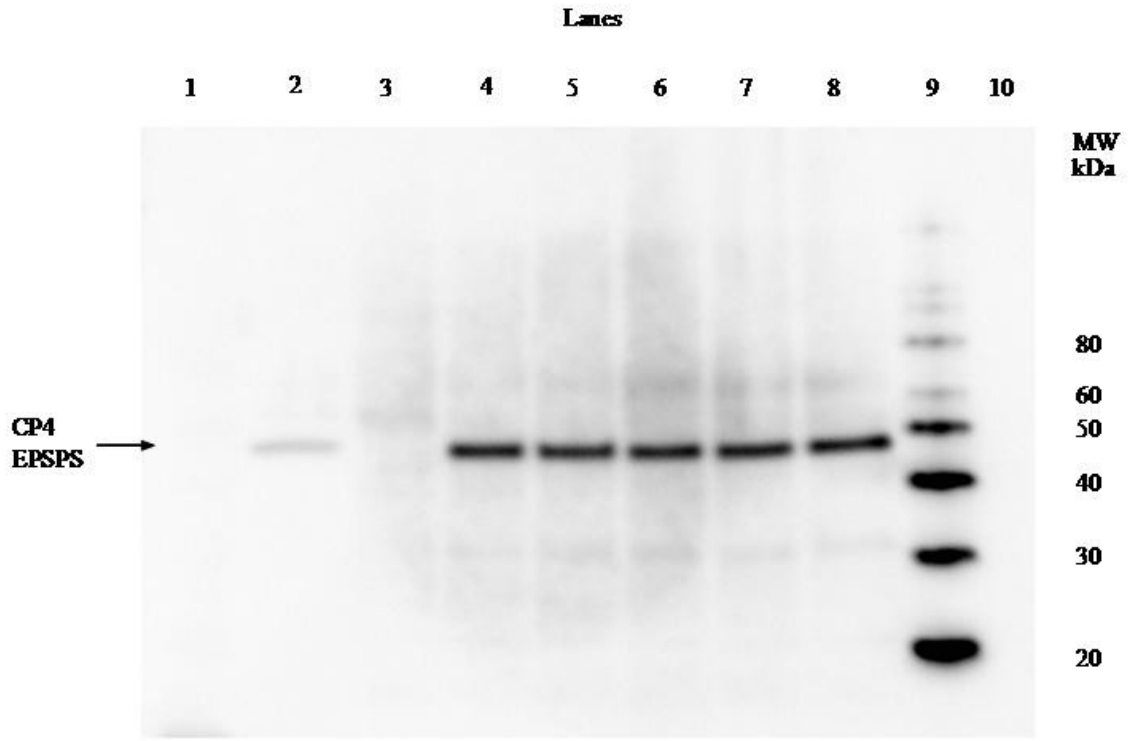


Figure 16. Presence of CP4 EPSPS Protein in Multiple Generations of MON 88302

Extracts from multiple generations of MON 88302 leaf tissues 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. The image represents a 30 second exposure.

Lane	Sample Description	Amount Loaded
1	Precision Plus Molecular Weight Marker	10 µl
2	*E. coli*-produced CP4 EPSPS protein (2 ng) (Molecular weight 43.8 kDa)	20 µl
3	Conventional Control	20 µl
4	R_2 Generation	20 µl
5	R_3 Generation	20 µl
6	R_4 Generation	20 µl
7	R_{5a} Generation	20 µl
8	R_{5b} Generation	20 µl
9	Magic Marker Molecular Weight Marker	0.5 µl
10	Blank	N/A

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 MON 88302 has been provided to FSANZ in MSL-22523.

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 88302. Molecular characterization data presented in this application demonstrate the absence of the *aadA* antibiotic resistant marker gene in MON 88302.

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)

Mode of action of CP4 EPSPS protein

The 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) family of enzymes is found in plants and microorganisms and their properties have been extensively studied (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). They catalyze one of the 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).

The EPSPS transgene (*cp4 epsps*) in MON 88302 is derived from *Agrobacterium* sp. strain CP4. It encodes a 47.6 kDa EPSPS protein that consists of a single polypeptide of 455 amino acids and is functionally identical to endogenous plant EPSPS enzymes, but has a reduced affinity for glyphosate relative to endogenous plant EPSPS (Padgette et al., 1996). In conventional plants, glyphosate binds to the endogenous EPSPS enzyme and blocks the biosynthesis of EPSP thereby depriving the plant of essential amino acids (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 (Padgette et al., 1996).

As explained in section A1(a), MON 88302 utilizes a *FMV/Tsf1* chimeric promoter sequence to drive CP4 EPSPS expression in different plant tissues including pollen. MON 88302 provides tolerance to glyphosate during the sensitive reproductive stages of growth, and enables the application of glyphosate at higher rates up to first flower.

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 *E. coli*-produced CP4 EPSPS protein has been assessed previously and the results are summarized by Harrison et al. (1996). For the existing CP4 EPSPS safety data set to be applied to CP4 EPSPS protein produced in MON 88302, the equivalence of the plant- and *E. coli*-produced protein was established. The equivalence of the plant- and *E. coli*-produced CP4 EPSPS proteins has been established previously for Roundup Ready crops such as soybean, corn, canola, sugar beet, alfalfa and cotton. To assess the equivalence between MON 88302-produced and *E. coli*-produced CP4 EPSPS protein, a small quantity of the CP4 EPSPS protein was purified from MON 88302 seed. The MON 88302-produced CP4 EPSPS protein was characterized and the equivalence of the physicochemical characteristics and functional activity between the MON 88302-produced CP4 EPSPS protein and the *E. coli*-produced CP4 EPSPS protein was assessed using a panel of analytical tests, including: 1) N-terminal sequence analysis of the MON 88302-produced CP4 EPSPS protein to establish identity, 2) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF

MS) analysis of peptides derived from tryptic digested MON 88302-produced CP4 EPSPS protein to establish identity, 3) western blot analysis using anti-CP4 EPSPS polyclonal antibodies to establish identity and immunoreactive equivalence between MON 88302-produced protein and the *E. coli*-produced protein, 4) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to establish equivalence of the apparent molecular weight between MON 88302-produced protein and the *E. coli*-produced protein, 5) glycosylation analysis of the MON 88302-produced CP4 EPSPS protein to establish the equivalence between the MON 88302-produced and *E. coli*-produced CP4 EPSPS proteins, and 6) CP4 EPSPS enzymatic activity analysis to demonstrate functional equivalence between MON 88302-produced and the *E. coli*-produced protein. A summary of the data obtained to support a conclusion of protein equivalence is below.

A comparison of the MON 88302-produced CP4 EPSPS to the *E. coli*-produced CP4 EPSPS protein confirmed the identity of the MON 88302-produced CP4 EPSPS protein and established the equivalence of the two proteins. The identity of the CP4 EPSPS protein isolated from the seed of MON 88302 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 88302-produced CP4 EPSPS protein matched the predicted amino acid sequence translated from the *cp4 epsps* coding sequence. The MALDI-TOF MS analysis yielded peptide masses consistent with the expected peptide masses from the translated *cp4 epsps* coding sequence. The MON 88302-produced CP4 EPSPS protein was detected on a western blot probed with antibodies specific for CP4 EPSPS protein. Furthermore, the immunoreactive properties and electrophoretic mobility of the MON 88302-produced CP4 EPSPS protein were shown to be equivalent to those of the *E. coli*-produced CP4 EPSPS protein by immunoblot. The apparent molecular weight, glycosylation status, and functional activity of the MON 88302-produced CP4 EPSPS protein and *E. coli*-produced CP4 EPSPS protein were also all found to be equivalent. Taken together, these data provide a detailed characterization of the CP4 EPSPS protein isolated from MON 88302 and establish its equivalence to the *E. coli*-produced CP4 EPSPS protein. Furthermore, because CP4 EPSPS proteins isolated from other Roundup Ready crops have been demonstrated previously to be equivalent to the *E. coli*-produced CP4 EPSPS protein, by inference, the MON 88302-produced CP4 EPSPS protein is equivalent to the CP4 EPSPS proteins expressed in other Roundup Ready crops, all of which have been reviewed by FSANZ. Please refer to MSL0022841 for more details.

N-terminal Sequence Analysis

N-terminal sequencing of the first 15 amino acids was performed on MON 88302-produced CP4 EPSPS protein. The expected sequence for the CP4 EPSPS protein deduced from the *cp4 epsps* gene present in MON 88302 was observed. The data obtained correspond to the deduced CP4 EPSPS protein beginning at amino acid positions 2 and 4 (Figure 17, Experimental Sequence 1 and 2, respectively). Hence, the sequence information confirms the identity of the CP4 EPSPS protein isolated from the seed of MON 88302.

Amino acid residue # from the N-terminus	→	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Expected Sequence	→	M	L	H	G	A	S	S	R	P	A	T	A	R	K	S	S	G	L
			|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence 1	→	-	L	H	G	A	X	X	X	P	A	T	X	X	X	X	X	^	^
					|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Experimental Sequence 2	→	-	-	-	G	A	S	X	R	P	A	T	A	X	K	S	X	G	X

Figure 17. N-Terminal Sequence of the MON 88302 CP4 EPSPS Protein
The expected amino acid sequence of the N-terminus of CP4 EPSPS protein was deduced from the *cp4 epsps* coding region present in MON 88302. The experimental sequences obtained from the MON 88302-produced CP4 EPSPS protein were compared to the expected sequence. The single letter IUPAC-IUB amino acid code is M, methionine; L, leucine; H, histidine; G, glycine; A, alanine, S, serine; R, arginine; P, proline; T, threonine; K. lysine; (X) indicates that the residue was not identifiable; (-) indicates the residue was not observed; (^) indicates not done, i.e., sequencing cycle was not conducted.

MALDI-TOF Tryptic Mass Map Analysis

The identity of the MON 88302-produced CP4 EPSPS protein was also confirmed by MALDI-TOF MS analysis of peptide fragments produced from tryptic digestion of the MON 88302-produced CP4 EPSPS protein. The ability to identify a protein using this method is dependent upon matching a sufficient number of observed tryptic peptide fragment masses with predicted tryptic peptide fragment masses. In general, protein identification made by 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 34 unique peptides identified that corresponded to the masses (Table 5) expected to be produced by tryptic digestion of the CP4 EPSPS protein. The identified masses were used to assemble a coverage map of the entire CP4 EPSPS protein (Figure 18). The experimentally determined mass coverage of the CP4 EPSPS protein was 85.5% (389 out of 455 amino acids). This analysis serves as additional identity confirmation for the MON 88302-produced CP4 EPSPS protein.

Table 5. Summary of the Tryptic Masses Identified for MON 88302-Produced CP4 EPSPS Protein Using MALDI-TOF MS

α-Cyano	α-Cyano	DHB	DHB	Sinapinic acid	Sinapinic acid	Expected Mass[1]	Diff[2]	Position[3]	Sequence
Extract 1	Extract 2	Extract 1	Extract 2	Extract 1	Extract 2				
		389.28				389.25	0.03	225-227	TIR
		474.32				474.27	0.05	228-231	LEGR
506.29						506.22	0.07	354-357	ESDR
		529.36				529.30	0.06	24-28	IPGDK
599.43	599.51	599.41				599.33	0.10	29-33	SISHR
616.44	616.48	616.41		616.24		616.34	0.10	128-132	RPMGR
629.44		629.45				629.29	0.15	201-205	DHTEK
629.44		629.45				629.34	0.10	383-388	GRPDGK
711.57	711.62	711.56	711.61			711.45	0.12	133-138	VLNPLR
		790.55				790.48	0.07	306-312	VRSSTLK
		790.55				790.41	0.14	139-145	EMGVQVK
		805.54				805.43	0.11	447-453	IELSDTK
835.54	835.58	835.53				835.39	0.15	62-69	AMQAMGAR
863.61	863.68	863.60				863.46	0.15	15-23	SSGLSGTVR
872.61	872.66	872.61	872.67	872.53		872.45	0.16	313-320	GVTVPEDR
872.61	872.66	872.61	872.67	872.53		872.52	0.09	358-366	LSAVANGLK
		930.66				930.51	0.15	169-177	VPMASAQVK
948.68	948.74	948.68	948.75			948.52	0.16	161-168	TPTPITYR
991.72		991.71				991.55	0.17	14-23	KSSGLSGTVR
1115.75	1115.83	1115.77	1115.86	1115.69		1115.57	0.18	295-305	LAGGEDVADLR
1357.94	1358.01	1357.97	1358.05	1357.89		1357.71	0.23	146-157	SEDGDRLPVTLR
1359.88	1359.96	1359.91	1360.00	1359.81	1359.87	1359.72	0.16	354-366	ESDRLSAVANGLK
1359.88	1359.96	1359.91	1360.00	1359.81	1359.87	1359.64	0.24	34-46	SFMFGGLASGETR
1559.11	1559.18	1559.13	1559.01			1558.83	0.28	47-61	ITGLLEGEDVINTGK
1647.10	1647.24	1647.16	1647.24			1646.84	0.26	389-405	GLGNASGAAVATHLDHR
1764.10	1764.26	1764.16		1764.06		1763.81	0.29	367-382	LNGVDCDEGETSLVVR
1994.31	1994.43	1994.35	1994.55	1994.21	1994.35	1993.97	0.34	206-224	MLQGFGANLTVETDADGVR
2183.54	2183.67	2183.57	2183.80	2183.45	2183.53	2183.17	0.37	275-294	TGLILTLQEMGADIEVINPR
2367.73	2367.87	2367.77	2367.85	2367.65	2367.8	2367.33	0.40	178-200	SAVLLAGLNTPGITTVIEPIMTR
2450.65	2450.83	2450.80		2450.51	2450.6	2450.23	0.42	24-46	IPGDKSISHRSFMFGGLASGETR
2450.65	2450.83	2450.80		2450.51	2450.6	2450.22	0.43	105-127	LTMGLVGVYDFDSTFIGDASLTK
3247.10 (Ave)	3247.05 (Ave)			3246.89 (Ave)	3246.97 (Ave)	3246.54 (Ave)	0.56	73-104	EGDTWIIDGVGNGGLLAPEAPLDFGNAATGCR
3251.94 (Ave)	3252.18 (Ave)	3252.06 (Ave)	3253.42 (Ave)	3252.58 (Ave)	3252.04 (Ave)	3251.75 (Ave)	0.19	321-351	APSMIDEYPILAVAAAFAEGATVMNGLEELR
		4191.34 (Ave)	4191.48 (Ave)	4191.89 (Ave)	4191.63 (Ave)	4190.89 (Ave)	0.37	234-274	LTGQVIDVPGDPSSTAFPLVAALLVPGSDVTILNVLMNPTR

[1]Only experimental masses that matched expected masses are listed in the table.
[2]The difference between the expected mass and the first column mass. Other masses shown within a row are also within 1 Da of the expected mass.
[3]Position refers to amino acid residues within the predicted CP4 EPSPS sequence as depicted in Figure 18.
DHB = 5-dihydroxybenzoic acid matrix, α-cyano = α-cyano-4-hydroxycinnamic acid matrix; Sinapinic acid = 3, 5-dimethoxy-4-hydroxycinnamic acid matrix; Ave = experimental mass average (for large peptides the monoisotopic mass is poorly resolved, therefore the mass average value is used for comparison).

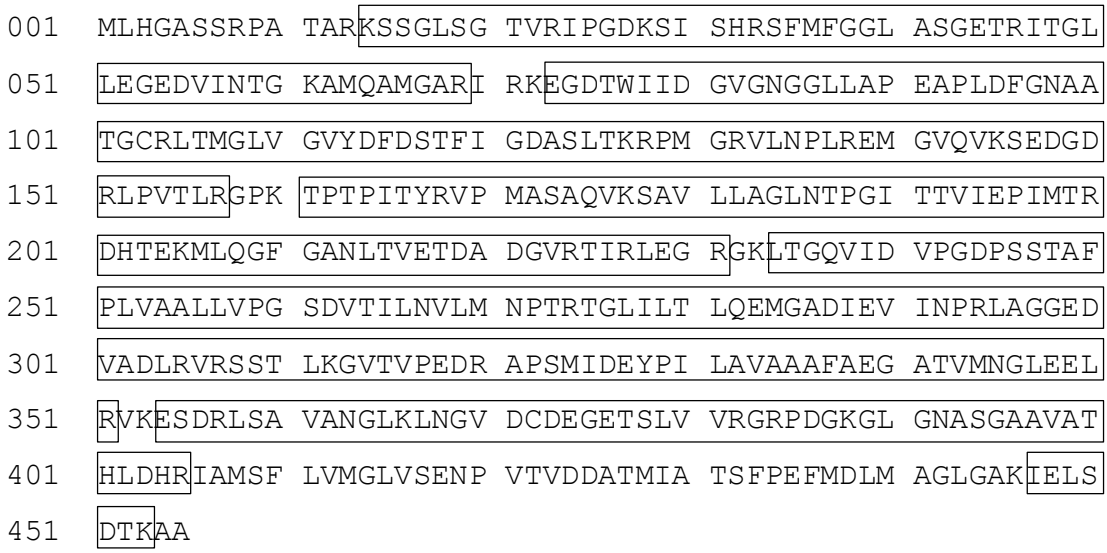


Figure 18. MALDI-TOF MS Coverage Map of the MON 88302-produced CP4 EPSPS Protein

The amino acid sequence of the mature CP4 EPSPS protein was deduced from the *cp4 epsps* gene present in MON 88302. Boxed regions correspond to regions covered by tryptic peptides that were identified from the MON 88302-produced CP4 EPSPS protein sample using MALDI-TOF MS. In total, 85.5% (389 of 455 total amino acids) of the expected protein sequence was covered by the identified peptides.

Western Blot Analysis of CP4 EPSPS Protein Isolated from the Grain of MON 88302 and Immunoreactivity Comparison to *E. coli*-Produced CP4 EPSPS Protein

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 seed of MON 88302 and 2) to determine the relative immunoreactivity of the MON 88302- and the *E. coli*-produced CP4 EPSPS proteins. The results demonstrated that the anti-CP4 EPSPS antibody recognized the MON 88302-produced CP4 EPSPS protein that migrated to an identical position as the *E. coli*-produced CP4 EPSPS protein (Figure 19). Furthermore, the immunoreactive signal increased with increasing amounts of CP4 EPSPS protein loaded.

Densitometric analysis was conducted to compare the immunoreactivity of MON 88302- and *E. coli*-produced CP4 EPSPS proteins**.** The average signal intensity (OD x mm^2) from the MON 88302-produced CP4 EPSPS bands and the *E. coli*-produced CP4 EPSPS bands at each amount of protein loaded are shown in Table 6. The percent differences in the average signal intensity from the MON 88302-produced CP4 EPSPS bands and from the *E. coli*-produced CP4 EPSPS bands for each amount analyzed was calculated. These values as well as the overall average percent difference (24.1%) are also shown in Table 6. The acceptance criterion for equivalence of immunoreactivity (±35%) of the MON 88302-produced CP4 EPSPS bands and *E. coli*-produced CP4 EPSPS bands was met. Thus, the western blot analysis established identity of the MON 88302-produced CP4 EPSPS and demonstrated that the MON 88302- and *E. coli*-produced CP4 EPSPS proteins have equivalent immunoreactivity with a CP4 EPSPS-specific antibody.

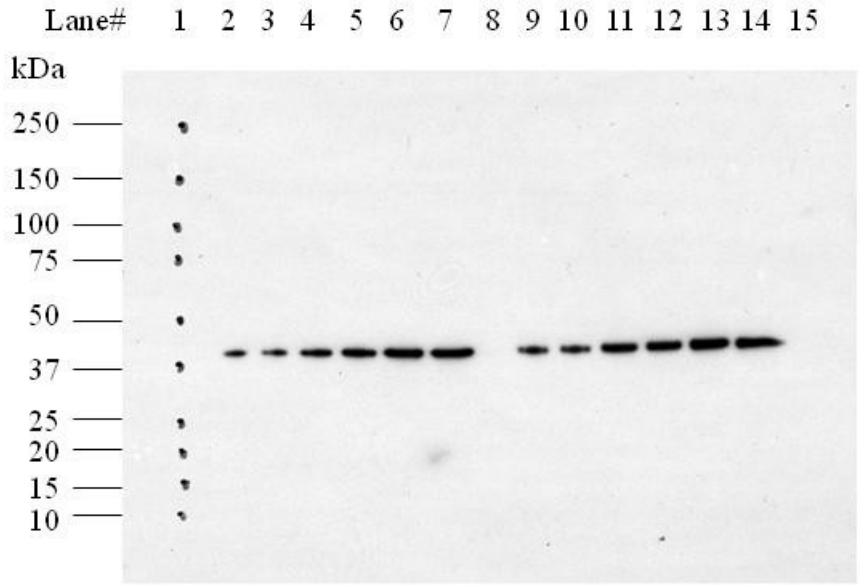


Figure 19. Western Blot Analysis of the MON 88302- and *E. coli*-produced CP4 EPSPS Protein

Aliquots of the MON 88302-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 (GE Healthcare, Piscataway, NJ). Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lane 1. The 1 min exposure is shown.

Lane	Sample	Amount (ng)
1	Precision Plus Protein Standards Dual color	-
2	*E. coli*-produced CP4 EPSPS protein	1
3	*E. coli*-produced CP4 EPSPS protein	1
4	*E. coli*-produced CP4 EPSPS protein	2
5	*E. coli*-produced CP4 EPSPS protein	2
6	*E. coli*-produced CP4 EPSPS protein	3
7	*E. coli*-produced CP4 EPSPS protein	3
8	Empty	-
9	MON 88302-produced CP4 EPSPS protein	1
10	MON 88302-produced CP4 EPSPS protein	1
11	MON 88302-produced CP4 EPSPS protein	2
12	MON 88302-produced CP4 EPSPS protein	2
13	MON 88302-produced CP4 EPSPS protein	3
14	MON 88302-produced CP4 EPSPS protein	3
15	Empty	-

Table 6. Comparison of Immunoreactive Signals from the MON 88302- and *E. coli*-produced CP4 EPSPS Proteins

Sample	Gel lane	Amount (ng)	Contour Qty (OD × mm^2)	Average Contour Qty[1]	Percent Difference[2] (%)	Average Difference[3] (%)
E. coli CP4 EPSPS	2	1	1.257	1.408	30.8	24.1
E. coli CP4 EPSPS	3	1	1.558			
MON 88302 CP4 EPSPS	9	1	2.064	2.033		
MON 88302 CP4 EPSPS	10	1	2.002			
E. coli CP4 EPSPS	4	2	3.296	3.748	26.5	
E. coli CP4 EPSPS	5	2	4.199			
MON 88302 CP4 EPSPS	11	2	4.979	5.101		
MON 88302 CP4 EPSPS	12	2	5.222			
E. coli CP4 EPSPS	6	3	6.264	6.407	14.9	
E. coli CP4 EPSPS	7	3	6.549			
MON 88302 CP4 EPSPS	13	3	7.737	7.527		
MON 88302 CP4 EPSPS	14	3	7.317			

[1]Average Contour Quantity = ∑(Contour Quantity)/2; contour quantity is average pixel density × band area.
[2]Percent Difference (%) = ((|Average Contour Quantity MON 88302–Average Contour Quantity *E. coli*|)/(Average Contour Quantity MON 88302)) × 100% .
[3]Average difference (%) = ∑ ⟦% difference⟧ /3.

MON 88302 CP4 EPSPS Protein Molecular Weight and Purity

For molecular weight and purity analysis, the MON 88302-produced CP4 EPSPS protein was separated using SDS-PAGE. The gel was stained with Brilliant Blue G Colloidal stain and analyzed by densitometry (Figure 20). The MON 88302-produced CP4 EPSPS protein (Figure 20, lanes 3-8) migrated to the same position on the gel as the *E. coli*-produced CP4 EPSPS protein (Figure 20, lane 2) and had an apparent molecular weight of 43.1 kDa (Table 7). The apparent molecular weight of the *E. coli*-produced CP4 EPSPS protein as reported on its Certificate of Analysis was 43.8 kDa (Table 7). The apparent molecular weights of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were considered equivalent if they were within 10% of one another. Because the experimentally determined apparent molecular weight of the MON 88302-produced CP4 EPSPS protein was within 10% of the *E. coli*-produced CP4 EPSPS protein (Table 7), the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were determined to have equivalent apparent molecular weights.

The purity of the MON 88302-produced CP4 EPSPS protein was calculated based on the six loads on the gel (Figure 20, lanes 3 to 8). The average purity was determined to be 99%.

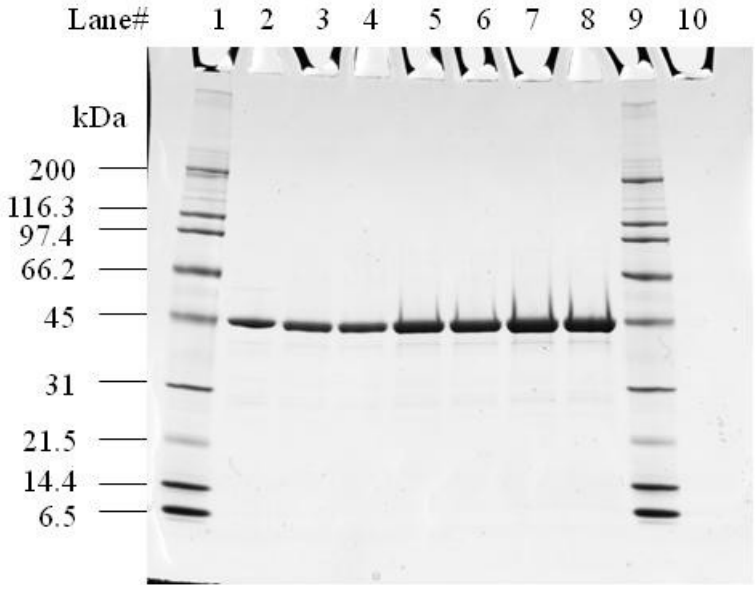


Figure 20. Molecular Weight and Purity Analysis of the MON 88302-produced CP4 EPSPS Protein

Aliquots of the MON 88302- 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 are shown on the left and correspond to the markers loaded in Lanes 1 and 9.

Lane	Sample	Amount (µg)
1	Broad Range Molecular Weight Markers	4.5
2	*E. coli*-produced CP4 EPSPS protein	1
3	MON 88302-produced CP4 EPSPS protein	1
4	MON 88302-produced CP4 EPSPS protein	1
5	MON 88302-produced CP4 EPSPS protein	2
6	MON 88302-produced CP4 EPSPS protein	2
7	MON 88302-produced CP4 EPSPS protein	3
8	MON 88302-produced CP4 EPSPS protein	3
9	Broad Range Molecular Weight markers	4.5
10	Empty	-

Table 7. Molecular Weight Comparison Between the MON 88302-Produced and *E. coli*-Produced CP4 EPSPS Proteins Based on SDS-PAGE

Molecular Weight of MON 88302-Produced CP4 EPSPS Protein	Molecular Weight of *E. coli*-Produced CP4 EPSPS Protein[1]	% Difference from *E. coli*-Produced CP4 EPSPS Protein
43.1 kDa	43.8 kDa	1.6%

[1]The molecular weight of the *E. coli*-produced CP4 EPSPS protein as reported on its Certificate of Analysis.

CP4 EPSPS Glycosylation Equivalence

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher et al., 1988) in a process known as glycosolation. 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 seed of MON 88302, the MON 88302-produced CP4 EPSPS protein was analyzed using an ECL Glycoprotein Detection Module (GE, Healthcare, Piscataway, NJ). Transferrin, a glycosylated protein, was used as a positive control in the assay. To assess equivalence of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins, the *E. coli*-produced CP4 EPSPS protein, previously been shown to be free of glycosylation (Harrison et al., 1996), was also analyzed. The positive control was clearly detected at expected molecular weight (~76 kDa) and the band intensity increased with increasing concentration (Figure 21, Panel A, lanes 2-5). In contrast, signals were not observed in the lanes containing the MON 88302- or *E. coli*- produced protein at the expected molecular weight for the CP4 EPSPS protein (Figure 21, panel A, lanes 6-9). To confirm that sufficient MON 88302- and *E. coli*-produced CP4 EPSPS proteins were present for glycosylation analysis, a second membrane (with identical loadings and transfer times) was stained with Coomassie Blue R250 for protein detection (Figure 21, Panel B). Both the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were clearly detected (Figure 21, Panel B, Lanes 6-9). These data indicate that the glycosylation status of MON 88302-produced CP4 EPSPS protein is equivalent to that of the *E. coli*-produced CP4 EPSPS protein and that neither is glycosylated.

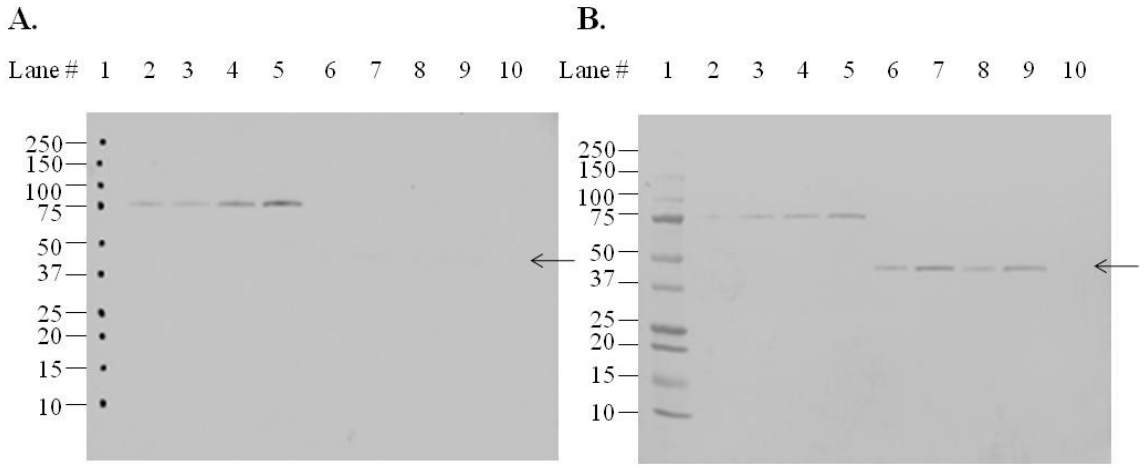


Figure 21. Glycosylation Analysis of the MON 88302-produced CP4 EPSPS Protein

Aliquots of the transferrin (positive control), *E. coli*-produced CP4 EPSPS protein and MON 88302-produced CP4 EPSPS protein were separated by SDS-PAGE (4-20%) and electrotransferred to PVDF membranes. (A) Where present, the labeled carbohydrate moieties were detected using the ECL-based system with exposure to Hyperfilm. A 2 min exposure is shown. (B) An equivalent blot was stained with Coomassie Blue R250 to confirm the presence of proteins. The signal was captured using a Bio-Rad GS-800 with Quantity One software (version 4.4.0). Approximate molecular weights (kDa) correspond to the Precision Plus, dual color markers (used to verify transfer and MW) in Lane 1. Arrows indicate the band corresponding to CP4 EPSPS protein.

Lane	Sample	Amount (ng)
1	Precision Plus, dual color MW markers	-
2	Transferrin (positive control)	50
3	Transferrin (positive control)	100
4	Transferrin (positive control)	150
5	Transferrin (positive control)	200
6	*E. coli*-produced CP4 EPSPS (negative control)	100
7	*E. coli*-produced CP4 EPSPS (negative control)	200
8	MON 88302-produced CP4 EPSPS	100
9	MON 88302-produced CP4 EPSPS	200
10	Empty	-

CP4 EPSPS Functional Activity

The functional activities of the MON 88302- and *E. coli*-produced CP4 EPSPS proteins were determined using a colorimetric assay that measures formation of inorganic phosphate (Pi) from the EPSPS-catalyzed reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). In this assay, protein-specific activity is expressed as units per milligram of protein (U/mg), where a unit is defined as one µmole of inorganic phosphate released from PEP per minute at 25 °C. The MON 88302- and *E. coli*- produced CP4 EPSPS proteins were considered to have equivalent functional activity if the specific activities were within 2-fold of one another.

The experimentally determined specific activities for the MON 88302- and *E. coli*-produced CP4 EPSPS proteins are presented in Table 8. The specific activities of MON 88302- and *E. coli*-produced CP4 EPSPS proteins were 4.93 U/mg and 2.79 U/mg of CP4 EPSPS protein, respectively. Because the specific activity of the MON 88302-produced CP4 EPSPS protein falls within the preset acceptance criterion (Table 8), the MON 88302-produced CP4 EPSPS protein was considered to have equivalent functional activity to that of the *E. coli*-produced CP4 EPSPS protein.

Table 8. CP4 EPSPS Functional Activity

MON 88302-produced CP4 EPSPS Protein[1] (U/mg)	E. coli-produced CP4 EPSPS Protein[1] (U/mg)	Previously set acceptance limits[2] (U/mg)
4.93 ± 0.36	2.79 ± 0.26	1.40 – 5.58

[1]Value refers to mean and standard deviation calculated based on n = 6 which includes three replicate assays spectrophotometrically.
[2]Within 2-fold of the *E.coli*-produced CP4 EPSPS specific activity (2.79 ÷ 2 U/mg to 2.79 x 2 U/mg)

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

CP4 EPSPS protein levels in various tissues of MON 88302 relevant to the risk assessment were determined by a validated enzyme-linked immunosorbent assay (ELISA). Forage, seed, leaf and root tissues of MON 88302 were collected from four replicate plots planted in a randomized complete block field design during the 2009 growing season from the following three field sites in the U.S.: Power County, Idaho; Wilkin County, Minnesota; and McHenry County, North Dakota, and the following three field sites in Canada: Portage la Prairie, Manitoba; Newton, Manitoba; and Saskatoon, Saskatchewan. These field sites were representative of canola producing regions suitable for commercial production. Forage, seed, over-season leaf (OSL-1 through OSL-4), and root (Root-1 and Root-2) tissue samples were collected from each replicated plot at all field sites (MSL0023090). Pollen tissue of MON 88302 was collected from three plots planted in a randomized completed block design in a greenhouse during 2010 in the U.S.(MSL0023598)

CP4 EPSPS protein levels were determined in all nine tissue types. The results obtained from ELISA are summarized in Table 9. CP4 EPSPS protein levels in MON 88302 across tissue types ranged from 8.2 to 500 µg/g dw. The mean CP4 EPSPS protein levels were determined across six sites with the exception of seed (5 sites), OSL-1 (5 sites), OSL-2

(3 sites), Root-2 (4 sites), and Pollen (1 site). The mean CP4 EPSPS protein levels were highest in leaf (ranging from OSL-1 at 180 µg/g dw to OSL-3 at 230 µg/g dw), followed by forage (170 µg/g dw), root (ranging from Root-2 at 38 µg/g dw to Root-1 at 82 µg/g dw), seed (27 µg/g dw), and pollen (9.0 µg/g dw).

Table 9. Summary of CP4 EPSPS Protein Levels in Canola Tissues from MON 88302 Grown in 2009 U.S. and Canadian Field Trials

Tissue[1]	Development Stage[2]	Days After Planting (DAP)	CP4 EPSPS Mean (SD) Range (µg/g fw)[3]	CP4 EPSPS Mean (SD) Range (µg/g dw)[4]	LOQ/LOD[5] (µg/g fw)
Forage	30 BBCH	37 - 57	18 (4.4) 14 - 28	170 (22) 120 - 210	0.91/0.28
Seed	99 BBCH	118 - 132	25 (5.2) 21 - 43	27 (5.6) 22 - 46	0.91/0.81
OSL-1	13-14 BBCH	23 - 40	23 (10) 10 - 45	180 (40) 110 - 250	0.91/0.098
OSL-2	17-19 BBCH	32 - 54	22 (5.9) 18 - 37	180 (41) 120 - 250	0.91/0.098
OSL-3	30 BBCH	37 - 57	31 (6.3) 20 - 41	230 (50) 130 - 300	0.91/0.098
OSL-4	60-62 BBCH	51 - 61	36 (14) 20 - 85	210 (80) 110 - 500	0.91/0.098
Root-1	30 BBCH	37 - 57	19 (4.1) 11 - 25	82 (17) 46 - 100	0.91/0.60
Root-2	71-73 BBCH	49 - 81	10 (3.3) 7.0 - 17	38 (14) 24 - 62	0.91/0.60
Pollen	60-69 BBCH	43-74	8.1 (0.64) 7.4 – 8.6	9.0 (0.71) 8.2 – 9.6	0.91/ND[6]

[1]OSL = over-season leaf.

[2]The development stage each tissue was collected. The canola growth stages are based on the Bayer, BASF, Ciba-Geigy and Hoechst Cereal Grain Growth Scale (BBCH) (BBCH, 2001).

[3]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a fresh weight basis (fw). The means, SD, and ranges (minimum and maximum values) were calculated for each tissue across all sites. The numbers of samples (n) figured into the calculations are as follows: forage n = 20, seed n = 16, OSL-1 n = 16, OSL-2 n = 9, OSL-3 n = 20, OSL-4 n = 2 0, Root-1 n = 19, Root-2 n = 11, and Pollen n =3.

[4]Protein levels are expressed as the arithmetic mean and standard deviation (SD) as microgram (µg) of protein per gram (g) of tissue on a dry weight basis (dw). The dry weight values were calculated by dividing the µg/g fw by the dry weight conversion factor obtained from moisture analysis data.

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

[6]LOD was not determined for pollen samples.

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

Several other Roundup Ready crops that produce the CP4 EPSPS protein have also been reviewed by FSANZ, including Roundup Ready soybean, Roundup Ready 2 Yield soybean, Roundup Ready canola, Roundup Ready sugar beet, Roundup Ready cotton, Roundup Ready Flex cotton, and Roundup Ready alfalfa. The CP4 EPSPS protein expressed in MON 88302 is identical to the CP4 EPSPS proteins in other Roundup Ready crops. Results from the protein characterization studies included in this application confirmed the identity of the MON 88302-produced CP4 EPSPS protein and established the equivalence of MON 88302-produced protein to the *E. coli*-produced CP4 EPSPS protein (section B2(b)) used previously to demonstrate the safety of the CP4 EPSPS protein produced in other Roundup Ready crops. The history of safe use of CP4 EPSPS is further supported by the lack of any documented reports of adverse effects of this protein 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.

Additionally, 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)); and 4) if the protein exerts any acute toxic effects in mammals (B3(c)). The CP4 EPSPS protein in MON 88302 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. By definition, homologous proteins 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 (please refer to MSL0022522). 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 $1x10^{-5}$ or less for any alignment was used to identify proteins from the TOX_2010 database with potential for significant shared structural similarity and function with CP4 EPSPS protein. As described above, the *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a lower degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences require an *E*-score of 1×10^{-5} or less to be considered to have sufficient sequence similarity to infer homology. The results of the search comparisons showed that no relevant alignments were observed against proteins in the TOX_2010 database.

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 75ºC (section B4(c)(ii)). The digestibility 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)(i)). Therefore, it is anticipated that exposure to functionally active CP4 EPSPS protein from the consumption of MON 88302 or foods derived from MON 88302 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 weeks) feeding study (Liener, 1994). The amino acid sequence of the CP4 EPSPS protein produced in MON 88302 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 toxicity of the CP4 EPSPS protein.

E. coli-produced 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 wt (bw) (Harrison et al., 1996). Additional groups of mice were administered comparable volume of the buffer or a comparable amount (mg/kg bw) of bovine serum albumin (BSA) to serve as vehicle or protein controls, respectively. 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 was considered to be 572 mg/kg bw, the highest dose tested (MSL0013077).

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, following the guidelines adopted by the Codex Alimentarius Commission (2009), an assessment of potential allergenicity of introduced proteins has been conducted, by comparing the characteristics of the introduced protein to characteristics of known allergens (Codex Alimentarius, 2009). 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 to known allergens based on the amino acid sequence (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 88302 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 (Padgette et al., 1996). The bacterial isolate, CP4, was identified by the American Type Culture Collection as an *Agrobacterium* species. *Agrobacterium* species are not commonly known for human or animal pathogenicity or allergenicity. According to a report of a joint FAO/WHO Expert Consultation (FAO/WHO, 2001), there is no known population of individuals sensitized to these bacterial proteins.

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, 2009) 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 88302 and known allergens, gliadins, and glutenins was assessed using the FASTA sequence alignment tool and an eight-amino acid sliding window search (Codex Alimentarius, 2009; Thomas et al., 2005). The data generated from these analyses confirm that the CP4 EPSPS protein does not share amino acid sequence similarities with known allergens, gliadins, or glutenins (MSL0022522).

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. By definition, homologous proteins have common secondary structures, and three-dimensional configuration, and, consequently, may share similar functions. The allergen, gliadin, and glutenin sequence database (AD_2010) was obtained from Food Allergy Research and Resource Program Database (FARRP_2010) and was used for the evaluation of sequence similarities shared between the CP4 EPSPS protein and all proteins. The

AD_2010 database contains 1,471 sequences. When used to align the sequence of the introduced protein to each protein in the database, the FASTA algorithm produces an *E*-score (expectation score) for each alignment. The *E*-score is a statistical measure of the likelihood that the observed similarity score could have occurred by chance in a search. A larger *E*-score indicates a low degree of similarity between the query sequence and the sequence from the database. Typically, alignments between two sequences which have an *E*-score of less than or equal to 1×10^{-5} are considered to have significant homology. Results indicate that the CP4 EPSPS protein sequence does not share significant similarity with sequences in the allergen database. No alignment met nor exceeded the threshold of 35% identity over 80 amino acids recommended by Codex Alimentarius (2009) 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 show 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 show that the CP4 EPSPS protein sequence lacks both structurally and immunologically relevant sequence similarities to known allergens, gliadins, and glutenins.

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 proteins to digestion in pepsin. The SGF assay protocol has been standardized based on results obtained from an international, multi-laboratory 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 protein was analyzed (MSL0017566) 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 in simulated digestive fluids. 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 using the standardized method published by the International Life Science Institute (ILSI) (Thomas et al., 2004), confirmed the *in vitro* digestibility of the *E. coli*-produced CP4 EPSPS protein in SGF. *E. coli*-produced CP4 EPSPS protein, shown to be physiochemically and functionally equivalent to the CP4 EPSPS protein produced in MON 88302 (section B2(b)), was used 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 sec, based on the results of visual inspection of colloidal blue stained SDS-PAGE gels (Figure 22). Western blot analysis confirmed that greater than 95% of the *E. coli*-produced CP4 EPSPS protein was digested in SGF within 15 sec (Figure 23). 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.

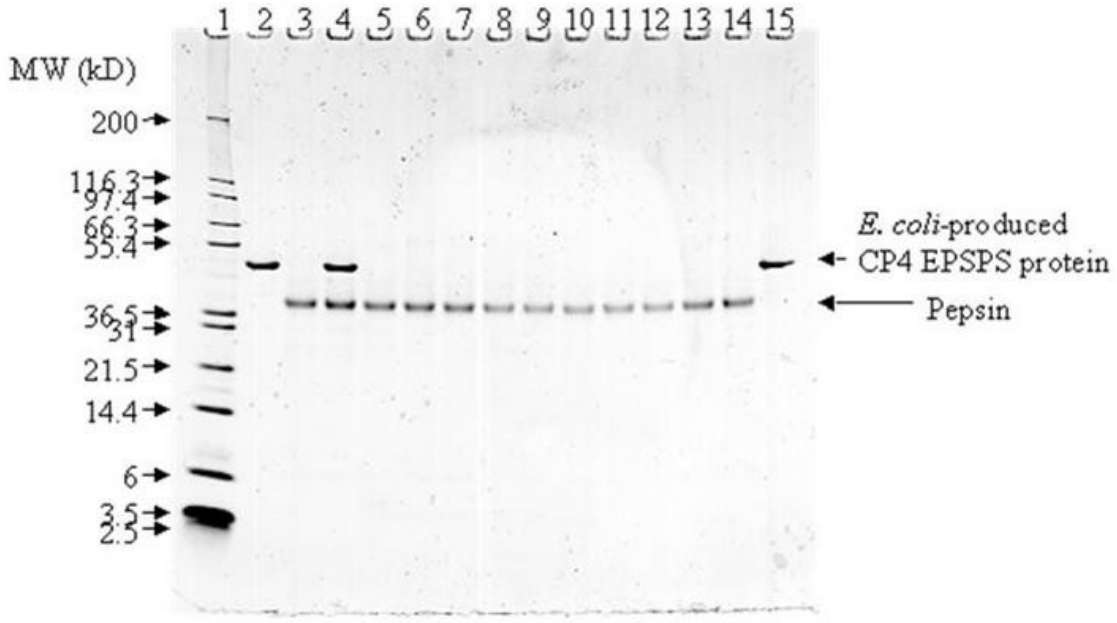


Figure 22. **Colloidal Blue Stained SDS-PAGE Gel Showing the Digestion of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid**
Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G Colloidal 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 (Invitrogen P/N LC 5677)	
2	Experimental control without pepsin	0
3	Experimental control without CP4 EPSPS	0
4	CP4 EPSPS protein in SGF	0
5	CP4 EPSPS protein in SGF	15 sec
6	CP4 EPSPS protein in SGF	30 sec
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

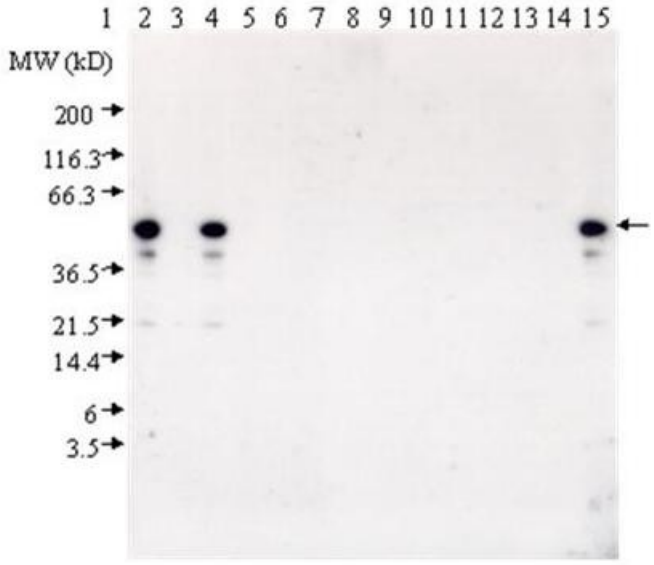


Figure 23. Western Blot Analysis of Purified *E. coli*-Produced CP4 EPSPS Protein in Simulated Gastric Fluid

Proteins were separated by SDS-PAGE using a 10-20% polyacrylamide gradient in a tricine buffered gel, electroblotted, and probed with anti-CP4 EPSPS goat serum. *E. coli*-produced CP4 EPSPS protein was loaded at 1 ng per lane based on 90% purity and pre-digestion concentrations. Lane 1 containing the molecular weight markers was cropped and the arrow on the right side of the image indicates the band corresponding to CP4 EPSPS protein.

Lane	Description	Incubation Time
1	Molecular weight markers (Invitrogen P/N LC5677)	
2	Experimental control without pepsin	0
3	Experimental control without CP4 EPSPS	0
4	CP4 EPSPS protein in SGF	0
5	CP4 EPSPS protein in SGF	15 sec
6	CP4 EPSPS protein in SGF	30 sec
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 used during processing of canola seed into oil and in canola oil refinement (Booth, 2004). 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.

Canola seed processing involves treatment with different temperature regimes, some of which are higher than 55 °C and of variable duration (Booth, 2004). Additionally, some steps, especially oil refinement and deodorization, are carried out at considerably higher temperatures, (e.g., 70 °C for 20 minutes and 240 °C for 20 minutes, respectively) (Booth, 2004). 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 ºC 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 24 and Figure 25, respectively). These data demonstrate that CP4 EPSPS behaves with a predictable tendency toward enzyme denaturation at elevated temperatures (MSL0023307). Therefore, in the unlikely event that canola oil contains protein, it is reasonable to conclude that CP4 EPSPS protein would not be consumed as an active protein in food products.

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 ºC)	6.03 ± 0.29	100%
25 ºC	4.88 ± 0.24	81%
37 ºC	5.08 ± 0.33	84%
55 ºC	4.22 ± 0.12	70%
75 ºC	< LOD[4]	< 3%[5]
95 ºC	< LOD[4]	< 3%[5]

[1] Mean specific activity determined from n = 2.
[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 ºC)	2.8 ± 0.26	100%
25 ºC	3.1 ± 0.23	110%
37 ºC	2.5 ± 0.05	88%
55 ºC	0.70 ± 0.09	25%
75 ºC	< LOD[4]	< 8%[5]
95 ºC	< LOD[4]	< 8%[5]

[1] Mean specific activity determined from n = 2.
[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.

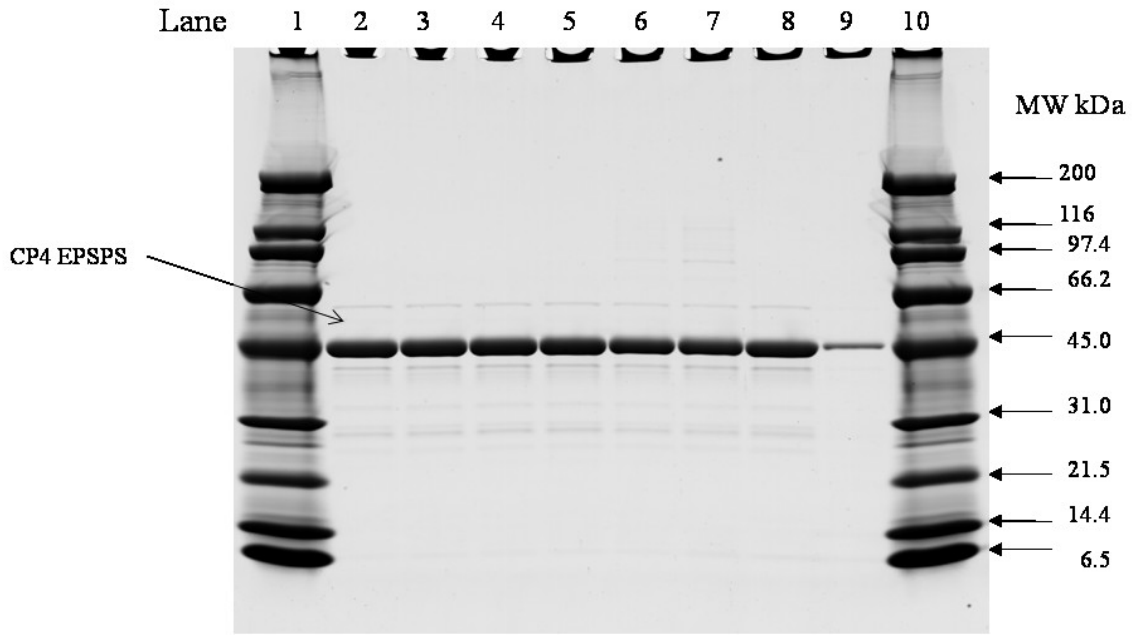


Figure 24. **SDS-PAGE of CP4 EPSPS Following Heat Treatment for 15 Minutes**
Heated-treated samples of CP4 EPSPS (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular 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 ºC)	3.2
3	CP4 EPSPS 25 ºC	3.2
4	CP4 EPSPS 37 ºC	3.2
5	CP4 EPSPS 55 ºC	3.2
6	CP4 EPSPS 75 ºC	3.2
7	CP4 EPSPS 95 ºC	3.2
8	CP4 EPSPS Reference	3.2
9	CP4 EPSPS Reference	0.32
10	Broad Range Molecular Weight Markers	4.5

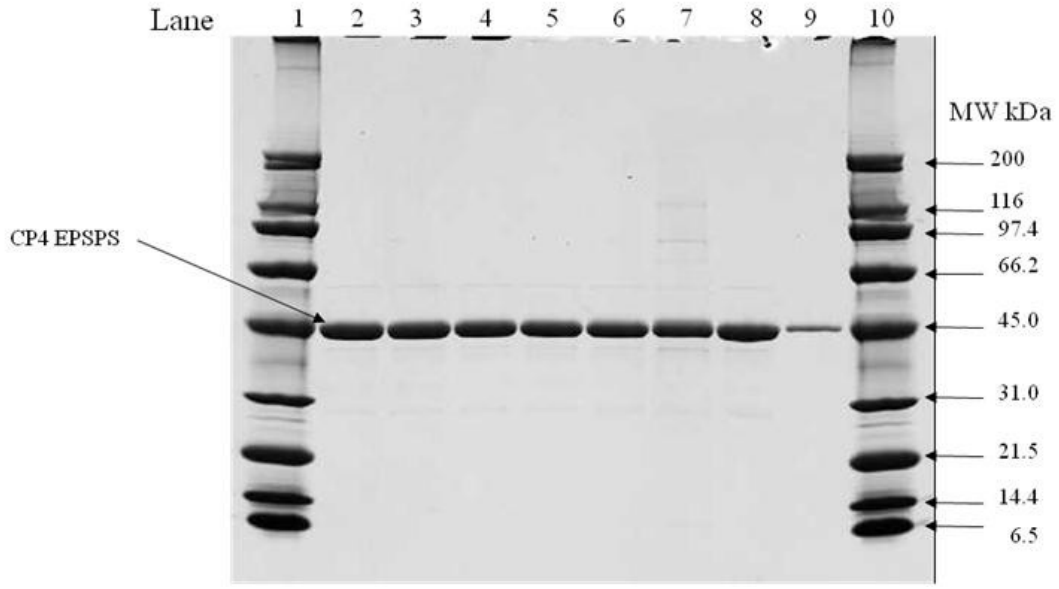


Figure 25. **SDS-PAGE of CP4 EPSPS Following Heat Treatment for 30 Minutes**
Heated samples of CP4 EPSPS protein (3.2 µg total protein) separated on a Tris-glycine 4-20% polyacrylamide gel under denaturing and reducing conditions. Gels were stained with Brilliant Blue G Colloidal. Approximate molecular 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 ºC	3.2
3	CP4 EPSPS 37 ºC	3.2
4	CP4 EPSPS 55 ºC	3.2
5	CP4 EPSPS 75 ºC	3.2
6	CP4 EPSPS 95 ºC	3.2
7	CP4 EPSPS Unheated Control (0 ºC)	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.

As described earlier, 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 these 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). Among tested tissues of MON 88302, seed is the most relevant to the assessment of food allergenicity since seed is the source of canola oil. The mean level of CP4 EPSPS protein in seed of MON 88302 is 27 µg/g dw. The mean percent dry weight of total protein in seed of MON 88302 is 23% (or 230,000 µg/g; Table 14). The percentage of CP4 EPSPS protein in MON 88302 seed is calculated as follows:

$$(27\ \mu g/g \div 230{,}000\ \mu g/g) \times 100\% \approx 0.01\% \text{ or } 100 \text{ ppm of total canola seed protein}$$

Therefore, the CP4 EPSPS protein represents a very small portion of the total protein in harvested seed of MON 88302. Additionally, the total protein content in oil extracted from canola seed is very low (<0.00002% or < 0.2 ppm, (Martín-Hernández et al., 2008). Canola oil is the predominant seed fraction that is used for foods, therefore the levels of CP4 EPSPS in oil from MON 88302 seed would be estimated to be 0.01% of <0.2 ppm total protein in the oil, essentially present in the oil.

B5 Toxicity of novel herbicide metabolites in GM herbicide-tolerant plants

Identification of novel metabolites or 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 ^{14}C-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 ^{14}C-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 ^{14}C-glyphosate via hydroponic solution also indicate that ^{14}C-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, canola 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 TRR in forage, hay, and grain of soybean. In the current glyphosate-tolerant canola product GT73 the *cp4 epsps* gene is present as well as the *goxv247* gene that produces the glyphosate oxidoreductase (GOX) enzyme. As a result of GOX, glyphosate is rapidly degraded to AMPA. 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 ^{14}C-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. In all glyphosate-tolerant crops 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 (MSL0022984) was conducted on canola MON 88302 grown in the US in 2009 at eight sites (one site was later removed from the study because the quality of the canola seed produced was determined to be not commercially acceptable). These sites represent major canola growing areas in the U.S. All sites contain three treated plots and one untreated plot. Glyphosate use patterns that were tested for the second-generation glyphosate tolerant canola MON 88302 are found in Table 12, Treatments 2 and 4 are the actual proposed use rates and patterns. Treatment 2 has two postemergence applications of 0.9 kg a.e./ha, one at each of the two appropriate stages of canola development (4-6 leaf stage and first-flower). Treatment 4 has the same proposed use rates, but with a slightly earlier timing for the last application (late bolting stage). Treatments 2 and 4 would also cover expected residues from an alternative use pattern, in which the two rates are combined into one application of 1.8 kg a.e./ha made at an earlier stage of development (4-6 leaf stage). The rate of the last application of Treatment 3 is twice that of the proposed use pattern (1.8 kg a.e./ha at first-flower versus 0.9 kg a.e./ha proposed) and, therefore, the data collected from this treatment are not relevant for determining an appropriate tolerance for canola and are not included in the summary table (Table 13) presented here. Those data for Treatment 3 were collected in the event that application of a full rate of 1.8 kg glyphosate a.e./ha was needed for a late-season weed control. However, this use pattern is not being proposed at this time.

Table 12. Applications of Glyphosate to Canola MON 88302

	Growth Stages/Target Application Rates (g a.e./ha)			
Treatment	**Pre-emergence**	**4-6 leaf**	**Late bolting**	**1st flower**
1(Untreated)	0	0	0	0
2	4250	900	0	900
3	4250	900	0	1800
4	4250	900	900	0

As shown in a Table 13 below, data were obtained on the residue levels of glyphosate and AMPA in canola seed, and has the same nominal glyphosate concentration and similar composition as Roundup WeatherMAX Herbicide (EPA Reg. No. 524-537), which is sold commercially in the U.S.

The glyphosate residue levels in seed from Treatment 2 were determined as an average of two field replicate samples and ranged from 0.24 to 6.3 ppm with a median value of 1.6 ppm. AMPA was detected at levels above 0.05 ppm in only two of seven sites with a maximum residue of 0.16 ppm. Total residue [defined as glyphosate + (1.523 × AMPA)] ranged from 0.24 to 6.5 ppm in Treatment 2.

The glyphosate residue levels in seed from Treatment 4 were also determined as an average of two field replicate samples and ranged from 0.08 to 2.4 ppm with a median value of 1.5 ppm. AMPA was detected at levels above 0.05 ppm in only one of seven sites with a maximum of 0.06 ppm. Total residue ranged from 0.08 to 2.5 ppm in Treatment 4.

While Treatments 2 and 4 both had the same application rates, the lower residues in Treatment 4 compared to Treatment 2 are consistent with the earlier in-crop application for the last application in Treatment 4 (late bolting vs. first flowerThese results are below the tolerance or Maximum Residue Limit (MRL) established by a number of national and

international regulatory agencies to support existing uses of glyphosate in both conventional and glyphosate tolerant canola or oil seed rape. For example, the United States EPA (U.S. EPA, 2008) and CODEX (Codex Alimentarius, 2011), have established MRLs of 20 ppm for glyphosate in canola. Monsanto Australia Limited is separately requesting that FSANZ consider amending Standard 1.4.2 – Maximum Residue Limits to harmonise the MRL tolerance for glyphosate on canola food imports to that which is set out in the Code of Federal Regulations of the United States of America and the Codex Alimentarius. Prior to this product being commercialised at a later date in Australia, Monsanto Australia Limited will also conduct local residue trials and submit an application to the Australian Pesticides and Veterinary Medicines Authority to ensure the MRL cultivation tolerance for glyphosate reflects new local use patterns associated with MON 88302 canola.

Table 12. Glyphosate and AMPA Residues in Canola Seed

Treatment	PHI,[1] days	Glyphosate, ppm		AMPA, ppm		Total, ppm[4]	
		Median	Range[2,3]	Median	Range[2,3]	Median	Range[2,3]
2	58-70	1.6	0.24-6.3	<0.05	<0.05-0.16	1.7	0.24-6.5
4	65-77	1.5	0.08-2.4	<0.05	<0.05-0.06	1.5	0.08-2.5

[1]PHI = Preharvest Interval, days between last application and sampling
[2]Range of site-averaged residues in canola seed sample analyses across all seven sites.
[3]Lower limit of method validation of glyphosate and AMPA is 0.05 ppm.
[4]Total ppm = [Glyphosate ppm] + [AMPA ppm × 1.523]

B6 Compositional Assessment

Several Roundup Ready crops that produce the CP4 EPSPS protein have been previously reviewed by FSANZ. The CP4 EPSPS protein expressed in MON 88302 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 88302 would affect nutritionally important nutrients, toxicants, and anti-nutrients present in seed from this new product.

Safety assessments of biotechnology-derived crops typically include comparisons of the composition of grain and/or other raw agricultural commodities of the biotechnology-derived crop to that of conventional counterparts (Codex Alimentarius, 2009). Compositional assessments are performed using the principles and analytes outlined in the OECD consensus document for canola composition (OECD, 2001).

A recent review of compositional assessments conducted according to OECD guidelines that encompassed a total of seven biotechnology-derived crop varieties, nine countries and eleven 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 and anti-nutrients and secondary metabolites that reflect the influence of environmental and genetic factors as well as extensive conventional breeding efforts to improve nutrition, agronomics, and yield (Harrigan et al., 2010; Mailer and Pratley, 1990; Marwede et al., 2004; Naczk et al., 1998; OECD, 2001; Pritchard et al., 2000; Reynolds et al., 2005; Ridley et al., 2004; Werteker et al., 2010).

Compositional equivalence between biotechnology-derived and conventional crops supports an "equal or increased assurance of the safety of foods derived from genetically modified plants" (OECD, 1998). OECD consensus documents on compositional considerations for new crop varieties emphasize quantitative measurements of essential nutrients and known anti-nutrients. This is based on the premise that such comprehensive and detailed analyses will most effectively discern any compositional changes that imply potential nutritional or safety (*e.g.*, anti-nutritional) concerns. Levels of the components in seed and forage of the biotechnology-derived crop are compared to: 1) corresponding levels in a conventional comparator, the genetically similar conventional line, grown concurrently, under field conditions, and 2) natural ranges generated from an evaluation of commercial reference varieties 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 well-documented variation in the concentrations of crop nutrients, toxicants, and anti-nutrients.

This section provides analyses of concentrations of key nutrients, toxicants, and anti-nutrients of MON 88302 compared with equivalent analyses of a conventional counterpart grown and harvested under the same conditions, as appropriate. In addition, commercial canola reference varieties were included in the composition analyses to establish a range of natural variability for each analyte, defined by a 99% tolerance interval. The production of materials for the compositional analyses used field designs to allow accurate assessments of compositional characteristics over a range of environmental conditions under which MON 88302 is expected to be grown. Design parameters included a sufficient number of trial sites to allow adequate exposure to the variety of conditions met in nature. Field sites

were replicated with an adequate number of plants samples, and the methods of analysis were sufficiently sensitive and specific to detect variations in the components measured and to allow statistically rigorous analyses. The information provided in this section also addresses the relevant factors in Codex Plant Guidelines, Section 4, paragraphs 44 and 45 for compositional analyses (Codex Alimentarius, 2009).

Compositional Equivalence of MON 88302 Seed to Conventional Canola

Compositional analysis comparing MON 88302 to the conventional control variety (Ebony) and commercial reference varieties demonstrated that MON 88302 is compositionally equivalent to conventional canola (MSL0022806 and RAR-2011-0237). Seed samples were collected from MON 88302 and the conventional control grown in a 2009 North American field production. Canola forage is rarely consumed by animals and is not a source of nutrition for humans. As such, the OECD consensus document on compositional considerations for canola (OECD, 2001) does not include analysis of canola forage, and so forage samples were not collected. The background genetics of the conventional control were similar to that of MON 88302, but did not contain the *cp4 epsps* expression cassette. Seven different commercial reference varieties were included across all sites of the field production to provide data on natural variability of each compositional component analyzed. The samples utilized for compositional analysis were obtained from two U.S. sites [Wilkin County, MN (MNCA) and McHenry County, North Dakota (NDVA)] and three Canadian sites [Portage la Prairie, Manitoba (MBPL); Newton, Manitoba (MBNW); and Saskatoon, Saskatchewan (SKSA)]. The sites were planted in a randomized complete block design with four replicates per site. MON 88302, the conventional control, and commercial reference varieties were treated with maintenance pesticides as necessary throughout the growing season. In addition to the conventional weed control programs, MON 88302 plots were treated at the 5-6 leaf stage with a glyphosate application at a target rate of 1.6 lb acid equivalents per acre (1800 g a.e./ha).

Compositional analyses were conducted as recommended for canola seed (OECD, 2001) to assess whether levels of key nutrients, toxicants and anti-nutrients in MON 88302 were equivalent to levels in the conventional control and to the composition of commercial reference varieties. Nutrients assessed in this analysis included proximates (ash, carbohydrates by calculation, moisture, protein, and total fat), fibers (acid detergent fiber [ADF], neutral detergent fiber [NDF], and total dietary fiber [TDF]), amino acids (18 components), fatty acids (FA; C8-C24,), vitamin E (α-tocopherol), and minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc) in seed. The toxicants assessed in seed included erucic acid and glucosinolates (alkyl glucosinolates [including 3-butenyl, 4-pentenyl, 2-hydroxy-3-butenyl, and 2-hydroxy-4-pentenyl glucosinolates], indolyl glucosinolates [including 3-indolylmethyl and 4-hydroxy-3-indoylmethyl], and total glucosinolates). The anti-nutrients assessed in seed included phytic acid and sinapine (as sinapic acid), and total tannins (as the sum of soluble and insoluble tannin fractions). The toxicant and anti-nutrient results are discussed together under the general heading of anti-nutrients. In all, 71 different components were measured. Of those 71 components, 18 nutrients and one toxicant (18 fatty acids, including erucic acid, and one mineral) had more than 50% of the observations below the assay limit of quantitation (LOQ) and, as a result, were excluded from the statistical analyses. Therefore, 52 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, expressed as percent fresh weight, and fatty acids, expressed as percent of total FA.

For MON 88302, six statistical comparisons to the conventional control were conducted for each compositional component. One comparison was based on compositional data combined across all five field sites (combined-site analysis) and five separate comparisons were conducted on data from each of the individual field sites. Statistically significant differences were identified at the 5% level ($\alpha = 0.05$). Data from the commercial reference varieties 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 canola varieties that have a history of safe consumption, and that were grown concurrently with MON 88302 and the conventional control in the same trial.

For the combined-site analysis, significant differences in nutrient, toxicant, and anti-nutrient components were further evaluated using considerations relevant to the safety and nutritional quality of MON 88302 when compared to the conventional control, which is the conventional counterpart with a history of safe consumption. Considerations used to assess the relevance of each combined-site statistically significant difference included: 1) the relative magnitude of the difference in the mean values of nutrient, toxicant, and anti-nutrient components of MON 88302 and the conventional control; 2) whether the MON 88302 component mean value is within the range of natural variability of that component as represented by the 99% tolerance interval of the commercial reference varieties grown concurrently in the same trial; 3) evaluation of the reproducibility of the statistically significant ($\alpha = 0.05$) combined-site component differences at individual sites; and 4) an assessment of the differences within the context of natural variability of commercial canola composition published in the scientific literature. If statistically significant differences detected in the individual site analyses were not observed in the combined-site analysis, they were not considered further for the compositional assessment of safety.

This analysis provides a comprehensive comparative assessment of the levels of key nutrients, toxicants, and anti-nutrients in seed of MON 88302 and the conventional control, discussed in the context of natural variability in composition of commercial canola. Results of the comparison indicate that the composition of the seed of MON 88302 is equivalent to that of the conventional control and within the natural variability of commercial reference varieties.

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

Nutrient Levels in Seed

In the combined-site analysis of nutrient levels in seed, the following components showed no significant differences in mean values between MON 88302 and the conventional control: proximates, two types of fiber (ADF and NDF), 18 amino acids (alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine), four fatty acids (16:0 palmitic acid, 20:1 eicosenoic acid, 24:0 lignoceric acid, and 24:1 nervonic acid), eight minerals (calcium, copper, iron, magnesium, manganese, phosphorus, potassium, and zinc), and vitamin E (α-tocopherol) (Table 14).

The components that showed significant differences in mean values between MON 88302 and the conventional control in the combined-site analysis were: total dietary fiber (TDF) and seven fatty acids (16:1 palmitoleic acid, 18:0 stearic acid, 18:1 oleic acid, 18:2 linoleic acid, 18:3 linolenic acid, 20:0 arachidic acid, and 22:0 behenic acid) (Table 13 and Table 14).

1) The statistically significant differences in nutrients were evaluated using considerations relevant to the nutritional quality of MON 88302 when compared to the conventional control: eight combined-site nutrient significant differences ($\alpha = 0.05$) between MON 88302 and the conventional control were attributable to TDF (expressed as % dry weight) and seven fatty acids (expressed as % total FA). The relative magnitudes of differences between the combined-site mean values for MON 88302 and the conventional control showed an increase for TDF, 18:2 linoleic acid, and 18:3 linolenic acid, (13.81%, 8.98%, and 20.01%, respectively) and a decrease for 16:1 palmitoleic acid, 18:0 stearic acid, 18:1 oleic acid, 20:0 arachidic acid, and 22:0 behenic acid (7.56%, 15.06%, 4.52%, 10.68%, and 6.01%, respectively). The relative differences in these components in the across-site analysis and at individual sites were between 3.48% and 28.69% (Table 14). The magnitudes of differences observed between MON 88302 and the conventional control were small relative to the natural variability of these components as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption as presented in the tables referenced above.
2) Mean values for all of the nutrient components found to be significantly different ($\alpha = 0.05$) from the combined-site analysis of MON 88302 were within the 99% tolerance interval established from the commercial references grown concurrently and were, therefore, within the range of natural variability of that component in commercial canola varieties with a history of safe consumption (Table 13).
3) Assessment of the reproducibility of the combined-site differences at the five individual sites demonstrated no significant differences for TDF; however, significant differences ($\alpha = 0.05$) were observed for 18:0 stearic acid, 18:1 oleic acid, and 18:2 linoleic acid at all five sites; significant differences for 16:1 palmitoleic acid and 18:3 linolenic acid at four sites, significant differences for 20:0 arachidic acid at three sites, and significant differences for 22:0 behenic acid at two sites (Table 13). The magnitudes of differences between the mean fatty acid values for MON 88032 and the conventional control were small relative to the variability of these components as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption, and relative to the variability of fatty acid components in canola due to environment (Pritchard et al.,

2000). Individual site mean values of MON 88302 for all nutrient components with significant differences fell within the 99% tolerance interval established from the commercial reference varieties grown concurrently and were, therefore, within the range of natural variability of that component in commercial canola varieties with a history of safe consumption (Table 14).

4) With the exception of TDF, for which no commercial reference values have been published, all of the compositional components identified as significantly different from the conventional control were within the natural variability of these components in commercial canola composition as published in the scientific literature (Table 16).

In summary, the combined-site statistical analysis identified eight significant differences ($\alpha = 0.05$) that were small in magnitude relative to their natural variability as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption.

Of these significant differences, only 18:0 stearic acid, 18:1 oleic acid, and 18:2 linoleic acid were observed consistently at all of the individual sites. All of the components identified as significantly different in the combined-site analysis and corresponding individual site analyses, were within the natural variability of commercial canola defined by the 99% tolerance interval established by the concurrently grown commercial reference varieties, and were within the published literature ranges (TDF does not have published reference data). Therefore, these significant differences are not meaningful to food and feed safety and nutrition. These findings support the conclusion that nutrients in seed from MON 88302 are compositionally equivalent to those in conventional canola varieties with a history of safe usage.

Toxicant and Anti-Nutrient Levels in Seed

According to OECD (2001), canola seed contains toxicants including erucic acid and glucosinolates, and anti-nutrients, including phytic acid, sinapine, and tannins. Erucic acid has been shown to have cardiopathic potential resulting in a weakening of the heart muscle in experimental animals (Bozcali et al., 2009; Chien et al., 1983). Glucosinolates in canola seed can be characterized into two main chemical groups, alkyl and indolyl, with alkyl being the most common (CCC, 2009). Upon enzymatic hydrolysis with myrosinase, certain glucosinolates form compounds that can depress growth and thyroid function (Bell, 1984). The standard for glucosinolates in canola seed is <18 µmoles/g (Szmigielska et al., 2000). Phytic acid is present in canola seed. Phytic acid chelates mineral nutrients, including calcium, magnesium, potassium, iron, and zinc, rendering them biologically unavailable to monogastric animals consuming the seed (Liener, 2000). Sinapine is the choline ester of sinapic acid, the primary phenolic component in canola seed. Sinapine imparts a bitter taste and reduces palatability of the seed (OECD, 2001). Sinapine levels were determined based on quantitation of the hydrolysis product, sinapic acid. Tannins are also present in canola seed (OECD, 2001). Soluble and insoluble tannin fractions were quantified individually, then summed and reported as total tannins.

MON 88302 levels of 22:1 erucic acid were below the level of quantitation (0.04% total FA) in canola seed, and therefore, 22:1 erucic acid was excluded from statistical analysis. In the combined-site analysis, no significant difference ($\alpha = 0.05$) was observed between MON 88302 and the conventional control (Table 13 and Table 15) for indolyl glucosinolates, total glucosinolates, phytic acid, sinapine, and total tannins (as the sum of soluble and insoluble tannin fractions). One statistically significant difference was identified for alkyl glucosinolates, and the net effect was a slight reduction of this anti-nutrient in MON 88302.

The following considerations show that this difference is not a meaningful concern from a food/feed nutritional or safety perspective:

1) The magnitude of the difference between the combined-site mean value for alkyl glucosinolates in MON 88302 and the conventional control showed a 27.59% decrease. This magnitude of difference was small relative to the natural variability of these components as determined by the 99% tolerance interval established by the concurrently grown commercial reference varieties with a history of safe consumption.

2) The MON 88302 mean alkyl glucosinolates value from the combined-site analysis was within the 99% tolerance interval established from the commercial reference varieties grown concurrently. The mean value was, therefore, within the range of natural variability for alkyl glucosinolates in commercial canola varieties with a history of safe consumption (Table 13 and Table 15).

3) Assessment of the reproducibility of the combined-site difference at the five individual sites was not consistent across sites. A significant difference for alkyl glucosinolates was observed at one of the individual sites. However, the mean value for alkyl glucosinolates in MON 88302 at this individual site was within the 99% tolerance interval established from the concurrently grown commercial reference varieties.

4) An assessment based on of the natural variability of alkyl glucosinolates in commercial canola varieties could not be made because a range was not available in the scientific literature.

In summary, the statistical analyses found a combined-site significant difference in alkyl glucosinolates that was lower than the conventional mean value, and not consistently observed at the individual sites. The mean alkyl glucosinolates value for MON 88302 was within the natural variability of commercial canola defined by the 99% tolerance interval established from the concurrently grown commercial reference varieties with a history of safe consumption, and the value was within the safety threshold for canola. Total glucosinolate levels in seed from MON 88302 ranged from 1.73 to 11.42 µmoles/g (Table 15), within the standard for canola. Thus, an evaluation of anti-nutrient components in seed supports the conclusion that MON 88302 is as safe as and compositionally equivalent to conventional canola.

Table 13.Summary of Differences (p < 0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Seed Fiber (% dw)						
Total Dietary Fiber	20.90	18.37	13.81	0.004	16.91 - 27.81	13.97, 24.85
Seed Fatty Acid (% Total FA)						
16:1 Palmitoleic	0.22	0.24	-7.56	0.008	0.20 - 0.26	0.17, 0.30
18:0 Stearic	1.68	1.98	-15.06	<0.001	1.54 - 1.87	0.90, 3.05
18:1 Oleic	62.82	65.79	-4.52	<0.001	60.51 - 65.20	56.13, 70.69
18:2 Linoleic	19.26	17.67	8.98	<0.001	17.78 - 20.66	12.60, 24.49
18:3 Linolenic	9.58	7.98	20.01	<0.001	8.71 - 11.23	6.96, 11.73
20:0 Arachidic	0.54	0.60	-10.68	<0.001	0.50 - 0.57	0.45, 0.80
22:0 Behenic	0.27	0.28	-6.01	0.016	0.24 - 0.29	0.19, 0.43

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in Combined-Site Analysis						
Seed Anti-nutrient						
Alkyl Glucosinolate (µmole/g dw)	3.68	5.08	-27.59	0.035	1.19 - 5.87	0, 29.02
Statistical Differences Observed in More than One Individual Site						
Seed Fatty Acid (% Total FA)						
18:0 Stearic Site MBNW	1.73	1.97	-12.23	0.028	1.64 - 1.87	0.90, 3.05
18:0 Stearic Site MBPL	1.58	1.87	-15.64	<0.001	1.55 - 1.59	0.90, 3.05
18:0 Stearic Site MNCA	1.67	1.86	-10.01	0.022	1.65 - 1.71	0.90, 3.05
18:0 Stearic Site NDVA	1.77	2.11	-16.06	0.004	1.71 - 1.84	0.90, 3.05
18:0 Stearic Site SKSA	1.66	2.08	-20.14	0.001	1.54 - 1.72	0.90, 3.05
18:1 Oleic Site MBNW	63.40	65.71	-3.51	0.004	62.94 - 64.03	56.13, 70.69

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Seed Fatty Acid (% Total FA)						
18:1 Oleic Site MBPL	62.06	64.30	-3.48	<0.001	61.82 - 62.35	56.13, 70.69
18:1 Oleic Site MNCA	61.67	64.86	-4.92	0.005	61.70 - 61.87	56.13, 70.69
18:1 Oleic Site NDVA	65.14	68.38	-4.74	0.003	64.90 - 65.20	56.13, 70.69
18:1 Oleic Site SKSA	61.91	65.69	-5.75	0.001	60.51 - 62.29	56.13, 70.69
18:2 Linoleic Site MBNW	19.27	17.89	7.71	0.011	18.82 - 19.66	12.60, 24.49
18:2 Linoleic Site MBPL	20.43	19.18	6.50	<0.001	20.13 - 20.66	12.60, 24.49
18:2 Linoleic Site MNCA	20.20	18.35	10.07	0.001	20.00 - 20.32	12.60, 24.49
18:2 Linoleic Site NDVA	17.86	15.71	13.67	0.009	17.78 - 18.02	12.60, 24.49

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Seed Fatty Acid (% Total FA)						
18:2 Linoleic Site SKSA	18.49	17.22	7.36	0.019	18.08 - 19.48	12.60, 24.49
Seed Vitamin (mg/100g dw)						
Vitamin E (a-tocopherol) Site MBNW	13.06	9.36	39.51	0.004	12.22 - 13.47	3.88, 17.28
Vitamin E (α-tocopherol) Site MBPL	11.50	7.63	50.83	<0.001	10.70 - 12.20	3.88, 17.28
Vitamin E (α-tocopherol) Site MNCA	13.39	10.82	23.73	0.006	12.58 - 14.62	3.88, 17.28
Vitamin E (α-tocopherol) Site NDVA	15.89	9.43	68.39	0.010	15.23 - 16.55	3.88, 17.28
Vitamin E (α-tocopherol) Site SKSA	1.49	6.91	-78.47	0.019	1.30 - 1.66	3.88, 17.28
Seed Anti-nutrient						
Sinapic Acid (% dw) Site MBNW	1.02	0.92	10.34	0.001	0.99 - 1.06	0.57, 1.13

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Seed Anti-nutrient						
Sinapic Acid (% dw) Site MBPL	0.97	0.86	12.04	<0.001	0.95 - 0.99	0.57, 1.13
Sinapic Acid (% dw) Site MNCA	1.06	0.96	10.66	0.001	1.02 - 1.08	0.57, 1.13
Sinapic Acid (% dw) Site NDVA	1.02	0.83	23.56	0.001	1.00 - 1.04	0.57, 1.13
Sinapic Acid (% dw) Site SKSA	0.22	0.81	-73.12	0.001	0.16 - 0.28	0.57, 1.13
Seed Fatty Acid (% Total FA)						
16:1 Palmitoleic Site MBNW	0.21	0.23	-9.71	0.015	0.20 - 0.21	0.17, 0.30
16:1 Palmitoleic Site MBPL	0.23	0.25	-10.10	0.008	0.22 - 0.23	0.17, 0.30
16:1 Palmitoleic Site MNCA	0.21	0.24	-10.88	0.001	0.21 - 0.21	0.17, 0.30
16:1 Palmitoleic Site NDVA	0.20	0.22	-11.05	0.036	0.20 - 0.20	0.17, 0.30

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Seed Fatty Acid (% Total FA)						
18:3 Linolenic Site MBNW	9.19	8.12	13.27	0.004	8.88 - 9.42	6.96, 11.73
18:3 Linolenic Site MBPL	9.28	7.74	19.89	<0.001	9.12 - 9.43	6.96, 11.73
18:3 Linolenic Site NDVA	8.82	7.31	20.67	<0.001	8.71 - 8.94	6.96, 11.73
18:3 Linolenic Site SKSA	10.78	8.38	28.69	<0.001	10.39 - 11.23	6.96, 11.73
Seed Fatty Acid (% Total FA)						
20:0 Arachidic Site MBPL	0.53	0.60	-11.73	<0.001	0.52 - 0.54	0.45, 0.80
20:0 Arachidic Site NDVA	0.57	0.65	-12.58	<0.001	0.56 - 0.57	0.45, 0.80
20:0 Arachidic Site SKSA	0.54	0.62	-13.28	<0.001	0.52 - 0.55	0.45, 0.80

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control)		MON 88302 Range	Commercial Tolerance Interval[5]
			Mean Difference (% of Control)	Significance (p-Value)		
Statistical Differences Observed in More than One Individual Site						
Seed Mineral						
Copper (mg/kg dw) Site MBNW	3.72	3.41	9.28	0.013	3.61 - 3.83	2.00, 4.43
Copper (mg/kg dw) Site MBPL	3.47	3.97	-12.50	0.016	3.35 - 3.56	2.00, 4.43
Copper (mg/kg dw) Site MNCA	4.40	4.11	6.91	0.027	4.16 - 4.57	2.00, 4.43
Seed Fatty Acid (% Total FA)						
22:0 Behenic Site MBPL	0.27	0.30	-13.00	<0.001	0.26 - 0.27	0.19, 0.43
22:0 Behenic Site NDVA	0.27	0.30	-9.83	0.007	0.27 - 0.27	0.19, 0.43
Seed Mineral						
Iron (mg/kg dw) Site MBPL	44.13	51.01	-13.48	0.001	42.80 - 45.09	23.39, 86.23
Iron (mg/kg dw) Site MNCA	42.57	50.64	-15.93	0.007	40.56 - 44.18	23.39, 86.23

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in More than One Individual Site						
Seed Mineral						
Potassium (g/100g dw) Site MBPL	0.70	0.77	-8.91	0.023	0.63 - 0.76	0.39, 0.96
Potassium (g/100g dw) Site SKSA	0.82	0.71	15.32	<0.001	0.77 - 0.90	0.39, 0.96
Zinc (mg/kg dw) Site MBPL	31.25	33.88	-7.76	0.024	30.45 - 32.05	20.19, 48.23
Zinc (mg/kg dw) Site SKSA	41.58	33.10	25.61	0.010	39.33 - 45.49	20.19, 48.23
Statistical Differences Observed in One Individual Site						
Seed Proximate (% dw)						
Carbohydrates Site MNCA	27.31	25.99	5.07	0.035	26.27 - 27.90	23.12, 30.77
Moisture (% fw) Site MNCA	5.52	6.69	-17.46	<0.001	5.37 - 5.61	4.33, 6.91
Protein Site SKSA	23.82	22.14	7.58	0.038	23.62 - 24.58	17.20, 30.08

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Individual Site						
Seed Proximate (% dw)						
Total Fat Site NDVA	48.04	45.17	6.35	0.014	47.20 - 48.87	39.65, 51.24
Seed Fiber (% dw)						
Acid Detergent Fiber Site MBPL	16.75	14.19	18.00	0.005	15.17 - 18.19	6.95, 23.92
Neutral Detergent Fiber Site MBPL	19.45	16.87	15.31	0.017	18.35 - 20.02	10.07, 25.94
Seed Amino Acid (% dw)						
Tyrosine Site MBPL	0.72	0.71	2.46	0.028	0.72 - 0.73	0.57, 0.81
Valine Site MNCA	1.15	1.24	-7.32	0.048	1.13 - 1.15	0.92, 1.55
Seed Fatty Acid (% Total FA)						
16:0 Palmitic Site SKSA	4.51	4.07	10.90	<0.001	4.46 - 4.57	2.84, 5.26
20:1 Eicosenoic Site SKSA	1.24	1.13	9.55	0.005	1.22 - 1.26	0.83, 1.68

Table 14. Summary of Differences (p<0.05) for the Comparison of Canola Seed Component Levels for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean[3]	Control[4] Mean	Mean Difference (MON 88302 minus Control) Mean Difference (% of Control)	Mean Difference (MON 88302 minus Control) Significance (p-Value)	MON 88302 Range	Commercial Tolerance Interval[5]
Statistical Differences Observed in One Individual Site						
Seed Fatty Acid (% Total FA)						
24:0 Lignoceric Site MBPL	0.16	0.19	-12.24	0.029	0.16 - 0.17	0.033, 0.25
24:1 Nervonic Site MBPL	0.13	0.16	-20.37	0.031	0.12 - 0.13	0.041, 0.18
Seed Anti-nutrient						
Alkyl Glucosinolate (µmole/g dw) Site SKSA	1.61	5.82	-72.32	0.005	1.19 - 2.17	0, 29.02
Indolyl Glucosinolate (µmole/g dw) Site SKSA	0.86	3.30	-73.88	0.001	0.49 - 1.31	1.37, 6.62
Total Glucosinolate (µmole/g dw) Site SKSA	2.53	9.22	-72.58	0.002	1.73 - 3.51	0, 32.20

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88302 treated with glyphosate.
[3]Mean = least-square mean.
[4]Control refers to the genetically similar, conventional control Ebony.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial reference varieties. Negative limits set to zero.

Table 14. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Proximate (% dw)						
Ash	3.96 (0.18) (3.31 - 4.45)	3.90 (0.18) (3.20 - 5.10)	0.055 (0.095) (-0.21 - 0.64)	-0.14, 0.25	0.565	3.32, 4.66 (2.98 - 4.52)
Carbohydrates	25.96 (0.68) (21.83 - 28.81)	26.13 (0.68) (23.91 - 28.73)	-0.17 (0.54) (-4.18 - 1.94)	-1.42, 1.09	0.765	23.12, 30.77 (22.53 - 29.96)
Moisture (% fw)	5.35 (0.34) (3.90 - 6.08)	5.45 (0.34) (4.41 - 6.98)	-0.10 (0.24) (-1.53 - 0.87)	-0.65, 0.45	0.688	4.33, 6.91 (4.09 - 8.48)
Protein	23.04 (0.70) (19.68 - 25.98)	23.14 (0.69) (20.29 - 27.02)	-0.10 (0.52) (-2.29 - 2.50)	-1.32, 1.11	0.847	17.20, 30.08 (18.68 - 28.32)
Total Fat	47.06 (0.83) (43.96 - 49.26)	46.82 (0.83) (43.65 - 50.24)	0.24 (0.52) (-2.28 - 4.10)	-1.00, 1.48	0.659	39.65, 51.24 (40.71 - 50.26)
Fiber (% dw)						
Acid Detergent Fiber	15.32 (1.36) (9.19 - 20.24)	14.47 (1.36) (8.94 - 18.71)	0.84 (0.41) (-2.71 - 3.57)	-0.14, 1.83	0.082	6.95, 23.92 (9.75 - 21.22)

Table 15. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fiber (% dw)						
Neutral Detergent Fiber	17.43 (1.38) (9.48 - 21.36)	16.70 (1.38) (11.56 - 19.58)	0.74 (0.57) (-2.74 - 4.43)	-0.58, 2.05	0.231	10.07, 25.94 (10.93 - 22.75)
Total Dietary Fiber	20.90 (0.79) (16.91 - 27.81)	18.37 (0.78) (14.58 - 23.00)	2.54 (0.84) (-0.49 - 9.96)	0.85, 4.23	0.004	13.97, 24.85 (12.64 - 26.47)
Amino Acid (% dw)						
Alanine	1.02 (0.025) (0.88 - 1.15)	1.04 (0.025) (0.93 - 1.19)	-0.015 (0.022) (-0.12 - 0.069)	-0.066, 0.035	0.502	0.77, 1.34 (0.87 - 1.27)
Arginine	1.45 (0.054) (1.23 - 1.72)	1.51 (0.054) (1.29 - 1.77)	-0.063 (0.032) (-0.27 - 0.15)	-0.13, 0.00082	0.052	1.10, 1.93 (1.23 - 1.96)
Aspartic Acid	1.65 (0.067) (1.40 - 1.93)	1.71 (0.067) (1.46 - 1.97)	-0.055 (0.043) (-0.37 - 0.12)	-0.16, 0.045	0.238	1.33, 2.12 (1.42 - 2.23)
Cystine	0.57 (0.027) (0.48 - 0.73)	0.58 (0.027) (0.49 - 0.79)	-0.0044 (0.015) (-0.054 - 0.053)	-0.040, 0.031	0.781	0.38, 0.83 (0.45 - 0.79)

Table 15. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Glutamic Acid	4.06 (0.18) (3.37 - 5.06)	4.24 (0.17) (3.64 - 5.26)	-0.19 (0.10) (-0.68 - 0.36)	-0.43, 0.049	0.103	2.73, 5.89 (3.26 - 5.43)
Glycine	1.14 (0.040) (1.02 - 1.32)	1.19 (0.040) (1.01 - 1.38)	-0.041 (0.025) (-0.18 - 0.044)	-0.10, 0.018	0.142	0.96, 1.47 (1.01 - 1.50)
Histidine	0.63 (0.023) (0.55 - 0.77)	0.65 (0.023) (0.57 - 0.78)	-0.015 (0.011) (-0.065 - 0.044)	-0.038, 0.0074	0.181	0.47, 0.86 (0.54 - 0.80)
Isoleucine	0.93 (0.028) (0.81 - 1.08)	0.96 (0.028) (0.82 - 1.12)	-0.024 (0.021) (-0.13 - 0.041)	-0.074, 0.026	0.299	0.70, 1.22 (0.78 - 1.15)
Leucine	1.64 (0.049) (1.40 - 1.90)	1.68 (0.049) (1.46 - 1.95)	-0.042 (0.039) (-0.25 - 0.086)	-0.13, 0.048	0.308	1.21, 2.18 (1.36 - 2.07)
Lysine	1.39 (0.041) (1.22 - 1.63)	1.41 (0.041) (1.25 - 1.65)	-0.019 (0.023) (-0.12 - 0.086)	-0.064, 0.027	0.410	1.02, 1.90 (1.20 - 1.68)

Table 15. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Methionine	0.46 (0.015) (0.40 - 0.54)	0.46 (0.015) (0.40 - 0.56)	-0.0018 (0.0089) (-0.038 - 0.034)	-0.022, 0.019	0.847	0.30, 0.65 (0.36 - 0.57)
Phenylalanine	0.98 (0.029) (0.84 - 1.11)	1.00 (0.028) (0.87 - 1.15)	-0.024 (0.024) (-0.17 - 0.044)	-0.079, 0.031	0.348	0.77, 1.26 (0.84 - 1.25)
Proline	1.40 (0.054) (1.20 - 1.71)	1.42 (0.054) (1.20 - 1.73)	-0.028 (0.027) (-0.16 - 0.17)	-0.093, 0.036	0.335	0.90, 2.01 (1.12 - 1.78)
Serine	1.02 (0.030) (0.87 - 1.14)	1.05 (0.030) (0.94 - 1.18)	-0.035 (0.019) (-0.17 - 0.052)	-0.080, 0.0095	0.105	0.81, 1.32 (0.88 - 1.30)
Threonine	0.98 (0.030) (0.86 - 1.11)	1.00 (0.030) (0.88 - 1.12)	-0.025 (0.018) (-0.12 - 0.065)	-0.066, 0.016	0.192	0.82, 1.20 (0.84 - 1.22)
Tryptophan	0.23 (0.010) (0.17 - 0.26)	0.24 (0.010) (0.19 - 0.31)	-0.013 (0.0093) (-0.063 - 0.036)	-0.032, 0.0059	0.172	0.13, 0.35 (0.17 - 0.32)

Table 15. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper l	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Amino Acid (% dw)						
Tyrosine	0.67 (0.019) (0.59 - 0.75)	0.69 (0.019) (0.61 - 0.77)	-0.017 (0.013) (-0.11 - 0.028)	-0.048, 0.015	0.249	0.57, 0.81 (0.60 - 0.84)
Valine	1.20 (0.035) (1.04 - 1.37)	1.22 (0.035) (1.05 - 1.41)	-0.025 (0.025) (-0.16 - 0.054)	-0.084, 0.034	0.352	0.92, 1.55 (1.01 - 1.46)
Fatty Acid (% Total FA)						
16:0 Palmitic	4.23 (0.078) (3.95 - 4.57)	4.10 (0.077) (3.94 - 4.41)	0.13 (0.067) (-0.22 - 0.48)	-0.027, 0.28	0.094	2.84, 5.26 (3.55 - 4.69)
16:1 Palmitoleic	0.22 (0.0081) (0.20 - 0.26)	0.24 (0.0081) (0.22 - 0.26)	-0.018 (0.0053) (-0.039 - 0.0074)	-0.030, -0.0059	0.008	0.17, 0.30 (0.19 - 0.27)
18:0 Stearic	1.68 (0.044) (1.54 - 1.87)	1.98 (0.044) (1.78 - 2.19)	-0.30 (0.031) (-0.48 - -0.059)	-0.37, -0.23	<0.001	0.90, 3.05 (1.50 - 2.64)
18:1 Oleic	62.82 (0.62) (60.51 - 65.20)	65.79 (0.62) (63.72 - 68.44)	-2.97 (0.31) (-4.30 - -1.52)	-3.69, -2.26	<0.001	56.13, 70.69 (57.86 - 68.53)

Table 15. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
18:2 Linoleic	19.26 (0.51) (17.78 - 20.66)	17.67 (0.51) (15.72 - 19.29)	1.59 (0.17) (0.40 - 2.42)	1.20, 1.97	<0.001	12.60, 24.49 (14.12 - 22.57)
18:3 Linolenic	9.58 (0.27) (8.71 - 11.23)	7.98 (0.27) (7.19 - 8.99)	1.60 (0.21) (0.76 - 2.64)	1.12, 2.07	<0.001	6.96, 11.73 (7.99 - 10.94)
20:0 Arachidic	0.54 (0.011) (0.50 - 0.57)	0.60 (0.011) (0.54 - 0.65)	-0.064 (0.0074) (-0.091 - -0.0032)	-0.081, -0.047	<0.001	0.45, 0.80 (0.53 - 0.71)
20:1 Eicosenoic	1.13 (0.024) (1.06 - 1.26)	1.09 (0.024) (1.00 - 1.18)	0.036 (0.017) (-0.042 - 0.14)	-0.0034, 0.076	0.068	0.83, 1.68 (1.04 - 1.56)
22:0 Behenic	0.27 (0.0072) (0.24 - 0.29)	0.28 (0.0072) (0.24 - 0.31)	-0.017 (0.0056) (-0.047 - 0.016)	-0.030, -0.0041	0.016	0.19, 0.43 (0.27 - 0.38)
24:0 Lignoceric	0.16 (0.016) (0.049 - 0.23)	0.16 (0.015) (0.045 - 0.22)	0.0038 (0.017) (-0.14 - 0.11)	-0.030, 0.038	0.823	0.033, 0.25 (0.044 - 0.21)

Table 15. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Fatty Acid (% Total FA)						
24:1 Nervonic	0.12 (0.015) (0.046 - 0.20)	0.11 (0.015) (0.045 - 0.17)	0.013 (0.014) (-0.072 - 0.081)	-0.020, 0.047	0.377	0.041, 0.18 (0.044 - 0.20)
Mineral						
Calcium (g/100g dw)	0.41 (0.030) (0.30 - 0.51)	0.40 (0.030) (0.28 - 0.49)	0.015 (0.012) (-0.068 - 0.081)	-0.0089, 0.039	0.210	0.16, 0.61 (0.25 - 0.53)
Copper (mg/kg dw)	3.78 (0.17) (3.27 - 4.57)	3.65 (0.17) (2.96 - 4.18)	0.14 (0.14) (-0.83 - 0.57)	-0.19, 0.46	0.361	2.00, 4.43 (2.52 - 4.93)
Iron (mg/kg dw)	48.73 (4.28) (40.55 - 69.61)	54.01 (4.24) (41.65 - 77.74)	-5.28 (2.89) (-20.41 - 14.87)	-11.85, 1.30	0.102	23.39, 86.23 (39.16 - 77.92)
Magnesium (g/100g dw)	0.37 (0.014) (0.31 - 0.42)	0.36 (0.014) (0.31 - 0.42)	0.0048 (0.0070) (-0.032 - 0.043)	-0.011, 0.021	0.508	0.32, 0.43 (0.30 - 0.45)
Manganese (mg/kg dw)	41.44 (2.02) (35.28 - 51.55)	40.34 (1.99) (33.12 - 50.97)	1.10 (1.83) (-8.36 - 12.63)	-2.62, 4.82	0.551	14.85, 61.05 (25.00 - 54.11)

Table 15. Statistical Summary of Combined-Site Seed Nutrient Content for MON 88302 vs. the Conventional Control (continued)

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Mineral						
Phosphorus (g/100g dw)	0.72 (0.042) (0.56 - 0.87)	0.72 (0.041) (0.56 - 0.93)	-0.0090 (0.022) (-0.095 - 0.16)	-0.055, 0.037	0.692	0.38, 1.06 (0.44 - 0.87)
Potassium (g/100g dw)	0.64 (0.053) (0.48 - 0.90)	0.64 (0.052) (0.53 - 0.81)	0.0016 (0.025) (-0.097 - 0.14)	-0.056, 0.060	0.951	0.39, 0.96 (0.50 - 0.92)
Zinc (mg/kg dw)	35.58 (1.78) (29.81 - 45.56)	33.01 (1.76) (28.46 - 40.66)	2.57 (1.83) (-4.50 - 11.44)	-1.66, 6.80	0.198	20.19, 48.23 (22.18 - 47.61)
Vitamin (mg/100g dw)						
Vitamin E (α-tocopherol)	11.06 (2.08) (1.30 - 16.55)	8.85 (2.08) (3.33 - 11.77)	2.21 (1.66) (-6.92 - 8.09)	-1.61, 6.03	0.218	3.88, 17.28 (2.62 - 14.84)

[1]dw = dry weight; fw = fresh weight; FA = fatty acid.
[2]MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI – confidence interval.
[4]Control refers to the genetically similar, conventional control Ebony.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table 15. Statistical Summary of Combined-Site Seed Anti-nutrient Content for MON 88302 vs. the Conventional Control

Analytical Component (Units)[1]	MON 88302[2] Mean (S.E.)[3] (Range)	Control[4] Mean (S.E.) (Range)	Difference (MON 88302 minus Control) Mean (S.E.) (Range)	95% CI[3] Lower, Upper	Significance (p-Value)	Commercial Tolerance Interval[5] (Range)
Anti-nutrient						
Alkyl Glucosinolate (µmole/g dw)	3.68 (0.43) (1.19 - 5.87)	5.08 (0.42) (2.45 - 8.28)	-1.40 (0.59) (-6.11 - 1.43)	-2.69, -0.11	0.035	0, 29.02 (2.32 - 28.33)
Indolyl Glucosinolate (µmole/g dw)	3.50 (0.51) (0.49 - 5.76)	3.89 (0.50) (1.83 - 5.89)	-0.39 (0.45) (-3.05 - 2.83)	-1.42, 0.64	0.408	1.37, 6.62 (1.84 - 7.18)
Phytic Acid (% dw)	1.95 (0.18) (1.20 - 2.58)	2.11 (0.18) (1.46 - 2.77)	-0.16 (0.083) (-0.67 - 0.68)	-0.33, 0.010	0.064	0.70, 3.52 (1.10 - 2.71)
Sinapic Acid (% dw)	0.86 (0.12) (0.16 - 1.08)	0.88 (0.12) (0.65 - 0.97)	-0.023 (0.11) (-0.76 - 0.21)	-0.27, 0.22	0.837	0.57, 1.13 (0.48 - 0.99)
Total Glucosinolate (µmole/g dw)	7.35 (0.87) (1.73 - 11.42)	9.08 (0.86) (4.38 - 12.72)	-1.73 (1.01) (-9.21 - 3.58)	-4.06, 0.61	0.127	0, 32.20 (5.52 - 31.98)
Total Tannins (% dw)	0.70 (0.11) (0.20 - 1.32)	0.69 (0.11) (0.31 - 1.11)	0.0036 (0.084) (-0.49 - 0.45)	-0.19, 0.20	0.966	0, 1.37 (0.14 - 1.18)

[1]dw = dry weight.
[2]MON 88302 treated with glyphosate.
[3]Mean (S.E.) = least-square mean (standard error); CI = confidence interval.
[4]Control refers to the genetically similar, conventional control Ebony.
[5]With 95% confidence, interval contains 99% of the values expressed in the population of commercial conventional references. Negative limits were set to zero.

Table 16. Literature Ranges for Components in Canola Seed

Component[1]	Literature Range[2]
Proximates (% dw)	
Ash	4.067 – 5.917[a]
Carbohydrates	N
Moisture (% fw)	3.177 – 8.045[a]; 7.4 – 10.0[b]
Protein	21.30 – 28.125[a]; 18.7 – 26.0[b]; 17.4 – 23.0[c]; 21.1 – 26.7[d]
Total Fat	35.59 – 44.93[a]; 24.0 – 43.6[b]; 42.0 – 49.5[d]
Fiber (% dw)	
Acid Detergent Fiber (ADF)	11.934 – 26.799[a];11.6[f]; 12.4[g]; 22.2[h]
Neutral Detergent Fiber (NDF)	18.653 – 34.720[a]; 17.8[f]; 16.49[g]; 31.3[h]
Total Dietary Fiber	N
Amino Acids (% dw)	
Alanine	0.93 – 0.96[b] ; 1.15 – 1.38[e]
Arginine	1.13 – 1.21[b]; 2.23 – 2.46[e]
Aspartic acid	1.54 – 1.59[e]
Cystine/Cysteine	0.52 – 0.54[b]
Glutamic acid	4.60 – 4.71[e]
Glycine	1.04 – 1.06[b]; 2.20 – 2.22[e]
Histidine	0.51 – 0.66[b]; 0.80 – 0.82[e]
Isoleucine	0.80 – 0.86[b]; 0.96 – 1.03[e]
Leucine	1.35 – 1.47[b]; 1.83 – 1.99[e]
Lysine	1.03 – 1.19[b]; 1.67 – 1.85[e]
Methionine	0.42 – 0.44[b]
Phenylalanine	0.75 – 0.82[b]; 0.90 – 1.03[e]
Proline	1.19 – 1.33[b]; 3.36 – 3.74[e]
Serine	0.90 – 0.94[b]; 1.44 – 1.55[e]
Threonine	0.87 – 0.94[b]; 1.28 – 1.30[e]
Tryptophan	0.23 – 0.27[b];
Tyrosine	0.51 – 0.59[b]; 0.81 – 0.92[e]
Valine	1.02 – 1.13[b]; 1.45 – 1.55[e]
Vitamins (mg/kg dw)	
Vitamin E (α-tocopherol)	71.1 – 108.4[i]

Table 17. Literature Ranges for Components in Canola Seed (continued)

Component[1]	Literature Range[2]
Minerals	
Calcium (% dw)	0.29 – 0.48[b]; 0.348 – 0.729[a]
Copper (mg/kg dw)	7[b]; 1.388 – 5.492[a]
Iron (mg/kg dw)	ND[b]; 0.0 – 965.6[a]
Magnesium (% dw)	0.29 – 0.31[b]; 0.272 – 0.402[a]
Manganese (mg/kg dw)	ND[b]; 33.813 – 64.757[a]
Phosphorus (% dw)	0.48 – 0.85[b]; 0.581 – 0.895[a]
Potassium (% dw)	0.83 – 0.91[b]; 0.681 – 1.016[a]
Sodium (% dw)	0.05[b]; 0.003 – 0.030[a]
Zinc (mg/kg dw)	62[b]; 0 – 126.953[a]
Fatty Acids (% total)	
16:0 Palmitic	3.3 – 6.0[b]
16:1 Palmitoleic	0.1 – 0.6[b]
18:0 Stearic	1.1 – 2.5[b]
18:1 Oleic	52.0 – 66.9[b]
18:2 Linoleic	16.1 – 24.8[b]
18:3 Linolenic	6.4 – 14.1[b]
20:0 Arachidic	0.2 – 0.8[b]
20:1 Eicosenoic	0.1 – 3.4[b]
20:2 Eicosadienoic	0.0 – 0.1[b]
20:3 Eicosatrienoic	N
20:4 Arachidonic	N
22:0 Behenic	0.0 – 0.5[b]
22:1 Erucic	0.0 – 2.0[b]
24:0 Lignoceric	0.0 – 0.2[b]
24:1 Nervonic	0.0 – 0.04[b]
Anti-nutrients	
Total Glucosinolates (µmol/g)	6 – 29[b]; 7.8 – 26.8[c]; 18 – 57[j]
Phytic Acid (% dw)	2.0 – 5.0[b]
Sinapine (% dw)	0.6 – 1.8[b]
Tannins (% dw)	1.5 – 3.0[b]; 0.68 – 0.77[k]

[1]fw = fresh weight; dw = dry weight; dm = dry matter; ND defined as below the level of detection; N defined as not reported.
[2]Literature Range = Values published for low erucic acid rapeseed (canola).
Citations = [a](Dairy One Forage Lab, 2010); [b](OECD, 2001); [c](Pritchard et al., 2000); [d](Barthet and Daun, 2005); [e](Wang et al., 1999); [f](NRC, 2001); [g](Mustafa et al., 2000); [h](Leupp et al., 2006); [i](Marwede et al., 2004);.[j](Mailer and Pratley, 1990), [k](Naczk et al., 1998).
Conversions: mg/100g dw × 10 = mg/kg dw; g/100g dw× 10 = mg/g dw.

B6(b) Levels of other GM-influenced constituents

As described in section B6(a), detailed compositional analyses of key components of MON 88302 have been performed and have demonstrated that MON 88302 is compositionally equivalent to conventional canola. The processing of MON 88302 is not expected to be any different from that of conventional canola. 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 important nutrients or endogenous toxicants that may be present in canola. Therefore, when MON 88302 and its progeny 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 canola.

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 88302 as described in section B6(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 88302 are as safe and nutritious as those derived from commercially-available, conventional canola for which there is an established history of safe consumption. Therefore, Monsanto believes that animal feeding studies will not add value to the safety of MON 88302.

Part 3 STATUTORY DECLARATION – AUSTRALIA

I, Michael Leader, 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.

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